Perphenazine and prochlorperazine decrease glioblastoma U-87 MG cell 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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Abstract. Glioblastoma multiforme is the most frequent type of malignant brain tumor, and is one of the most lethal and untreatable human tumors with a very poor survival rate. Therefore, novel and effective strategies of treatment are required. Integrins play a crucial role in the regulation of cellular adhesion and invasion. Integrins and α-tubulin are very important in cell migration, whereas E-cadherin plays a main role in tumor metastasis. Notably, drugs serve a crucial role in glioblastoma treatment; however, they have to penetrate the blood-brain barrier (BBB) to be effective. ABC transporters, including ATP binding cassette subfamily B member 1 (ABCB1) and ATP binding cassette subfamily G member 2 (ABCG2), are localized in the brain endothelial capillaries of the BBB, have a crucial role in the development of multidrug resistance and are modulated by phenothiazine derivatives. The impact of perphenazine and prochlorperazine on the motility of human Uppsala 87 malignant glioma (U87-MG) cells was evaluated using a wound-healing assay, cellular migration and invasion were assessed by Transwell assay, and the protein expression levels of ABCB1, ABCG2, E-cadherin, α-tubulin and integrins were determined by western blotting. The present study explored the effects of perphenazine and prochlorperazine on the levels of ABCB1, ABCG2, E-cadherin, α-tubulin and integrins (α3, α5, and β1), as well as on the migratory and invasive ability of U87-MG cells. The results suggested that perphenazine and prochlorperazine may modulate the expression levels of multidrug resistance proteins (they decreased ABCB1 and increased ABCG2 expression), E-cadherin, α-tubulin and integrins, and could impair the migration and invasion of U-87 MG cells. In conclusion, the decrease in migratory and invasive ability following treatment with phenothiazine derivatives due to the increase in ABCG2 and E-cadherin expression, and decrease in α-tubulin and integrins expression, may suggest that research on perphenazine and prochlorperazine in the treatment of glioblastoma is worth continuing.

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Introduction

Glioblastoma multiforme (GBM) is the most frequent malignant brain tumor leading to 225,000 deaths per year (according to the data from 2018), which translates into 30% of all central nervous system tumors (CNST), 45% of malignant CNST as well as 80% of primary malignant CNST (1). 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 (2). 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 (1).

During glioblastoma therapy one of the goals is to alter epidermal growth factor receptor (EGFR)/phosphoinositide 3-kinase (PI3K)/phosphatase and tensin homolog deleted on chromosome ten (PTEN)/neurofibromatosis type 1 (NF1)/rat sarcoma oncogene (RAS), tumor protein p53 (TP53)/mouse double minute 2 homolog (MDM2)/mouse double minute 4 homolog (MDM4)/alternate open reading frame encoding protein p14 (p14ARF), retinoblastoma protein 1 (RB1)/cyclin-dependent kinase 4 (CDK4)/cyclin-dependent kinase inhibitor 4A (p16INK4A)/cyclin-dependent kinase inhibitor 2B (CDKN2B), and isocitrate dehydrogenase 1 (IDH1)/isocitrate dehydrogenase 2 (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 (3-5).

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 (5,6). Moreover, integrins have a role in metastasis and angiogenesis of various tumors, which makes the integrin inhibitors potentially useful in glioblastoma therapy (5). 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 (6). Furthermore, α3β1, α5β1, α9β1, and β8 integrins affect migration and/or invasion of glioblastoma cells (7,8). Alpha-tubulin, by controlling dynamics of focal adhesion for lamellipodial extension after the tubulin acetylation, also influences cellular migration (9). E-cadherin epithelial cell adhesion protein has the main role in tumor metastasis (10) 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 (BBB) (3). Drugs used in the treatment of newly diagnosed or recurrent glioblastoma should penetrate the BBB or exhibit intracerebral activity (11). Perphenazine and prochlorperazine used in this study penetrate BBB (12), and they have different biological activities such as sedative, antiemetic (13,14), anticancer activities (15). Therefore, we decided to continue the previous investigation of an anticancer activity of perphenazine and prochlorperazine against U-87 MG cells in the present study. 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) and ATP-binding cassette subfamily G member 2 (ABCG2), was analyzed. ABCB1 is also called glycoprotein P (P-gp) or multi-drug resistance 1 (MDR1), while ABCG2 is also referred to as a breast cancer resistance protein (BCRP). Those transporters are responsible for moving biologically important substrates (amino acids, cholesterol) across the cell membranes, and for impeding the penetration of the BBB by many chemotherapeutic agents, actively transporting them back into the bloodstream (16).

