Perphenazine And Prochlorperazine Decrease Glioblastoma Cells Migration And Invasion: Analysis of ABCB1 And ABCG2 Transporters, e-cadherin, α-tubulin, And Integrins (α3, α5, and β1) Expression in U87-MG Cells

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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 (up to 18 months). Thus, novel and effective strategies of treatment are still required since resistance and metastasis are major problems of anticancer chemotherapy. Interestingly, ABC transporters, which play a crucial role in the development of multidrug resistance, are modulated by phenothiazine derivatives, while cancer metastasis, migration, and invasion are regulated by cadherins, α-tubulin, and integrins.

Methods: The impact on the motility of human glioblastoma U87-MG was performed by wound healing assay, cellular migration and invasion were performed by transwell assay, while ABCB1, ABCG2, E-cadherin, α-tubulin, and integrins (α3, α5, and β1) content were determined by Western blot.

Results: The present 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 migration and invasion ability of human glioblastoma (U87-MG) cells suggesting that phenothiazine derivatives impair multidrug resistance proteins (ABCB1 and ABCG2), E-cadherin, α-tubulin, and integrins amount as well as impair migration and invasion of the U87-MG cell line.

Conclusions: The study demonstrated that an increase of ABCG2 and E-cadherin as well as a decrease of α-tubulin, and integrins amount may explain the decrease of migration and invasion ability after phenothiazine derivatives treatment. Moreover, only prochlorperazine significantly reduces the rate of cell migration. Thus, the drug may be considered for the development of new and effective glioblastoma therapy.

Introduction

Glioblastoma multiforme (GBM) is the most frequent malignant brain tumor leading to 225,000 deaths per year in the entire world and representing 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 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 only (Alphandéry et al. 2018).

The ATP-binding cassette (ABC) transporters are involved in diverse regulation processes that are responsible for the efflux of substances from tumor cells. In the brain endothelial capillaries that build the brain-blood barrier (BBB) are localized the ATP-binding cassette drug efflux transporters: 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). The transporters move not only biologically important substrates across the cell membranes (amino acids, cholesterol) but also are involved in reducing the brain entry of many chemotherapeutic agents, as they can actively transport

them back into the blood (Balça-Silva et al. 2018). Thus, novel and effective GBM therapy should overcome the impediment posed by the BBB and the ABC efflux transporters.

The main role in tumor metastasis plays E-cadherin epithelial cell adhesion protein. In many cancers during metastasis the high levels of E-cadherin cause that cells are more invasive and metastasize. Moreover, E-cadherin plays an important role “in collective cell migration behaviors that facilitate invasion and metastasis”. Noteworthy, E-cadherin can also enhance cell survival (Na et al. 2020). In case of cell migration very important is alpha-tubulin, which controls dynamics of focal adhesion for lamellipodial extension after the tubulin acetylation (Lee et al. 2018). Moreover, the integrins which are cell surface receptors interact with extracellular matrix molecules and play important role in cellular migration, invasion, proliferation, and survival. Especially, α6β4, α5β1, αvβ6, αvβ3, αvβ5, and α7 integrin are up-regulated in different tumors (including glioblastoma) and are related to poor patient prognosis (Masoumi et al. 2021). The α3 integrin possesses laminin motif-binding, α5 integrins possesses arginine-glycine-aspartic acid (RGD) motif binding, while β1 possesses collagen, laminin, and RGD motif-binding. Noteworthy, integrins not only “directly support cell adhesion and migration” but also α3β1 and α5β1 integrins are related to the invasive types of glioma cells (Ellert-Miklaszewska et al. 2020). Mainly, α3β1, α5β1, α9β1, and β8 integrins are related to migration and/or invasion of glioblastoma cells (Malric et al. 2017).

Previously it has been determined cytotoxic activity of perphenazine and prochlorperazine towards U87-MG cells. The obtained EC_{50} values were related to the concentrations detected in human plasma (Otręba and Buszman 2018). This study aimed was to investigate the effect of perphenazine and prochlorperazine on ABCB1 and ABCG2 amount, migration and invasion ability of human glioblastoma (U87-MG) cells as well as expression of the proteins related to the cellular migration and invasion: E-cadherin, α-tubulin, and integrins (α3, α5, and β1). In conclusion, the present data indicate that phenothiazine derivatives may be effective in GBM treatment based on the effect of the drug on migration and invasion ability as well as ABCB1, ABCG2, E-cadherin, α-tubulin, and integrins (α3, α5, and β1) content.

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, 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 blot analysis according to the method described earlier (Otręba et al. 2019) with slight modification. Cells were treated with various concentrations of perphenazine, prochlorperazine, elacridar, or medium for 24 h. 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 prestained 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 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 slight modification. In brief, some 1x106 U87-MG were incubated with supplemented growth medium for 24 h to approximately 80–90% confluence in a 35mm plate (Sarstedt, Germany). Then 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 post-scratching using 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). The wound closure was calculated using the formula (Grada et al. 2017):

$$Wound\ closure\ (\%) = [\frac{\mathrm{At-0h} - \mathrm{At-\Delta h}}{\mathrm{At-0h}}]\ x100\%$$

At-0h is the area of the wound measured in time t_0

At-Δh is the area of the wound measured h hours after t_0

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 migration and invasion transwell assay was performed according to the method described by Bernhart et al. (2013), Limame et al. (2012) as well as the Corning cell migration, chemotaxis, and invasion assay protocol with slight modification.

