



MS-5, a Naphthalene Derivative, Induces the Apoptosis of an Ovarian Cancer Cell CAOV-3 by Interfering with the Reactive Oxygen Species Generation

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Abstract

Reactive oxygen species (ROS) are widely generated in biological processes such as normal metabolism and response to xenobiotic exposure. While ROS can be beneficial or harmful to cells and tissues, generation of ROS by diverse anti-cancer drugs or phytochemicals plays an important role in the induction of apoptosis. We recently identified a derivative of naphthalene, MS-5, that induces apoptosis of an ovarian cell, CAOV-3. Interestingly, MS-5 induced apoptosis by down-regulating the ROS. Cell viability was evaluated by water-soluble tetrazolium salt (WST-1) assay. Apoptosis was evaluated by flow cytometry analysis. Intracellular ROS (H₂O₂), mitochondrial superoxide, mitochondrial membrane potential (MMP) and effect on cycle were determined by flow cytometry. Protein expression was assessed by western blotting. The level of ATP was measured using ATP Colorimetric/Fluorometric Assay kit. MS-5 inhibited growth of ovarian cancer cell lines, CAOV-3, in a concentration- and time-dependent manner. MS-5 also induced G1 cell cycle arrest in CAOV-3 cells, while MS-5 decreased intracellular ROS generation. In addition, cells treated with MS-5 showed the decrease in MMP and ATP production. In this study, we found that treatment with MS-5 in CAOV-3 cells induced apoptosis but decreased ROS level. We suspect that MS-5 might interfere with the minimum requirements of ROS for survival. These perturbations appear to be concentration-dependent, suggesting that MS-5 may induce apoptosis by interfering with ROS generation. We propose that MS-5 may be a potent therapeutic agent for inducing apoptosis in ovarian cancer cell through regulation of ROS.

Key Words: Reactive oxygen species, Apoptosis, Anti-cancer effect

INTRODUCTION

Reactive oxygen species (ROS) have been implicated in the regulation of various cellular functions including cancer survival and apoptosis. While many studies report the apoptosis of cancer cells accompanied by the increased generation of ROS (Zhou *et al.*, 2014; Ko *et al.*, 2016; Zhang *et al.*, 2016; Li *et al.*, 2017), some compounds have been implicated to induce the apoptosis of cancer cells while reducing the generation of ROS (Liu *et al.*, 2014). It is suggested that there is a delicate balance of ROS generation that keeps the cancer cells proliferating: too much ROS or less than necessary ROS may lead to the death of cancer cell (Trachootham *et al.*, 2009). Interestingly, even the same compound seems to ex-

ert the opposite effect on the regulation of ROS level. For example, dihydromyricetin induces apoptosis of head and neck squamous cell carcinoma by raising the ROS level (Fan *et al.*, 2016), while it reduces the generation of ROS in HepG2 cells resulting in the apoptosis (Liu *et al.*, 2014).

Ovarian cancer is the second most common gynecological cancer, and about 21,000 cases occur every year in the USA. Due to the lack of early symptoms, timely detection of ovarian cancer is difficult, and many patients seek medical care when the disease is already in stage 3, or even later. The mortality rate of ovarian cancer is very high because of late detection, regrowth, and recurrence after initial therapy (Menon, 2007; Siegel *et al.*, 2016; Oronsky *et al.*, 2017). Development of better therapeutic treatment (Kim *et al.*, 2017) and early detection

