Valspodar-modulated chemotherapy in human ovarian cancer cells SK-OV-3 and MDAH-2774

Maciej Zalewski[1], Julita Kulbacka[2], Jolanta Saczko[2], Małgorzata Drag-Zalesinska[3], Anna Choromanska[4*]

[1]Department of Gynecology and Obstetrics, Wroclaw Medical University, Wroclaw, Poland, [2]Department of Molecular and Cellular Biology, Wroclaw Medical University, Wroclaw, Poland, [3]Department of Human Morphology and Embryology, Wroclaw Medical University, Wroclaw, Poland, [4]Department of Medical Biochemistry, Wroclaw Medical University, Wroclaw, Poland

ABSTRACT

Overcoming drug resistance in ovarian cancer is the overarching goal in gynecologic oncology. One way to increase drug cytotoxicity without increasing the drug dose is to simultaneously apply multidrug resistance modulator. Valspodar is the second generation P-glycoprotein 1 modulator capable of reversing multidrug resistance in different cancers. In this study, we evaluated the effect of valspodar and cisplatin co-treatment on cell viability, cell death and oxidative status in ovarian cancer cells. Two human ovarian cancer cell lines SK-OV-3 and MDAH-2774 were treated with cisplatin, valspodar, or cisplatin + valspodar for 24 or 48 hours. Untreated cells were used as control group. Cell viability was evaluated by MTT assay. Cell death was assessed by TUNEL and comet assay. Lipid peroxidation (malondialdehyde) and protein thiol groups were analyzed as oxidative stress markers. The expression of mitochondrial superoxide dismutase (MnSOD) was assessed by immunocytochemistry. Valspodar effectively reduced the resistance of SK-OV-3 cells to cisplatin, as demonstrated by increased oxidative stress, decreased cell viability and increased apoptosis in SK-OV-3 cells co-treated with valspodar and cisplatin compared to other groups. However, valspodar did not significantly affect the resistance of MDAH-2774 cells to cisplatin. Stronger staining for MnSOD in MDAH-2774 vs. SK-OV-3 cells after co-treatment with cisplatin and valspodar may determine the resistance of MDAH-2774 cell line to cisplatin.

KEY WORDS: Ovarian carcinoma; cisplatin; valspodar; oxidative stress
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INTRODUCTION

Ovarian carcinogenesis is a complex and multifactorial process. Considerable diversity in histological appearance of ovarian cancer makes it difficult to understand the mechanism of its development. Ovarian cancer is frequent, along with breast, cervical, colorectal, lung, stomach, and endometrial cancer [1], and its incidence is rising. It commonly occurs shortly before or after menopause [2]. Newly diagnosed ovarian cancer patients are treated with a combination of surgery and chemotherapy. The purpose of primary surgical procedure is to confirm the diagnosis, determine the stage of lesion progression and whether complete or optimal cytoreduction of the tumor was achieved. The extent of surgery and complementary therapy depend mainly on the clinical stage of ovarian cancer [3]. Despite high response rates after initial treatment with cytotoxic drugs, the five-year survival rate of ovarian cancer patients is at most 30%. Similarly, the response rate after secondary treatment in patients with relapse remains disappointingly low, due to the selection of tumor cell clones that are resistant to cytotoxic drugs. This phenomenon is described as multidrug resistance (MDR) and it is related to a decreased susceptibility of cancer cells to apoptosis. MDR of cancer cells is the main obstacle for successful treatment with chemotherapy [4]. Overcoming drug resistance in ovarian cancer is the overarching goal in gynecologic oncology. One way to increase drug cytotoxicity without increasing the drug dose is to simultaneously apply MDR modulator. By targeting MDR genes modulators can change the expression of proteins that cause resistance of cancer cells. P-glycoprotein 1 (P-gp) is a membrane protein known to cause MDR in cancer [5,6]. Strategies to inhibit or bypass P-gp and thus sensitize cancer cells to chemotherapy are constantly being studied for improvement. To date, three generations of P-gp modulators have been developed [7]. Valspodar is the second generation P-gp modulator, which acts by binding to P-gp in the cell membrane [8]. The antitumor efficacy of concurrent administration

*Corresponding author: Anna Choromanska, Department of Medical Biochemistry, Wroclaw Medical University, Chalubinskiego 10, 50-368 Wroclaw, Poland. Phone: +48 71 784 18 87; Fax: +48 71 784 00 85. E-mail: anna.choromanska@umed.wroc.pl

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of cytotoxic drugs and modulators has been reported by many preclinical and clinical studies. For example, concurrent treatment with valspodar and mitoxantrone resulted in a greater reduction of tumor size in mice compared to treatment with anticancer drug alone [9]. Furthermore, valspodar has shown the capacity to modulate ovarian cancer resistance in phase I/II, II, and III clinical trials [10,11].

