European Review for Medical and Pharmacological Sciences 2015; 19: 2084-2090

Underlying mechanism of 2-methoxyestradiol-induced apoptosis and growth arrest in SKOV3 human ovarian cancer cells

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Abstract. – OBJECTIVE: In this study, we sought to investigate the effects of 2-methoxyestradiol (2-ME) on cisplatin-induced apoptosis and growth inhibition in SKOV3 ovarian cancer cells.

MATERIALS AND METHODS: Cells were treated with 2-ME, carboplatin, or both, the control group, and cell viability and growth inhibition assays were performed using the MTT method. Apoptosis was detected by flow cytometry analysis. Reverse transcription polymerase chain reaction and western blotting were used to monitor the mRNA and protein expression of the pro-apoptotic genes bax and caspase-3 and the anti-apoptotic gene bcl-2. The phosphorylation of Bcl-2 protein was monitored by western blotting.

RESULTS: Cell viability was inhibited by all three treatments in a time-dependent manner. Importantly, the combination treatment resulted in significantly reduced cell growth compared with the other groups. The mRNA and protein expression of Bax and caspase-3 were increased in the combination treatment group, and the expression of Bcl-2 was decreased in the combination treatment group as compared with the other two groups. The ratio of bax to Bcl-2 mRNA in the combination treatment group was higher than that in the carboplatin-treated group. Finally, phosphorylation of Bcl-2 protein was increased stronger in the combination treatment group compared with the carboplatin-treated group.

CONCLUSIONS: 2-ME promoted the growth inhibitory and apoptosis-inducing effects of platinum-based agents in SKOV3 ovarian cancer cells. The mechanism mediating this effect may be related to the phosphorylation of Bcl-2 protein, which reduces the formation of dimers and, thereby, increases apoptosis. Moreover, 2-ME promoted the mRNA and protein expression of Bax, thereby, increasing the Bax/Bcl-2 expression ratio and activating the mitochondrial apoptosis pathway.

Key Words:
2-methoxyestradiol, Bax/Bcl-2 ratio, Ovarian cancer.

Introduction

Approximately 200,000 women are diagnosed with ovarian cancer each year, and nearly 125,000 die from this disease annually[1]. Ovarian cancer is generally treated by a combination of surgery and chemotherapy. However, the 5-year survival rate still remains less than 40% in patients who underwent surgery followed by adjuvant chemotherapy[2].

A combined chemotherapy using platinum-based agents such as carboplatin is common practice for the treatment of epithelial ovarian cancer and platinum has been used as the first-line of chemotherapy since the 1980s. Contextually, the high mortality rate in patients with ovarian cancer can be partly attributed to the gradual development of resistance to chemotherapy[3,4]. Therefore, further development of effective treatments is necessary to improve prognoses in patients with ovarian cancer[5-7].

2-Methoxyestradiol (2-ME) is an estrogen metabolite with antitumor activity and has been shown to exhibit potent apoptotic activity in a wide variety of tumor types such as prostate, pancreatic, and breast cancer. In the present study, we examined the effects of 2-ME on carboplatin-induced growth arrest and induction of apoptosis in SKOV3 ovarian cancer cells. Our data will provide a clinical and experimental basis for the development of more effective combinatorial chemotherapies for the management of ovarian cancer.

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Materials and Methods

Reagents

2-ME, Maly'5a, and MTT cell proliferation assay kit were purchased from Sigma (St. Louis, MO, USA). 2-ME was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mM and stored at 4°C. It was diluted in the culture medium to its final concentration before use. The concentration of DMSO in the medium was less than 0.1%. Carboplatin was purchased from Qilu Pharmaceutical Company Ltd. (Jinan, China). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA), and the ReverTra Ace qPCR 127 kit was purchased from Toyobo Co., Ltd (Osaka, Japan). Cellular apoptosis analysis kit and BCA protein assay kit were from Beyotime Institute of Biotechnology (Shangai, China). The following rabbit polyclonal antibodies were used for western blot analysis: anti-Bcl-2 (1:100, Abcam, Cambridge, UK), anti-Bax (1:200, Abcam), caspase-3 (1:100, Abcam), and anti-phospho-Bcl-2 (Ser70) (1:200, Cell Signaling Technology, Danvers, MA, USA).

Cell Culture

SKOV3 cells were cultured in a medium containing 10% fetal bovine serum and 100 IU/mL penicillin/streptomycin at 37.4°C in a humidified atmosphere with 5% CO_2. Experiments were performed using cells in the logarithmic growth phase.

