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Original Article

DHMEQ enhances the cytotoxic effect of cisplatin and carboplatin in ovarian cancer cell lines

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Abstract: Ovarian cancer (OvCa) is one of the most lethal gynaecological malignancies. It is diagnosed mostly in advanced stages. Due to a lack of appropriate early detection markers and non-ambiguous symptoms, the five-year survival rate is significantly reduced. Despite a primary good response to platinum-based therapy, approximately 70% of patients will develop a chemoresistance phenotype. The activation of the NF-κB signalling pathway plays a crucial role in this process. It is responsible for increasing cell viability, cell cycle progression and induces growth and migration of neoplastic cells. A few independent studies have yet suggested a high correlation between activation of NF-κB and poor outcome in OvCa patients. Thus, developing inhibitors of the NF-κB pathway has become a new target of cancer therapies. One of the promising compounds is DHMEQ (dehydroxymethylepoxyquinomicin). Our preliminary studies indicated that DHMEQ combined with cisplatin (CDDP) or carboplatin (CBP) enhanced apoptosis in the A2780 cell line and caused cell cycle arrest in the G2/M phase in the SKOV3 cell line, but not in the normal cell line MRC-5 pd19. Moreover, the combination of those agents caused decreased motility of cells, especially with the CBP. However, the invasion of cells was not changed significantly. The analysis of drug interactions using CompuSyn software has revealed that observed effect of the doses used in the study was antagonistic, but the DRI guidelines and *in vitro* observation of biological response indicate that a combination of DHMEQ with CDDP or CBP could be a novel proposal in ovarian cancer treatment.

Keywords: Ovarian cancer, NF-κB, DHMEQ, cisplatin, carboplatin

Introduction

Ovarian cancer (OvCa) is still a leading cause of death among gynaecological malignancies and the fifth cause of death among all malignancies, with a five-year survival rate below 30%. Among OvCa histological types, high grade (Grade 3) serous epithelial cancer is a common histological type also known as the most aggressive [1]. Ovarian cancer is a heterogeneous disease, which consists of two types. Types I OvCa (low grade) is thought to develop from extra ovarian benign lesions that embed in the ovary and then convert into malignant disease (a series of mutations are responsible for malignant transformation). This kind of tumor is less aggressive in comparison with type II (high grade), which in turn is thought to develop in the Fallopian tube and then spread in the peritoneal cavity [2]. The vast majority of patients are diagnosed at advanced stages of the disease when cancer is disseminated within the entire abdomen. Treatment consists of a combination of cytoreductive surgery followed by adjuvant chemotherapy. Since the majority of patients are diagnosed at advanced stages, the effectiveness of surgical treatment is insufficient. The gold standard for the first-line chemotherapy is based on two agents-paclitaxel and platinum derivatives (either cisplatin or carboplatin) [3].

The estimated number of new ovarian cancer cases in the USA in 2019 reached 22,530 and

13,980 patients are expected to die of this disease [4, 5]. Based on global Cancer Statistics predictions, 295,414 new cases of OvCa are expected to be diagnosed in 2018 through 185 countries and 84,799 patients are expected to die of this disease [6].

Despite improvements in therapeutic approaches, OvCa five-year survival rates have not increased for a few decades [7]. The main reason is late diagnosis mostly due to a lack of specific symptoms and early-stage biomarkers. Another cause is the development of intrinsic or induced chemoresistance phenotype of ovarian cancer cells that become insensitive to the cytotoxic agents used in treatment [8-11].

One of the mechanisms involved in the development of the resistant phenotype is an activation of the NF-κB pathway [12]. The proteins of the NF-κB family modulate a wide range of genes responsible for immune surveillance, cell differentiation, proliferation, apoptosis, angiogenesis and cell cycle progression [13-16]. Enhanced activity of these proteins leads to poor clinical outcomes of ovarian cancer treatment [17, 18]. Thus, the NF-κB pathway seems to be a promising target in anticancer therapy. More than 800 agents, both natural and synthetic, have already been identified as either activators or inhibitors of that pathway [19].

One of those drugs is DHMEQ (*dehydroxymethylepoxyquinomicin*). That compound is a modified antibiotic epoxyquinomycin and its inhibitory activity towards the NF-κB pathway was described. It forms covalent bonds with specific cysteine residues of the DNA binding site of RelA, c-Rel and RelB of the NF-κB family proteins [20]. DHMEQ interrupts the ability of NF-κB family proteins to bind to DNA, which causes inhibition of the NF-κB signalling pathway [20]. Compared to other inhibitors, DHMEQ neither targets gene products of the NF-κB pathway nor inhibits proteasome that prevents the degradation of IKKβ. The enhancement of the cytotoxic effect of DHMEQ is non-toxic in mice and rodents. The agent exerts direct antitumour effects in vitro and in vivo and shows significant chemo- and immuno-sensitizing activity in resistant tumour cells [21].

In this study, we show that NF-κB inhibitor (DHMEQ) enhances the cytotoxic effect of platinum derivative (both cisplatin and carboplatin) in A2780 and SKOV3 cell lines that represent a primary platinum-sensitive and metastatic ovarian cancer.

Materials and methods

Derivation of DHMEQ

Racemic DHMEQ is a modified epoxyquinomicin C, which was synthesized from 2,5-dimethoxyaniline in a five-step reaction as previously described [22]. DHMEQ in the form of a pure lyophilisate was kindly provided by Prof. Kazuo Umezawa.

Cell culture

Epithelial ovarian cancer cell lines (A2780 and SKOV3, American Type Culture Collection (ATCC), VA, USA) were used in all experiments. They represent different clinical stages of ovarian cancer-A2780 is the primary platinum-sensitive ovarian cancer line and SKOV3 represents treated metastatic ovarian cancer. The cells were maintained in a humidified atmosphere (5% CO_2, 37°C). They were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS) (Biowest, France) and 1% Penicillin/Streptomycin (Biochrom, France). In this study, the normal cell line MRC-5 pd19 (European Collection of Authenticated Cell Cultures (ECACC), UK) was used and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (both manufactured by Biowest, France), 1% Penicillin/Streptomycin (Biochrom, France) and 1× non-essential amino acids (NEAA) (Merck KGaA, Germany). The cells from early passages (2-6 after thawing) were used for analysis.

MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used for estimation of IC_{50} of racemic DHMEQ (stock 20 mg/ml suspended in DMSO [Amresco, OH, USA]), cisplatin (CDDP) (Ebewe, Austria) (1 mg/ml) and carboplatin (CBP) (Accord, UK) (10 mg/ml). The drugs were added in two-fold serial dilution (starting concentrations: 100 μg/ml of DHMEQ, 12.5 μM of CDDP and 100 μM of CBP) onto a 96-well plate with previously seeded cells (500-6000 cells per well). After 24, 48 and 72 h the medium was replaced with one containing 0.5 mg/ml of MTT (ThermoFisher

inc., MA, USA). After two hour of incubation at 37°C, the reagent was removed and 100 µl of DMSO were added into the wells to suspend the developed crystals. The plate was incubated for 10 minutes at 37°C and absorbance was measured at 565 nm using DeNovix Spectrophotometer (DeNovix Inc., PA, USA). Measurement results were normalized to the absorbance level of the cells incubated without the drugs. Additionally, the cells were treated with DMSO to exclude toxicity of DHMEQ solvent. The IC_{50} was estimated in GraphPad Prism ver. 6.0 (GraphPad Software, Inc. CA, USA), using a non-linear regression model.

Clonogenic survival assay

To assess the influence of DHMEQ on cell survival, a clonogenic assay was performed. A2780 (200 cells per well) and SKOV3 (150 cells per well) cells were collected after 24, 48 and 72 h of exposure to DHMEQ (IC_{50}=72 h) and seeded onto a six-well plate. After five (A2780) and seven (SKOV3) days, when the colonies reached above 50 cells, the clonogenic assay was finalized. The cells were washed with phosphate-buffered saline (PBS) (Biowest, France) and fixed with 70% ethanol (POCh, Poland) for 10 minutes. Next, they were stained with Coomassie Blue for 15 minutes. The plates were washed with water and left overnight to dry. The next day, images of the plates were taken with ChemiDoc (Biorad, CA, USA) and colonies were counted using GeneTools ver. 4.3.7 (Syngene, UK). Further, the survival fraction (SF) was calculated by normalization of plating efficiency (PE) of the treated cells to PE non-treated cells.