Cisplatin is one of the most commonly used chemotherapy drugs in ovarian cancer, despite its severe side effects and development of resistance of cancer cells to cisplatin cytotoxic effect. Nevertheless, promising results in the treatment of ovarian cancer were reported [12] with cisplatin used either alone as a liposome-encapsulated drug or in combination with other chemical agents, such as withaferin, trichostatin A, and 5-aza-2′-deoxycytidine [13].

In this study, we evaluated the effect of valspodar and cisplatin co-treatment on cell viability, cell death, and oxidative status in human ovarian cancer cells.

MATERIALS AND METHODS

Cell culture

We used two human ovarian cancer cell lines SK-OV-3 (ovarian cancer cells resistant to diphtheria toxin, cisplatin, and adriamycin) and MDAH-2774 (ovarian endometrioid adenocarcinoma, sensitive to cisplatin). The SK-OV-3 cell line was a kind gift from Prof. J. Golab from the Department of Immunology, Center of Biostructure Research at Medical University of Warsaw. The MDAH-2774 cell line was purchased from ATCC® (Manassas, VA, USA). Cells were cultured as a monolayer in culture flasks (Falcon). Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St Louis, MO, USA) was used as the culture medium with the addition of 2 mM L-glutamine, 10% fetal bovine serum (FBS, Sigma-Aldrich, St Louis, USA) and 50 μg/ml streptomycin (Sigma-Aldrich, St Louis, MO, USA). The cells were grown at 37°C in 5% CO_2. After 48 hours the cells were removed from culture flasks by trypsinization (trypsin 0.25% and EDTA 0.02%; Sigma-Aldrich, St Louis, MO, USA) and washed with PBS.

Chemotherapy

Cisplatin was used as a chemotherapeutic drug and valspodar as an MDR modulator; both were purchased from Sigma-Aldrich (St Louis, MO, USA). The concentration range of cisplatin and modulator was chosen based on previous studies [14-16] and it was 10–50 μM for cisplatin and 0.5–8 μM for valspodar. SK-OV-3 and MDAH-2774 were treated with cisplatin, valspodar, or cisplatin+valspodar for 24 or 48 hours. Untreated cells were used as control group.

Cytotoxicity evaluation – MTT assay

Cells were incubated with cisplatin and/or valspodar for 24 hours or 48 hours. After incubation, cell viability was assessed by MTT assay (Sigma-Aldrich, St Louis, USA), which is a colorimetric assay that indirectly measures mitochondrial dehydrogenase activity. SK-OV-3 and MDAH-2774 cells were grown on 96-well plates at a concentration of 1×10^4 cells per well. The absorbance was measured at 570 nm on a microplate reader (Enspire, PerkinElmer, USA). Cell viability was expressed as a percentage of untreated control cells.

Lipid peroxidation

Lipid peroxidation levels were assessed by measuring the level of malondialdehyde (MDA), which is a final product of lipid peroxidation. In this assay, MDA reacts with thiobarbituric acid (TBA) and forms MDA-TBA adduct. The level of MDA-TBA complex was measured spectroscopically at 535 nm, and the concentration of MDA was determined using a set of MDA standards of known concentration [17].

The level of thiol groups

The level of protein thiol groups was assessed according to the Ellman method [18].

Immunocytochemical analysis of mitochondrial superoxide dismutase (MnSOD)

Immunocytochemistry was performed using the avidin-biotin complex method. The cells were plated onto microscope slides. After incubation with cisplatin and/or valspodar for 24 hours the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA). Then, they were permeabilized with 0.1% Triton X-100 in PBS and blocked with 10% goat serum in PBS (to exclude non-specific bonds). The polyclonal primary antibody SOD2 (Santa Cruz Biotechnology, Inc., USA) was diluted to 1:100 concentration and applied to microscope slides, followed by an overnight incubation at 4°C. To visualize the peroxidase activity, the samples were incubated with diaminobenzidine-H_2O_2 mixture. Microscopic analysis was performed on an Olympus BX51 (Japan) and 100 cells from any chosen field were evaluated. The reaction was considered positive if it occurred in at least 5% of the assessed cells. The intensity of the reaction was evaluated as: negative (-), weak (+), moderate (++), and strong (+++).

Neutral comet assay

The comet assay was carried out under neutral conditions, as described by Collins et al. [19]. For visual scoring, 100–200 nuclei from each slide were evaluated.

TUNEL assay

Qualitative analysis of apoptotic activity was performed by the TUNEL method using an ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore, Germany), according to the manufacturer's instructions. This assay is based on the most important feature of apoptosis, DNA fragmentation, and was performed on fixed sections of 180-base-pair long DNA fragments. These small DNA fragments contain free 3'-OH ends that attach digoxigenin-labeled nucleotides in a reaction catalyzed by DNA nucleotidylexotransferase (TdT - terminal deoxynucleotidyl transferase).