MTT Assay

Cell proliferation was measured using the MTT assay. Briefly, SKOV3 cells were plated in 96-well plates at a density of 5×10^4 cells/well. After 24 h, cells were treated individually with 2-ME and carboplatin, or in combination at indicated concentrations. After 24, 48, or 72 h, 20 µL of MTT solution was added to each well and plates were incubated for an additional 4 h. Following this, the MTT solution was removed and 150 µL DMSO was added; the plates were shaken gently for 5 min to solubilize formazan crystals. The absorbance in each well at 490 nm was measured using a Rayto RT2100C instrument (Shenzhen, China).

The inhibition rate was calculated as: (1 – average absorbance of treatment group/average absorbance of control group) × 100.

The half-maximal inhibitory concentration (IC_{50}) was calculated for SKOV3 cells using the results from this MTT assay.

Flow Cytometry

SKOV3 cells were treated with the indicated concentrations of 2-ME, carboplatin, or 2-ME plus carboplatin for 48 h. Cells were then collected, centrifuged, and resuspended in phosphate-buffered saline (PBS) at a concentration of 1×10^6 cells/mL. One hundred microliters of the solution was transferred to a 5 mL culture tube, and 5 µL Annexin V was added following which cells were incubated in the dark at 4°C for 15 min. Ten microliters of propidium iodide (PI) was added and cells were incubated again in the dark at 4°C for 15 min prior to analysis using a fluorescence-activated cell sorter (FACS) instrument (Eppendorf, Hamburg, Germany).

Semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis

Total RNA was extracted from SKOV3 cells treated with 2-ME, carboplatin, or a combination of 2-ME plus carboplatin for 48 h using TRIzol reagent (Invitrogen, Tokyo, Japan). Equal amounts of total (1 µg) were reverse-transcribed with oligo-(dT) 18 primers (Takara, Japan). The transcribed cDNA mixture was then subjected to PCR using TaqDNA and normalized to the expression of β-actin as a housekeeping gene. PCR products were subjected to electrophoresis on 1.5% agarose gels and stained with ethidium bromide for visualization.

The PCR primers (Shanghai BioSune Biological Engineering Technology Service Co.) were as follows: *bcl-2* forward, 5′-CGTGCGGTCATGTGTGTGG-3′ and reverse, 5′-CGGTTCAGGTACTCAGTCATCC-3′; *bax* forward, 5′-CCCGAGAGGTCTTTTTCCGAG-3′ and reverse, 5′-CCAGCCCATGATGGTTAGACG-3′; *caspase-3* forward, 5′-CATGCAAGCGAATCAATGGACT-3′ and reverse, 5′-CTGTACCAGACCGAGATGTCA-3′; and *β-actin* forward, 5′-CCAACCGCGAGAAGATGA-3′ and reverse, 5′-CCAGAGGCGTACAGGGATAG-3′.

Western Blot Analysis

SKOV3 cells were treated with the indicated concentrations of 2-ME, carboplatin, or 2-ME plus carboplatin for 48 h. Protein concentrations were determined using the BCA method (Beyotime, Shanghai, China), and equal amounts of protein (30 µg) were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels. Proteins were then transferred to polyvinylidene difluoride membranes, blocked

for 2 h with 5% milk in Tris-buffered saline with Tween (TBST), and incubated with primary antibodies against Bax, caspase-3, Bcl-2, or phospho-Bcl-2. After washing thrice with TBST, the membranes were incubated with secondary antibodies (Zhongshan, Beijing, China) for 1 h followed by washing thrice with TBST. Protein bands were detected using a Super ECL Plus kit (Applygen Technologies, Beijing, China).

Statistical Analysis

SPSS v18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All values are shown as means ± standard errors of the mean (SEMs). Data were statistically compared using Student's *t*-tests, and differences with *p* values of less than 0.05 were considered significant.

Results

Effects of 2-ME, Carboplatin, and 2-MS Plus Carboplatin on the Growth of SKOV3 Cells

MTT assay was performed in order to measure the effects of 2-ME, carboplatin, and 2-ME plus carboplatin on the growth of SKOV3 cells (Figure 1). All three lines of treatment was observed to cause a concentration- and time-dependent inhibition of SKOV3 cell growth. While individual treatments with 2-ME (1, 2, and 4 μM) and carboplatin (1, 2, and 4 μM/mL) inhibited SKOV3 growth over the 72 h study period, their combination (2 μM 2-ME plus 2 μg/mL carboplatin) exerted significantly greater inhibitory effects on SKOV3 cell growth, achieving an inhibition rate of 41.69% ± 0.32% ($p < 0.05$ versus the other two groups). The control, which was treated only with the DMSO vehicle, exhibited less than 11.2% inhibition throughout the assay, demonstrating no significant effect of vehicle on cell growth. These data suggested that 2-ME enhanced the antitumor effects of carboplatin in SKOV3 cells.