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 case of invasion assay, the insert membrane was covered by 45 µl of the Geltrex diluted 1:1 v/v in medium with 1% FBS. Inserts with Geltrex were kept for 45 minutes at 37oC before use. Then 25,000 U87-MG cells were seeded into inserts for 48h in 100 µl of growth medium with 1% FBS or a growth medium with 1% FBS containing bacitracin (2.5 mM), perphenazine (0.5 µM), or prochlorperazine (0.5 µM). The lower compartment was filled with 600 µl of the growth medium with 10% FBS, growth medium with 1% FBS, or growth medium (1 or 10%) 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 inserts were washed in PBS and incubated with methanol for 20 minutes. The cells were next washed in PBS and stained 0.1% crystal violet for 10 minutes. Next, inserts were washed in PBS until the water runs clear and non-migrated cells from the upper part of the insert were removed using 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 up 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 prepared a standard curve using the inserts without Geltrex as a control to calculate total invasion due to the Corning cell migration, chemotaxis, and invasion assay protocol. The standard curve was performed as above description, but using the different number of cells were used: 0, 500, 1000, 2500, 5000, 1000, 15000, 20000, 25000, 30000, 35000, 40000, 45000, and 50000 cells. Moreover, the standard curve was prepared in two ways: invasion from the growth medium with 1% FBS

the growth medium with 10% FBS as well as from the growth medium with 1% FBS the growth medium with 1% FBS.

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 migrated/invaded from the growth medium with 1% FBS the growth medium with 1% FBS called random migration) from results of cells migrated/invaded from the growth medium with 1% FBS the 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 blot analysis according to the method described in point 2.2 with slight modification. Bacitracin was used as an inhibitor of U87-MG cell migration (Li et al. 2016). The obtained proteins were separated on 10% SDS-PAGE along and finally 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 are 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 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 were expressed *via* western blot after a 24h treatment of glioblastoma cells by different concentrations of perphenazine, prochlorperazine, and elacridar (as negative control) (Fig. 1A). The Full-length immunoblots with the molecular mass marker are shown in Supplementary Figure S1. The Western blot raw data represented in this figure is found in Supplementary Files S1 and S2.

Elacridar significantly decreased the ABCB1 level by 45.7 % in comparison to DMSO control containing medium and 0.5% DMSO (Fig. 1B). Perphenazine in the concentration of 0.1 μM significantly reduced the ABCB1 amount by 30.9 %. In contrast, perphenazine (0.1 μM and 1.0 μM) increase ABCG2 amount by 29.4 % and 50.7 %, respectively (Fig. 1C). A similar situation was observed in case of prochlorperazine: significant reduction of the ABCB1 amount by 30.9% in the concentration of 0.1 μM as well as a significant increase of ABCG2 amount in the concentration 0.1 μM and 1.0 μM by 34.9 % and 140.2 % compared with its control group, respectively (Fig. 1D).

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

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

Figure 2A shows original photos of wound healing after times 0, 3, 6, 9, 12, and 24 hours. In all the cases the wound closure 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. In case of control and 1.0 uM perphenazine from 3 up to 24 hours incubation significant stimulation of wound closure and reduction of total wound area from 22.0 % to 74.7 % as well as from 27.0 % to 69.3 % were observed, respectively in comparison to t_0 group (Fig. 2B). Moreover, a significant increase of the wound closure was observed after the 6-hour treatment of U87-MG cells with perphenazine in the concentration of 1.0 μM in comparison to prochlorperazine in the same concentration.

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 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 transwell migration and invasion

The raw transwell migration and invasion assay data represented in Figs. 3 and 4 are found in Supplementary Files S4, S5, and S6.

In case of invasion from the growth medium with 1% FBS the growth medium with 1% FBS, in 45000 cells and 50000 cells samples we observed a high decrease of cell amount, thus we finished the standard curve at 40000 cells up till it is still linear. The standard curves are present in Fig. 3.

The transwell invasion assay showed a significant decrease in invasion by 22.91, 11.31, and 12.19 % respectively for bacitracin (2.5mM), perphenazine (0.5 μM), and prochlorperazine (0.5 μM) in comparison

to control (Fig. 4A). The analysis of internal control showed that 2.71% of cells invade randomly. Moreover, phenothiazine and prochlorperazine significantly increase the percentage of invaded cells by 5.65 and, 6.85% respectively in comparison to control (Fig. 4C).

In case of 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 control (Fig. 4B). The observed decrease of U87-MG cell migration caused by bacitracin was not significant. The analysis of internal control showed that 1.97 % of cells migrate randomly. Moreover, phenothiazine and prochlorperazine significantly increase the percentage of invaded cells by 6.80 and, 10.30% respectively in comparison to 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) were expressed via western blot after a 24h treatment of glioblastoma cells by different concentrations of perphenazine, prochlorperazine, and bacitracin (as negative control) (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 is found in Supplementary File S1.