Flow cytometric analysis of apoptosis

To test the effect of the drugs on cell viability, A2780 and SKOV3 cells were exposed to DHMEQ (IC_{50}=72 h) for 24, 48 and 72 h. Additionally, the effect of DHMEQ combined with CDDP (IC_{50}=72 h) or CBP (IC_{50}=72 h) for 72 and 96 h were tested in OvCa cell lines and MRC-5 pd19. Then, 1×10^5 of the cells were suspended in PBS and washed. The cells were stained with Annexin V, PI (Apoptosis kit, ThermoFisher Scientific, MA, USA) or 7-AAD (BD Bioscience Pharmigen, CA, USA). The procedure was performed according to the manufacturer's instructions. Briefly, the cells were suspended in 1× Annexin V binding buffer. After washing, they were incubated with an antibody against Annexin V conjugated with APC or PE. Next, 7-AAD or PI was added into the cell suspension and measurement was conducted within one hour (Accuri C6, Becton Dickinson, USA or CytoFLEX S, Beckmann Coulter, CA, USA). Untreated cells were used as a control.

Flow cytometric analysis of the cell cycle

To investigate the distribution of cell cycle phases, the OvCa cells were exposed to DHMEQ (IC_{50}=72 h) for 24, 48 and 72 h. To determine the cell cycle phases in OvCa cell lines and MRC-5 pd19 after exposition to DHMEQ combined with CDDP (IC_{50}=72 h) or CBP (IC_{50}=72 h), cells were cultured for 72 and 96 h. Then, 2×10^5 of cells were suspended in PBS and fixed by 70% ice-cold ethanol added to continuously shaken cells to obtain a single cells suspension without aggregates. The cells were stored at -20°C until analysis. Thawed samples were washed twice with PBS and incubated with PBS containing iodium propide (20 µg/ml) (Cayman Chemicals, MI, USA), and RNAse I (500 µg/ml) (Panreac AppliChem, Germany) for one hour at 37°C. Then the analysis was performed (Accuri C6, Becton Dickinson, CA, USA).

Wound healing assay

To establish the effect of DHMEQ and appropriate drug combinations on the motility of cells, a wound healing assay was used. The SKOV-3, A2780 and MRC-5 pd19 cells were seeded onto a 24-well plate and cultured until reached full confluency. Then, in the middle of the plate, the scratch was performed using 10 µl (A2780) or 200 µl (SKOV-3 and MRC-5 pd19) pipette tips, the well was washed three times using (PBS) and then were co-cultured with the estimated concentrations (IC_{50}=72 h) of DHMEQ, CDDP and CBP, in combination or separately. The pictures were taken using an inverted microscope Axio Vert.A1 (Carl Zeiss, Germany). The area of the closed wound was calculated using MRI wound healing tool, a patch dedicated for ImageJ ver 1.52q (National Institutes of Health, MD, USA).

Matrigel invasion assay

Cells A2780 (1×10^6 of cells), SKOV3 (5×10^5 of cells) and MRC-5 pd19 (5×10^5) was co-cultured with DHMEQ and its combination with CDDP or

CBP for 72 h (IC_{50}=72 h) to assess their effect onto invasive potential. Then, cells were washed, suspended in the serum-free medium and placed onto previously coated with Matrigel™ (Beckton Dickinson, Germany) 8 μm membrane of the 24-well insert at appropriate dilution with serum-free medium for each cell line (1:20 for SKOV3; 1:40 for A2780 and MRC-5 pd19). After 18 hours the insert was twice washed with PBS and fixed for 20 minutes with 100% methanol (POCh, Poland). Then the membrane was washed and stained using 0.1% crystal violet (Sigma-Aldrich, MO, USA). Further, the membrane was washed three times in deionized water and the top of the membrane was scratched using a cotton swab. At least five of the areas of the bottom of the membrane was documented and cells that migrated through the membrane were counted using Cell Counter of ImageJ ver 1.52q (National Institutes of Health, MD, USA).

Combination index (CI) and dose reduction index (DRI)

CI and DRI for DHMEQ combined with CDDP or CBP were calculated based on a simulation generated *via* CompuSyn software according to Chou-Talay et al. 2006 [23]. The cells (500-1500 per well of a 96 well plate) were exposed to two-fold serial dilutions of the drugs (starting concentrations: four-fold of IC_{50} estimated at 72 h for DHMEQ, CDDP and CBP) alone or combined. Then, survival fraction (SF) was determined by normalization of absorbance results to the non-treated population of the cells. The CompuSyn software fraction affected (Fa) was determined as Fa=1-SF.

Statistical analysis

All experiments were performed in triplicate. The comparisons between groups were analyzed using GraphPad Prism ver. 6.0 (GraphPad Software, Inc. CA, USA). The one-way analysis of variance (ANOVA) was used and for multiple comparisons post-hoc Tukey's test was performed. The accepted significance level was $P<0.05$.

Results

DHMEQ affects the viability and cell cycle phases of ovarian cancer cells

First, to establish the effects of DHMEQ in the ovarian cancer cell lines, cytotoxicity IC_{50} values were estimated by MTT-assay. We observed declined proliferation in both cell lines, A2780 and SKOV3, in a time-dependent manner (**Figure 1A**). However, IC_{50} was higher in the metastatic OvCa cell line (SKOV3) compared to the primary OvCa cell line (A2780). Then we analyzed the effects of DHMEQ (IC_{50} at 72 h) on the viability of both ovarian cancer cell lines via clonogenic assay for 24, 48 and 72 h (**Figure 1B**). After 24 h exposure to DMSO and DHMEQ, the SKOV3 line showed a slight decrease in viability. However, this effect diminished after 48 and 72 h, when no changes in the colony formation were observed. Contrary to that, A2780 cells exposed to DHMEQ or DMSO did not present any effects after 24 h but after 48 and 72 h, the number of their colonies dropped in comparison with the non-treated cells. Next, the effect of DHMEQ on the cell cycle phases distribution was tested (**Figure 2A**). After 24 h the assay demonstrated a decrease in the cell population at G1/G0 with a parallel increase of cell number at S and G2/M vs. control populations of SKOV3 cells. After 48 h only at phase S, the decreased percentage of cells in DHMEQ treated SKOV3 cells was observed. After 72 h we saw a slight elevation in the percentage of cells at G2/M vs. the controls. In the A2780 cell line exposed to DHMEQ for 24 h, we noticed more cells at G1/G0 phase and fewer cells at S and G2/M phase in comparison with the control. At 48 h after exposure to DHMEQ, the level of cells at phase S was decreased with a parallel increase of cells at phase G2/M in comparison with control. At 72 h the A2780 cells exhibited an increased level of cells at S and G2/M phase indicating G2/M cell cycle arrest.

Further, the influence of DHMEQ, on the survival of OvCa cell lines was confirmed by Annexin-V and PI staining (**Figure 2B**). SKOV3 cells exposed to DHMEQ for 24 h did not exhibit any changes. However, after 48 h showed a significant rise in the percentage of early apoptotic cells and late apoptotic cells in comparison with the non-treated cell population. After 72 h of exposure to DHMEQ, SKOV3 cells exhibited a difference in necrotic and late apoptotic population *vs* non-treated cells. On the other hand, the number of A2780 cells with positive responses to Annexin-V rose significantly in a time-dependent manner.