Effects of 2-ME, Carboplatin, and 2-ME Plus Carboplatin on Apoptosis in SKOV3 Cells

Next, effects of the different chemotherapeutic agents either individually or in combination on apoptosis in SKOV3 cells were analyzed. Similar to its effect on cell growth, 2-ME was also observed to induce apoptosis in a concentration-dependent manner as assessed by AnnexinV-PI double staining (Figure 2). Maximum apoptotic cells, 14.36% ± 0.062%, were observed after a treatment with 4 μM 2-ME for 48 h. Carboplatin was also observed to induce apoptosis, albeit at a reduced rate of 7.88% ± 0.05% at 2 μg/mL. The combination of 2-ME (2 μM) plus carboplatin (2 μg/mL) increased the percentage of apoptotic cells to 12.75% ± 0.01%, which was higher than either of the individual treatments with identical concentrations. This clearly demonstrated a synergistic effect of the combination treatment on apoptosis. Importantly, the control cells exhibited an apoptosis rate of only 4.49% ± 0.08%, which was significantly lower as well as different than all treatment groups ($p < 0.01$).

Effects of 2-ME, Carboplatin, and 2-ME Plus Carboplatin on mRNA and Protein Expression Levels of pro- and Anti-apoptotic Targets

Based on the effects of 2-ME and carboplatin on apoptosis rates, we next examined the effects of these agents on the expression of several markers of apoptosis. As shown in Figures 3 and 4, changes in the expression levels of pro- and

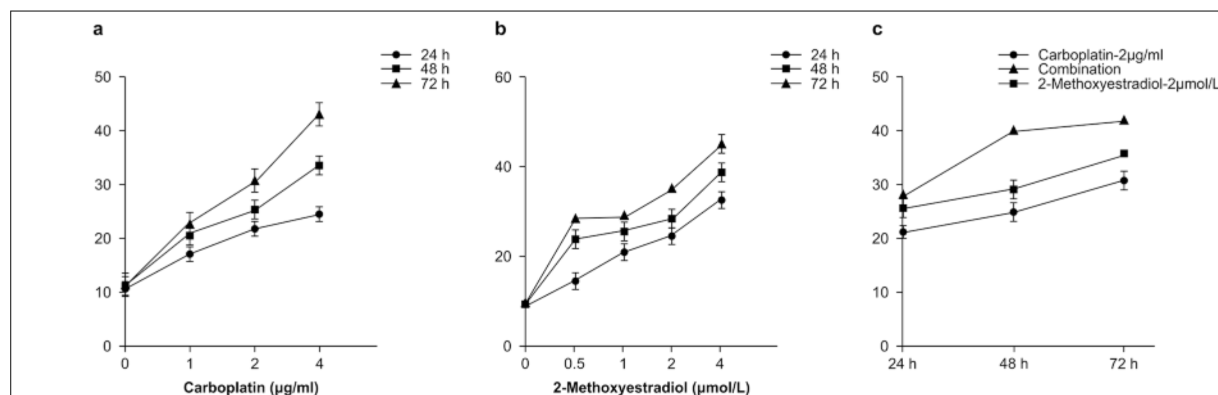

Figure 1. Effects of treatment with different drugs on the proliferation of SKOV3 cells.