The western blot analysis of E-cadherin shown a significant increase of the protein amount by 45.3 and 32.8 % after treating U87-MG cells with perphenazine in the concentration 0.25 and 0.5 μM, respectively (Fig. 5B). Prochlorperazine in the concentration of 0.25 μM also significantly increase 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 shown a significant decrease of 54.3 and 65.7 % after incubation of U87-MG cells with perphenazine in the concentration 0.5 and 1.0 μM, respectively (Fig. 5B). In case of prochlorperazine, the significant increase of α-tubulin by 27.5 % was observed after incubation of U87-MG cells with perphenazine in the concentration 0.25, while in the concentration of 1.0 μM significant decrease by 60.2 % was observed (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) shown a significant decrease of α3 integrin by 34.2 and 27.1 % after incubation of U87-MG cells with perphenazine in the concentration 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. Analysis of α5 integrin shown that perphenazine did not significantly decrease the level of α5 integrin (Fig. 5B). In case of prochlorperazine, only in the concertation of 1.0 μM significant decrease of α3 and β1 by 53.1 and 38.1 % was noticed, respectively. Analysis of α5 integrin shown that prochlorperazine also did not significantly decrease the level of α5 integrin (Fig. 5C). Moreover, bacitracin also did not significantly impair the level of all the analyzed integrins (Fig. 5D).

Discussion

Our previous study has shown that perphenazine and prochlorperazine induce a concentration-dependent loss in human glioblastoma U87-MG viability; additionally, the obtained EC_{50} values for the drugs (0.98 and 0.97 μM, respectively) (Otręba and Buszman 2018) correlate with the concentrations detected in human plasma. 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 (Otręba and Buszman 2018). In the present study, we tested the impact of perphenazine and prochlorperazine in the U87-MG cell line on a level of ABC transporters, trying to explain the differences observed in the cellular viability. Obtained results demonstrating that the analyzed phenothiazine derivatives can reduce ABCB1 and increase ABCG2 content in glioblastoma cells. Since ABCB1 also regulates cell proliferation, and the knockdown of ABCB1 suppresses cell proliferation (Muriithi et al. 2020), it can explain the decrease of cellular viability after perphenazine and prochlorperazine treatment because in our study we observed a decrease of ABCB1 amount. It was found also that prochlorperazine significantly reduces the rate of cell migration.

ABCB1 and ABCG2 transporters are localized in the brain endothelial capillaries (Balça-Silva et al. 2018) and are related to multidrug resistance (Liu et al. 2016). In certain glioblastomas, high expression of ABCB1 and ABCG2 has been reported to associate with poor prognosis. It is believed that the transporters that limit drug accumulation are upregulated in the tumor cells thereby limiting drug uptake (Liu et al. 2016). Interestingly, the resistance to TMZ, which is the drug of choice in treating glioblastoma, may be related to the excretion of the drug from the brain by these transporters (Wijaya et al. 2017). It was confirmed by a study, which showed the increase TMZ accumulation in the tumors of mouse GBM model during elacridar treatment (non-selective inhibitor of ABCB1 (Chen et al. 2017) as well as in mice that were deficient for ABCB1 and ABCG2 genes (Lin et al. 2014). The ABCG2 transporter protects tissues against deadly xenobiotic exposures by the contribution of the absorption, distribution, and elimination of the drugs and endogenous compounds (Gupta et al. 2020). Moreover, the ABCG2 expression enhances stress-induced autophagy and cell survival in multiple tumor cell types (Wesołowska 2011).

Antipsychotic phenothiazines such as chlorpromazine (Abdallah et al. 2015, Wesołowska 2011), prochlorperazine (Wesołowska 2011), thioridazine, and fluphenazine (Abdallah et al. 2015) impair drug efflux mediated by ABCB1 demonstrating an inhibitor activity. Noteworthy, verapamil and thioridazine not only block ABCG2 or ABCB1 (P-gp) but also can reverse the resistance of doxorubicin-resistant sarcoma 180 cells completely. Spengler et al. (2016) suggest that thioridazine, in concentrations greater than that

clinically employed for the therapy of severe psychosis, induces apoptosis of the MDR lymphoma cells and inhibits the activity of their over-expressed ABCB1 transporter. Wang et al. (2008) showed that antipsychotic drugs (e.g. chlorpromazine) exhibited moderate to mild inhibitory effects on BCRP (ABCG2) activity and its transport efficiency in breast cancer MCF7 and MCF7/MX100 cells. The authors based on their previous findings suggest that several antipsychotics (chlorpromazine, risperidone, quetiapine, paliperidone, and clozapine) are dual inhibitors of both P-gp and BCRP. Interestingly, Riganti et al. (2014) demonstrated that TMZ dose-dependent decreases ABCB1 level, without affecting ABCG2 in the immortalized primary human brain microvascular endothelial cell line (hCMEC/D3). It confirms our findings in the ABCB1 amount observed in the concentration of 0.1 μM of perphenazine and prochlorperazine. Moreover, in case of both drugs significant increase of the P-gp amount was dose-dependent. Interestingly the observed decrease of ABCB1 amount was not caused by cell death or proliferation disturbances, since previous results of the WST-1 assay showed that perphenazine in the concentration of 0.1 μM does not affect U-87MG cells viability (Otręba and Buszman 2018).

Pan et al. (2017) measured protein level and gene expression of ABCG2 in four different human malignant glioma cell lines (A172, U87, SHG-44, and U251). Western blot analysis showed that U87 has the lowest ABCG2 amount among all the cell lines, whereas no significant differences are found in the mRNA expression levels of MRP1 and MDR1 in the four GBM cells.