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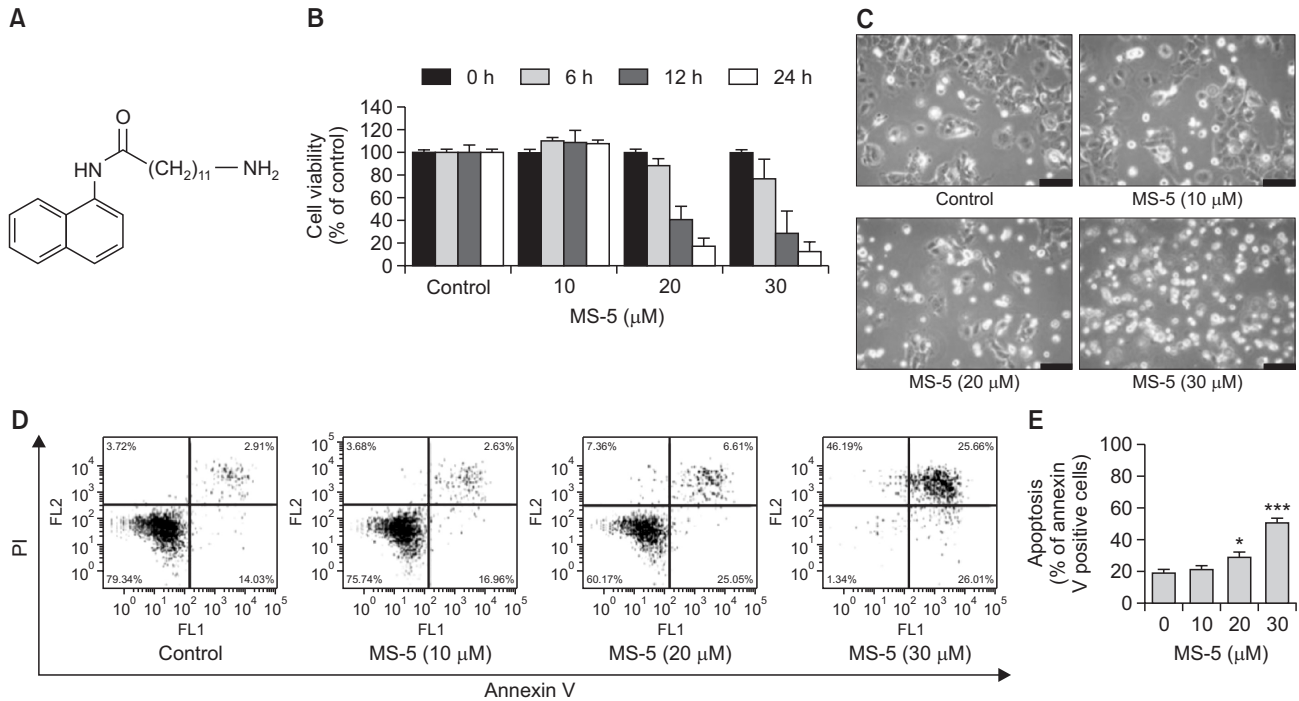


Fig. 1. Cytotoxic effects of MS-5 in CAOV-3 cells. (A) The chemical structure of MS-5. (B) The viability of CAOV-3 cells treated with different concentrations of MS-5 for 0 (negative control) 6, 12, and 24 h. (C) Microscopic morphology of CAOV-3 cells treated with MS-5 (0, 10, 20, 30 μM) for 24 h (x100, scale bars represent 200 μm). (D) Flow cytometry analysis of CAOV-3 cells treated with MS-5 for 24 h and staining with Annexin V and PI. (E) The statistical analysis of apoptosis. The apoptotic index (%) data are representative of three independent experiment. * $p < 0.05$, *** $p < 0.001$, compared to control.

is necessary for better outcome in ovarian cancer patients. CAOV-3 is a cancer cell line of the epithelial ovarian cancer type, which accounts for 90% of ovarian cancers (Choi *et al.*, 2007). It has been reported that the generation of ROS is involved in the regulation of growth in CAOV-3 cells (Muniyan *et al.*, 2015). In this report, we identified a derivative of naphthalene, MS-5 (Fig. 1A), that shows cytotoxic effect on CAOV-3 cells by reducing the generation of ROS production.

MATERIALS AND METHODS

Materials

N-acetylcysteine (NAC) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against cleaved caspase-9, -7, -3, poly (ADP-ribose) polymerase (PARP), Bcl-2, Bax, cytochrome c, cyclin D1, and survivin were from Cell Signaling Technology (Beverly, MA, USA). Antibodies against CDK2, cyclin E, p16, p21 and p27 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cox4 and horse-radish peroxidase-conjugated secondary antibodies were purchased from Thermo scientific (Rockford, IL, USA). The antibody against β-actin was from Sigma-Aldrich.

Fluorescence dyes 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA), 3,3'-Dihexyloxycarbocyanine iodide (DiOC₆(3)), propidium iodide (PI) and MitoSOX Red were procured from Invitrogen (Carlsbad, CA, USA). MS-5 (99% pure) was synthesized and purified and the structure of the compound was confirmed by NMR and mass spectrometry. MS-5 was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich), and the

stock solution was stored at -20°C.

Cell culture and treatment

Human ovarian carcinoma cells CAOV-3 were from American Type Culture Collection. The cultured cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin G and 100 mg/mL streptomycin) at 37°C in a humidified incubator containing 5% CO₂ and 95% air. Unless otherwise specified, cells were seeded at 1×10⁶ cells into 100 mm dish.