Materials and methods

Cell culture and reagents. The human glioblastoma cells U-87 MG were obtained from the Sigma Aldrich (USA)-European Collection of Authenticated Cell Cultures (ECACC) 89081402. The U-87 MG cell line was authenticated by ECACC by STR profiling with the use of PowerPlex 16 HS PCR amplification kit. Glioblastoma cells were cultured in Dulbecco's modified Eagle medium (DMEM), constituting a basal medium, which was supplemented with fetal bovine serum (FBS) (10%), neomycin (10 μg/ml), amphotericin B (0.25 μg/ml), and penicillin G (100 U/ml) at 37˚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, FBS EU professional heat-inactivated and growth medium DMEM with 4.5 g/l Glucose, L-glutamine, and 3.7 g/l $NaHCO_3$ 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 (17). 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). In short, $1x10^6$ cells were seeded on tissue culture dish of 35 mm in diameter (Sarstedt, Germany) and incubated to about 80-90% confluence. Then the 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 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 h at room temperature in a blocking buffer.

Proteins were detected by incubation with primary antibodies: MDR1/ABCB1 (E1Y7B) Rabbit monoclonal antibody (mAb), ABCG2 rabbit Ab, and β-actin rabbit Ab (Cell Signaling Technology, USA) at 1:1,000 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 1:2,500 (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 ABCG2 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. In case of ABCG2, protein densitometry of two bands was used to calculate a relative amount of the protein.

Wound healing assay. The assay was performed according to the method described previously Otręba *et al* (2019) with a slight modification (17). In brief, some $1x10^6$ U-87 MG were incubated with supplemented growth medium for 24 h to approximately 80-90% confluence (18-20) on a 35-mm 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 growth medium containing 10% FBS (21), perphenazine, or prochlorperazine solutions were added. The wound area was

photographed after 0, 3, 6, 9, 12, and 24 h after scratching with the use an inverted microscope Nikon TS100F (Nikon Corporation, Japan) equipped with a Canon EOS 450D digital camera (Canon Inc, Japan). At each time point, three photos of each dish were taken and dishes were immediately placed in the incubator. 10% FBS was used during the wound healing assay because cell viability would be affected by serum starvation-the analyzed drugs used in concentration of 1.0 μM, causing about 50% decrease of viability. 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) (22). The wound closure was calculated using the following formula (23,24):

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

$A_{t=0h}$ 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 24 h was calculated using the formula (24):

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

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 (25,26). 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 min at 37˚C before the use. Then 25,000 U-87 MG cells were seeded into inserts for 48 h 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 U-87 MG cell migration and invasion (27). 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 48 h 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 min. After fixation, the inserts were washed in PBS and incubated with methanol for 20 min. The cells were next washed in PBS and stained with 0.1% crystal violet for 10 min. Then, 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 min 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=590 nm 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, 1,000, 2,500, 5,000, 1,000, 15,000, 20,000, 25,000, 30,000, 35,000, 40,000, 45,000, and 50,000 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 from Fig. S1. 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 western blot analysis of ABCB1 and ABCG2 section with a slight modification. The positive control of E-cadherin, α-tubulin levels was bacitracin (1.25, 2.5, and 5.0 mM 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 1:1,000 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. The R^2 values were determined using the Excel 2013 RSQ function (Microsoft Corporation); the RSQ function returns the square of the Pearson product-moment correlation coefficient through data points in known y's and x's. 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 24 h-treatment of glioblastoma cells under different concentrations of perphenazine, prochlorperazine, and elacridar (as a negative control) (Fig. 1A). The full-length immunoblots with a molecular mass marker are shown in Fig. 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 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 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 Fig. 2A-C.

Fig. 2A shows original photos of wound healing after a given period of time, i.e. 0, 3, 6, 9, 12, and 24 h. In all the cases the calculations showed an increase in wound closure (Fig. 2B). After 6, 9, 12 and 24 h of treatment with 1.0 μM prochlorperazine, the wound closure in human glioblastoma cell cultures increased from 24.6 to 62.7% in comparison to t_0. For the control and perphenazine (1.0 μM), after 3 to 24 h of 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). The significant difference between the effect of perphenazine and prochlorperazine on wound closure was observed only after 6 h. The analysis of the rate of cell migration showed a significant difference between the control and prochlorperazine after 24 h-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. As long as invasion from the growth medium with 1% FBS to the growth medium with 1% FBS, in 45,000 cells and 50,000 cells samples is concerned, we observed a high decrease in the cell amount, thus we finished constructing the standard curve at 40,000 cells when it was still linear. The standard curves are presented in supplementary Fig. S1.

The Transwell invasion assay showed a significant decrease in invasion by 22.91, 11.31, and 12.19% for bacitracin (2.5 mM), perphenazine (0.5 μM), and prochlorperazine (0.5 μM) in comparison to the control, respectively (Fig. 3A). 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. 3C). 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. 3B). The observed decrease of U-87 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. 3C).

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 24 h-treatment of glioblastoma cells with different concentrations of perphenazine, prochlorperazine, and bacitracin (as the negative control) are presented in Fig. 4A. The full-length immunoblots with a molecular mass marker are shown in Fig. S3.

The western blot analysis of E-cadherin showed a significant increase of the protein amount by 45.3 and 32.8% after treating U-87 MG cells with perphenazine in the concentration of 0.25 and 0.5 μM, respectively (Fig. 4B). 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. 4C). 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. 4D).