We also evaluated the effect of perphenazine and prochlorperazine on wound closure, transwell migration, and transwell migration of human glioblastoma cell line 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 for control and perphenazine ($p < 0.01$) as well as between t_0 and 6, 9, 12 as well as 24 hours for control, perphenazine, and prochlorperazine ($p < 0.01$). Moreover, stronger stimulation of U87-MG migration after perphenazine (1.0 μM) treatment was observed after 6-hour treatment in comparison to prochlorperazine. The analysis of the rate of cell migration after 24 h incubation showed the tendency that the U-87MG cells in perphenazine or prochlorperazine migrate slower in comparison to control. Interestingly, only in case of prochlorperazine, the difference is statistically significant ($p < 0.01$) and suggests that the cells migrate 1.3 times slower in prochlorperazine (1,0 μM) in comparison to control.

Moreover, we analyzed the transwell migration of U87-MG cells using the drug concentration causing only about a 30% decrease in cell viability. The study showed that both of the analyzed drugs can decrease migration and invasion of the cells. Noteworthy, the analysis of internal control shown 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, respectively than the growth medium with 10% FBS. It may also explain that less 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 cell migration/invasion from the growth medium with 1% FBS to the growth medium with 10% FBS.

The observed strong effect of perphenazine and prochlorperazine on viability and migration of human glioblastoma may be related to ABCB1 and/or ABCG2 amounts, since the proteins may affect cell migration. P-Glycoprotein contains a Pim-1 phosphorylation consensus sequence, which mediates the phosphorylation of Ser683 in P-glycoprotein, protects ABCB1 from degradation, and allows its glycosylation and cell-surface expression. Overexpression of the serine/threonine protein kinase Pim-1 is often observed in human malignancy tumors (acute myeloid leukemia, acute lymphoblastic leukemia, prostate cancer, and gastric cancer (Katayama et al. 2014), breast cancer (Brasó-Maristany et al. 2016), glioblastoma multiforme (Herzog et al. 2015). Noteworthy, Pim-1 promotes tumor cell growth by promoting cell cycle progression, cell migration, and protein translation and by the suppression of apoptosis (Brasó-Maristany et al. 2016). Moreover, Liang et al. (2015) showed that nuclear ABCG2 in lung cancer is involved in a transcription regulation of the E-cadherin–encoding gene (CDH1). CDH1 is a key cell-cell adhesion gene. Thus, ABCG2 could affect cell migration, since the ABCG2 overexpression enhances E-cadherin expression as well as increased nuclear ABCG2 expression (Liang et al. 2015). Especially if E-cadherin prevents loss of cell-cell adhesion and cell junctions, which allow cells to invasion and migration (Na et al. 2020). Nucleus localization of ABCG2 was also confirmed in glioblastoma multiforme (Bhatia et al. 2012) as well as relative expression of E-cadherin by Western blot was shown in U87-MG cells (Zhang et al. 2015). Thus, ABCG2 may also affect migration in U87-MG. Our studies have shown a significant increase of ABCG2 after prochlorperazine treatment (0.1 and 1.0 μM) of U-87 MG cells as well as E-cadherin after perphenazine (0.25 and 0.5 μM) and prochlorperazine treatment (0.25 μM) what is in line with the above suggestions.

Interestingly, the alteration of alpha-tubulin acetylation “may be involved in cancer cell migration, invasion, and cancer metastasis” (Lee et al. 2015). It is possible since the acetylation may promote cellular migration by the increase in the vicinity of focal adhesions (Bance et al. 2019).

The α3 integrin is mainly localized in invading cells and cells surrounding vessels of glioblastoma. Moreover, the overexpression of α3 leads to the increase of glioma migration and/or invasion, while “downregulation of α3 integrin inhibits glioma invasion concomitant with a change in the phosphorylation level of the extracellular signal-regulated kinase (ERK) 1/2 pathway”. Thus, α3 integrin plays important role in the cellular invasion by ERK 1/2 activation (Nakada et al. 2013). In case of α5 integrin, it plays important role in cellular migration, invasion, and adhesion of many tumors including glioblastoma. The α5β1 integrin is also called the critical regulator of cell migration and invasion since it can affect cytoskeleton rearrangement, cell adhesion, and the production of matrix metalloproteinase (MMP). The integrin may promote cancer cell invasion and metastasis 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). Nakada et al. 2013 showed that overexpression of the α3 integrin in glioblastoma cells (U87-MG, SNB19, and U251) increases cell migration. Noteworthy, in U87-MG cells the authors observed the highest expression of α3 integrin (1.3 fold) and the lowest invasion of glioblastoma cells (1.5 fold). The analysis of invasion showed that the invasion of U87-MG cells was stronger in α3 integrin overexpressing cells, which suggests that α3 integrin may be an invasion promotor. 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”. Those observations are in line with our results since we observed a decrease of migration and invasiveness of U87-MG cells after treatment with perphenazine or prochlorperazine on the concentration 0.5 μM. In the same concentration, we did not notice the increased level of, which may explain a decrease in cellular migration and invasion.