Cell viability assay

To determine cell viability, the colorimetric WST-1 assay (DoGen Bio Co., Seoul, Korea) was used. Cells (2×10³/well) were incubated, in triplicate, in a 96 well plate, and exposed to varying concentrations of MS-5 compound for different time periods. The cells treated with DMSO only served as a negative control. The volume of cultured cells including medium was 100 μL, and the final concentration of DMSO was kept lower than 0.1%. After treatment of cells with MS-5, cells were incubated with WST-1 solution for 2 h, and cell viability was assessed by measuring absorbance at 450 nm with microplate reader (BMG Labtech, Offenburg, Germany). The relative cell viability (%) was calculated relative to the negative control.

Cell cycle analysis

For cell cycle analysis, the cells were then treated with fresh media containing 0, 15, and 30 μM MS-5 for 24 h. Next, the cells were trypsinized, washed twice with cold phosphate buffered saline (PBS) and fixed with cold 80% ethanol at 4°C

overnight. The cells were then washed twice with PBS and incubated with propidium iodide solution (10 µg/ml propidium iodide and 5 µg/ml RNase A) at room temperature (RT) for 30 min. Cells were subsequently analyzed by flow cytometry using Cube 8 flow cytometer (Partec, Münster, Germany). Each measurement was conducted on 10,000 events and analyzed with FCS express 4 (Partec).

Annexin V staining

Annexin V staining was done using Annexin V-FITC staining kit (BD Biosciences, San Jose, CA, USA) following the manufacturer's instruction. Briefly, cells treated with MS-5 were washed with PBS and resuspended in 1x binding buffer containing Annexin V and propidium iodide. Fluorescence intensity was measured by flow cytometry.

Western blot analysis

Cells were harvested and lysed with RIPA buffer containing the protease inhibitor cocktail, 2 mM Na₃VO₄, and 10 mM NaF (pH 7.5), and the resulting protein samples were quantified by using the Coomassie protein assay reagent (Thermo scientific). Equal amounts of protein extracts were denatured by boiling for 5 min. The proteins were resolved by 10-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% skim milk in TBST buffer (10 mM Tris, 150 mM NaCl, pH 7.5 and 0.1% Tween 20) for 1 h at RT. The membranes were washed 3 times for 10 min each with TBST buffer, and incubated with the respective primary antibody for overnight at 4°C. Next, the membranes were washed three times and incubated with horse-radish peroxidase-conjugated secondary antibodies for 2 h at RT. Chemiluminescence detection was carried out by using ECL kit (GE Lifesciences, Piscataway, NJ, USA).

Measurement of reactive oxygen species and mitochondrial membrane potential

To measure reactive oxygen species, CAOV-3 cells in 60-mm culture dish were treated with MS-5 for the indicated time before staining with either 2' 7'-dichlorodihydrofluorescein diacetate (H₂DCFDA, 10 µM) or MitoSOX Red (5 µM). Mitochondrial membrane potential (MMP) assay was performed with MS-5 treated and untreated cells using DiOC₆(3) (5 nM). Fluorescence signals were detected using a Cube 8 flow cytometer.

Measurement of ATP production

The intracellular level of ATP was measured using ATP Colorimetric/Fluorometric Assay kit from BioVision (Mountain View, CA, USA) following the manufacturer's instructions. Briefly, cells were treated with MS-5 (15, or 30 µM) for 6 h, 12 h and 24 h. Cells were lysed and centrifuged at 12,000 rpm for 5 min. The supernatant (100 µL) was transferred to a 24-well plate, and then mixed with ATP detection working solution (100 µL). Luminescence signals were measured by a microplate reader (BMG Labtech). The protein concentration of each group was also determined using a Coomassie protein assay reagent (Thermo scientific). The relative ATP level was expressed as ATP value/protein value.