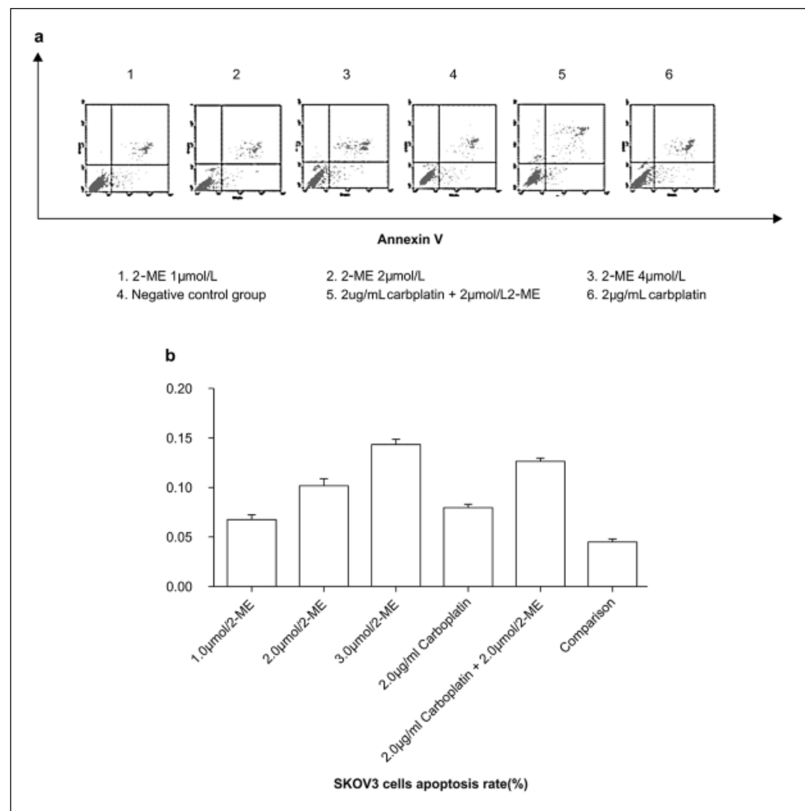

Figure 2. Effects of treatment with different drugs on the apoptosis rate of SKOV3 cells.

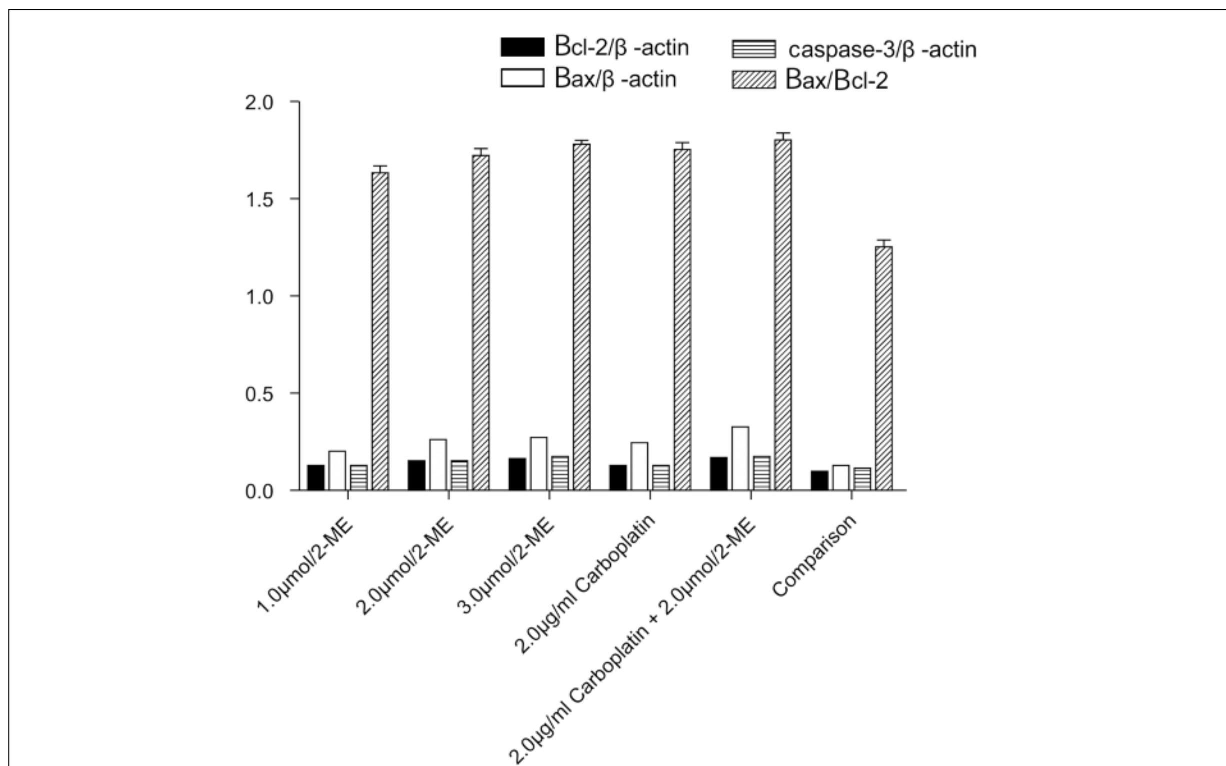

Figure 3. Effects of treatment with different drugs on the mRNA expression of bax, caspase-3, and Bcl-2 and the ratio of Bax/Bcl-2 mRNA in SKOV3 cells.