Furthermore, α5β1 integrin also precipitates in the aggressiveness of solid tumors, the high expression of the protein may decrease patient survival, which makes it an important factor in therapy resistance (Renner et al. 2016). Thus, the noticed in our study trend to decrease of α5 and β1 integrins may be a good prognosis in glioblastoma therapy.

Finally, Li et al (2016) showed that bacitracin can be used as an anti-invasive agent, which is in line with our results.

In the future, we plan to use PCR assay to confirm variations of the proteins as well as use more human glioblastoma cell lines to get more generalized conclusions about the possibility of use phenothiazine derivatives in glioblastoma treatment.

Conclusion

The significant reduction of cellular migration activity caused may be explained by the decrease of the ABCB1 level and increase of ABCG2 level in U-87MG cells treated with perphenazine or prochlorperazine. Data presented here and the obtained previously EC_{50} value related to the prochlorperazine concentrations detected in human plasma indicate that prochlorperazine possesses better anticancer activity *in vitro* than perphenazine, especially if only prochlorperazine significantly reduces the rate of cell migration. The results obtained showed the decrease of multidrug resistance proteins (ABCB1 and ABCG2) amount and modulate E-cadherin, α-tubulin, and integrins (α3, α5, and β1) levels as well as impair of migration and invasion of U87-MG cells. Since phenothiazine derivatives decrease viability, migration, and invasion of U87-MG glioblastoma next studies determining the type of cell death should be performed near future. Noteworthy, prochlorperazine possess also antiemetic activity. Thus, it is considered that the drug possesses a better potential for the development of new and effective glioblastoma therapy.

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

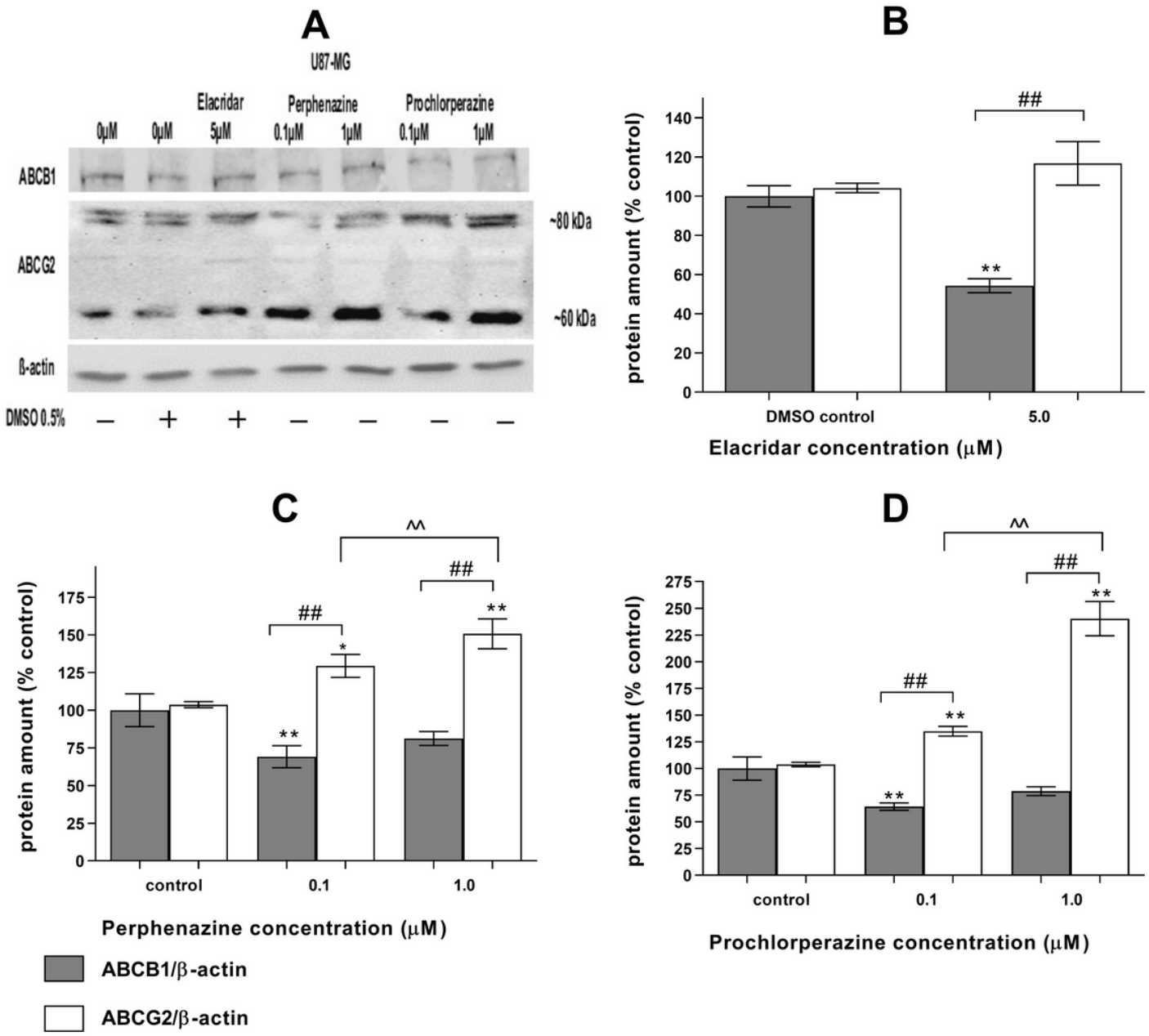

Figure 1

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 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 molecular mass marker are shown in Supplementary Figure S1. The raw Western blot data represented in this figure is found in Supplementary File S1.

