RESEARCH ARTICLE

Cellular Mechanisms of a New Pyrazinone Compound that Induces Apoptosis in SKOV-3 Cells

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Abstract

   We screened a small molecular library that was designed and independently synthesized *in vitro* and found a new drug (MY-03-01) that is active against ovarian cancer. We established that MY-03-01 effectively inhibited SKOV-3 cell survival in a dose-dependent manner, based on cell viability rates, and that it not only induced SKOV-3 apoptosis by itself, but also did so synergistically with paclitaxel. Secondly, when MY-03-01 was applied at 40 μM, its hemolytic activity was less than 10%, compared with the control, and there was almost no damage to normal cells at this concentration. In addition, we used DAPI staining and flow cytometry to show that MY-03-01 could significantly induce apoptosis of SKOV-3 cells. Finally, we found that MY-03-01 likely induced SKOV-3 apoptosis by activating caspase3 and caspase9 through the mitochondrial pathway.

Keywords: Apoptosis - flow cytometry - hemolytic activity - ovarian cancer - paclitaxel - small molecular inhibitor

Introduction

   During the last 50 years, ovarian cancer has been one of the most lethal gynecological cancers worldwide (Chauhan et al., 2009; Rouzier et al., 2010; Siegel et al., 2011). Because the ovarian embryonic development, tissue anatomy, and endocrine function are very complex, and diagnostic methods are limited, the tumors of patients may be already malignant once diagnosed (Rogalska et al., 2013). There is a high incidence of ovarian cancer (Abuharbeid et al., 2005), which increases with age. Of the patients who present, 70% have middle to advanced disease, resulting in a low 5-year survival rate, and 20–40% of patients present with stage III or IV cancer (Zamboni et al., 2008). However, the current treatment options for ovarian cancer are very limited, and are mainly based on surgery and chemotherapy. Chemotherapy is a common anticancer treatment that uses anti-proliferative molecules to kill cancer cells (Descôteaux et al., 2012).

   The first-line chemotherapy drugs for ovarian cancer mainly contain carboplatin and paclitaxel (McGuire et al., 1996; du Bois et al., 2005; Metzger-Filho et al., 2010). These chemotherapeutic age-nts can also affect healthy cells, leading to severe side effects (Sac-hdeva, 1998). Also used is gleevec, one of several new generation, orally administered small molecule drugs developed to target cancer. These drugs inhibit protein kinases of the PDGF receptor. Gleevec can not only inhibit the activity of PDGF, but also restrain the activity of two other kinases, BCR-ABL and c-Kit. The FDA approved gleevec for use in clinical treatment of ovarian cancer. Gefitinib (iressa), also a small molecular compound, is an orally-administered epidermal growth factor receptor-tyrosine kinase (EGFR-TK) antagonist. Phase II clinical studies showed that gefitinib treatment of recurrent ovarian cancer had an efficacy rate of 4%, while the EGFR-positive efficacy rate was 9%. Unfortunately, the effects of these EGFR-TK agonists are limited (Wagner et al., 2007). To solve this problem, finding new drugs that can efficiently induce apoptosis in ovarian cancer cells without damaging normal cells is imminent. This focus currently receives high activity in research worldwide.

   In the present study, we assessed the efficacy of a novel small molecular drug, MY-03-01, on the SKOV-3 human ovarian cancer cell line by testing the cell viability rate, apoptotic death, and western blot.

Materials and Methods

Materials

   Human ovarian cancer SKOV-3 cells were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Rabbit blood stabilized with dipotassium ethylene diamine tetraacetate (EDTAP) was obtained from the Experimental Animal Center of Jilin University (Changchun, China). MY-03-01 was requested from Shenyang Pharmaceutical University (Shenyang, China), and dissolved in DMSO.

