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Novel MGMT inhibitors increase the sensitivity of glioma MGMT-positive cells to treatment with alkylating agents *in vitro*

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Aim. Novel non-nucleoside inhibitors of MGMT (O^6-methylguanine-DNA methyltransferase) have shown high efficacy and low level of cytotoxicity in human cancer cells HEp-2. But it is important to investigate the impact of new inhibitors in other human cancer cells, especially, those which have different levels of *MGMT* expression. This study was performed using two glioma cell lines: T98G with a high level of *MGMT* expression and U251MG with a methylated promoter in the *MGMT* gene. **Methods.** Western-blot analysis of the level of MGMT. The autophagy level and number of alive and dead cells were measured with fluorescence microscopy and fluorescence spectrophotometry after Monodansylcadaverine dying for autophagosomes and dying by Live-Dead Imaging Cell Kit respectively. **Results.** The new inhibitors significantly reduce MGMT level, but this effect could be observed only after 24-hour treatment. Longer treatment has a weaker inhibiting effect compared with standard inhibitor O^6-benzylguanine. The combined treatment by the inhibitors and nitrosoguanidine induces a high autophagy level in T98G cells, whereas in U251MG cells a low level of autophagy was observed. The analyzed inhibitors do not elevate the level of dead cells in both cell lines. However, the combined treatment leads to a high death rate only in T98G cells. **Conclusions.** The analyzed compounds have inhibiting activity against MGMT in glioma cells. The inhibitors don't affect glioma cells' survival and autophagy level however make them more sensitive to an alkylating agent.

Keywords: O^6-methylguanine-DNA methyltransferase (MGMT), new inhibitors, glioma cells, autophagy, alkylating agent.

Introduction

Glioma is a type of primary brain tumors characterized by a variety of tumor subtypes. Currently, the global prevalence of glioma diagnosis is 7 cases per 100,000 individuals [1]. One of the most common treatments for malignant gliomas is alkylating chemotherapy, which is based on the damage of cancer cell DNA by forming a mutagenic adduct of O^6-methylguanine [2, 3]. However, the existing chemotherapeutic approaches are still not effective, so the search and development of new, more effective drugs with lower cytotoxicity is an extremely urgent task. One of the known biological barriers that reduce the efficacy of chemotherapy is the cell's repair systems, in particular the repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT). This enzyme removes alkyl residues from DNA, thereby counteracting the therapeutic effect of alkylating agents [3–5]. Thus, MGMT inhibition increases the efficiency of chemotherapy [4].

In the third phase of clinical trials, the standard inhibitor O^6-benzylguanine (BG) was found to cause increased cytotoxicity of chemotherapy, especially for hematopoietic cells. Therefore, it is necessary to search for alternative inhibitors that could be effective and nontoxic. Recently, we have shown that new nonnucleoside compounds: 5-(5-Chloro-2-hydroxy-benzylidene)-4-thioxo-thiazolidin-2-one (inhibitor 41) and 5-Benzo[1,3]dioxol-5-ylmethylene-thiazolidin-2, 4-dione (inhibitor 41B) are effective and low cytotoxic inhibitors for human cell line HEp-2, which have derived from an epidermoid carcinoma of the larynx. According to the molecular docking data these compounds interact with MGMT enzyme as substrates, but their affinity could be stronger than the native substrate of MGMT — O^6-methylguanine. Therefore, it is a competitive type of inhibition [6].

Therefore, in the present study we have focused on the effects of new inhibitors (41, 41B) on the MGMT protein level in a glioma cells model. Moreover, we analyzed the sensitivity of glioma cells to the alkylator treatment in combination with the novel MGMT inhibitors. The alkylating compound temozolomide (TMZ) is commonly used to treat glioblastoma. Studies have shown that apoptosis often occurs in glioma cells under the influence of TMZ. Moreover, this process is associated with autophagy, which is explained by the cell's response to the cytotoxic effect of TMZ [6–8]. It is known that apoptosis and autophagy regulate each other, and they can also overlap and regulate each other through Atg5, Beclin-1, Bcl-2, p53, *etc.* [10–12]. These processes are inextricably linked, therefore studies of autophagy in cancer cells permit us to expand our knowledge of cancer metabolism and treatment.

Autophagy plays a key role in various processes that can rescue the cell or lead to apoptosis [9, 12]. In cancer cells, autophagy could have two opposing effects on the cancer development. In the early stages of cancer, autophagy inhibits the tumor growth and its progression and has an antimetastatic effect. Meanwhile, in advanced cancer, autophagy only promotes the cancer development by increasing the cell survival [9, 13].