The analysis of α-tubulin showed a significant decrease of 54.3 and 65.7% in U-87 MG cells with perphenazine in the concentration of 0.5 and 1.0 μM, respectively (Fig. 4B). 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. 4C). 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. 4D).

The analysis of integrins (α3, α5, and β1) showed a significant decrease of α3 integrin by 34.2 and 27.1% after incubation of U-87 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 U-87 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. 4B). 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

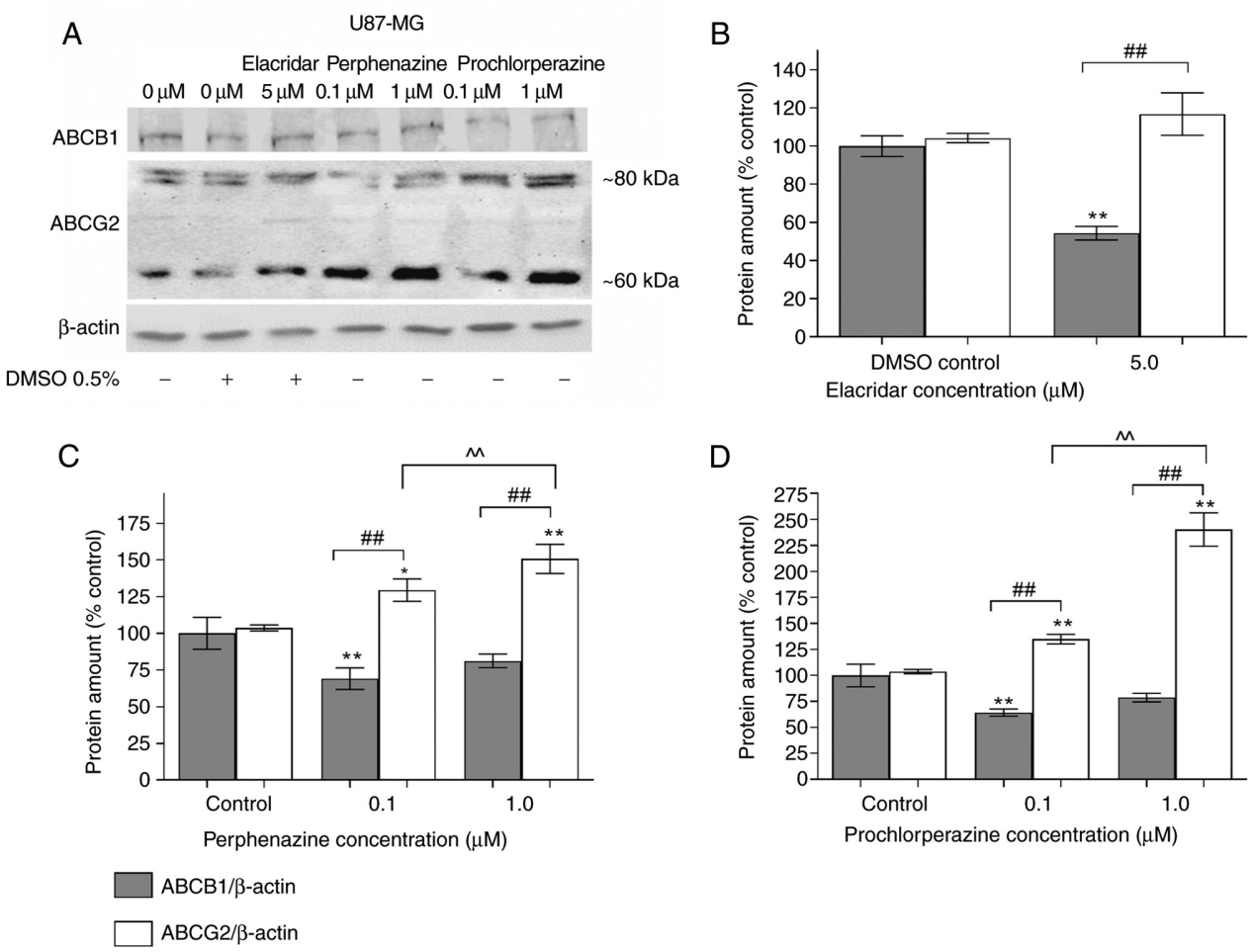

Figure 1. Western blot analysis and graph of the relative amounts of selected proteins, including loading controls in U-87 MG cells. (A) Representative blots of ABCB1, ABCG2 and β-actin, as well as ABCB1 and ABCG2 relative amounts after (B) elacridar treatment, (C) 24-h perphenazine treatment and (D) 24-h 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. control; $^{\#\#}$P<0.01; $^{\wedge\wedge}$P<0.01.

integrin showed that prochlorperazine also did not significantly decrease the level of α5 integrin (Fig. 4C). Moreover, bacitracin also did not significantly influence the level of all analyzed integrins (Fig. 4D).

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), and the ability of drugs to penetrate the blood-brain-barrier. 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 (28). 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 U-87 MG cells.