   Annexin-V-FITC Apoptosis Detection Kit I was obtained from BD Biosciences (BD Biosciences, San

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Diego, CA, USA). PVDF membrane was purchased from Millipore. 3-(4,5-dimethylth- iazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4,6-diamid- ino-2-phenylindile (DAPI), Paclitaxel, propidium iodide (PI), p-coumaric acid (PCA), and luminol (5-Amino-2,3-dihydro- 1,4-phthalazinedione-free acid) were obtained from Sigma-Aldrich (Sigma Aldrich, Shanghai, China). DMEM was purchased from Invitrogen (Shanghai, China). Fetal bovine serum (FBS) and G418 were purchased from GIBCO-BRL (Gibco, NZL, USA). All other chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich). Antibodies against PARP, Procaspase3, Procaspase8, Pro-caspase9, β-actin, HRP-labeled goat anti-rabbit IgG, and HRP-labeled goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The SKOV-3 cell line was cultured in DMEM medium supplemented with 10% FBS, and 2 mM glutamine, at 37 °C and 5% CO_2.

Cell proliferation assay

The effect of MY-03-01 on cell viability rate was determined by an MTT assay. In brief, 7000 cells per well were seeded in 96-well plates and incubated overnight. The medium was removed and replaced with fresh DMEM, alone or supplemented with MY-03-01 at concentrations from 1 to 100 µM. Treated cells were incubated up to 24 h at 37 °C. Then each well was incubated with 20 µl of MTT reagent for 4 h in 5% CO_2 incubator. After the removal of culture medium, 150 µl DMSO was loaded into each well to dissolve formazan crystals. The MTT assay was also performed with paclitaxel, added at a concentration of 0.1 to 100 µM. A concentration of 1 µM was selected for use with different concentrations of MY-03-01, and the two were added to stimulate SKOV-3 cells for 24 h. The amount of MTT that is converted to formazan indicates the number of viable cells. The results were assessed in a 96-well plate reader by measuring the absorbance at wavelength of 495 nm. The viability of SKOV-3 cell was calculated with equation (1): cell viability (%) = (OD of experimental group) / (OD of control)(1) In equation (1), OD is the optical density that was absorbed and detected; for OD values with said detection unit, OD = 1 g (1/trans), wherein the light transmitting material is evaluated for the detection of trans values.

Hemolysis activity assay

The hemolytic properties of MY-03-01 were examined by spectrophotometry. To obtain red blood cells (RBCs), 5 mL of rabbit blood was added to 10 mL of physiological saline (PS); RBCs were then isolated from serum by centrifugation at 1200 × g for 8 min. The RBCs were further washed six times with 40 mL of PS. Following the last wash, the remaining RBCs were dispersed in 30 mL PS. MY-03-01, suspended in 0.4 mL of PS at different concentrations, was separately mixed with 0.4 mL of RBCs suspended in PS. The mixtures were then incubated at 37 °C in a thermoregulated water bath for 1.5 h. RBCs were then centrifuged at 3000 × g for 10 min, and 100 µL of supernatant from each sample was transferred to a 96-well plate. Free hemoglobin in the supernatant was measured with a Bio-Rad 680 microplate reader (Bio-Rad, CA, USA) at 540 nm. PBS and ddH_2O were used as negative and positive controls, respectively. All hemolysis experiments were carried out in triplicate. The hemolysis ratio (HR) of RBCs was calculated with equation (2): HR (%) = (Asample-Anegative control) / (Apositive control-Anegative control) × 100 (2) In equation (2), Asample, Anegitive control, and Apositive control denote the absorbencies of the sample, negative control, and positive control, respectively.

Nuclear staining with 4,6-diamidino-2-phenylin- dile (DAPI) was conducted

After treating with MY-03-01, the cells in a 24-well plate were harvested, washed with ice-cold phosphate buffered saline (PBS) and fixed with 70% ice-cold ethanol for 5 min at 4 °C. The fixed cells were then washed with PBS (0.01M, pH 7.4) and stained with 300 µl of DAPI (2.5M) solution for 5 min at room temperature. The nuclear morphology of the cells was examined by a fluorescent microscope. All experiments were performed in triplicate.