The level of *MGMT* expression in cancer cells is an important biomarker for determining

the dose and duration of the inhibitors administration in chemotherapy regimens. Only 45 % of patients with glioblastoma have a methylated *MGMT* gene promoter or a low expression level of this gene. The remaining 55 % of patients have a high level of the *MGMT* expression in cancer cells and respond poorly to the standard alkylating chemotherapy. The latter patients are highly chemoresistant and require the combination chemotherapy with MGMT inhibitors [14, 15]. Therefore, it is also important to investigate the differential effect of the new inhibitors on glioma cells with different levels of MGMT. That's why in this study we performed experiments with two glioma cell lines with different levels of the *MGMT* expression to compare the influence of the inhibitors in combination with the alkylating agent on this process.

Materials and Methods

New MGMT inhibitors

Two new MGMT inhibitors were used in this study, namely 5-(5-Chloro-2-hydroxy-benzylidene)-4-thioxo-thiazolidin-2-one (inhibitor 41) and 5-Benzo[1,3]dioxol-5-ylmethylene-thiazolidin-2, 4-dione (inhibitor 41B). The inhibitors were designed by flexible molecular docking and synthesized at the Biomedical Chemistry Department of IMBG NAS of Ukraine [16]. In the following experiments, the compounds were dissolved in dimethylsulfoxide (DMSO).

Cell cultures

The study was conducted using two human glioma cell lines: T98G with a constitutively high level of *MGMT* expression (MGMT+ line) and U251MG without expressed *MGMT* (MGMT– line). The cells of both lines were cultured in DMEM (DMEM powder (1×) high glucose (4.5 g/L) with L-glutamine, PPA) with 10 % of inactivated fetal bovine serum (FBS, Biowest) and 1 % benzylpenicillin/streptomycin at 37 °C and in the gas phase of air with 5 % CO2.

Western blot analysis

T98G and U251MG cells were treated with the inhibitors 41 and 41B at a concentration of 10 μM. The standard MGMT inhibitor O^6-benzylguanine (10 μM) was used as a negative control. Untreated cells were used as a positive control. After the treatment cells were cultured for 24, 48, and 72 hours under standard conditions for the following analysis.

The cells were washed with ice-cold PBS, lysed with RIPA buffer, and proteins were quantified using a Bio-Rad Protein Assay Kit (USA, #5000001). The proteins (40 μg) from each sample were separated by SDS-PAGE and electroblotted onto a PVDF membrane. The membrane was blocked in 5 % non-fat milk/TBST for one hour and probed with the primary antibody for MGMT (1:1000) (NovusBiologicals, USA, #NB100-168), and then with horseradish peroxidase-conjugated secondary antibodies (1:5000) (Sigma-Aldrich, USA, #A9044). The chemiluminescence signal was detected using ECL and X-ray films (Fujifilm, USA). The proteins were quantified by densitometry using Image J soft [https://imagej.net/ij/index.html].

Analysis of autophagy level by MDC staining

The cells of both lines were seeded into 96-well plates at a concentration of 4000 cells per well.

After one day incubation under standard conditions, the cells were treated with the analyzed inhibitors (10 μM), standard inhibitor BG (10 μM), and DMSO (vehicle control). On the second day of incubation, the medium was changed to serum-free and the alkylating agent N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) was added at different concentrations (0.5, 0.05, 0.005, and 0.0005 μg/mL) and incubated for one hour. Then 10 % FBS was added and incubated for another 2 hours. The untreated cells were used as a negative control. There was also a set of cells treated with MNNG only at different concentrations. As a positive control, the cells were incubated for 12 hours with 20 μM of the autophagy inducting factor FCCP (Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) (Sigma-Aldrich, USA).

After all treatments, the cells were washed with PBS, stained with 50 μM monodansylcadaverine (MDC) (Sigma-Aldrich, USA), and incubated at 37 °C for 15 min. The fluorescence was measured using a Tecan Infinite 200 PRO at excitation/emission: 332/518 nm, followed by visualization using a Leica DM2000 LED microscope. The images were analyzed using the LAS X software. The data were normalized to the total number of live cells stained with Hoechst 333 dye (excitation/emission: 350/461 nm).