DHMEQ increases the response of ovarian cancer cells to platinum-based drugs

To verify NF-κB pathway inhibition by DHMEQ combined with platinum-based agents, IC_{50} for

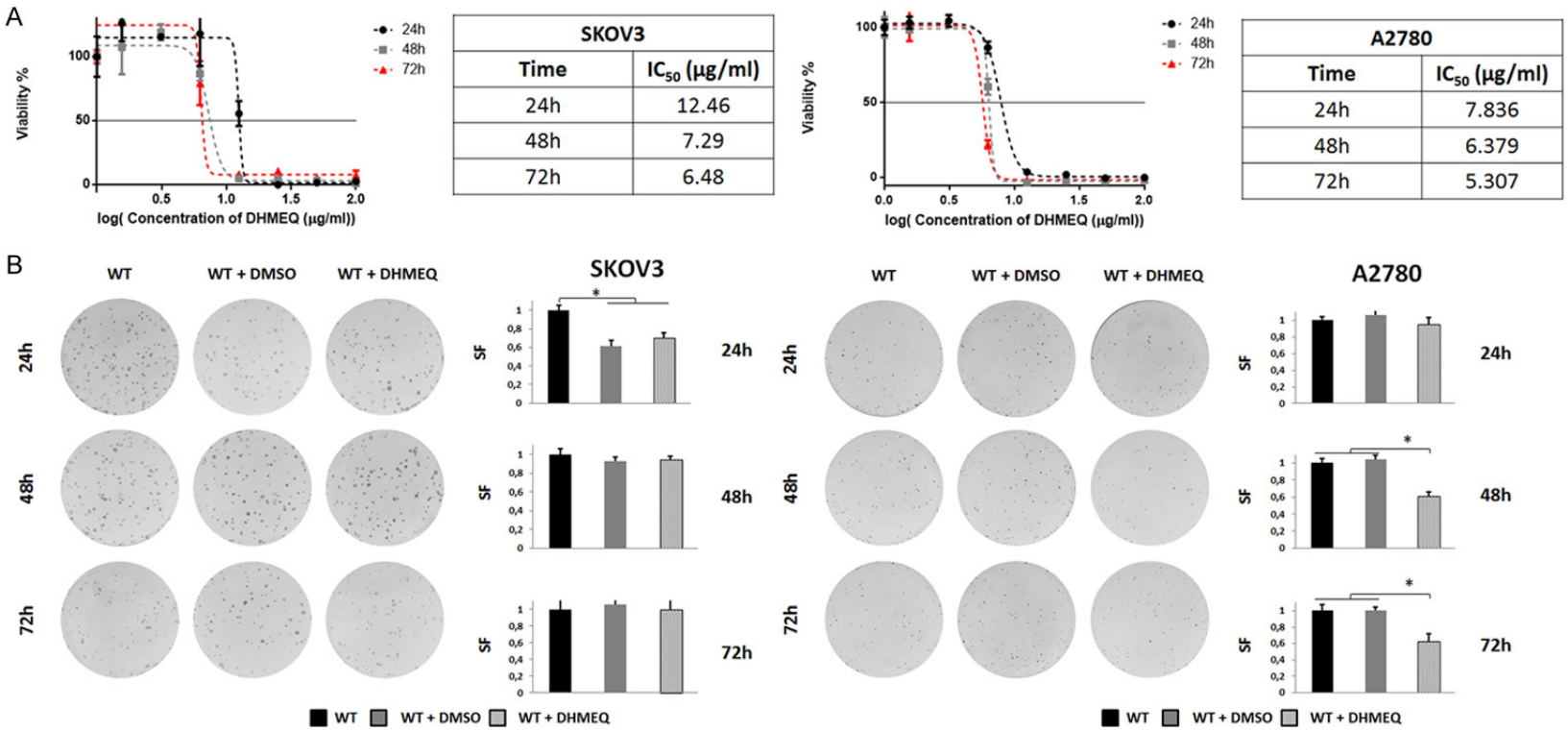

Figure 1. DHMEQ exerted cytotoxic effects in the ovarian cancer cell lines. A. SKOV3 and A2780 cell lines treated with DHMEQ (dose range: 100-1.56 µg/ml) to determine IC_{50} at 24, 48 and 72 h *via* MTT assay. IC_{50} depended on the time of incubation with SKOV3 and A2780, which indicated the time-dependent increase in cytotoxic properties of DHMEQ. Data are presented as mean ± SD. B. SKOV3 and A2780 cell lines exposed to DHMEQ (IC_{50} at 72 h) for 24, 48 and 72 h. A clonogenic assay was performed daily. A2780 cells were more sensitive to DHMEQ (as manifested by their declined survival after 72 h) than SKOV3 cells that showed an increased sensitivity at 24 h. Results are presented as mean ± SD. The asterisk represents significance at $P<0.05$. The clonogenic assay pictures show representative wells from a 6-well plate.

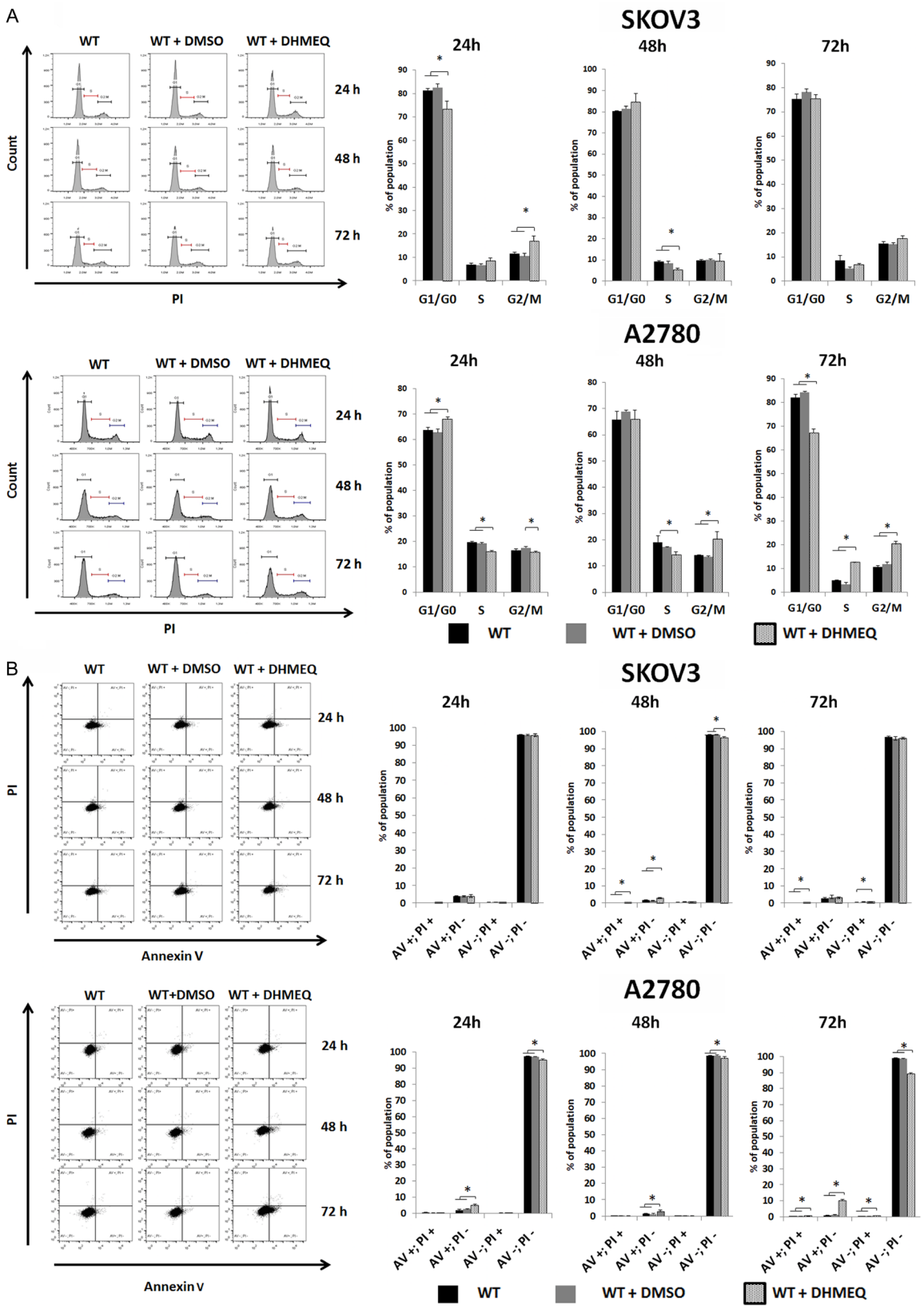

Figure 2. DHMEQ causes cell cycle arrest at the M/G2 phase and decreases the viability of OvCa cell lines. A. SKOV3 and A2780 cells treated with DHMEQ (concentration IC_{50} at 72 h). Cell cycle phases distribution was analyzed after 24, 48 and 72 h. In the SKOV3 cell line, the strongest effect was observed at 24 h and it was maintained for the rest

of the analysis period. Contrary to that, in the A2780 cell line, the effect of DHMEQ on the cell cycle arrest in G2/M increased every day. Results represent mean ± SD. The asterisk represents statistical significance at $P<0.05$. The left panel displays representative histograms of the cell cycle. B. DHMEQ decreases the viability of SKOV3 cells at 24 h but the effect weakens over time. In A2780 cells incubated with DHMEQ viability decreases with increasing exposure to the drug. Results are presented as a mean of percentage population, error bars indicate SD. The asterisk represents statistical significance at $P<0.05$. The left panel displays representative cytograms generated during flow cytometric analysis of viability with Annexin V (AV) and propidium iodide (PI).