Statistical analysis

All samples were analyzed in triplicates. Statistical significance was determined by unpaired Student's *t*-test vs. untreated control group, where $p \leq 0.05$ was assumed as statistically significant. Statistical analysis was performed in Microsoft Excel 2010.

RESULTS

To investigate the effect of a combined cisplatin + valspodar treatment on cell viability, cell death and oxidative status in ovarian cancer cells, SK-OV-3 and MDAH-2774 were treated with cisplatin, valspodar, or cisplatin + valspodar for 24 hours or 48 hours. Untreated cells were used as control group.

Cytotoxicity evaluation – MTT assay

In the first stage, SK-OV-3 and MDAH-2774 cells were incubated with cisplatin (10–50 μM) or valspodar (0.5–8 μM) for 24 hours to evaluate their cytotoxic effects (Figure 1A and B). After 24 hours of incubation, cisplatin did not induce cytotoxic effects in cells within the tested concentration range. In SK-OV-3 and MDAH-2774 cells treated with 50 μM cisplatin, the viability of cells was 78% and 90%, respectively. Similarly, treatment with valspodar did not cause a significant decrease in cell viability within the tested concentration range. At the highest valspodar concentration (8 μM), the viability of SK-OV-3 and MDAH-2774 was 87% and 96%, respectively. For the subsequent experiments, the highest drug and modulator concentrations were used.

The co-treatment of SK-OV-3 and MDAH-2774 cells with cisplatin and valspodar for 24 hours increased the effectiveness of cisplatin in ovarian cancer cells. However, SK-OV-3 cell line was much more sensitive to the combination of cisplatin and valspodar compared to MDAH-2774 cells. As the incubation time increased, the viability of SK-OV-3 cells decreased. I.e., after 24 hours of incubation the cell viability was 61% (Figure 2A), and after additional 24 hours of incubation (in total 48-hour incubation) the cell viability decreased below 32% (Figure 2B). Under the same conditions, the viability of MDAH-2774 cells was 85% after 24-hour incubation with cisplatin and valspodar and 52% after 48-hour incubation (Figure 2A and B).

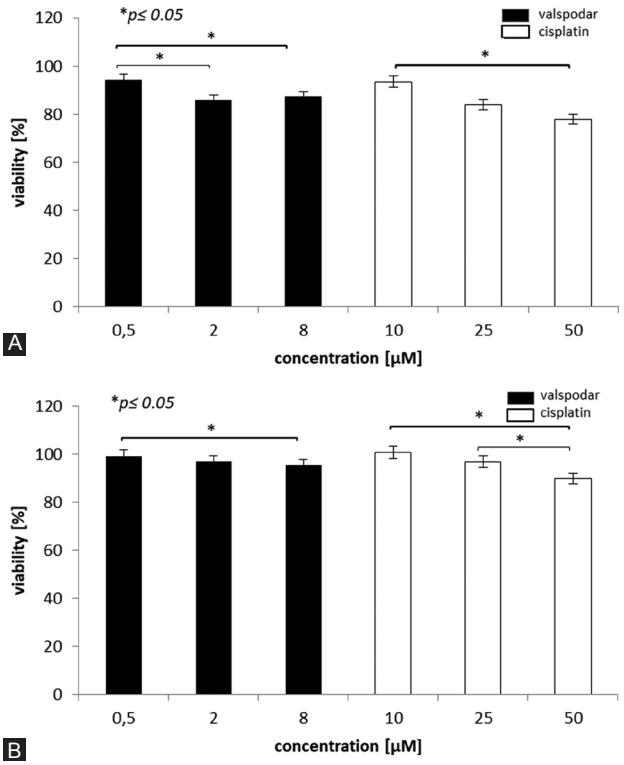

FIGURE 1. Cytotoxicity of cisplatin and valspodar in SK-OV-3 (A) and MDAH-2774 (B) ovarian cancer cell lines after 24-hour incubation. The effects of cisplatin and valspodar on cell viability were determined by MTT assay. Each column shows cell viability (%), error bars are expressed as mean ± SD for n ≥ 3; *$p \leq 0.05$. Mitochondrial metabolic function was expressed as the percentage of viable treated cells in relation to untreated control cells. The 24-hour incubation with cisplatin did not induce cytotoxic effects in ovarian cancer cells. In SK-OV-3 and MDAH-2774 cells treated with 50 μM cisplatin, the viability of cells was 78% and 90%, respectively. Similarly, treatment with valspodar did not cause a significant decrease in cell viability. At the highest valspodar concentration, the viability of SK-OV-3 and MDAH-2774 was 87% and 96%, respectively.