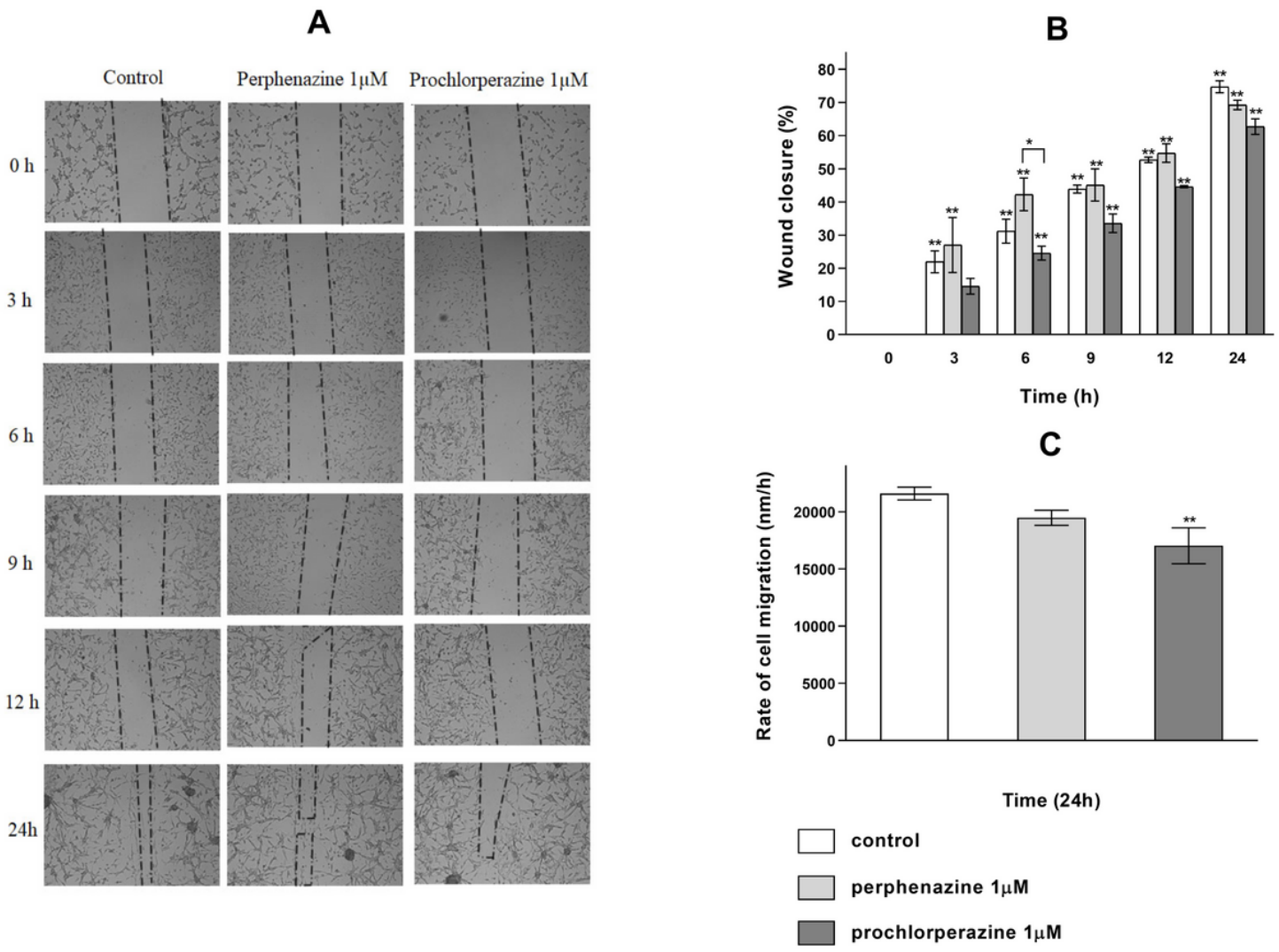

Figure 2

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 post-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) 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 in the same time point. Rate of cell migration of glioblastoma cells after 24h incubation (C) 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 control.