CDDP and CBP after 72 h was assessed for OvCa and MRC-5 pd19 cell lines (normal lung fibroblasts cell line, commonly used and accepted as a reference non-malignant cell line for drug testing) (**Figure 3A**). Additionally, the selectivity index (SI) was assessed, which describes the toxicity of tested compounds against cancer cell lines in comparison with normal cell lines, DHMEQ is a more selective drug for both OvCa cell lines but in SKOV3 it is even better than tested both CDDP and CBP. In the case of the A2780 cell line, CDDP is more selective in comparison with other compounds. However, the SKOV3 cell line is less sensitive to platinum-based drugs than A2780. Further, we used flow cytometry (Annexin V and 7-AAD or PI) (**Figure 3B-D**) to test the viability of cells exposed to single drugs or a combination thereof. After 72 h, the percentage of viable cells was lower in the SKOV3 line treated with CDDP, CBP, DHMEQ and their combination compared to control. The viability of the cells exposed to the drug combinations was slightly lower but the differences were not significant. After 96 h, the cell viability decreased significantly in all studied variants vs. the non-treated cells. Moreover, the combination of DHMEQ and platinum-based drugs resulted in an increased percentage of apoptotic cells in comparison with platinum-based drugs alone (not significant). However, the A2780 line treated with DHMEQ combined with either CDDP or CBP showed a significantly higher percentage of apoptotic cells as compared to the control and cells exposed to DHMEQ alone. Similar trends were noticed at 72 and 96 h after the exposure of A2780 cells to the studied drugs and their combinations. In the case of the MRC-5 pd19 cell line (**Figure 3D**), after 72 h, the effect of combined agents revealed enhanced apoptosis as a manifestation of a higher percentage of Annexin V positively stained cells. However, the platinum-based drugs alone caused an increased percentage of necrotic cells population in comparison with other studied variants. After 96 h, a similar trend was observed in the case of the apoptotic and necrotic population of cells. However, the viability of cells was significantly decreased in CDDP or CBP cells treated alone.

The next step was an investigation of cell cycle phases distribution in tested cell lines using flow cytometry (**Figure 4A-C**). After 72 h DHMEQ combined with either CDDP or CBP enhanced G2/M arrest in SKOV3 cells (**Figure 4A**). Over 70% of the cells experienced an arrest in the G2/M phase following 96 h treatment with platinum-based drugs and their combination with DHMEQ vs. the non-treated or DHMEQ only treated cells. Both combinations of DHMEQ with platinum-based drugs enhanced the cellular response to the treatment. Differences between the cells treated with CDDP alone and CDDP plus DHMEQ were significant. In A2780 cells exposed to the drugs for 72 h showed fewer cells at G1 and enlarged population at S and G2/M vs. control (**Figure 4B**). DHMEQ used alone increased the cytotoxic effects in A2780 cells, whereas a combination of DHMEQ with platinum-based drugs induced a non-significant enhancement in cytotoxicity in comparison with cells treated with CDDP or CBP alone. However, after 96 h of exposition of A2780 cells to the tested agents, we noticed a decrease in their percentage at G0/G1 accompanied by an increase in the number of cells at the G2/M phase. Moreover, the cells exposed to DHMEQ combined with platinum-based drugs, especially CBP, responded stronger to the treatment. In MRC-5 pd19 cells, the cell cycle analysis revealed accumulation of cells at phase G2/M at 72 h and 96 h after their exposure to the tested agents in comparison with the control or DHMEQ variant. This arrest was observed in platinum-based drugs alone and in combination with DHMEQ. However, the differences between the usage of single and combined agents were not observed.

A combination of DHMEQ and platinum-based drugs decrease the motility of A2780 cells

Since the NF-kB signalling pathway is responsible for numerous biological processes related to cancer invasiveness, we decided to test the

A

	SKOV3	SI	A2780	SI	MRC-5 pd 19
DHMEQ (DQ) [μg/ml]	6.48 (95% CI: 5.99-6.89)	2.03	5.31 (95% CI: 2.88-10.46)	2.48	13.15 (95% CI: 10.90-15.86)
Cisplatin (CDDP) [μg/ml]	0.926 (95% CI: 0.743-1.152)	0.80	0.127 (95% CI: 0.076-0.204)	5.86	0.744 (95% CI: 0.672-0.836)
Carboplatin (CBP) [μg/ml]	18.87 (95% CI: 15.63-22.79)	0.19	2.42 (95% CI: 2.10-2.80)	1.50	3.62 (95% CI: 3.22-4.07)

$$SI = \frac{IC_{50}\ MRC{-}5\ pd19\ cell\ line}{IC_{50}\ A2780\ or\ SKOV3\ cell\ line}$$