Previously, Otręba and Buszman (2018) showed that perphenazine and prochlorperazine in the concentration of 0.5 and 1.0 μM reduced U-87 MG cells viability by 32 and 54.5% as well as 30.5 and 56.3%, respectively after 24 h-incubation (29). In the present study, we observed a decrease in ABCB1 amount after 24 h-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 (30). 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 our previous results of the WST-1 assay (29) showed that perphenazine in the concentration of 0.1 μM did not affect U-87 MG cells viability.

The main role of ABCB1 (P-gp) and ABCG2 (BCRP) transporters, localized in the brain endothelial capillaries (16), is related to multidrug resistance (31). The ABCG2 transporter protects tissues against deadly xenobiotic exposures by the contribution to the absorption, distribution, and elimination of the drugs and endogenous compounds (32). Thus, high expression of ABCB1 and ABCG2 has been reported to be related with poor prognosis in certain glioblastomas (31).

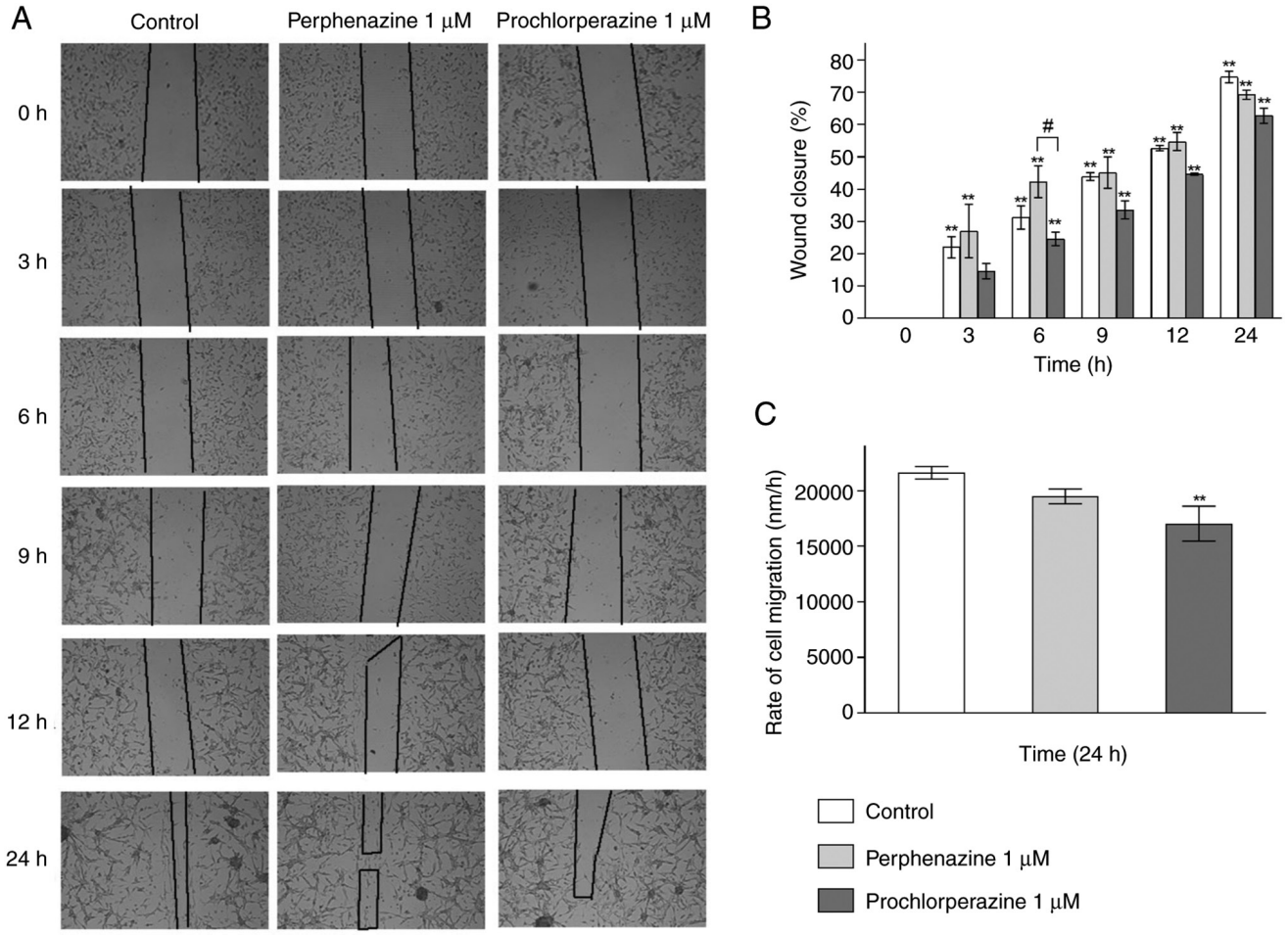

Figure 2. Impact of perphenazine and prochlorperazine on glioblastoma cell migration. *In vitro*, wound-healing assay U-87 MG cells (A) were incubated for 24 h with perphenazine or prochlorperazine in the concentration of 1 μM. Cells were photographed after 0, 3, 6, 9, 12, and 24 h after scratching by Nikon TF100 inverted microscope x4 magnification. Representative cell images from each group at the indicated time points are shown. (B) Wound closure of U-87 MG cells is expressed as a percentage of time t_0. Mean values ± SEM from three independent experiments (n=3) performed in triplicate are presented. **P<0.01 vs. the t0 group. $^{\#}$P<0.05. (C) The rate of cell migration of glioblastoma cells after 24 h incubation is expressed as nm per hour. Mean values ± SEM from three independent experiments (n=3) performed in triplicate are presented. **P<0.01 vs. control.