Lipid peroxidation and protein thiol groups

Significant changes in the level of oxidative stress markers following the treatments were observed only in SK-OV-3 cells. After 24-hour incubation of SK-OV-3 cells with cisplatin or cisplatin + valspodar, the level of MDA increased from 0.35 μM/l in untreated control cells to 0.42 μM/l in cisplatin and 0.47 μM/l in cisplatin + valspodar treated cells (Figure 3A). The level of MDA in treated MDAH-2774 cells in all groups was comparable to MDA level in control group [0.41μM/l] (Figure 3A).

A decrease in the level of thiol groups was observed only in SK-OV-3 cell line. In untreated control cells, the level of -SH

groups was 23.1 nmol/mg of protein. In SK-OV-3 cells treated with valspodar for 24 hours, a slight decrease in thiol groups (22.2 nmol/mg of protein) was observed compared to control. The 24-hour treatment of SK-OV-3 cells with cisplatin caused higher decrease in the level of thiol groups (20.7 nmol/mg of protein) compared to valspodar and control groups. However, a significant reduction in thiol group levels was observed in SK-OV-3 cells co-treated with cisplatin and valspodar for 24 hours (17.7 nmol/mg of protein, Figure 3B), compared to the other SK-OV-3 cell groups. In MDAH-2774 cells, the level of protein thiol groups remained constant in all treatment groups and comparable to that in control group (19 nmol/mg of protein, Figure 3B).

Immunocytochemical analysis of MnSOD

The detection of MnSOD expression in SK-OV-3 and MDAH-2774 cell lines is shown in Figure 4 and semi-quantitative results are presented in Table 1. A slight increase in MnSOD protein expression was observed in SK-OV-3 and MDAH-2774 cells treated with cisplatin for 24 hours compared to control cells. Treatment with valspodar alone did not change the expression of MnSOD in both cell lines. This expression was at the level of control. Distinct differences in the staining intensity were observed in MDAH-2774 cells co-treated with cisplatin and valspodar for 24 hours compared to control cells. In SK-OV-3 cells, the co-treatment with cisplatin and valspodar for 24 hours did not cause significant changes in staining intensity compared to cells treated with cisplatin alone.

Neutral comet assay

Under neutral non-denaturing conditions, the comet assay detects double-strand DNA breaks and it is therefore suitable for the detection of apoptosis. With this method, we evaluated the percentage of apoptotic cells in treated SK-OV-3 and MDAH-2774 cells (Figure 5A and B). In both cell lines, we observed the highest number of damaged cells in groups co-treated with cisplatin and valspodar for 24 hours. Furthermore, this effect was more pronounced in SK-OV-3 cells (47% apoptotic cells, Figure 5A) than in MDAH-2774 cells (18% apoptotic cells: 62% undamaged cells, Figure 5B).

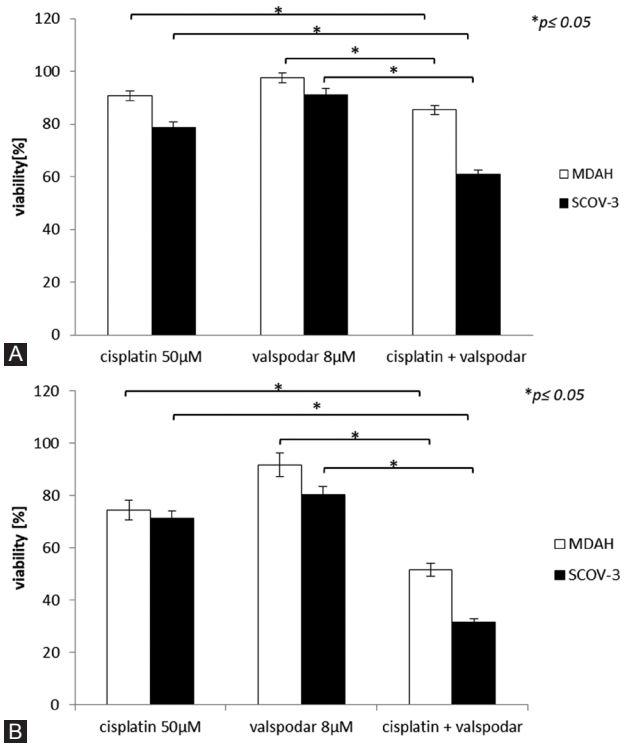

FIGURE 2. Cytotoxicity of cisplatin (50 μM), valspodar (8 μM) and cisplatin + valspodar in SK-OV-3 and MDAH-2774 ovarian cancer cells after 24 hours (A) and 48 hours (B) of incubation. Cell viability was determined by MTT assay. Each column shows cell viability (%), error bars are expressed as mean ± SD for n ≥ 3; *$p \leq 0.05$. Mitochondrial metabolic function was expressed as the percentage of viable treated cells in relation to untreated control cells. SK-OV-3 cell line was more sensitive to the combination of cisplatin and valspodar compared to MDAH-2774 cells. After 24 hours of incubation, SK-OV-3 cell viability was 61%, and after additional 24 hours of incubation the cell viability decreased below 32%. Under the same conditions, the viability of MDAH-2774 cells was 85% after 24-hour incubation and 52% after 48-hour incubation.