Preparation of mitochondrial sub-fraction

The cells were suspended in 200 µL modified lysis buffer

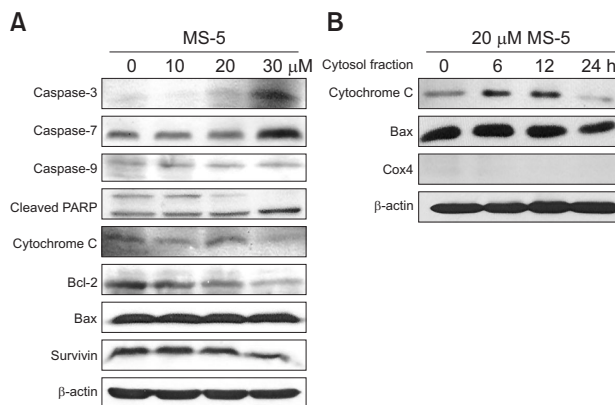


Fig. 2. Effect of MS-5 on cellular markers of apoptosis in CAOV-3 cells. Cells were treated with indicated concentrations of MS-5 for 24 h. (A) Western blot analysis of cell lysates for cleaved caspase-3, -7, and -9, cleaved PARP, cytochrome c, Bcl-2, Bax, and Survivin. β-actin was used as a loading control and Cox4 was used as a loading control for mitochondrial fraction. (B) The cytoplasmic level of caspase-3 was assayed by Western blotting after separation of cytosolic and mitochondrial fractions.

(20 mM Hepes-KOH, pH 7.5, 0.1 mM EGTA, 250 mM sucrose, the protease inhibitor cocktail, 2 mM Na₃VO₄, and 10 mM NaF) and incubated on ice for 10 min. The cells were then lysed with Dounce homogenizer, and lysate was centrifuged at 3,000 rpm for 5 min at 4°C to remove unbroken cells, large plasma membrane pieces and nuclei. The supernatant was further centrifuged at 13,200 rpm for 10 min. The supernatant and pellet were used to prepare a cytosolic fraction and the mitochondrial fraction respectively.

Statistical analysis

When necessary, data were expressed as mean ± SD of at least three independent experiments, and statistical analysis for single comparison was performed using the Student's *t*-test and *p*-value less than 0.05 was considered statistically significant.

RESULTS

MS-5 induces changes in cell morphology and viability

To determine whether MS-5 is cytotoxic to CAOV-3 cells, we carried out the WST-1 assay. MS-5 showed a growth inhibition of CAOV-3 cancer cells in a concentration- and time- dependent manner (Fig. 1B). At 20 µM, MS-5 inhibited the cell viability approximately 80% compared to the untreated cells after 24-hour incubation. Under microscope, CAOV-3 cells treated with MS-5 (10, 20, or 30 µM) showed shrinkage, rounding, and detachment from the culture dish. The morphological changes observed 24 h after treatment of MS-5 are shown in Fig. 1C.

MS-5 induces the apoptosis of CAOV-3 cells

As MS-5 showed the cytotoxic effect on CAOV-3 cells accompanied with apoptotic morphological changes, flow cytometry analysis was performed to see if the MS-5 induced the apoptosis. CAOV-3 cells treated with MS-5 for 24 h were analyzed after Annexin V/propidium iodide staining to quantify the population of cells undergoing apoptosis (both Annexin V

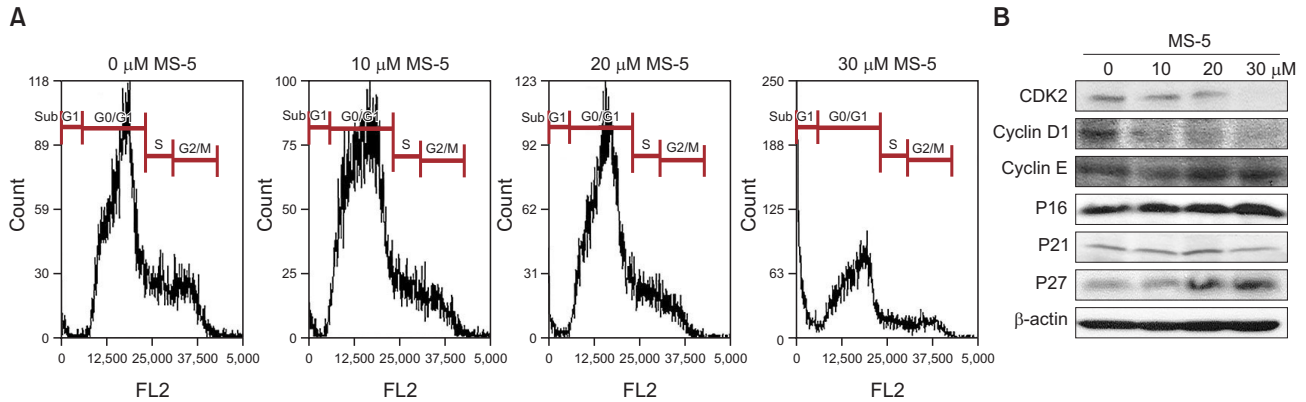


Fig. 3. Cell cycle arrest induced by MS-5 treatment. (A) The cell cycle distribution was analyzed by flow cytometry after PI staining. (B) Cells were treated with indicated concentrations of MS-5 for 24 h. Western blot analysis of cleaved CDK2, cyclin D1, cyclin E, p16, p21, and p27 protein levels. β -actin was used as a loading control.