SI < 1 high toxicity
SI ≥ 1 low toxicity
SI > 3 highly selective agents

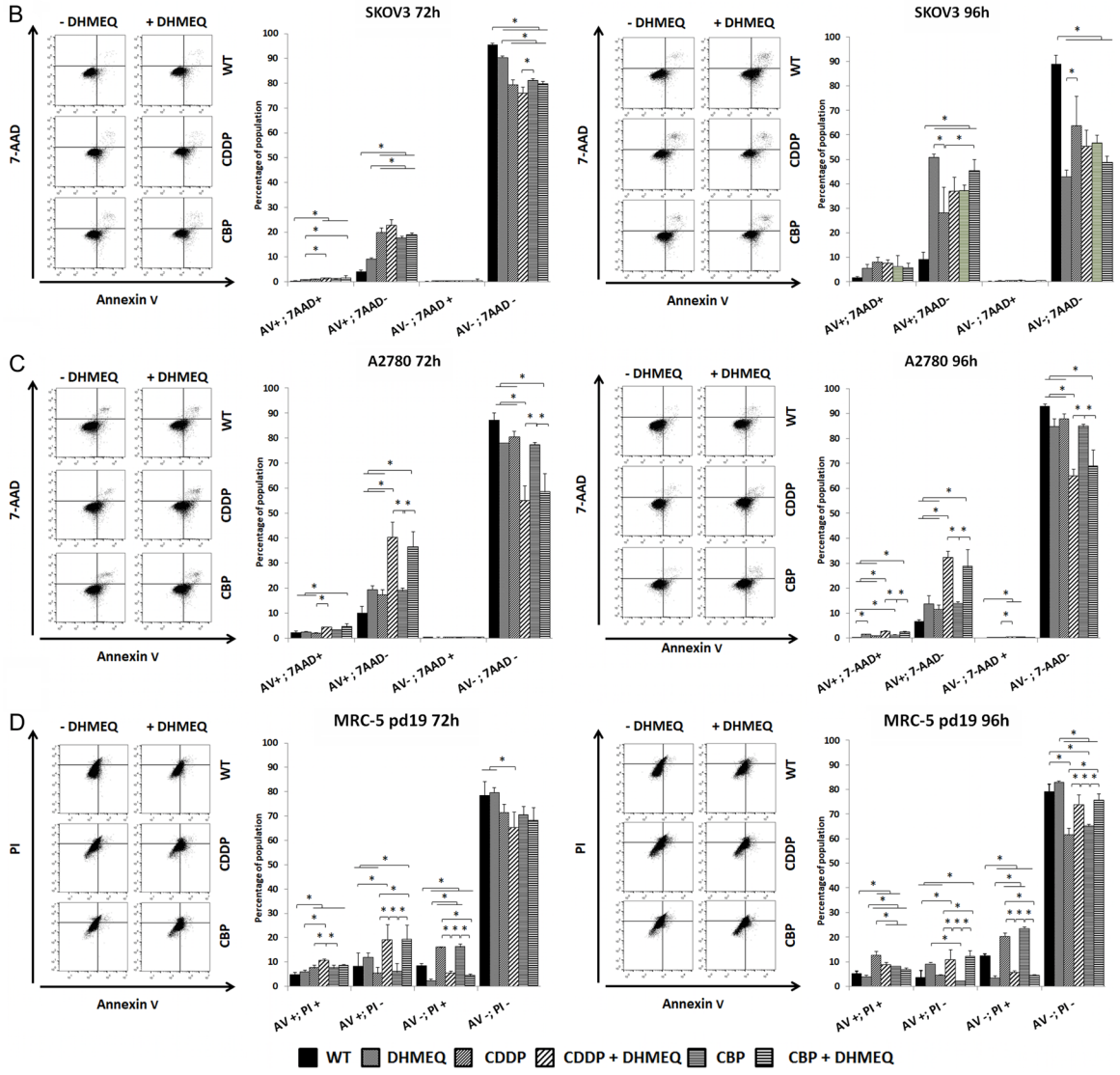

Figure 3. DHMEQ sensitizes ovarian cancer cells to platinum-based drugs. A. IC_{50} estimated *via* MTT assay at 72 h for DHMEQ (DQ), cisplatin (CDDP) and carboplatin (CBP) for OvCa and MRC-5 pd19 cell lines. The calculation of selectivity index (SI) has revealed moderate selective properties of DHMEQ against OvCa cells. SKOV3 cell line is more resistant to platinum-based drugs than A2780. B. Flow cytometry analysis of apoptosis in SKOV3 cell line exposed to DHMEQ and its combination with CDDP or CBP. Platinum-based drugs and their combinations with DHMEQ at 72 and 96 h revealed slight enhancement of cytotoxic effect in the cells exposed to combined variants (DHMEQ + CDDP and DHMEQ + CBP). Data are presented as mean ± SD. Statistical significance at $P<0.05$. The X-axis represents the percentage of the population. Left panels represent example cytograms generated during analysis. C. Flow cytometry analysis of apoptosis in A2780 cell line exposed to DHMEQ and its combination with CDDP or CBP. Platinum-based drugs and their combinations with DHMEQ at 72 and 96 h exerted a stronger effect in the cells exposed to combined variants than cisplatin alone. Data are presented as mean ± SD. Statistical significance at $P<0.05$. The X-axis represents the percentage of the population. Left panels represent example cytograms generated during analysis. D. Flow cytometry analysis of apoptosis in MRC-5 pd19 cell line exposed to DHMEQ and its combination with CDDP or CBP. DHMEQ alone and in combination with platinum-based drugs did not cause the strong reduction of viability of cells. Data are presented as mean ± SD. Statistical significance at $P<0.05$. The X-axis represents the percentage of the population. Left panels represent example cytograms generated during analysis.

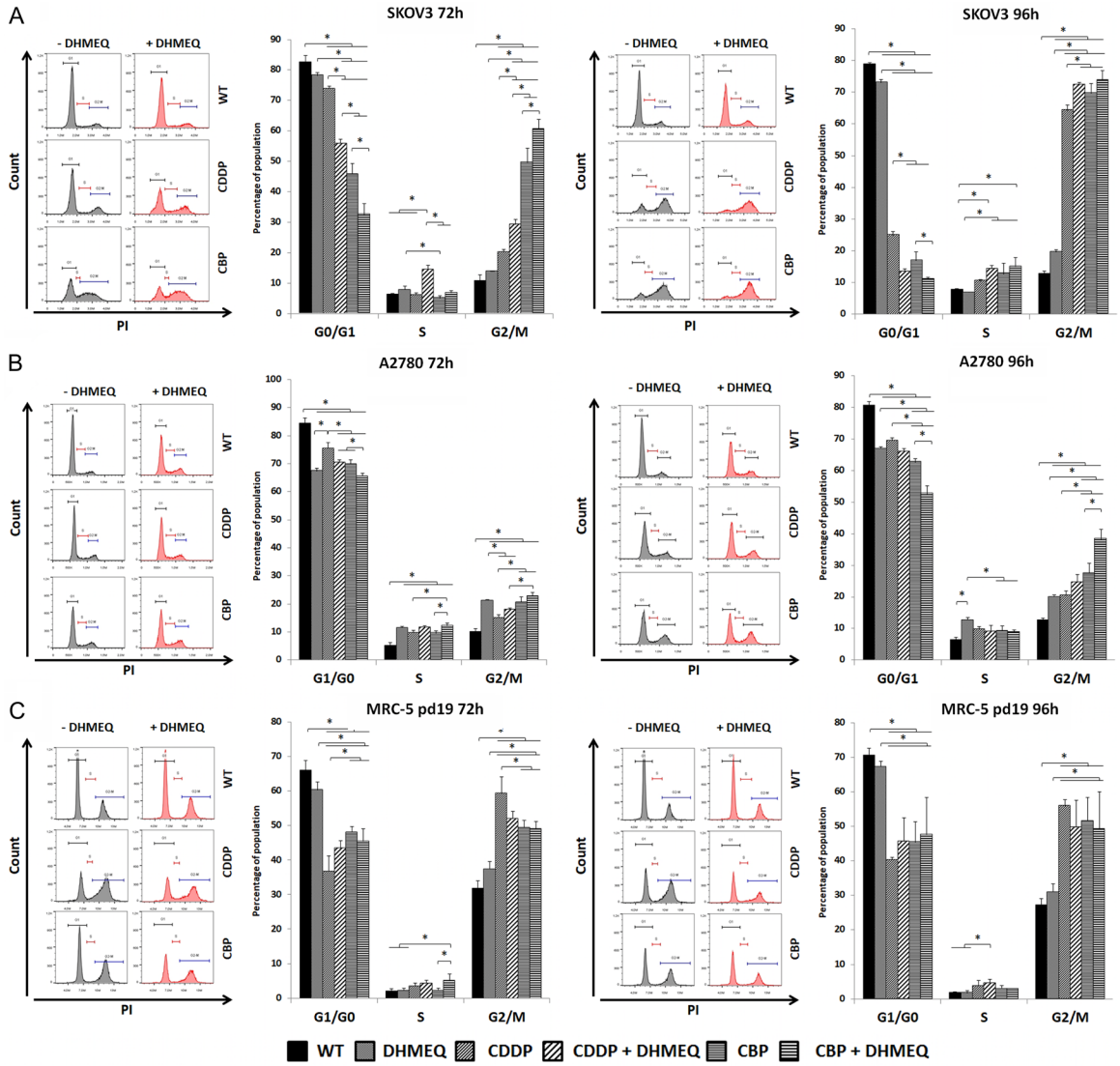

Figure 4. The combination of DHMEQ with platinum-based drugs enhances the cell cycle arrest in G2/M. A. Cell cycle phases distribution in SKOV3 cells incubated with DHMEQ, platinum-based drugs or their combination for 72 and 96 h (IC_{50} at 72 h). The combination of platinum-based drugs with DHMEQ enhanced the cell cycle arrest at the G2/M phase. Left panels show representative histograms, data are presented as mean percent ± SD of the determined cell cycle phases. B. Cell cycle phases distribution in A2780 cells incubated with DHMEQ, platinum-based drugs or their combination for 72 and 96 h (IC_{50} at 72 h). The combination of platinum-based drugs with DHMEQ slightly enhanced the cell cycle arrest at the G2/M phase, especially for DHMEQ + CBP variant. C. Cell cycle phases distribution in MRC-5 pd19 cells incubated with DHMEQ and CDDP or CBP in combination and separately. The arrest of the G2/M phase was observed only in platinum-based agents alone and combined with DHMEQ, but the enhanced effect was not observed in those combinations. Left panels show representative histograms, data are presented as mean percent ± SD of the determined cell cycle phases.