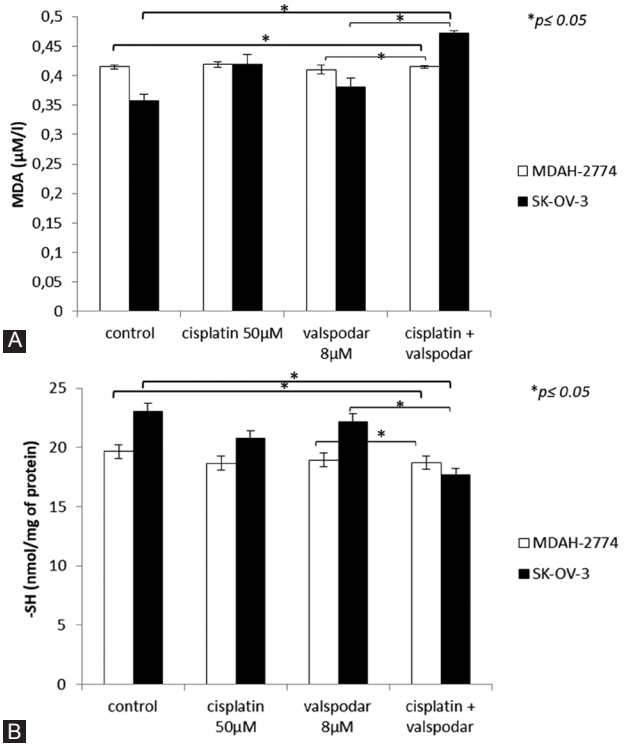

FIGURE 3. The level of oxidative stress markers after 24-hour incubation with cisplatin (50 μM), valspodar (8 μM) or cisplatin + valspodar in SK-OV-3 and MDAH-2774 ovarian cancer cells. Graph A depicts MDA levels, graph B depicts the level of thiol groups. Error bars are expressed as mean ± SD for n = 3. *Statistically significant at $p \leq 0.05$. Significant changes in the level of oxidative stress markers following the treatments were observed only in SK-OV-3 cells.

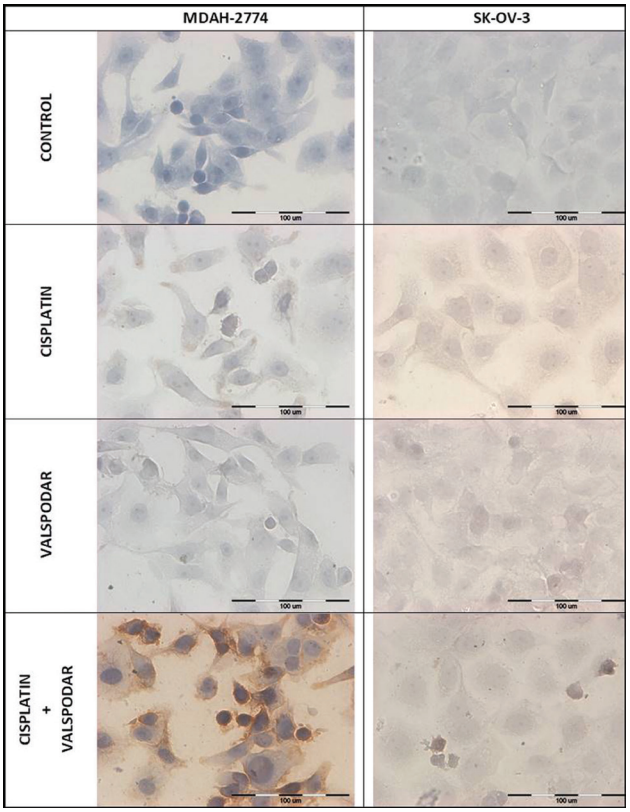

FIGURE 4. Immunocytochemical analysis of MnSOD expression in SK-OV-3 and MDAH-2774 ovarian cancer cell lines after 24-hour incubation with cisplatin, valspodar, or cisplatin + valspodar. Results represent the percentage of positively stained cells and are expressed as the mean number ± SD of cells counted in three fields (×400). The staining reaction was evaluated as: (-) negative, no reaction, (+) weak, (++) moderate, and (+++) strong. Distinct differences in the staining intensity were observed in MDAH-2774 cells co-treated with cisplatin and valspodar for 24 hours compared to control cells. In SK-OV-3 cells, the co-treatment with cisplatin and valspodar for 24 hours did not cause significant changes in staining intensity compared to cells treated with cisplatin alone. MnSOD: Mitochondrial superoxide dismutase.