+/propidium iodide - and Annexin V +/propidium iodide +, Fig. 1D). The results showed that the treatment of cells with MS-5 dramatically increased the percentage of apoptotic cells as compared with untreated control cells (Fig. 1E).

MS-5 induces the modulation of Bcl-2/Bax balance

Treatment of CAOV-3 cells with MS-5 induced activation of caspase-9, caspase-7 and caspase-3, and cleavage of PARP. As this result indicates the involvement of the mitochondrial apoptotic pathway, we examined the levels of Bcl-2 family proteins. We did not observe the increase in the level of Bax, a pro-apoptotic protein. However, the level of anti-apoptotic proteins, Bcl-2 and survivin were downregulated by treatment with MS-5 (Fig. 2A). The shift in Bcl-2/Bax balance correlated with the release of cytochrome c into the cytosol (Fig. 2B).

MS-5 induces G₁ cell cycle arrest in CAOV-3 cells

The effects of MS-5 on cell cycle profile were monitored by flow cytometry and Western blotting. Flow cytometry analysis indicated that MS-5 caused significant accumulation of CAOV-3 cells in G₁ phase (Fig. 3A). There was a concentration dependent increase of cells in G₁ phase with a concomitant decrease of the cells in S and G₂/M phase. Interestingly, Western blotting analysis showed that there is a decrease in the levels of CDK2, cyclin D1, whereas the level of protein p27 increased by the treatment of MS-5. The levels of cyclin E, p16, and p21 remained relatively unchanged (Fig. 3B).

MS-5 induces the modulation of ROS generation

The effect of MS-5 on ROS generation was examined as the accumulation of ROS can induce cell death. To measure the generation of intracellular ROS upon MS-5 treatment, we used the oxidation-sensitive dye H₂DCFDA. When analyzed with H₂DCFDA, the decrease in the generation of ROS was observed in the cells treated with MS-5 (Fig. 4A). Interestingly, we could observe the increase in the generation of ROS when MitoSOX Red was used for mitochondrial superoxide analysis (Fig. 4B).

MS-5 reduces the mitochondrial membrane potential (MMP) and the intracellular level of ATP

To evaluate the dysfunction of mitochondria, we analyzed MMP and the intracellular levels of ATP in MS-5 treated cells.

To investigate whether MMP decreased in CAOV-3 cells treated with MS-5, fluorescent dye DiOC₆(3) was used. We could observe that MMP was reduced by 25% at 20 μ M MS-5 (Fig. 4C). Likewise, the intracellular levels of ATP in MS-5-treated CAOV-3 cells dramatically decreased in a concentration-dependent manner (Fig. 4D).

DISCUSSION

In the present study, we showed that MS-5 could induce apoptosis in CAOV-3 cells. We could observe that the cell proliferation decreased as we increased the concentration of MS-5. The decrease in proliferation was well correlated with the increase in the rate of apoptosis. The apoptotic effect of MS-5 coincided with the decline in the production of intracellular ROS as probed by H₂DCFDA. Our results support the idea that there is a minimum requirement of ROS generation for cancer cells to grow normally (Trachootham *et al.*, 2009).

While the mechanism by which MS-5 perturbs the ROS-regulated signal transduction has to be revealed, our results indicate that the mitochondria-dependent apoptotic pathway is involved. We observed the cleavage of caspase-7, caspase-9 and caspase 3, as well as PARP. We also confirmed that the balance of Bax and Bcl-2 was disturbed by MS-5 treatment. While the protein level of Bax remained constant, the level of Bcl-2 decreased upon MS-5 treatment, probably resulting in the release of cytochrome c. In addition, we noticed, from the cell cytometry analysis, the accumulation of cells in G₁ phase with the treatment of MS-5. The cell cycle is a series of events that direct the growth and proliferation of cells divided into four stages, G₁, S, G₂ and M (Widrow *et al.*, 1998; Buolamwini, 2000). We monitored the levels of several proteins involved with the cell cycle progress. First, we observed the decrease in the level of CDK2 and cyclin D₁. The expression and activation of cyclin-dependent kinases (CDKs) is the key part of cell cycle regulation (Dickson and Schwartz, 2009). We suspect the increased level of p27 contributed the inhibition of CDK activity and G₁ phase arrest, possibly binding to cyclin E-Cdk2 complex (Polyak *et al.*, 1994a, 1994b; Satoh and Kaida, 2016).