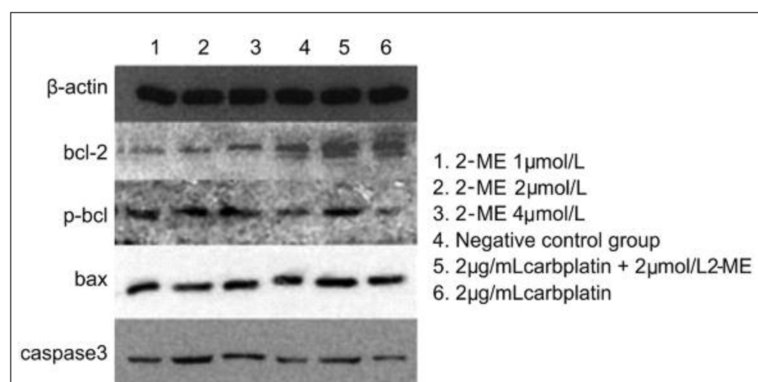

Figure 4. Effects of treatment with different drugs on the protein expression of Bax, caspase-3, and Bcl-2 and the phosphorylation of Bcl-2 in SKOV3 cells.

anti-apoptotic targets were consistent with the results of the apoptosis assays described above. Both 2-ME and carboplatin upregulated the mRNA and protein levels of Bax and caspase-3 in a concentration-dependent manner. Importantly, the combination of 2-ME plus carboplatin resulted in a significant increase in the upregulation of mRNA and protein levels of both Bax and caspase-3 than either of the individual agents, supporting the synergistic effects of the combined treatment. Interestingly, while these treatments did not significantly alter the expression of the anti-apoptotic target Bcl-2, the levels of phosphorylated Bcl-2 were observed to increase in a concentration-dependent manner. Moreover, the ratio of *bax*/*bcl-2* mRNA increased following a concentration-dependent 2-ME treatment. A further increase was observed following the addition of 2-ME plus carboplatin. Taken together, these results suggested that the combination treatment of 2-ME plus carboplatin led to activation of the mitochondrial apoptotic pathway.

Discussion

Epithelial ovarian cancer is the leading cause of death among gynecological cancers and close to 70% of patients afflicted with an advanced-stage disease experience recurrence[8]. While ovarian cancers are often sensitive to the first round of chemotherapeutics, resistance to chemotherapy, possibly due to high levels of Bcl-2 expression, is common in ovarian cancers[9]. Therefore, improving the ovarian cancer treatment options may mean developing therapies that resensitize cells to standard chemotherapeutics[10]. In this study, we examined the synergistic effects of 2-ME, a novel potential anticancer drug, and the standard first-line treatment, carboplatin. Our data demonstrated 2-ME to be a potent inhibitor of cancer cell growth, which enhanced the effects of carboplatin in SKOV3 cancer cells. Thus, 2-ME may exhibit therapeutic applications in the treatment of ovarian cancer.

A novel strategy against ovarian carcinoma is to overcome resistance by re-establishing sensitivity to apoptotic agents[10]. Apoptosis occurs through two main pathways: the extrinsic or death receptor pathway, which is trigged through the Fas death receptor; and the mitochondrial pathway, also called the intrinsic pathway. Previously, we demonstrated that the intrinsic pathway plays a major role in determining the sensitivity of ovarian cancer to pro-apoptotic reagents such as chemotherapeutics[10]. In the current study, we found that 2-ME and carboplatin induced apoptosis through the mitochondrial pathway, which proceeds through the Bd-2 family of proteins[9,11]. In this pathway, the outer mitochondrial membrane becomes permeable in response to apoptotic stimuli, which leads to the release of cytochrome c. Cytochrome c binds to and activates procaspase-3, which is subsequently cleaved into caspase-3, the key executor caspase of the mitochondrial apoptotic pathway[12].