TABLE 1. Immunocytochemical analysis of MnSOD expression in SK-OV-3 and MDAH-2774 ovarian cancer cells

Groups		Staining intensity	Positively stained cells [%]
SK-OV-3	Untreated control	-	0
	Cisplatin	+	95
	Valspodar	-	0s
	Cisplatin+Valspodar	+	100
MDAH-2774	Untreated control	-	0
	Cisplatin	+	95
	Valspodar	-	0
	Cisplatin+Valspodar	+++	100

MnSOD: Mitochondrial superoxide dismutase

TUNEL analysis

TUNEL assay was used to confirm the induction of apoptosis in treated SK-OV-3 and MDAH-2774 cells (Table 2). The staining was the strongest in cells treated with cisplatin and valspodar for 24 hours (Figure 6). Apoptotic cells were also observed in SK-OV-3 and MDAH-2774 cells treated with

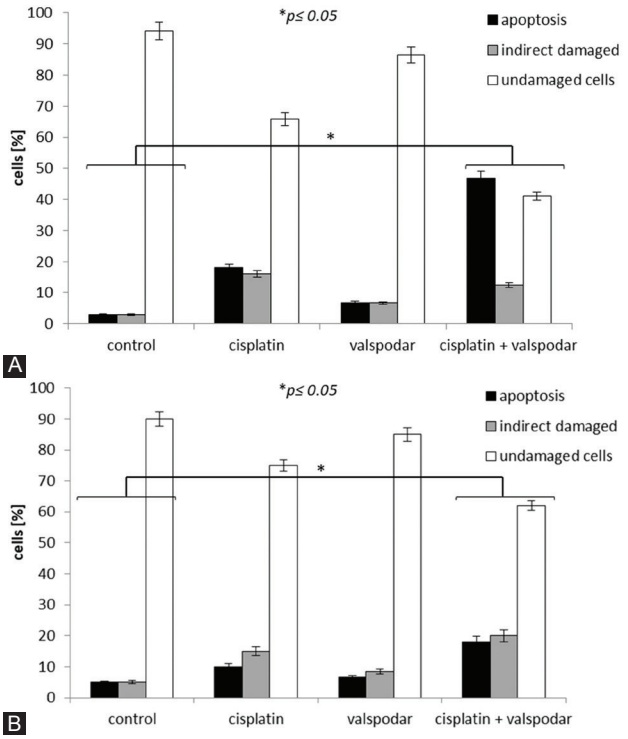

FIGURE 5. Percentage of apoptotic, indirectly damaged and undamaged SK-OV-3 (A) and MDAH-2774 (B) ovarian cancer cells after 24-hour incubation with cisplatin, valspodar or cisplatin + valspodar. Error bars are expressed as mean ± SD for n = 3. *Statistically significant at $p \leq 0.05$. In both cell lines, the highest number of damaged cells was observed in groups co-treated with cisplatin and valspodar for 24 hours. This effect was more pronounced in SK-OV-3 cells (47% apoptotic cells) than in MDAH-2774 cells (18% apoptotic cells).

cisplatin, but to a lesser extent than in cells co-treated with cisplatin and valspodar. The TUNEL assay results confirmed the results of neutral comet assay.

DISCUSSION

It is estimated that approximately 70% of ovarian cancer cases are diagnosed at an advanced stage of the disease. The response rate to chemotherapy is 60–80% and the 5-year survival rate is 15–20%. Classical treatment approaches to ovarian cancer do not yield the expected results. The main obstacle is the resistance of primary or secondary cancer cells to chemotherapy or radiotherapy [12]. The effectiveness of cytotoxic drugs depends on proteins involved in the defense mechanisms of cells. The level and conformational changes of these proteins and mutations in the associated genes affect the cell response to treatment. Examples of such proteins are p53, glutathione S-transferase pi (GSTP), heat shock protein (HSP) family, and SOD [14]. Cancer MDR refers to insensitivity of cancer cells to multiple anticancer drugs. MDR can be primary and secondary. Primary resistance is defined as the insensitivity of cancer cells to initial drug treatment, which may be caused by a lack of appropriate receptors on