influence of DHMEQ onto motility and invasion in Matrigel of OvCa and normal cell lines (**Figure 5A**, **5B**). In SKOV3 the closure of the scratch was slightly inhibited in all variants combined with DHMEQ (**Figure 5A**). However, a significant delay of gap coverage was observed in CBP combined with DHMEQ than in CBP treated alone. In A2780 cells, the combination of DHMEQ with CDDP caused the reduced motility of cells in comparison with control but the biggest gap among studied variants was observed in DHMEQ used with CBP. In the case of MRC-5 pd19 cells, their motility was slightly inhibited by all drugs in comparison with the control (statistically non-significant). The invasion Matrigel assay did not reveal any significant changes in

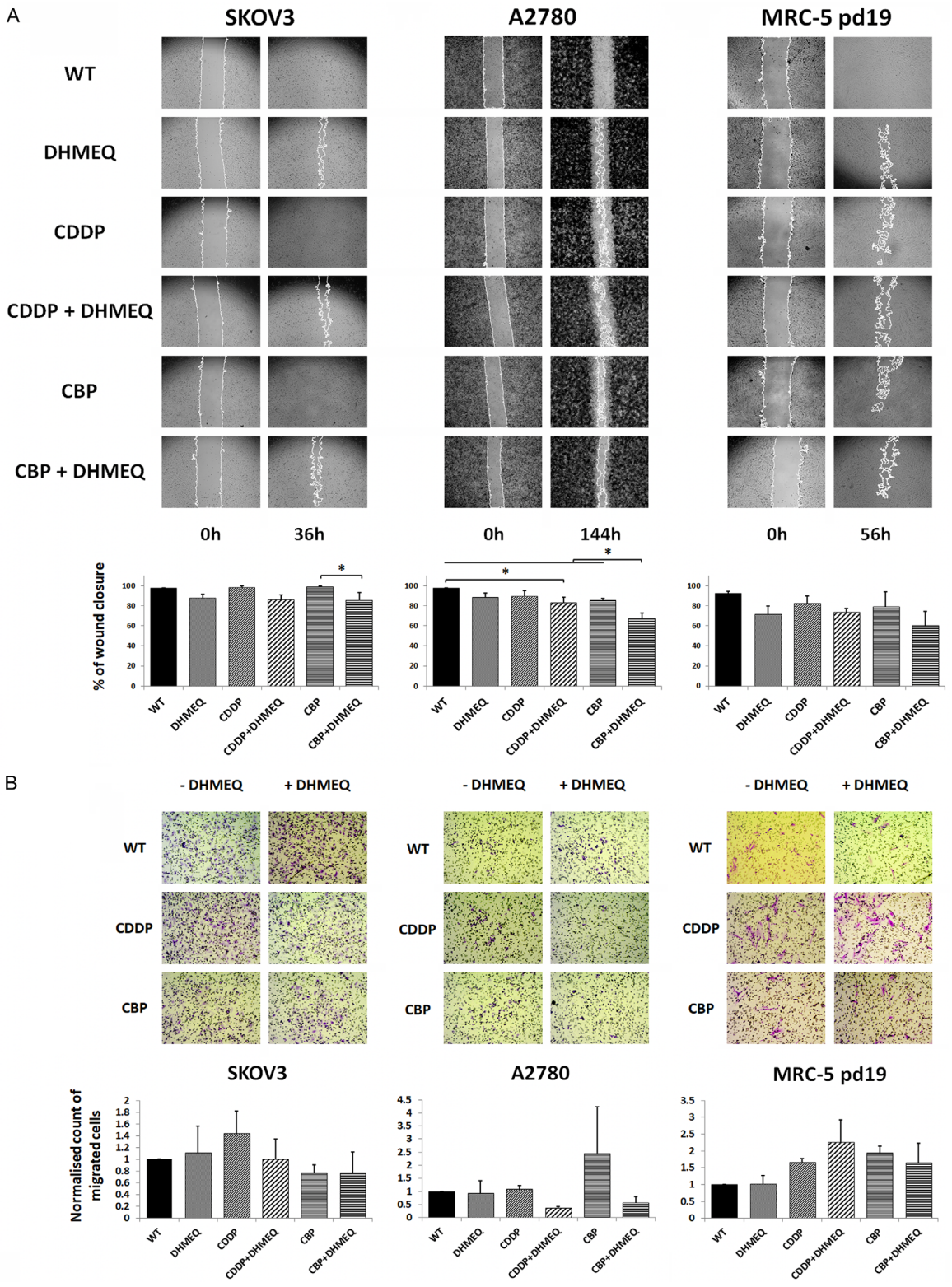

Figure 5. The effect of DHMEQ and its combination with platinum-based drugs on motility and invasive potential of SKOV3, A2780 and MRC-5 pd19 cell lines. A. The representative pictures of wound closure indicating the motility of studied cells (SKOV3, A2780, MRC-5 pd19) treated by DHMEQ, CDDP or CBP and their combinations. The pictures were taken under 40× magnification. The graphs represent the mean percentage of wound closure calculated from 3 independent experiments, error bars indicate the SD. The significant changes were acknowledged at $P<0.05$. B. The representative picture of invasion through the Matrigel of cells exposed to tested agents, pictures were taken under 100× magnification. The graphs represent the mean of a normalized count of migrated cells, error bars indicate ± SD.

tested cell lines (**Figure 5B**). Only in the A2780 cell line, the combination of CDDP or CBP with DHMEQ caused a decreasing trend of invasive potential (statistically non-significant).

A combination of DHMEQ and platinum-based drugs cause antagonistic effects in OvCa cell lines at low doses and a slightly synergistic effect at higher doses

To assess the therapeutic effect of the drug combination, the combination index (CI) and dose reduction index (DRI) were calculated using CompuSyn software (**Figure 6A**, **6B**; **Table 1**). The combination of DHMEQ with either CDDP or CBP enhanced growth inhibition in SKOV3 cells vs. the cells treated with individual drugs. The CI for DHMEQ combined with CDDP showed an antagonistic effect at low DHMEQ doses but with Fa>0.8 a synergistic effect was observed. Treatment of SKOV3 cells with DHMEQ and CBP resulted in a slight antagonistic effect, the CI value of which increased with the concentration of the combined drugs. The DRI for DHMEQ combined with CDDP indicated a possibility for CDDP dose reduction to achieve similar biological effects. This possibility was much lower for CBP.

The growth of A2780 cells (**Figure 6B**) was slightly more affected by treatment with DHMEQ alone than its combination with CDDP or CBP. The combination of DHMEQ with CBP inhibited the growth of A2780 cells more efficiently than DHMEQ plus CDDP. A simulation of CI indicated an antagonistic effect of DHMEQ combined with CDDP and a synergistic effect for Fa<0.8 for DHMEQ combined with CBP. Apart from the antagonistic effect of DHMEQ plus CDDP, the DRI value for CDDP increased along with CDDP dose, which could reduce the cytotoxic effect of the drug. However, the DRI values for DHMEQ were below 1, and its dose had to be enlarged to achieve similar biological effects. For DHMEQ combined with CBP, the DRI index for both drugs exceeded 1. Thus, a decrease in their concentrations enabled us to achieve similar biological effects.

Discussion

Despite remarkable improvements in ovarian cancer treatment and clinical outcomes since platinum derivates have been first introduced into clinical practice, about 70% of patients fail to succeed due to disease relapse [24, 25]. Activation of NF-κB strongly correlates with the development of a resistant phenotype through enhancement of antiapoptotic response, increased motility, migration abilities and upregulation of proangiogenic proteins [15, 26, 27]. Enhanced NF-κB activity and overexpression of Bcl-2 are responsible for the development of platinum-based antineoplastic resistance in ovarian cancer [28-30]. Moreover, constitutive activation of NF-κB dependent pathways results in increased production of IL-6 and IL-8 by ovarian cancer cells. This leads to immunosuppressive conditions within the tumour microenvironment, which in turn contributes to treatment failure [31, 32]. One of the potential therapeutic approaches is decreasing the activity of the NF-κB signalling pathway. For that purpose, DHMEQ, a molecule that targets the components of NF-κB, mainly RelB and p65, might be used. In epithelial ovarian cancer, it showed the ability to reduce the production of IL-6 and IL-8. Intraperitoneal administration of DHMEQ (5 mg kg^{-1} per day) in a murine model caused reverse immunosuppression of dendritic cells and macrophages and resulted in decreased tumour volume [33]. However, the effects of DHMEQ combined with platinum-based drugs on apoptosis and cell cycle distribution in ovarian cancer were not evaluated. In our preliminary study, we investigated SKOV3 and A2780 cell lines exposed to DHMEQ plus cisplatin or carboplatin-drugs commonly used in OvCa treatment. Additionally, we used the MRC-5 pd19 cell line, which represents a normal cell line. We are aware that these cells are originated from lung fibroblasts, but is commonly used in antineoplastic drugs assays, including OvCa [34-36]. MRC-5 pd19 cell line is also a recognized model of NF-κB activity and signalling in DNA damage response experiments [37].