A previous study[13] pointed out the interesting result that the Bcl-2 family of proteins regulates the release of cytochrome c. Bcl-2 proteins are critical for the induction of apoptosis and have been shown to control the mitochondrial pathway, which is frequently triggered in response to chemotherapeutic agents. Elevated levels of Bcl-2 in tumor cells may contribute to chemoresistance, thereby increasing the stability of the mitochondrial membranes against apoptotic insults. Thus, Bcl-2 dimerizes with Bax and contributes to the anti-apoptotic pathway[14,15]. Interestingly, the current and a previous study links 2-ME to induction of the mitochondrial pathway, potentially via the

Bcl-2-dependent pathway[16]. Further, 2-ME has been shown to promote the phosphorylation of Bcl-2 protein in several cancer cell lines[17]. Consistent with this finding, we detected phosphorylated Bcl-2 in ovarian carcinoma cells incubated with 2-ME for 48 h. Moreover, 2-ME showed a concentration-dependent increase in Bcl-2 phosphorylation, consistent with the observed effects of 2-ME on apoptosis. Additionally, the combination of carboplatin and 2-ME caused a further upregulation of Bcl-2 phosphorylation. Phosphorylation of Bcl-2 reduces heterodimer formation with Bax molecules and impairs its anti-apoptotic effects, thereby leading to initiation of caspase activation and apoptosis induction[18,19]. Based on these lines of evidence, the possible mechanism of 2-ME action may proceed via 2-ME mediated phosphorylation of Bcl-2 leading to interference between the binding of Bcl-2 and Bax. Bax may then form homodimers (rather than heterodimers with Bcl-2), thus, inducing cell death through the mitochondrial pathway.

Sumie et al[9] reported a 2-ME mediated upregulation of Bax. Additionally, 2-ME was shown to increase the Bax/Bcl-2 ratio, leading to the induction of apoptosis[13]. The current study supported the finding that treatment with 2-ME leads to upregulation in Bax expression. We also found that increasing concentrations of 2-ME led to an increase in the ratio of Bax/Bcl-2. Further, 2-ME also enhanced the effects of carboplatin on Bax/Bcl-2 ratio, and a combination treatment with 2-ME and carboplatin also led to upregulated expression of Bax compared to the effects produced by carboplatin alone. Taken together, these results supported the findings of Sumie et al[9], and confirmed the synergistic effects produced by a combination of 2-ME and carboplatin on the induction of apoptosis in SKOV3 ovarian cancer cells.

Mitochondrial cytochrome c release leads to the activation of the caspase cascade involving a combination of the proteolytic enzymes caspase-9 and caspase-3, which lead to the progression of apoptosis[13]. Cuello et al[20] reported that 2-ME mediates the intrinsic mitochondrial apoptotic pathway by affecting the reaction between caspases-8 and -9. Indeed, we found that 2-ME enhanced the levels of caspase-3 mRNA and protein in a concentration-dependent manner. Moreover, 2-ME enhanced the effects of carboplatin on induction of caspase-3 in SKOV3 cells. Since caspase-3 is considered to be the final protein in the caspase cascade[21], its elevated expression signaled further evidence concerning a change in the Bax/Bcl-2 ratio that lead to activation of the mitochondrial apoptosis pathway.

Based on recent studies[22], substantial inhibitory effects of 2-ME on tumor growth and induction of apoptosis in a variety of cell lines and solid tumors are now known. Our previous results demonstrated the 2-ME mediated selective induction of cell death in ovarian cancer without affecting corresponding normal tissues[10]. In the current report, we further showed that 2-ME inhibited the growth of SKOV3 cells and induced apoptosis in these cells. Therefore, we postulate that 2-ME may have potential applications as a novel therapeutic agent in the treatment of ovarian cancer treatment. We suggest that clinical trials using this agent should be performed. Here, we tested the combinatorial effects of 2-ME and carboplatin on SKOV3 cells. A combination of these drugs exhibited a substantial synergistic effect and improved the sensitivity of SKOV3 cells to carboplatin. Thus, a combination of these drugs may reduce the dose of carboplatin required to achieve the similar effects, thereby reducing the side effects associated with this common chemotherapeutic agent.

Our results suggested that the mechanism of carboplatin mediated induction of apoptosis might differ from that observed during combined treatment with 2-ME and carboplatin. A combination treatment induced stronger inhibition of anti-apoptotic proteins and a greater induction of pro-apoptotic proteins and may help to overcome resistance to chemotherapy by targeting different aspects of the apoptotic pathway.

Conclusions

Our findings supported the pleiotropic effects of 2-ME on enhancing the sensitivity of carboplatin in epithelial ovarian cancer cells. This *in vitro* study offers a basis for further preclinical studies on the benefits of 2-ME in combination with carboplatin for the treatment of ovarian cancer.

Acknowledgements

We would like to thank our colleagues for their help and guidance.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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