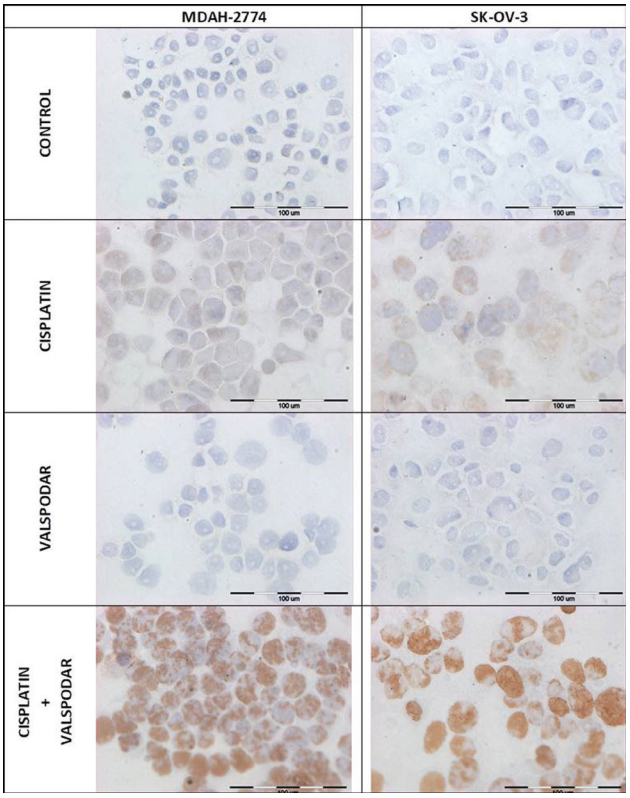

FIGURE 6. TUNEL analysis of SK-OV-3 and MDAH-2774 ovarian cancer cell lines after 24-hour incubation with cisplatin, valspodar or cisplatin + valspodar. Results are presented as the percentage of positively stained cells and expressed as the mean number ± SD of cells counted in three fields (×400). The staining was the strongest in cells treated with cisplatin and valspodar for 24 hours in both cell lines.

TABLE 2. TUNEL analysis of SK-OV-3 and MDAH-2774 ovarian cancer cells

Groups	Positively stained cells [%]	
	MDAH-2774	SK-OV-3
Untreated control	0	0
Cisplatin	75	75
Valspodar	0	0
Cisplatin+Valspodar	100	100

Results are expressed as the mean number of cells counted in three fields.

the surface of cancer cells. In secondary (acquired) resistance, cancer cells develop resistance as the result of adaptation to initial drug treatment [16]. MDR is associated with the activity of membrane transporters that pump cytotoxic drugs out of cells [15,16]. For this reason, the focus has been on finding effective modulators of these proteins to increase the effectiveness of chemotherapy. An ideal MDR modulator selectively blocks the transport of a drug and does not interact with normal cells [20]. Evaluation of valspodar efficacy in modulating cancer cell response to current chemotherapy drugs may contribute to finding an effective therapy for ovarian cancer. Previous studies investigating the efficacy of valspodar in reversing MDR of cancer cells showed contradictory results in different types of cancer, which was the reason why such studies have been halted. Nevertheless, Duraj et al. showed that in human ovarian cancer cells resistant to paclitaxel valspodar reverses the sensitivity of resistant cells to paclitaxel [21]. In addition, some promising results were obtained in studies on patients with ovarian cancer resistant to anthracyclines and cisplatin. For example, increased effectiveness of therapy with doxorubicin and cisplatin was observed when these drugs were given in combination with valspodar [13]. A study comparing cyclosporin A and valspodar as drug resistance modulators in Chinese hamster ovary (CHO) cell lines resistant to colchicine showed that valspodar has a much higher efficacy as a modulator compared to cyclosporin A [22]. Similar results were obtained by Naito et al. in human ovarian cancer cell line A-2780, who demonstrated that a lower dose of valspodar compared to cyclosporine A is required to reduce the resistance of cells to vincristine and adriamycin [23]. Moreover, in comparison to cyclosporine A, valspodar causes less side effects, i.e., it does not cause nephrotoxicity and does not have a negative impact on the cardiovascular system [24]. Watanabe et al. compared the reversal efficacy of three MDR modulators, valspodar, verapamil, and cyclosporine A, in adriamycin (ADM)-resistant P388-bearing mice and colon adenocarcinoma 26-bearing mice. They showed higher potency of valspodar to reverse MDR *in vivo* [25]. These previous results were the basis for our *in vitro* assessment of valspodar as an MDR modulator in ovarian cancer cells.