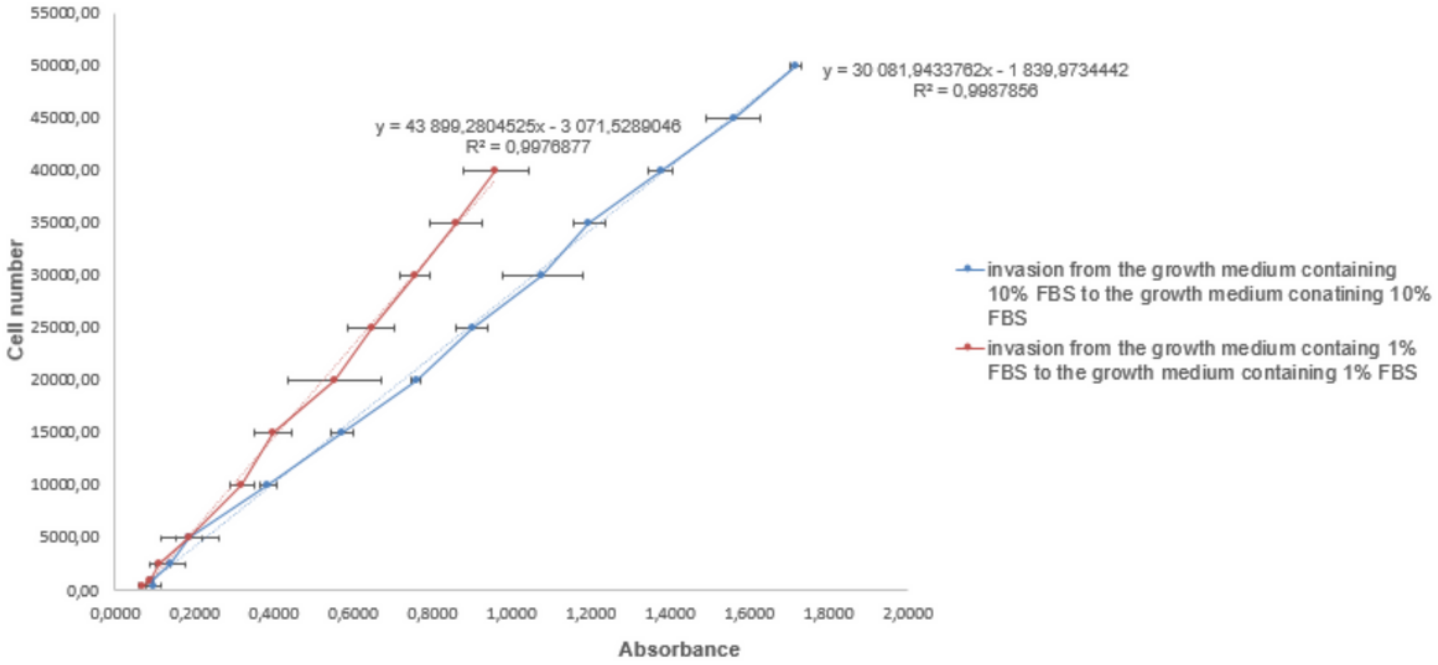

Figure 3

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.