Live/dead cell assay

The amount of live and dead cells after the treatments was analyzed using the LIVE/DEAD Cell Imaging Kit (ThermoFisher Scientific, #R37601). The 24-hour fasting period was used as a positive control in this treatment scheme. The cells were stained for 15 minutes at 37 °C according to a standard protocol. Using a Tecan Infinite 200 PRO, the fluorescence of live cells was measured at excitation/emission: 488/515 nm, and dead cells at excitation/emission: 570/602 nm, followed by visualization with a Leica DM2000 LED microscope. The images were analyzed using the LAS X software. The level of the dead cell fluorescent signal was normalized to summed signals of the total number of cells (dead and live).

Statistical analysis

The data were analyzed using software applications, including Origin 8.1, Microsoft Excel 2016, and Image J. Various statistical measures were used such as mean (M), standard error (SEM), and standard deviations (SD). Each experiment was iterated 2-4 times, with each iteration consisting of 2-8 identical treatments. One-way ANOVA was used for Western blotting results, while two-way ANOVA was used to calculate the P value (p) and effect size (Ƞ) for MDC staining and live/dead assay results. Significance was determined at a threshold of $p < 0.05$.

Results and Discussion

MGMT protein levels. First, we analyzed how the inhibitors affected the MGMT repair enzyme level in glioma cells. We used the T98G cell line, where MGMT is constitutively expressed at high levels, in contrast to the U251MG cell line. Using Western blot analysis, we found that T98G cells (MGMT+) demonstrated high level of MGMT protein, in contrast to U251MG cells (MGMT–), as expected (data not shown). Based on this, the T98G cell lines were used for further analysis of the developed inhibitors' effects.

As a result, we found that both compounds under study are indeed inhibitors of the repair enzyme MGMT, as they reduce the level of MGMT protein in glioma cells (Fig. 1).

Our data indicate that new MGMT inhibitors significantly reduce MGMT protein level in the glioma cell line compared to the control. However, their inhibitory activity is characterized by a shorter time point compared to the effect of BG. However, the new inhibitors show the greatest effectiveness during the first 24 hours of treatment. That is when the lowest MGMT level was observed. While the standard inhibitor BG had a prolonged effect on the amount of protein in the cells. Low MGMT level was observed even after 72 hours of treatment with BG (Fig. 1). These data may indicate a different mechanism of action of the inhibitors under study.

Autophagy level. The sensitivity of cancer cells to chemotherapy depends on several factors, including the level of autophagy and the balance between autophagy and apoptosis. Therefore, as a next step, we analyzed the level of autophagy in glioma cells of two lines after the combined treatment with inhibitors and alkylating agent MNNG. In this series of experiments, the U251MG cell line was used as a MGMT control. As a result, we have found that the new inhibitors and the standard inhibitor BG alone did not increase autophagy in either cell line (Fig. 2).

In combination with the alkylating agent MNNG, the new inhibitors, as well as the