Cisplatin induces the generation of reactive oxygen species (ROS) in cells and increased ROS levels lead to apoptosis [26]. The accumulation of free radicals in a cell causes damage to two main components of the cell membrane: proteins and lipids [17]. Our analysis of MDA and protein thiol groups in treated ovarian cancer cells showed significant oxidative damage to SK-OV-3 cells co-treated with cisplatin and valspodar for 24 hours, which was also confirmed by the cell viability assay. This indicates that valspodar enhanced the cytotoxic effect of cisplatin in SK-OV-3 cell line. However, MDAH-2774 cells showed markedly higher resistance to cisplatin, and no significant changes were observed in the levels of MDA and thiol groups in MDAH-2774 cells after co-treatment with cisplatin and valspodar compared to untreated control group. Yang et al. showed that costunolide induces apoptosis (increases caspase activity) in three platinum-resistant ovarian cancer cell lines (MPSC1, A-2780, and SK-OV-3) through ROS production [26]. Al-Eisawi et al. demonstrated that the treatment of resistant A-2780 cells with a combination of platinum drug [cisplatin, carboplatin or trans-bis(3-hydroxypyridine) dichloroplatinum(II)] and the proteasome inhibitor bortezomib induces oxidative stress as a major factor leading to cell death [27]. In our study, large differences in the response of SK-OV-3 and MDAH-2774 cells to the

combination of cisplatin and valspodar may be due to markedly increased MnSOD expression in MDAH-2774 cells after the co-treatment with the drug and modulator. This indicates that antioxidant mechanisms are activated in cells to neutralize free radicals and prevent oxidative damage of cell proteins and lipids. High expression of MnSOD is associated with a weak or lack of cell response to anticancer therapy [28]. Our results showed a slight increase in MnSOD expression after treatment of SK-OV-3 and MDAH-2774 cells with cisplatin and strong staining for MnSOD in MDAH-2774 cells after co-treatment with cisplatin and valspodar. Low MnSOD expression in SK-OV-3 cells was correlated with a stronger cytotoxic effect of cisplatin and valspodar. Similar to our results, Piotrowska et al. showed that a strong cytotoxic effect of resveratrol derivative in A-2780 cell line was correlated with low expression of MnSOD, while high expression of MnSOD in SK-OV-3 cells was associated with increased cell viability after the treatment [29]. In the study of Yeung et al., human ovarian cancer cell line OVCAR-3 overexpressing MnSOD had a markedly higher survival rate after doxorubicin or paclitaxel treatment compared to cells with low MnSOD expression [30].

Our analysis of the type of cell death induced by cisplatin and valspodar co-treatment showed apoptotic cell death in most of the examined cells. The results of the TUNEL assay were similar for both cell lines, while the comet assay indicated a higher resistance of MDAH-2774 cells to cisplatin and valspodar co-treatment (18% of apoptotic cells in MDAH-2774 cell line vs. 47% in SK-OV-3 cell line). Apoptosis may be triggered by several pathways. Piotrowska et al. indicated that anticancer 3,4,4',5-tetramethoxystilbene induces the intrinsic (mitochondrial) apoptotic pathway in SK-OV-3 cells and the extrinsic apoptotic pathway in A-2780 cells [29]. Previous studies suggested that valspodar can induce apoptosis in cancer cells via sphingomyelin hydrolysis or *de novo* ceramide synthesis [31]. Cabot et al. showed a significant increase in ceramide levels in MDR human breast adenocarcinoma cell line MCF-7 after incubation with valspodar [32]. Similar results were obtained with KB-V-1 MDR human epidermoid carcinoma cells [33] and SK-OV-3 ovarian cancer cells [34]. Moreover, high levels of glucosylceramide (glycosylated ceramide) are associated with MDR in different cancer cell lines [35], and conversely, the inhibition of ceramide glycosylation increases the sensitivity of resistant cells to chemotherapeutic agents [32]. Morad et al. demonstrated that the inhibition of acid ceramidase (AC) in human pancreatic carcinoma cell lines PANC-1 and MIA-PaCa-2 converts the cytostatic effects of valspodar to cytotoxic [36]. AC inhibitors may result in cell cycle arrest [37], apoptosis or blockage of tumor growth *in vivo* [38]. An important aspect of our further research will be the characterization of apoptotic pathways induced in cells after co-administration of cisplatin and valspodar. Because of high level of oxidative stress in SK-OV-3 cell line, it is highly likely that in these cells apoptosis was associated with the activation of caspase-12 or sphingomyelin pathway [39]. Furthermore, the characterization of apoptotic pathways in treated MDAH-2774 cells may provide clues on how to increase the sensitivity of these cells to chemotherapeutic agents.

The application of valspodar in combination with cisplatin significantly enhances the cytotoxic effects of the drug. While some studies indicated that valspodar has only a modulatory effect on proteins involved in MDR, others suggested its direct anticancer effects [9]. The mechanistic details of synergistic valspodar-cisplatin action in cancer require further investigation.

Our study showed that the oxidative status of ovarian cancer cells and associated molecular pathways influence their resistance to chemotherapeutic drugs. Overexpression of MnSOD in ovarian cancer cells decreased oxidative stress and reduced the cytotoxic effect of cisplatin. Our preliminary *in vitro* results may serve as the basis for future *in vivo* and clinical studies.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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