Lots of chemicals induce the increase in the generation of ROS, and the oxidative stress is related with apoptosis in many cases (Biliński *et al.*, 1989; Yakes and VanHouten,

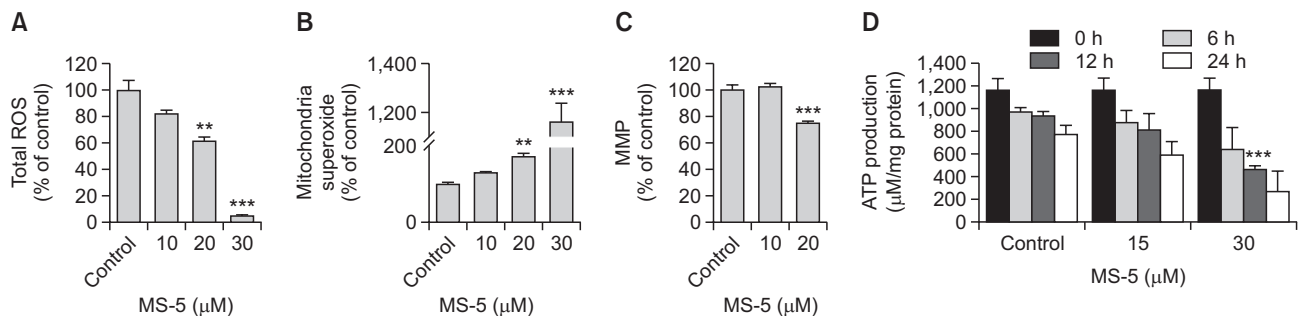


Fig. 4. Effects of MS-5 on total ROS, mitochondrial superoxide, mitochondrial membrane potential (MMP), and ATP production in CAOV-3 cells. CAOV-3 cells were treated with 20 μM MS-5 for 0, 6, 12 and 24 h, and the level of total ROS (A), Mitochondrial superoxide (B), and MMP (C) were measured by flow cytometry. (D) The levels of ATP in MS-5 treated cells were markedly decreased compared with their levels in the control. Data are representative of three independent experiments. ***p*<0.01, ****p*<0.001, compared to control group.

1997; Cabisco *et al.*, 2000). Hydrogen peroxide has been reported to induce apoptosis and this apoptotic effect could be blocked by antioxidant (Singh *et al.*, 2007; Min *et al.*, 2008; Halasi *et al.*, 2013). In this study, we observed the decrease, not the increase, of the ROS generation while MS-5 significantly induced apoptosis in CAOV-3 cells. The decrease in the intracellular ROS level was accompanied by the decrease in MMP and the intracellular ATP, indicating that mitochondrial function has been assaulted. It is noticeable that mitochondrial superoxide level was significantly increased by treatment of MS-5. Considering that hydrogen peroxide and superoxide are the main ROS species, this implies that the decrease comes from the reduction in the generation of hydrogen peroxide. Although further study is necessary to understand the molecular mechanism underlying the MS-5-induced apoptosis, our data demonstrates that MS-5 induces the apoptosis of ovarian cancer CAOV-3 cells through the perturbation of the ROS-regulated signal transduction. The detailed mechanism might elucidate a new target for the treatment of ovarian cancer. Our results added the repertoire of chemicals inducing apoptosis by decreasing the generation of ROS. We suspect that MS-5 perturbed the minimum requirement of ROS for cell survival. This perturbation was demonstrated in a concentration-dependent manner, and MS-5 somehow interfered ROS-regulated signal transduction thereby inducing apoptosis and G₁ phase arrest in CAOV-3 cells.

In conclusion, MS-5, a derivative of naphthalene, induced apoptosis and G₁ phase arrest of CAOV-3 cells by interfering with the ROS-regulated signal transduction, and elucidation of molecular mechanism may lead to a new target for the treatment of ovarian cancer.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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