The study examined the influence of inhibition of the NF-κB signalling pathway on cell viability, cell cycle progression, motility and invasion in the presence of the drugs. Additionally, the combination index of simultaneous administration of DHMEQ either CDDP or CBP in OvCa was verified with the Chou-Talay method [23]. Recently Momeny et al. demonstrated the usefulness and enhanced effect of cytotoxic drugs (cisplatin, carboplatin, paclitaxel and erlotinib) in combination with another inhibitor of NF-κB signalling pathway, named Bay 11-7082, in *in vitro* model compound acts as an inhibitor of IκBα phosphorylation. Similarly to our results,

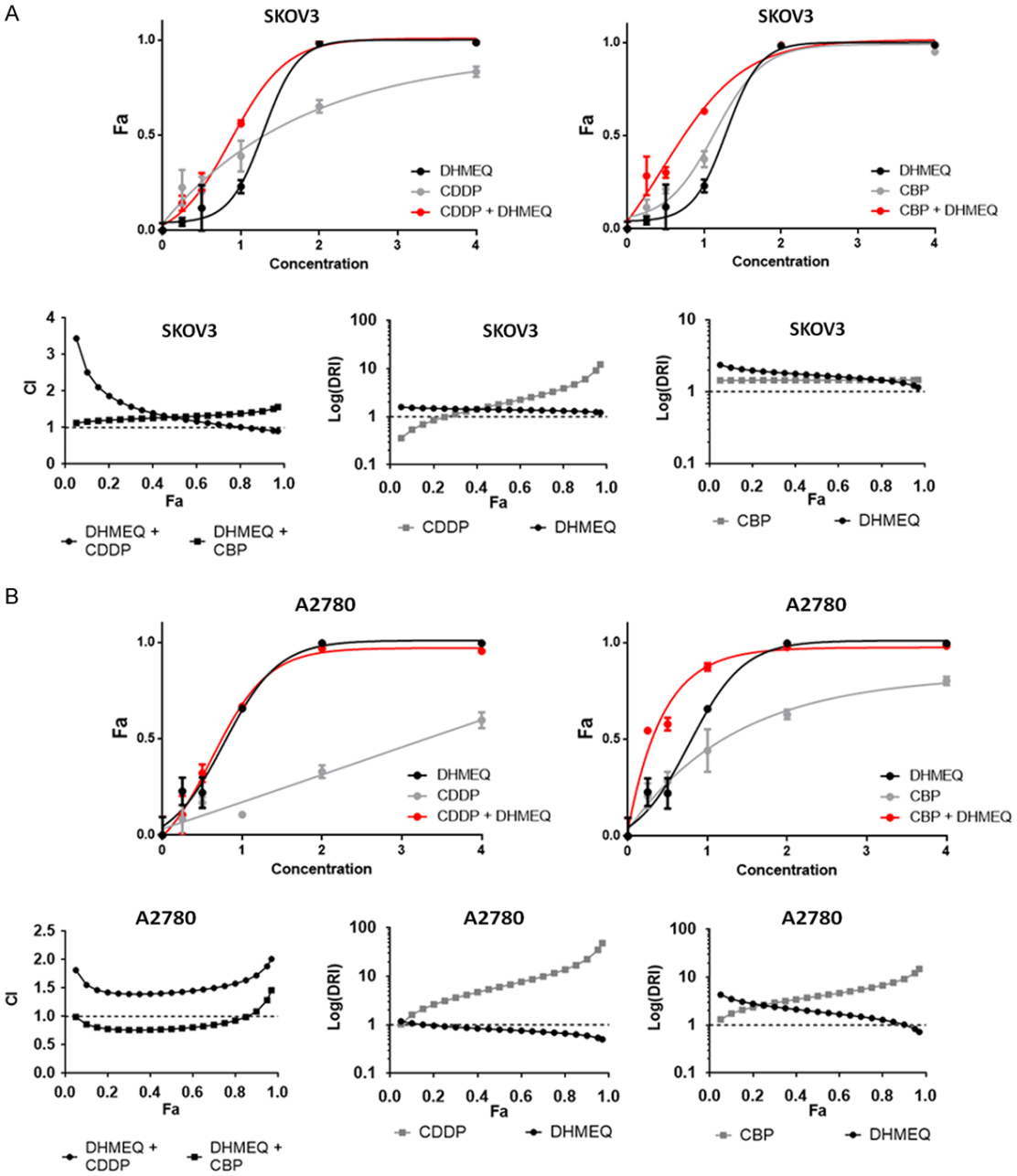

Figure 6. DHMEQ combined with platinum drugs exerts mostly antagonistic effects but it could limit the side effects of platinum drugs by decreasing their concentration and maintaining the same therapeutic effect. A. Survival curve of SKOV3 cells after 72 h incubation with DHMEQ, CDDP and a combination thereof (top left graph). Decreased viability of cells after exposure to the drug combination was observed. Survival curve of SKOV3 cells after 72 h incubation with DHMEQ, CBP and their combination (top right graph). Decreased viability of cells after exposure to the drug combination was observed. CompuSyn generated graphs for CI (bottom left graph) indicating the slightly antagonistic effect of the combination of DHMEQ and CDDP and slightly synergistic effect at higher doses of DHMEQ combined with CBP. B. Survival curves of A2780 cells after 72 h incubation with DHMEQ, CDDP and their combination (top left graph). Decreased viability of cells after exposure to the drug combination was observed. Survival curves of A2780 cells after 72 h incubation with DHMEQ, CBP and their combination (top right graph). The enhanced effects of combined drugs were indicated. CompuSyn generated graphs for CI (bottom left graph) indicating the slightly antagonistic effect of the combination of DHMEQ and CDDP and slightly synergistic effect at higher doses of DHMEQ combined with CBP.

Table 1. CI and DRI values estimated for the simultaneous combination of DHMEQ with either CDDP or CBP. Ovarian cancer cell lines (A2780 and SKOV3) were exposed to these compounds for 72 hours

A2780					
DHMEQ DOSE (μg/ml)	CDDP DOSE (μg/ml)	DHMEQ + CDDP			
		Fa	CI	DRI DHMEQ	DRI CDDP
21.24	0.51	0.95	2.68	0.38	26.53
10.62	0.25	0.97	1.17	0.86	78.82
5.31	0.13	0.67	1.60	0.67	8.42
2.66	0.06	0.32	1.50	0.82	3.57
1.33	0.03	0.11	1.60	1.02	1.61
A2780					
DHMEQ DOSE (μg/ml)	CBP DOSE (μg/ml)	DHMEQ + CBP			
		Fa	CI	DRI DHMEQ	DRI CBP
21.24	9.68	0.98	1.93	0.53	16.17
10.62	4.84	0.98	1.10	0.95	22.99
5.31	2.42	0.87	1.10	1.03	7.82
2.66	1.22	0.58	1.17	1.18	3.09
1.33	0.61	0.55	0.63	2.26	5.39
SKOV3					
DHMEQ DOSE (μg/ml)	CDDP DOSE (μg/ml)	DHMEQ + CDDP			
		Fa	CI	DRI DHMEQ	DRI CDDP
25.92	3.70	0.99	0.48	2.19	49.72
12.96	1.85	0.99	1.13	0.94	16.25
6.48	0.93	0.56	1.77	0.94	1.40
3.24	0.46	0.21	2.41	1.12	0.66
1.62	0.23	0.14	1.68	1.92	0.86
SKOV3					
DHMEQ DOSE (μg/ml)	CBP DOSE (μg/ml)	DHMEQ + CBP			
		Fa	CI	DRI DHMEQ	DRI CBP
25.92	75.50	0.99	1.92	0.90	1.25
12.96	37.75	0.99	0.91	1.88	2.65
6.48	18.87	0.63	2.02	1.04	0.95
3.24	9.44	0.30	1.74	1.32	1.02
1.62	4.72	0.28	0.90	2.56	1.97

the A2780 cell line showed an enhanced apoptotic response and decreased clonogenicity in comparison with the SKOV3 cell line. Bay 11-7082 induced cell death by declining the expression of Bcl-2 known as a marker of anoikis [38]. These findings suggest that DHMEQ might exert a similar effect. Watanabe et al. claim that DHMEQ is more beneficial than Bay 11-7082 in adult T-cell leukaemia due to higher specificity and more efficient induction of apoptosis. Moreover, Bay 11-7082 also activates p38, Jun N-terminal kinase-1 and tyrosine phosphorylation of 130 to 140 kDa unknown protein, which decreases its therapeutic value due to decreased target specificity [39, 40].