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 (33,34). Additionally, in 2017, Pan *et al* measured ABCG2 protein level and gene expression in four different human malignant glioma cell lines (A172, U-87, SHG-44, and U-251). 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 (35). Interestingly, phenothiazine derivatives such as chlorpromazine (36-38), prochlorperazine (38), thioridazine (39), and fluphenazine (36) impair drug efflux mediated by P-gp or BCRP. Inhibition properties of those drugs in the mentioned mechanism has their own significance due to the possibility of using phenothiazine derivatives in glioblastoma treatment. Moreover, elacridar inhibits ABC transport activity, but it can also downregulate the expression of P-gp and BCRP. Abdallah *et al* (2021) observed that elacridar (5 μM) significantly downregulated the expression of P-gp by 40% and BCRP by 53% (Fig. 4), and suggested NF-κB pathway as a potential mechanism for BBB disruption. Data from the *in vivo* studies showed downregulation of P-gp and BCRP, and upregulation of the receptor for advanced glycation end products (RAGE), which accompanied activation of NF-κB pathway in mouse brains (40). Our study has confirmed that elacridar impairs P-gp and BCRP levels. We also observed two bands of ABCG2 protein. It may be assumed that two bands could be visible on western blot of ABCG2 due to different glycosylated forms of the protein. Diop and Hrycyna (2005) found that replacing asparagine with glycine in three possible N-linked glycosylation sites of ABCG2 (418, 557, and 596) changed molecular mass. ABCG2 (N418Q) and ABCG2 (N557Q) migrated as a range of bands between the 50 and 75 kDa, while ABCG2 (N596Q) migrated as a single species at about 60 kDa. On the other hand, the authors noticed that the presence of two bands might be caused by the time of incubation. The half-lives of each of the ABCG2 proteins are similar in analyzed cells (about 4 to 5 h). Interestingly, the incubation time up to 3 h resulted in 1 visible band of ABCG2, while 9 to 20 h of incubation resulted in 2 visible bands confirming different varieties of ABCG2 (41). In our study cells were incubated for 24 h before the western blot analysis, consequently 2 bands 60 and 80 kDa could be