Cell apoptosis and cycle arrest studies

Cell apoptosis and cycle arrest were studied using flow cytometry. Cells (1×10^6) were grown in 6-well plates containing 2 ml media and allowed to attach overnight at 37 °C. Medium (2 ml) containing 5, 10, 20, or 40 µM of MY-03-01 was then added to the 6-well plate, and the cells were incubated for 24 h at 37 °C. Cells (1×10^6) were washed with PBS (0.01 M, pH 7.4) and then resuspended in binding buffer according to the manufacturer's protocols. Next, cell cycle distribution and induction of apoptosis were determined by analyzing 15,000 ungated cells using a FACScan cytometer and Cell Quest software (FACSCalibur; Becton-Dickinson, San Jose, CA, USA). Cells treated with medium alone were used as the negative control. After 24 h, the cells were harvested and washed twice with cold PBS (0.01 M, pH 7.4). For analyzing the cell cycle, the cells were fixed in ice-cold 70% ethanol and then stored at 4 °C overnight. Prior to analysis, the cells were washed twice with PBS, suspended in 0.5 mL of cold PI solution containing 10 µL RNase A (25 mM), 10 µL PI (50 mM) and then incubated at 37 °C for an additional 30 min in the dark. All experiments were performed in triplicate.

Western blotting analysis

The protocol for western blot has been described previously (Zhang et al., 2012). Cells were treated with different concentration of MY-03-01, washed twice with ice-cold PBS, and gently lysed for 1 h in ice-cold cell lysis buffer (Dingguo, Beijing, China). Lysates were centrifuged at 12000 ×g for 10 min at 4 °C. Supernatants were collected, and protein concentrations were determined for western blotting analysis. An equal amount of protein was subjected to electrophoresis on an SDS-polyacrylamide gel and transferred to a PVDF membrane by electroblotting. The blots were blocked in phosphate buffered saline (PBS) containing 10% non-fat
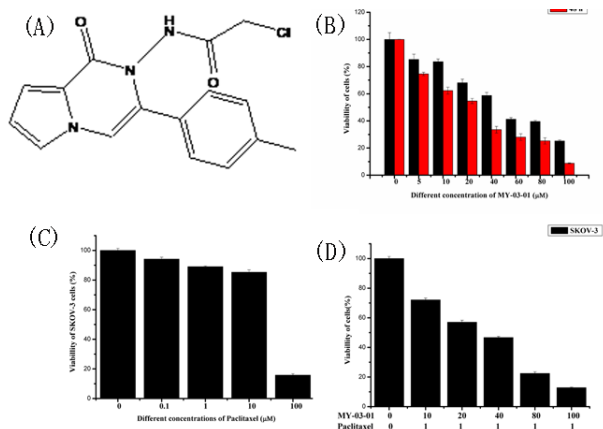
Figure 1. (A) Chemical structure of 2-chloro-N-(2-(1-oxo-3-(p-toly) pyrrolo[1,2-a]pyrazin-2(1H)-yl) acetamide (MY-03-01). (B) Effects of MY-03-01 and paclitaxel on cell growth. MY-03-01 inhibited the proliferation of SKOV-3 cells. Cells were seeded at a density of 7000 cells per well and then treated with 5, 10, 20, 40, 60, 80 and 100 μM of MY-03-01 for 24 h and 48 h respectively. Cell viability was then determined by MTT assay. (C) SKOV-3 cells were treated with 0.1, 1, 10 and 100 μM of paclitaxel for 24h, then were assessed by MTT. (D) Different concentrations of MY-03-01 each with 1μM Taxol synergy effects. The significance was determined by a Student's t-test ($p<0.05$, compared with control)

Figure 2. Percentage of RBCs Hemolysis Incubated with MY-03-01 and ddH$_2$O as Positive Controls. The concentrations of MY-03-01 were 5, 10, 20 and 40 μM separately. Data were presented as mean standard deviation (n=3)