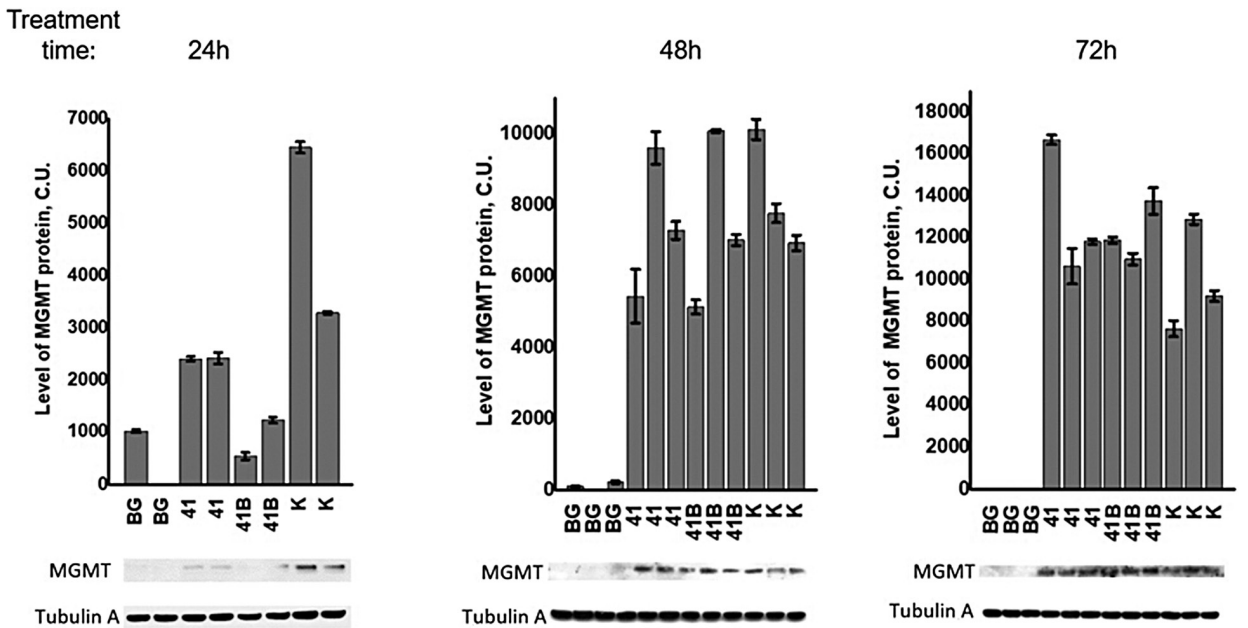

Fig. 1. Level of MGMT protein in T98G cells after treatment with new inhibitors (41 and 41B) and standard inhibitor BG with different treatment times (24h, 48h, and 72h) according to Western blot analysis. Densitometry of MGMT normalized to tubulin A. The data are expressed as the mean ±SD of arbitrary fold of change relative to the control levels. $p<0.05$.

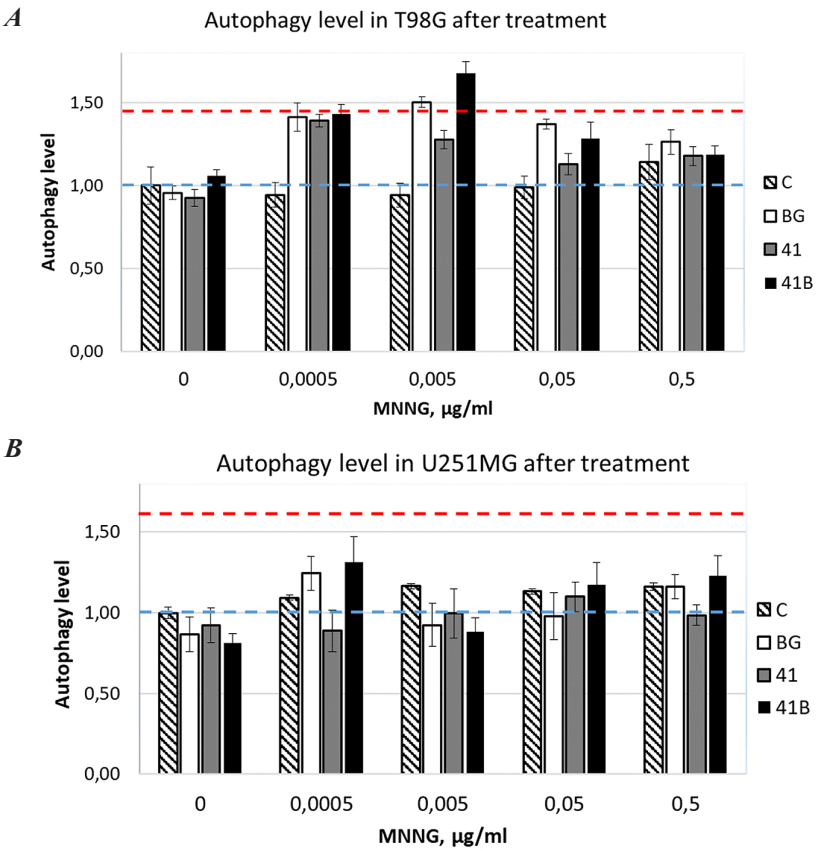

Fig. 2. Relative quantification of monodansylcadaverine (MDC) staining in T98G (***A***) and U251MG (***B***) cells after treatment with the standard inhibitor BG, novel inhibitors 41 and 41B, and control (C) in combination with the alkylating agent MNNG at different concentrations (0.0005, 0.005, 0.05, and 0.5 µg/ml), and without MNNG. Red dashed line (upper dashed line) — positive control, blue dashed line (lower dashed line) — negative control. The data are expressed as the mean ±SD of arbitrary fold of change relative to control values. $p < 0.05$; ***A*** — Η(inhibitor) = 0,69, Η(MNNG) = 0.12, Η(interaction) = 0.23; ***B*** — Η(inhibitor) = 0.74, Η(MNNG) = 0.10, Η(interaction) = 0.25.