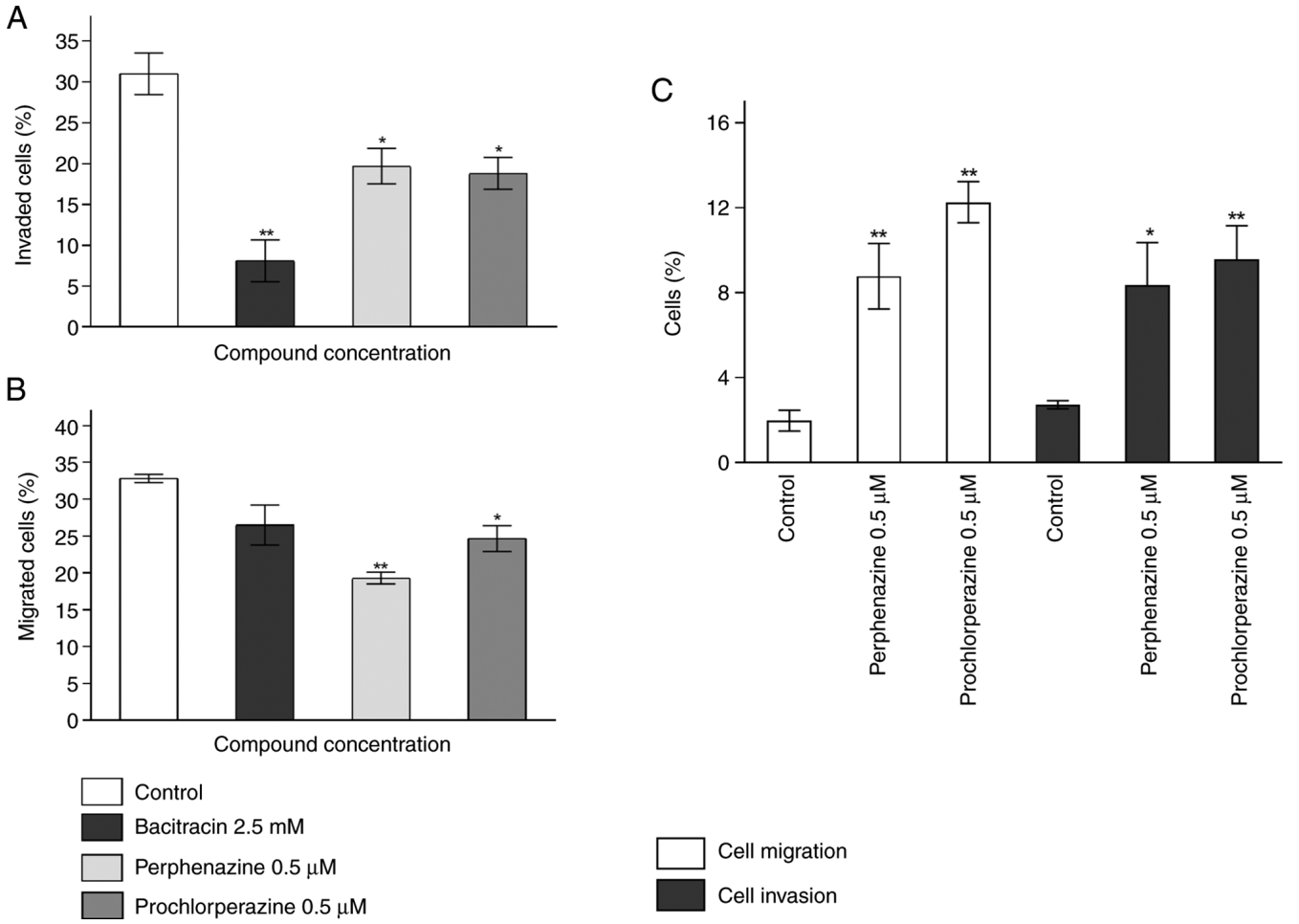

Figure 3. The impact of perphenazine and prochlorperazine on glioblastoma migration and invasion determined with the Transwell assay. (A) Transwell invasion assay of U-87 MG cells is expressed as a percentage of invaded cells. (B) Transwell migration assay of U-87 MG cells is expressed as a percentage of migrated cells. (C) The internal control of cell migration and invasion from a starvation medium to medium containing 1% FBS and perphenazine or prochlorperazine, as verification to determine if the analyzed drugs were chemoattractants or not. The control 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. control.

visible. 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 GBM tumors are less sensitive to apoptosis, leading in consequence to the frequent recurrences (42). 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 h only for the control and perphenazine (P<0.01), while statistically significant differences were recorded between t_0 and 6, 9, 12 and 24 h 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 h-treatment in comparison to the use of prochlorperazine. The analysis of the rate of cell migration after 24 h-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. The observed difference between perphenazine and prochlorperazine concerning the rate of cell migration may be caused by differences in their chemical structure (Fig. 5) and interaction with dopamine receptors. According to the International Union of Basic and Clinical Pharmacology (IUPHAR) the main target of perphenazine is DRD2, while DRD2 and DRD3 are the targets of prochlorperazine (43). Perphenazine has a 3-[4-(2-hydroxyethyl)piperazin-1-yl]propyl group at N-10 which interacts mainly with D2 receptor. On the other hand, prochlorperazine has a 3-(4-methylpiperazin-1-yl) propyl group at the N-10 position and interacts mainly with D2 and D3. Thus, prochlorperazine may stimulate more strongly the rate of migration than perphenazine since the migration of glioblastoma cells depends on D2 and D3 receptors.

Since regulation of invasion is an important objective in glioblastoma treatment, by employing the Transwell assay we analyzed migration and invasion of U-87 MG cells using the drug concentration (0.5 μM) which caused about a 30% decrease of cell viability according to our previous study results (29). We decided to use such a concentration to minimalize the cytotoxicity effect, since we used growth medium with 1% of FBS. In this case using the concentration of 1.0 μM, causing about 50% decrease of viability (29), would be very risky because it could