milk and 0.1% Tween-20 (blotting grade) for 3 h, then probed with the desired antibodies overnight at 4 °C. Membranes were subsequently incubated with appropriate HRP-conjugated secondary antibody for 45 min and visualized by Western blotting detection reagents (Trans Gene, Beijing, China).
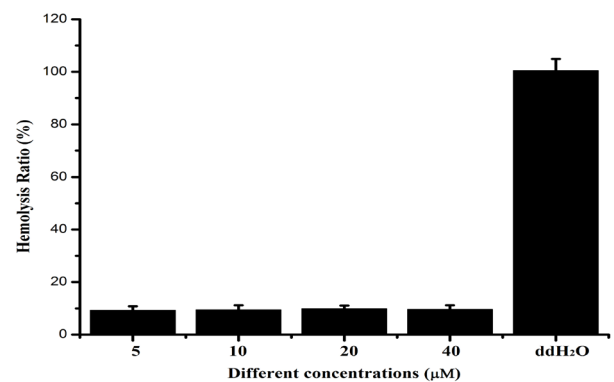
Statistical analysis

All data are presented as the mean±SD. Significant differences among groups were determined by using the unpaired Student's t-test. A *p*-value of <0.05 was considered to be statistically significant. The results shown in each of the figures in this article are representative of at least three independent experiments.

Results and Discussion

Ovarian cancer is difficult to treat (Rogalska et al., 2011). Taxanes are widely used in chemotherapy, but their clinical success is limited owing to the emergence of resistant tumor cells. For this reason, finding a new small molecule compound for treating ovarian cancer is imminent (Rogalska et al., 2013).

MY-03-01 inhibits SKOV-3 cell growth

In the present study, 2-chloro-N-(2-(1-oxo-3-(p-toly)-pyrrolo- [1,2-a]py-razin-2(1H)-yl)acetamide (MY-03- 01) is a new synthetic small molecular, selected from our own synthetic library (shown in Figure 1A) (Meng et al., 2013). To examine the exact effect of MY-03-01 on cell growth, we conducted a dose escalation experiment as shown in Figure 1B. The results indicated that MY-03-01 inhibited the viability of SKOV-3 cells in a dose-dependent manner. There was a significant decrease in cell viability of SKOV-3 observed after 24 h of treatment with 40 μM MY-03-01,

and this inhibition rate was 25% higher than that with 5 μM MY-03-01. After treating with the same concentration of MY-03-01 for 48 h, the IC$_{50}$ was 18.1644 μM.

Currently, paclitaxel is one of the primary ovarian cancer chemotherapy drugs, but some ovarian cancers show resistance to paclitaxel. In this study, when paclitaxel was incubated with SKOV-3 cells for 24 h at a concentration of 1 μM, the inhibition rate was 11.03% (Figure 1C). When SKOV-3 cells were concurrently treated with both MY-03-01 and paclitaxel, the inhibition rate was higher than that for MY-03-01 alone, and had better effects than using paclitaxel alone (Figure 1D). According to other reports, SKOV-3 cells are the least sensitive to small molecule anticancer drugs compared with other ovarian cancer cell lines. A series of new O-methylated analogues of resveratrol and have been tested in vitro model to select derivatives with the highest cytotoxic activity. Screening results show that 3,4,4'5-tetramethoxystilbene has the highest effect on SKOV-3 cell lines, and after 72 h, its IC$_{50}$ value is greater than 20 μM (Piotrowska et al., 2012). In this study, after 48 h of SKOV-3 cell line treatment with MY-03-01, the IC$_{50}$ was only 18.16 μM. Curcumin can also induce apoptosis of SKOV-3 cells lines, but the concentration of curcumin needed to be as high as 100 μM. Although SKOV-3 cells were treated after 24 and 48 h, the viability rate was still greater than 50% (Wahl et al., 2007). When the concentration of MY-03-01 was 40 μM, 24 and 48 h treatments showed a viability rate for SKOV-3 cells was lower than 50% (Figure 1B). As mentioned above, paclitaxel is one of the main chemotherapy drugs for the treatment of ovarian cancer, but resistance is often observed during treatment. However, when SKOV-3 cell lines were co-treated with MY-03-01 and paclitaxel, the induction of apoptosis was better than for each individual drug (Figure 1C and 1D).