standard BG inhibitor, increase the level of autophagy in the cells. Interestingly, a significant increase in autophagy levels was observed in T98G cells (MGMT+). In contrast, U251MG cells (MGMT–) showed only a small increase in autophagy level (Fig. 2). It was expected that the level of autophagy would be higher in the latter since the cells lack MGMT, which prevents the cytotoxic effect of the alkylating agent. However, in the cells treated with MNNG alone, this was exactly what happened: in T98G cells (MGMT+), autophagy was as low as in the control, and in U251MG cells (MGMT–), autophagy became higher and increased with the concentration of the alkylating agent (Fig. 2). Probably, the inhibitors affect some other components of the metabolic pathways in the cells of different lines.

The spectrophotometric data were generally in agreement with the microscopic analysis. T98G cells have a higher number of autophagosomes than U251MG cells after the combined treatment with each inhibitor and MNNG. The inhibitors by themselves almost do not cause an increase in autophagy level (Fig. 3).

Cell death after treatment with the investigated compounds. In general, T98G cells were more sensitive to the cytotoxic effects of the alkylating agent in combination with the inhibitors than U251MG cells. However, the

inhibitors themselves hardly increased the level of dead cells compared to the control in both lines (Fig. 4). Notably, slightly lower levels of dead cells were detected after treatment with the new inhibitors 41 and 41B than after treatment with BG, confirming that the new inhibitors are less cytotoxic than the standard inhibitor BG.