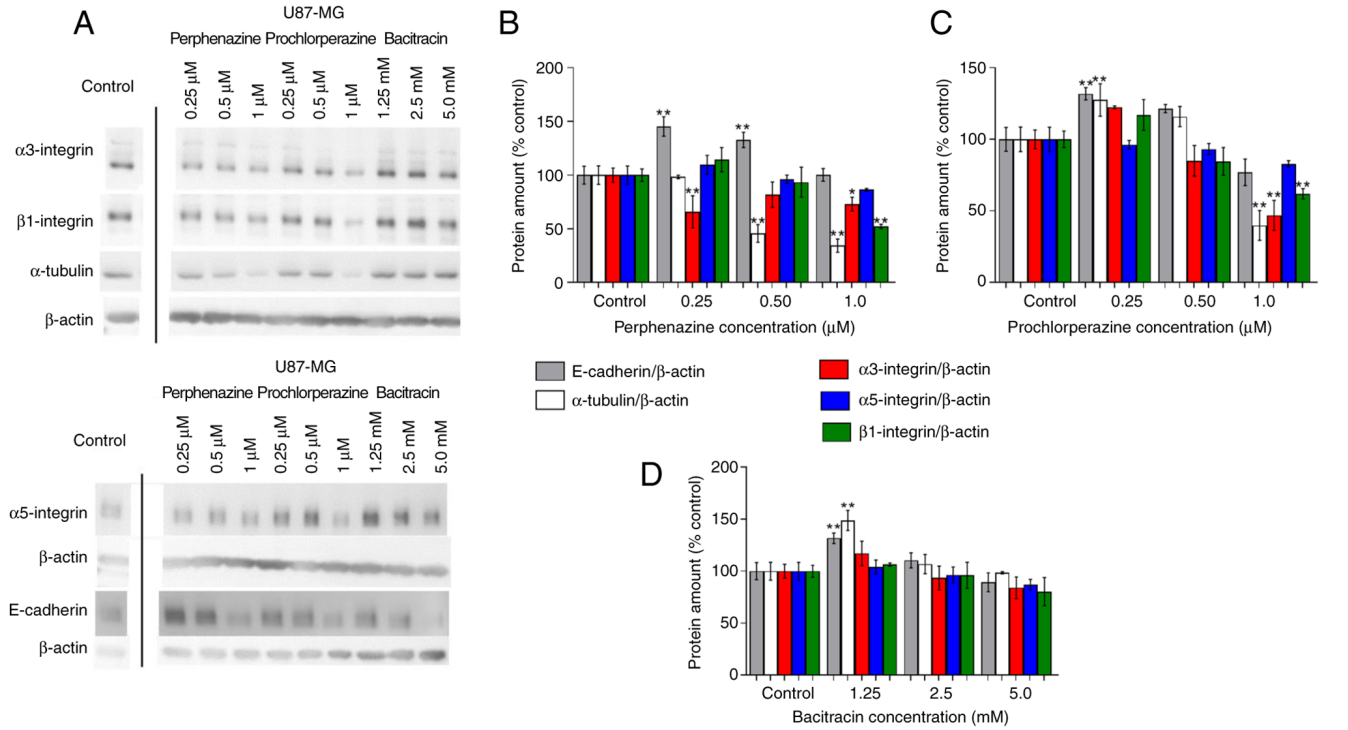

Figure 4. Western blot analysis and a graph of the relative amounts of selected proteins, including loading controls in U-87 MG cells. (A) Representative blots of E-cadherin, α-tubulin, integrins (α3, α5, and β1), and β-actin. E-cadherin, α-tubulin, integrins (α3, α5, and β1) relative amounts after (B) 24 h perphenazine treatment, (C) 24 h prochlorperazine treatment, (D) 24 h bacitracin treatment, expressed as % of the control. Lanes were not continuous on the gel. Mean values ± SD from three independent experiments (n=3) are presented. *P<0.05, **P<0.01 vs. the control samples.

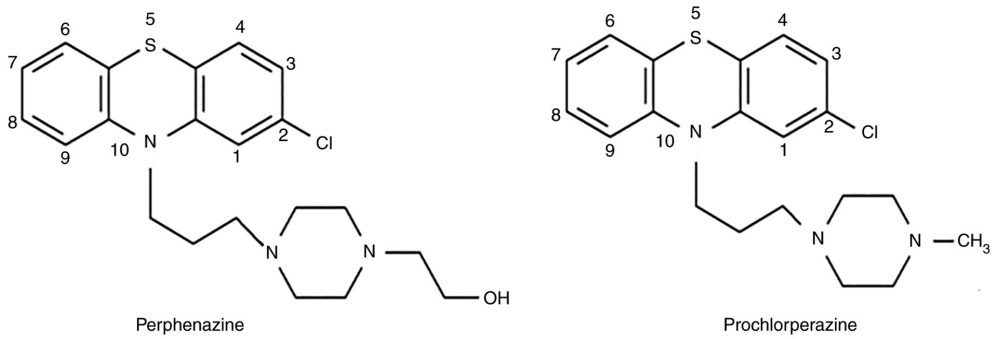

Figure 5. The structure of perphenazine and prochlorperazine (29).

result in death of majority or all analyzed cells. Using of 0.5 μM concentration is a potential limitation of the study since the results of migration/invasion could have been affected by the effect of the drugs on cell viability. The present 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 (4), while in 2020 the same authors showed that perphenazine reduced migration of malignant or non-malignant SVZ cells to glioblastoma (44). Thus, the ability of phenothiazines (perphenazine and prochlorperazine) to decrease migration and invasion of U-87 MG cells may be related to dopamine receptors activity, which was confirmed, by Aaberg-Jessen (2013) (45), Bartek and Hodny (2014) (12), Caragher *et al* (2019) (46), Weissenrieder *et al* (2020) (47), Bhat *et al* (2020) (48), and Agrawal *et al* (2021) (49). Bartek and Hodny (2014) described in detail the anti-glioblastoma activity mechanism of the dopamine receptor subtype 2 (DRD2) antagonists in combination with epidermal growth