Hemolysis activity

In recent years, many cytotoxic compounds have been designed to obtain more specific anticancer drugs and to minimize toxic side effects (Descôteaux et al., 2012). Characterization of in vitro blood compatibility of the MY-03-01 compound is important for evaluating whether normal cells are poisoned in addition to the cancer cells. To evaluate the damage inflicted by MY-03-01 on normal

Control   10 μM

20 μM   40 μM
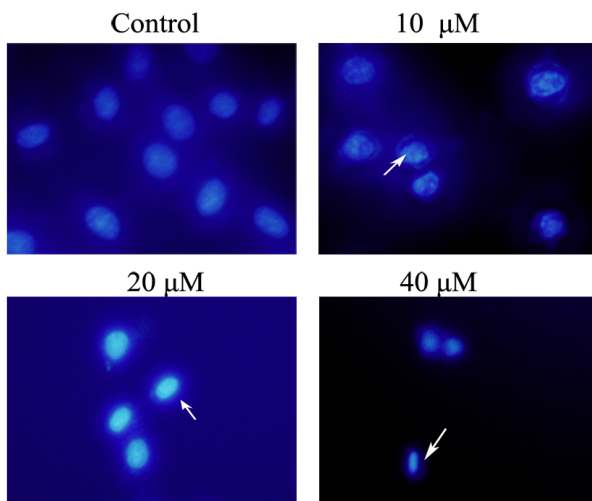
Figure 3. MY-03-01 Induced the Cell Apoptosis in SKOV-3 Cells. Cells were treated with 10, 20 and 40 μM of MY-03-01 for 24 h. The cells were then fixed and stained with 4,6-diamidino-2-phenylindile. The stained nucleus were observed under a fluorescent microscope using a blue filter

cells, hemolysis activity experiments were conducted. Hemolysis activity refers to the phenomenon of red blood cell rupture. Many physical and chemical factors can cause hemolysis, such as bile salts, detergents, etc. We needed to make sure that MY-03-01 will not cause cell death, and the hemolysis assay is a preliminary way to do so. The blood compatibility of MY-03-01 was assessed by a hemolysis assay. An enhanced hemolytic rate (HR) results in a higher level of broken RBCs. The RBCs for the assay were exposed to MY-03-01 at different concentrations for 1.5 h, and positive controls is ddH$_2$O. As shown in Figure 2, when the concentration of MY-03-01 was 40 μM, its hemolytic activity was less than 10% compared with the control, which suggested that there was almost no damage to normal cells. Because we did not buy the positive drugs of testing cell toxicity, it is better able to explain the problem if coupled with the result of the positive drug control.

MY-03-01 induction of apoptosis

Apoptosis is one of the main types of programmed cell death and involves a series of biochemical events leading to cellular morphological changes and cell death. The results of the present study confirmed that MY-03-01 could induce apoptosis in the SKOV-3 cell line. This finding was supported by two methods: DAPI staining and Annexin V/PI staining by flow cytometry. To determine whether MY-03-01 played a direct role in SKOV-3 apoptosis, we conducted morphological analysis of nuclei using DAPI staining. As shown in Figure 3, chromatin condensation and the formation of apoptotic bodies in cells were observed after 24 h of MY-03-01 treatment. When the concentration of MY-03-01 was 10 μM or higher, leaflets within the cell membrane with high phosphatidylserine (PS) content began to form, a phenomenon that is typical of apoptosis. Therefore, we assessed the effect of MY-03-01 on SKOV-3 cell apoptosis under identical treatment conditions using FACS analysis. The dose-dependent apoptotic effects of MY-03-01 are clearly shown in Figure 4A. As indicated in FACS analysis scatter grams, Annexin V/PI staining of control cells showed a large population of viable cells. Annexin V/PI staining can differentiate between early apoptosis, late apoptosis, and dead cells. Treatment of cells with MY-03-01 at 20 μM for 24 h resulted in a strong shift from live cells to early apoptotic cell populations, late apoptotic cell populations, and dead cell populations.