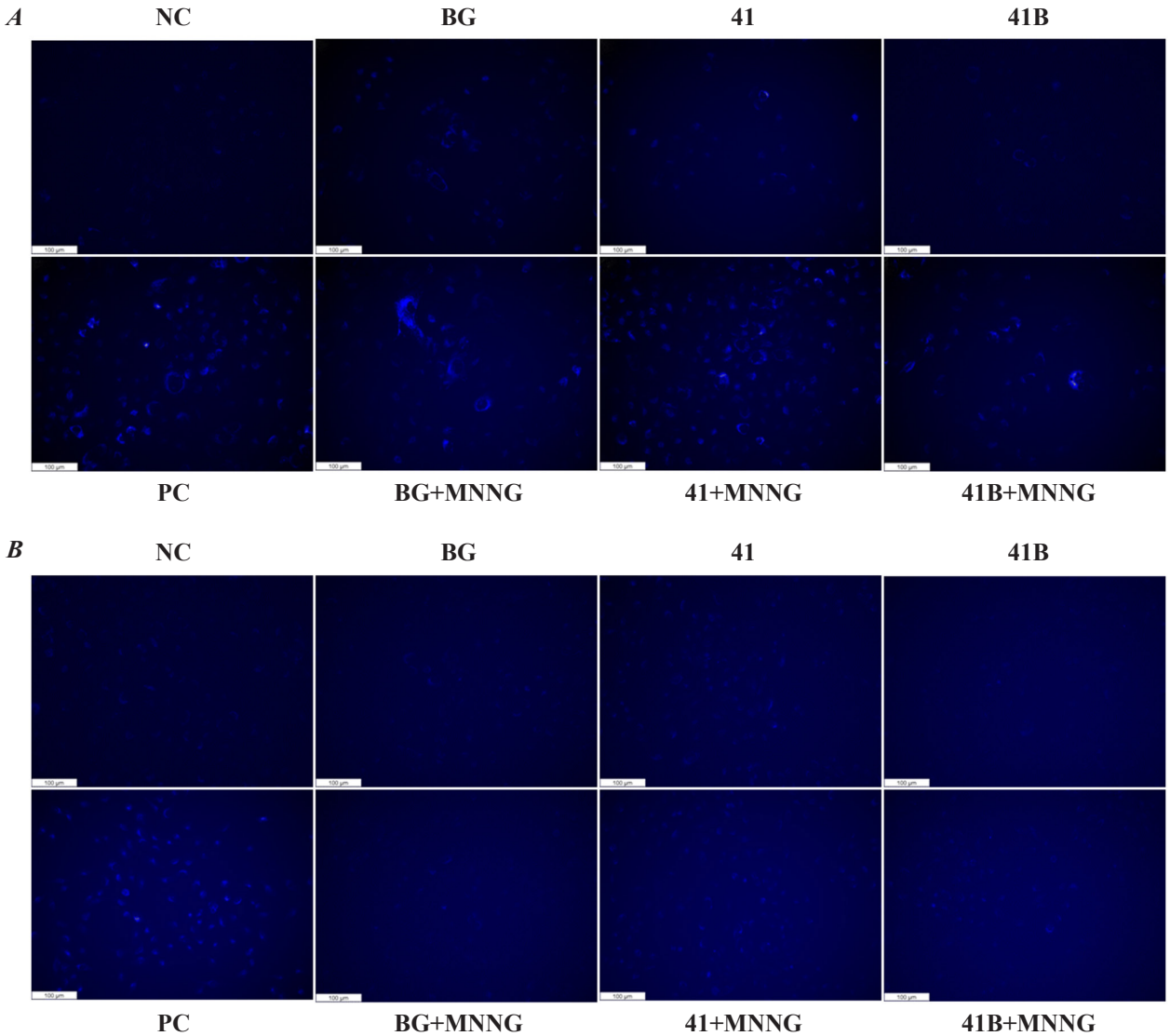

Fig. 3. Representative micrographs of autophagosome staining by MDC in T98G (**A**) and U251MG (**B**) cells. NC — negative control; PC — positive control; BG — treatment with standard inhibitor BG; 41, 41B — treatments with novel inhibitors 41 and 41B, respectively; BG/41/41B+MNNG — treatment with BG, 41, 41B inhibitors, respectively, in combination with alkylating agent MNNG (0.005 μg/ml). The experiment was performed with 4-6 identical treatments.

The spectrophotometric data were generally consistent with the fluorescence microscopy data. Significantly more dead cells were observed in T98G cells after the combined treatment than in U251MG cells. Treatment with the inhibitors alone did not cause much cell death (Fig. 5).

Autophagy occurs as a result of cell damage caused by external factors [7, 8]. Therefore, it could be considered as an indicator of the cytotoxicity of compounds that enter the cell. Based on the results of autophagy studies (as shown in Fig. 2 and 3), we can conclude that the new inhibitors 41 and 41B are not harmful to glioma cells of both lines at a concentration of 10 μM. However, the inhibitors' ability to inhibit MGMT increases the cytotoxic effect of the alkylating compound MNNG, which is necessary for effective alkylating chemotherapy.

The results of the autophagy assay are confirmed by the results of the glioma cell viability assay (Fig. 4, 5). Comparing the data, it can be seen that the cells treated with the inhibitors only did not show a significant level of autophagy, on the contrary, a shallow level of cell death was observed. This confirms the low cytotoxicity of these compounds and demonstrates their safety for glioma cells.

We found that the inhibitors had a positive impact on the cytotoxic effect of MNNG, but