In our study, we observed enhanced apoptosis in the A2780 cell line but not in SKOV3 after exposure to DHMEQ. This is related to the properties of DHMEQ and the origin of those cell lines. In a study on hepatocellular carcinoma DHMEQ induced oxidative stress, i.e. elevated production of reactive oxygen species (ROS), which in turn led to increased formation of γH2AX foci and consequently apoptosis [41]. As a model of primary non-treated ovarian cancer A2780 cell line has less efficient repair mechanisms against DNA damage than the resistant SKOV3 cell line [42]. However, in both cell lines increased response to platinum drugs combined with DHMEQ was observed. Similar find-

ings were reported for bladder cancer, where a combination of DHMEQ (3 μg/ml) and IC_{50} of cisplatin or carboplatin caused a synergistic effect. Moreover, DHMEQ combined with paclitaxel decreased tumour volume and eliminated resistance in cisplatin-resistant bladder cancer [43]. In hepatoma cell lines DHMEQ induced apoptosis and cell cycle arrest in G0/G1 phase by downregulation of cyclin D1. Disparate effects were observed in our study, where G2/M arrest dominated [44]. Despite different mechanisms of DHMEQ influence on the cell cycle phases distribution, the changes were not significant even in combination with cisplatin and carboplatin. The target of platinum-based drugs is quite similar to that of ionizing radiation. They induce double and single-strand damage of DNA [45]. Similarly to our results, prostate cancer cells treated with DHMEQ alone or combined with irradiation (4Gy) experienced a massive arrest in the G2/M phase. It was explained by the fact that cdc2 kinase is strictly controlled by p21, p53 and 14-3-3σ, and their expression was upregulated by the combined treatment [46]. What interesting, in our experiment we have shown that DHMEQ is selective against cancer cell lines in comparison with MRC-5 pd19. Thus, DHMEQ did not enhance the cytotoxicity of combined treatment but increased G2/M cell cycle arrest. However, its dose was approximately twice as much used in comparison with OvCa cell lines. There is a lack of data about a comparison of selectivity of DHMEQ against malignant vs. normal cells. In the example of keloid tumour and normal skin fibroblasts, their exposure to DHMEQ has led to significantly decreased growth of keloid fibroblasts at concentrations 5 μg/ml and 10 μg/ml compared to normal fibroblasts [47]. Based on the *in vivo* models of several malignant diseases (i.e. prostate, bladder, breast, thyroid, and bile duct cancer) the lack of toxicity of DHMEQ against normal tissues through peritoneal administration was observed [43, 48-53].

One of the issues related to the progression of malignant disease is the acquired ability of cancer cells to migrate and invade the local and distal sites of the organism. We indicated that the combination of DHMEQ with CBP decreased the motility of cells in both OvCa cell lines. However, did not affect significantly the invasion of cells through the matrigel-coated membrane. In general, there is a lack of data about the influence of the combination of DHMEQ with antineoplastic drugs on the invasiveness of malignant cells. A few studies suggested that DHMEQ used as a single agent can decrease the metastatic potential of cancer cells. In the example of ovarian cancer cell lines (RMG1 and ES-1), DHMEQ caused the inhibition of cellular invasiveness by decreasing the expression of proteins involved in the CXCL12/CXCL4 signalling pathway (related to NF-κB activity), which upregulation correlates with the poor prognosis of several cancers [54, 55]. Moreover, its usage has led to a decrease in the expression of proteins associated with metastasis such as metalloproteinase 9 (MMP-9) or urokinase-type plasminogen activator (uPA) [54]. A similar effect was observed in nasopharyngeal carcinoma, where DHMEQ used alone caused downregulation in a dose-dependent manner MMP-9, Intercellular Adhesion Molecule 1 (ICAM-1) and Vascular Endothelial Growth Factor (VEGF) [56]. The study of Pires group revealed that the NF-κB pathway is also responsible for the control of epithelial-mesenchymal transition (EMT) in breast cancer and the invasive potential could be inhibited using DHMEQ [57]. Furthermore, its addition to triple-negative breast cancer cell line spheres caused the limited formation of invasive sprouts via downregulation of MMP-2 [58].

In our experiment, we calculated the interactions between drugs using the Chou-Talalay method. We indicated that CBP affected the cell growth of the primary platinum-sensitive ovarian cancer cell line (A2780) more effectively (compared with CDDP) when combined with DHMEQ. On the other hand, CDDP was more effective (but only at high doses) in combination with DHMEQ for metastatic ovarian cancer (SKOV3). We did not observe a strong response of simultaneously administered agents as expected, which correlates with the CI calculations for the used IC_{50} dose. Only at higher doses of cisplatin, the effect was synergistic. This correlates with the data of studies regarding cholangiocarcinoma and osteosarcoma, where the simultaneous combination of DHMEQ with cisplatin caused a stronger reduction of cancer cells viability than drugs tested alone [59, 60]. The simultaneous administration seems to be an adequate combination since the damage caused by the administration of

platinum-based drugs activate the NF-κB signalling pathway, which could prevent from development of resistance to this kind of treatment, especially in ovarian carcinoma [61, 62].

The application of NF-κB signalling pathway inhibitors is a promising approach that could improve the response to platinum-based drugs and overcome the resistance of ovarian cancer [15]. However, Yang et al. pinpointed the dualistic role of NF-κB in ovarian cancer. It could act as an enhancer or suppressor through the regulation of MAPK or cellular apoptosis. This means that permanent activation of NF-κB inhibited tumour growth in ovarian cancer cells but enhanced growth in chemoresistant cell lines. The team also showed higher survival rates and better response to cytotoxic drugs in a group of patients diagnosed with high-grade serous ovarian cancer that featured increased nuclear expression of p65 [63]. Another exception to the rule that overexpression of NF-κB in cancer cells correlated with the poor outcome is data published by Baba et al. [41]. They demonstrated that strong cytoplasmic expression of NF-κB in triple-negative breast cancer before neoadjuvant chemotherapy corresponded to a good prognosis and was not associated with a response to neoadjuvant chemotherapy. Thus, in triple-negative breast cancer, high cytoplasmic NF-κB/p65 expression may be considered a good prognostic factor [64]. That is why appropriate markers should match the type of patients, in whom the application of those inhibitors will benefit the treatment.

The ambiguous role of NF-κB revealed in different cancer cell lines mean that its inhibitors should not be considered as universal anticancer drugs, since their antineoplastic activity may be detected in selected cancer types. There is a need for the development of predictive factors, which indicate the benefits of the implementation of NF-κB inhibitors during anticancer treatment. Of clinical interest is a phenomenon of taking dormant cancer cells at phase G0/G1 and pushing them into the cell cycle, in turn, exposes them to phase-specific cytotoxic agents like platinum derivatives and halts cell cycle at the stage G2/M.

Conclusions

Since intrinsic or acquired platinum resistance of ovarian cancer is still the most challenging issue, any approach that could solve the problem is welcome.

NF-κB is a promising and attractive target for the further development of novel diagnostic and therapeutic modalities in ovarian cancer treatment.

A combination of DHMEQ with CDDP or CBP could be a new solution in ovarian cancer treatment. However, their combination ratio and schedule of administration (sequential or simultaneous) should be further evaluated to achieve more efficient therapeutic effects.

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Disclosure of conflict of interest

None.

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