Finally, growth inhibition effects of MY-03-01 were evaluated to determine if apoptosis was correlated with the induction of cell cycle arrest. Cell cycle distribution was examined in cells treated with different concentrations of MY-03-01. The initial amount of cells in G2 phase appeared normal when compared with the control group (Figure 4B). After 24 h treatment with 20 μM MY-03-01, the percentage of G2/M phase cells increased from 8.29% (control) to 9.87%. G2 values showed almost no change when MY-03-01 concentration increased. This indicated that MY-03-01 may not induce apoptosis through inducing cell cycle arrest of SKOV-3 cells.
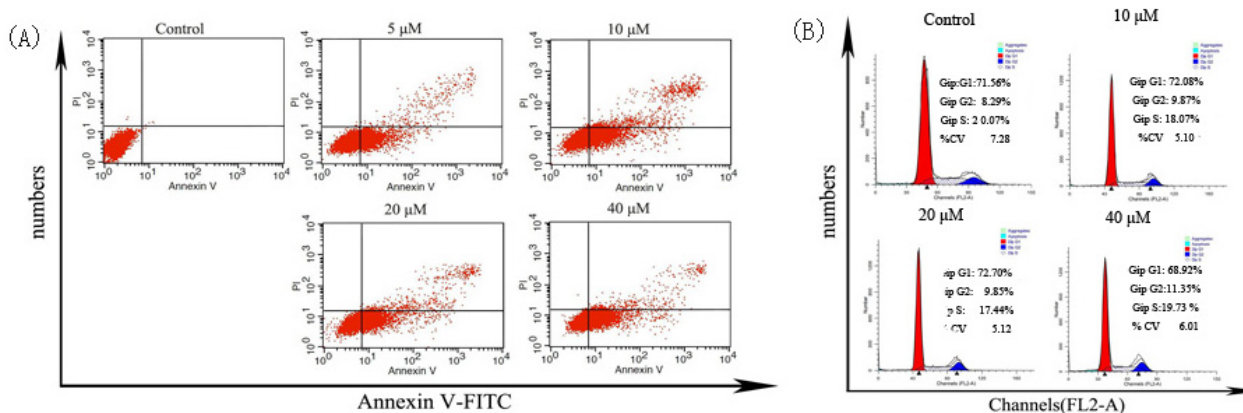
Figure 4. (A) MY-03-01 induced the cell apoptosis. Cells treated with 5, 10, 20 and 40 μM of MY-03-01 for 24 h, and were assessed for apoptosis by staining with Annexin V-FITC and propidium iodide (PI). (B) MY-03-01 triggered cell cycle arrest. The DNA content of cells treated with MY-03-01 for 24 h was analyzed by a flow cytometer. Cell-cycle distributions after treatment with 10, 20 and 40 μM MY-03-01 for 24 h separately. Cell-cycle distributions were assessed by PI staining. The DNA contents of 25000 events were analyzed by flow cytometry. The results were shown from one of three experiments with similar results. Each point represents the mean±SD of three independent experiments
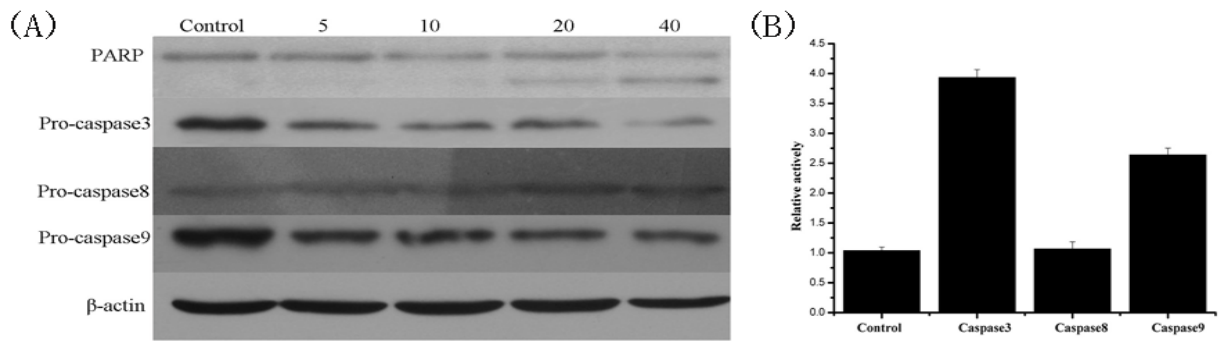
Figure 5. MY-03-01 Triggered Apoptosis by Activating the Caspases. (A) Cells were incubated with 0, 5, 10, 20 and 40 μM of MY-03-01 for 24 h. Equal amounts of cell lysates were then resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane, and probed with anti-poly (ADP-ribose) polymerase(PARP), pro-caspase3, pro-caspase-8, pro-caspase-9 and β-actin antibodies. The proteins were then visualized using detection system. Actin was used as an internal control. (B) Cells were incubated with 40μM of MY-03-01 for 24 h. Equal amounts of cell lysates were assayed for caspase3, caspase8 and caspase9 activity using DEVD-pNA, IETD-pNA and LEHD-pNAas substrates, respectively. The conentrations of the fluorescent productsreleased were then measured. Results represent the mean±SD of triplicate determinations. The significance was determined by a Student's t-test ($p<0.05$, compared with control.)