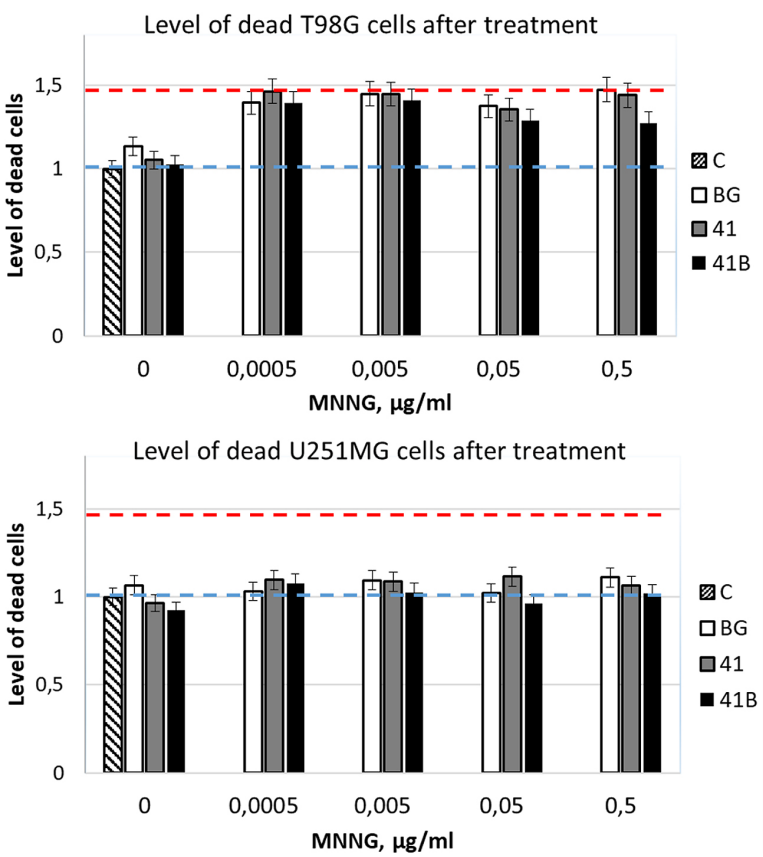

Fig. 4. Relative quantification of fluorescence signal level of T98G (***A***) and U251MG (***B***) dead cells after treatment with standard inhibitor BG, new inhibitors 41 and 41B in combination with alkylating agent MNNG at different concentrations (0.0005, 0.005, 0.05 and 0.5 μg/ml), and without MNNG. Control (C) — untreated cells. Red dashed line (upper dashed line) — positive control, blue dashed line (lower dashed line) — negative control. The data are expressed as the mean ±SD of arbitrary fold of change relative to control levels. $p < 0,05$; ***A*** —Η(inhibitor) = 0.22, Η(MNNG) = 0.64, Η(interaction) = 0.05; ***B*** — Η(inhibitor) = 0.33, Η(MNNG) = 0.21, Η(interaction) = 0.39.