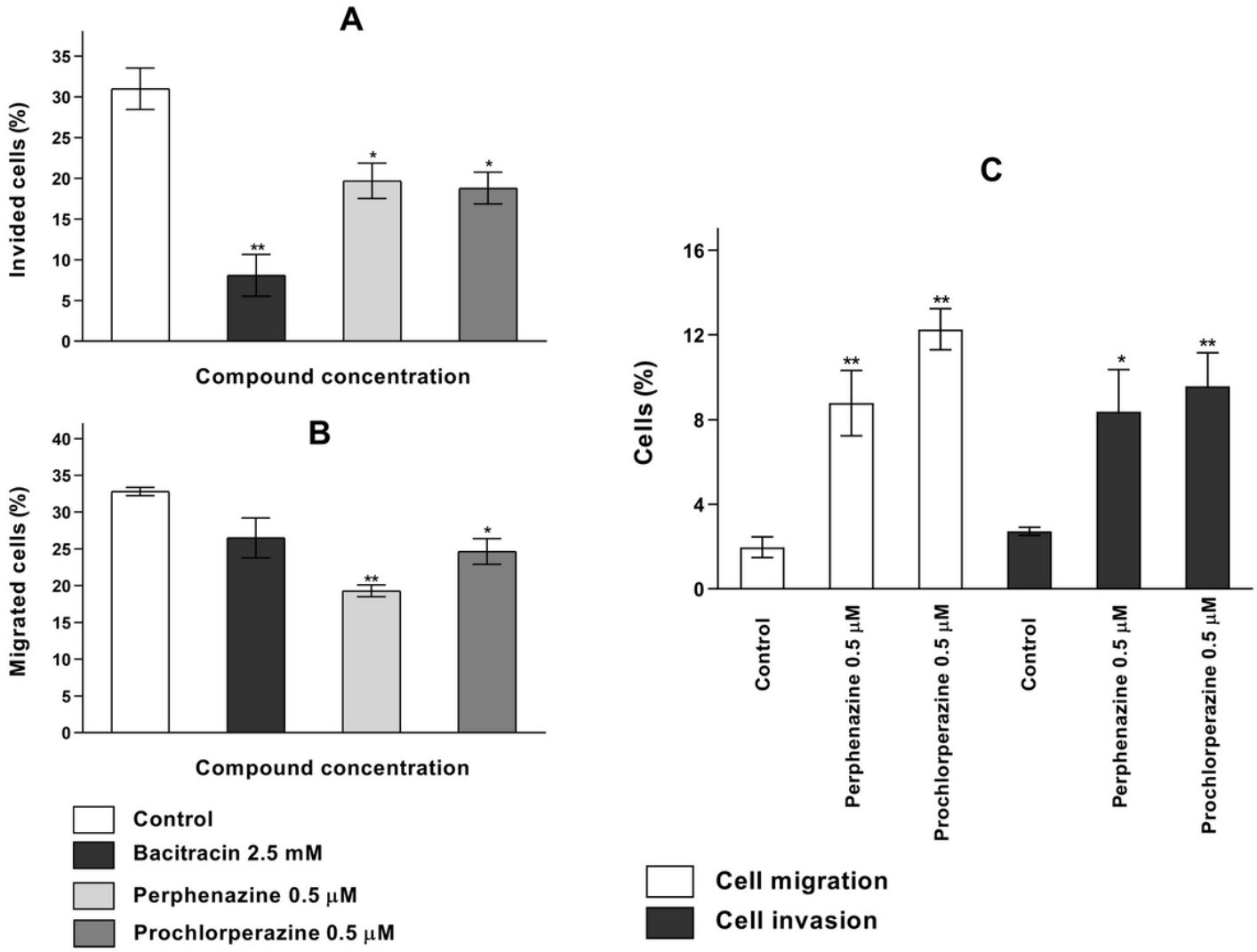

Figure 4

The impact of perphenazine and prochlorperazine on glioblastoma transwell migration and invasion. Transwell invasion assay on U87-MG cells (A) expressed as a percentage of invaded cells. Transwell migration assay of U87-MG cells (B) expressed as a percentage of migrated cells. Internal control of migrated and invaded cells (C) 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 control.

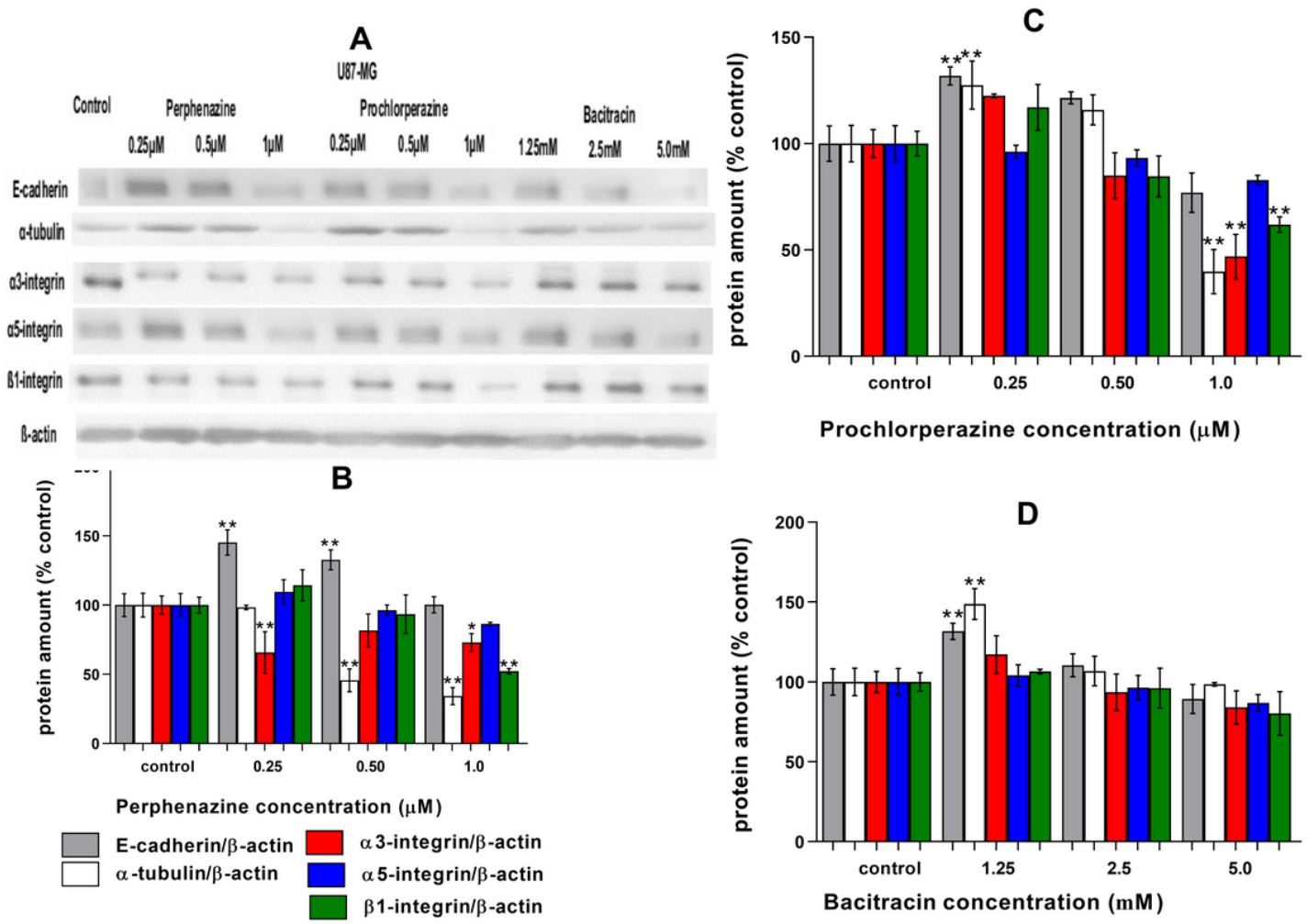

Figure 5

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, expressed as % of 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 molecular mass marker are shown in Supplementary Figure S2. The raw Western blot raw data represented in this figure is found in Supplementary File S1.

Supplementary Files

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- SupplementaryFileS4woundhealingtranswellassay.pzf
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