Mechanism of MY-03-01-induced apoptosis

The main mediators of apoptosis are cysteine proteases belonging to the family of caspases (Petrucci et al., 2007). Two main pathways for induction of apoptosis have been described, consisting of induction through a complex signaling sequence and through the activation of caspases (Degterev et al., 2003). Mitochondria play a very important role in multicellular organisms. If mitochondria cease functioning, cells stop aerobic respiration, and rapid cell death ensues. Targeting mitochondrial apoptotic proteins in different ways can adjust mitochondrial function. The apoptotic proteins can pass through the mitochondrial membrane pores, causing mitochondrial swelling, or the permeability of the mitochondrial membrane can increase and cause leakage of apoptotic effectors (Dejean et al., 2006). In the mitochondrial apoptotic pathway, cytochrome C is released and bind to apoptotic protease activating factor 1 (Apaf-1), ATP, then finally pro-caspase9, which leads to formation of apoptotic bodies. Pro-caspase9 will be cut to generate caspase9. In the end, caspase3 is activated to induce apoptosis. Since caspase3 and caspase9 are both key factors in the mitochondrial pathway of apoptosis, we investigated the effect of MY-03-01 on the protein levels of pro- caspase3, pro-caspase8, pro-caspase9, and PARP. PARP is a core member of apoptotic caspases. PARP will be cut when the caspase is activated, and while caspase8 is not involved in the mitochondrial pathway of apoptosis caspase3 and caspase9 are involved; all three of these caspases cleave PARP when active. Results showed that pro-caspase3 and pro-caspase9 levels were reduced in a dose-dependent manner, and the level of pro-caspase8 did not change in the treated cells (Figure 5A). PARP was cleaved under the treatment of MY-03-01 in a dose dependent manner. Next, caspase3, caspase8 and caspase9 activities determined by colorimetric assays indicated that the caspase3 and caspase9 were activated by MY-03-01, while caspase8 was not (Figure 5B). In this study, we found that caspase3 and caspase9 were evidently activated through MY-03-01. Obviously, MY-03-01 induced apoptosis by activating caspase3 and caspase9 in SKOV-3 cells. The results of

this study show that MY-03-01 has more advantages for ovarian cancer treatment than previous small molecule anti-cancer drugs, and provides a new clue for the clinical treatment of ovarian cancer in the future.

In conclusion, a small molecular compound (MY-03-01) showed great potential in the treatment of ovarian cancer. In this study, MY-03-01 could effectively induce apoptosis in SKOV-3 cells, and the mechanism of apoptosis was through the activation of caspase3 and caspase9. In addition, it is encouraging to notice that MY-03-01 had almost no toxicity in normal cells. MY-03-01 showed synergy with paclitaxel in killing SKOV-3 cells, which suggested that the synergistic effect was significantly better than using each of them alone. The low toxicity and good effects make the MY-03-01 a promising drug for the therapy of ovarian cancer. Moreover, because the MY-03-01 is cheap, easy to obtain at the industrial scale, ecology friendly and has the potential to be commercialized, it can serve as a platform for future drug delivery applications.

Acknowledgements

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