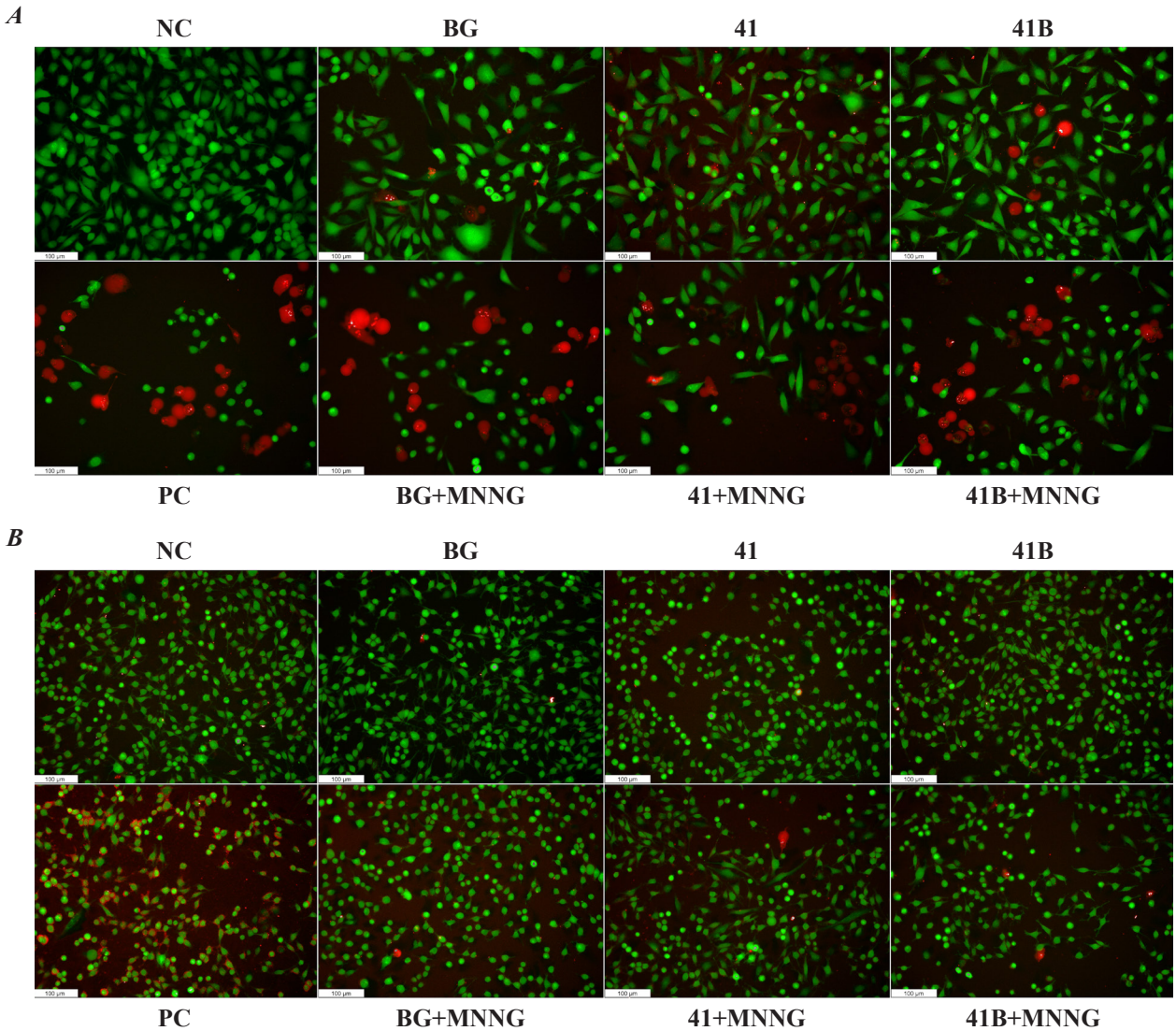

Fig. 5. Representative micrographs of live (green) and dead (red) staining of T98G (***A***) and U251MG (***B***) cells. NC — negative control; PC — positive control; BG — treatment with standard inhibitor BG; 41, 41B — treatments with novel inhibitors 41 and 41B, respectively; BG/41/41B+MNNG — treatment with BG, 41, 41B inhibitors, respectively, in combination with alkylating agent MNNG (0.005 μg/ml). The experiment was performed with 4-6 identical treatments.

only in T98G (MGMT+) cells. The level of autophagy was high in these cells after the combined treatment, leading to a higher mortality rate. Interestingly, the number of dead cells increased with increasing autophagy. This is explained by the effectiveness of the new inhibitors, which enhance the cytotoxic effect of the alkylating agent to a level where the

glioma cells' defense mechanisms can not cope, leading to the cell death. The cells subsequently undergo apoptosis after an attempt to rehabilitate themselves during autophagy [17]. It has been shown that many components of autophagy act as apoptotic factors to mediate the cell death [18, 19]. Thus, autophagy may be a necessary step for the induction of certain apoptotic pathways in cancer cells.

Conclusions

In conclusion, our data indicate that the analyzed inhibitors 41 and 41B at 10μM concentration reduce the level of repair enzyme MGMT, however, this inhibitory effect has a short time (24h) compared to the standard inhibitor BG.

New inhibitors did not affect the survival and autophagy level of glioma cells but they made them more sensitive to alkylating agent MNNG. It suggests that the analyzed compounds may have a therapeutic effect during the combined alkylating chemotherapy of the patients with high MGMT gliomas.

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Нові інгібітори MGMT підвищують чутливість MGMT-позитивних гліомних клітин до лікування алкілуючими агентами *in vitro*

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Мета. Нові ненуклеозидні інгібітори MGMT (O^6-метилгуанін-ДНК-метилтрансфераза) продемонстрували високу ефективність та низьку цитотоксичність в ракових клітинах людини HEp-2. Однак, важливо дослідити вплив нових інгібіторів на інші ракові клітини, особливо ті, що мають інший рівень експресії *MGMT*. Це дослідження було проведено з двома лініями клітин гліоми: T98G з високим рівнем експресії *MGMT* та U251MG з метильованим промотором у гені *MGMT*. **Методи.** Вестерн-блот аналіз рівня MGMT. Вимірювання рівня аутофагії та кількості живих і мертвих клітин за допомогою флуоресцентної мікроскопії та спектрофотометрії після фарбування автофагосом Монодансилкадаверином та фарбування живих і мертвих клітин за допомогою Live-Dead Imaging Cell Kit. **Результати.** Нові інгібітори значно знижують рівень MGMT, але цей ефект спостерігається лише після 24-годинної обробки. Більш тривала обробка має слабший інгібувальний ефект порівняно зі стандартним інгібітором O^6-бензилгуаніном. Комбінована обробка інгібіторами та нітрозогуанідином індукує високий рівень аутофагії в клітинах T98G, тоді як у клітинах U251MG спостерігається низький рівень аутофагії. Досліджувані сполуки не збільшують кількість мертвих клітин в обох лініях. Однак, комбінована обробка призводить до більшого цитотоксичного ефекту, проте лише в клітинах T98G. **Висновки.** Досліджувані сполуки володіють інгібувальною активністю щодо MGMT у гліомних клітинах. Інгібітори не впливають на виживаність та рівень автофагії клітин гліоми, проте роблять їх більш чутливими до дії алкілуючих сполук.

Ключові слова: O^6-метилгуанін-ДНК-метилтрансфераза (MGMT), нові інгібітори, клітини гліоми, аутофагія, алкілувальна сполука.

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