factor receptor (EGFR) inhibitors, which impair cellular growth and survival by mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling cascade (12). In 2013 Aaberg-Jessen *et al* observed that primary glioblastoma spheroids limited glioma invasion (45). 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. The authors noticed also that anti-glioma chemotherapy may increase DRD2 protein expression, leading to a four times higher increase in sphere formation capacity (46). In 2020, Weissenrieder *et al* reported that they saw clear spheroid formation effects at selective concentrations of DRD2 modulators. The authors found that 7-day treatment of U-87 MG cells with thioridazine (0.1 μM) decreased spheroid proliferation and invasive capacity as well as reduced spheroid formation, and significantly reduced Sox2 expression. Thus, the ability of DRD2 to form spheres in U-87 MG cell line may be due to other factors (cell-cell adhesion or EGFR signaling) that may contribute to spheroid formation, but it does not depend on alteration of marker expression (47). 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 lengthening of mouse survival. The authors observed the loss of radiation-induced Nanog mRNA expression, GSK3 activation, and reduction in p-Akt, Sox2, and β-catenin levels. In the *in vivo* study the authors noted reduction of the sphere-forming capacity in the surviving tumor cells after trifluoperazine treatment, while the *in vitro* study using HK-308 and HK-374 cells showed that trifluoperazine (1 μM) treatment combined with radiation had an additive inhibitory effect on self-renewal, and formed as many spheroids as saline used during radiation. 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 (48). Agrawal *et al* (2021) found that dopamine induces 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 (49).

Therefore, it is possible that the first generation of antipsychotics (perphenazine and prochlorperazine), which penetrate the blood-brain-barrier (12), as DRD2 receptors antagonists (44) block the receptor 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) (50-52).

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 (50). 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 (53). Arrillaga-Romany *et al* (2020) used a small-molecule DRD2 antagonist (ONC201) that penetrated the BBB 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 (51). 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 (52).

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 bone morphogenetic proteins (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 (54). The overexpression of the serine/threonine protein kinase Pim-1 is often observed in different human malignancy tumors including glioblastoma multiforme (55). Thus, the observed significant decrease in the ABCB1 level may explain a decrease in U-87 MG migration. In the case of ABCG2, Liang *et al* (2015) showed that in lung cancer ABCG2 (56), localized also in the nucleus of glioblastoma multiforme (57), 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 (56). E-cadherin prevents the loss of cell-cell adhesion and cell junctions, which promotes cellular invasion and migration (10). The relative expression of E-cadherin with the use of western blot was shown in U-87 MG cells by Zhang *et al* (2015) (58). 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 U-251 and U-87 cells through activation of SMAD signaling, which finally leads to the suppression of tumor cell invasion (28). Therefore, a significant increase in ABCG2 level after perphenazine and prochlorperazine (0.1 and 1.0 μM) treatment of U-87 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 a decrease in the migration and invasion of the analyzed glioblastoma cells. Our results confirm also that E-cadherin is a negative regulator of U-87 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 (59). 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 U-87 MG and U-373 MG cells migration and invasion. The authors noticed also lower expressions of α-tubulin-mediated mitophagy-associated proteins (60). This confirms our results since we observed a decrease in the α-tubulin level after perphenazine (1.0 μM) and prochlorperazine (0.5 and 1.0 μM) treatment of U-87 MG cells accompanied by a decline in the U-87 MG migration and invasion.

Nakada *et al* (2013) found that the overexpression of α3 integrin in glioblastoma cells: U87-MG, surgical neurology branch-19 (SNB19), and U251 increased cellular migration and/or invasion via the ERK 1/2 pathway, while the decrease of α3 integrin inhibited glioma invasion. The authors also observed that the invasion of U-87 MG cells was stronger in α3 integrin overexpressing cells, which suggests that α3 integrin may be an invasion promotor (61). 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 U-87 MG cells after 24 h 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 (62). Mallawaartchy *et al* (2015) showed a high level of α5 integrin in U-87 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' (63). 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 (64). Those observations are in line with our results. We observed a decrease in migration and invasion of U-87 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 U-87 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 U-87 MG migration and invasion.

In the future, we are planning to use polymerase chain reaction (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 U-87 MG glioblastoma next studies determining the type of cell death should be performed in near future.

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 U-87 MG cells. Our study showed correlation between the cellular migration and/or invasion and cellular levels of ABCB1, ABCG2, E-cadherin, α-tubulin, and integrins (α3, α5, and β1). 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 U-87 MG cells. These findings provided additional insights into a potential use of phenothiazine derivatives in the treatment of glioblastoma, and suggested the purpose of the next research which should include other glioblastoma cell lines and new methods in order to draw more general conclusions about anti-glioblastoma effects of phenothiazine derivates.

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Availability of data and materials

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

Authors' contributions

MO, JS, AKD and ARS conceived and designed the research. MO, JS and ARS contributed reagents and/or analytical tools. MO conducted experiments, analyzed data and wrote the manuscript. MO, JS, AKD and ARS edited the manuscript. MO, JS, AKD and ARS have read and approved the manuscript. MO and ARS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

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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