ABT-737 reverses cisplatin resistance through ROS-ASK1-JNK MAPK signaling pathway and Ca\textsuperscript{2+} signaling in human ovarian cancer cells

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Abstract

ABT-737, is a BH3-only protein mimetic, which can effectively inhibit the anti-apoptotic proteins Bcl-xL and Bcl-2. A large number of studies have shown that ABT-737 can induce a variety of tumor cell apoptosis, and also enhance cisplatin induced tumor cell apoptosis. However, the mechanism of ABT-737 enhances the sensitivity of ovarian cancer cells to cisplatin is still unclear and needs further study. Our results showed that ABT-737 can significantly increase the sensitivity of A2780/DDP cells to cisplatin. We detected that ABT-737 could significantly increase the activation levels of JNK and ASK1 in A2780/DDP cells induced by cisplatin. Inhibition of JNK and ASK1 pathway could significantly reduce cisplatin sensitivity increased by ABT-737 in A2780/DDP cells, and inhibition of ASK1 pathway could significantly reduce the activation level of JNK. We further detected that ABT-737 could obviously increase the level of reactive oxygen species (ROS) in A2780/DDP cells induced by cisplatin, and the inhibition of ROS could significantly reduce the activation levels of JNK and ASK1, as well as could significantly reduce cisplatin sensitivity increased by ABT-737 in A2780/DDP cells. Moreover, calcium chelators can significantly reduce cisplatin sensitivity increased by ABT-737 in A2780/DDP cells, the result is consistent with the current reports. These results suggested that ROS-ASK1-JNK signaling axis and calcium signaling play an important role in ABT-737 reversing cisplatin resistance in ovarian cancer. This might be a novel molecular mechanism of ABT-737 enhances the sensitivity of ovarian cancer cells to cisplatin through regulating ROS-ASK1-JNK signaling axis.

Introduction

Ovarian cancer is one of the malignant tumors that seriously threaten women's health all over the world. Due to the hidden anatomical site of the ovary, the clinical symptoms of ovarian cancer are not typical. About 75% of patients have been found and diagnosed as stage III or IV of the International Federation of gynecology and Obstetrics (FIGO)[1-3]. At present, the standard treatment of ovarian cancer is cytoreductive surgery and first-line chemotherapy. The sensitivity of patients with initial chemotherapy is about 50-80%, but most patients will relapse in about 2 years of complete remission, and the recurrence rate is as high as 80%. Patients with recurrent ovarian cancer are resistant to almost all chemotherapy drugs. More importantly, the 5-year survival rate of patients is only 30%-40%[4, 5]. Cisplatin is one of the most widely used chemotherapy drugs, which has a significant effect on ovarian cancer. However, cisplatin resistance is a major obstacle to achieving satisfactory effects in ovarian cancer treatment[6]. Thus, there is an urgent need for strategies to increase the cisplatin sensitivity of ovarian cancer.

Bcl-2 and Bcl-xL are two kinds of important anti-apoptotic protein in Bcl-2 family. They locate in mitochondria and regulate the permeability of mitochondrial outer membrane. They play an important regulatory role in apoptosis. The high expression of these two proteins is related to the occurrence and development, low survival rate, radiotherapy and chemotherapy resistance of a variety of tumors. They are effective tumor treatment targets[7]. The overexpression of Bcl-2 is correlated with poor responses to primary chemotherapy and decreases survival rate in ovarian cancer patients[8, 9]. In addition, Bcl-2 overexpression can also participate in cisplatin resistance in ovarian cancer. The overexpression of Bcl-2
protein can significantly reduce inhibition of proliferation and apoptosis of ovarian cancer cells induced by cisplatin[10]. Increased Bcl-xL protein expression was observed in recurrent compared to primary ovarian cancers and was associated with clinical resistance to chemotherapy and decreased survival[11, 12]. Some studies have shown that inhibiting Bcl-xL expression can effectively induce ovarian cancer cell death[13, 14]. Therefore, targeting anti-apoptotic protein Bcl-2 and Bcl-xL may be one of the important means to improve the chemotherapy tolerance of ovarian cancer patients and improve the five-year survival rate of ovarian cancer patients.

ABT-737, BH3 only protein simulant, is an effective small molecule inhibitor of Bcl-xL and Bcl-2. It can specifically inhibit the binding of Bcl-2/Bcl-xL to Bak/Bax by competing with BH3 domain, and then induce apoptosis through mitochondrial pathway[15]. A large number of studies have shown that ABT-737 has good antitumor activity in a variety of tumor types[16]. ABT-737 is not only play a synergistic cytotoxic role in different cancer cells, including ovarian, lung, and bladder cancer, but also induces significant apoptosis in different cancers[17]. In addition, it has been reported that ABT-737 could enhance cisplatin induced tumor cells apoptosis[17]. However, the mechanism of whether ABT-737 enhance the sensitivity of ovarian cancer cells to cisplatin remains unclear and it needs further study.

Although the role of ABT-737 in enhancing cisplatin sensitivity of cisplatin-resistant ovarian cancer cells has been reported, its specific molecular mechanism has not been fully explored. Some studies have shown that ABT-737 can induce the accumulation of a large number of reactive oxygen species (ROS) in the body and destroy the redox balance of organisms, so as to induce oxidative stress of a variety of tumor cells, including ovarian cancer, and then induce tumor cells apoptosis. These results suggested that there is a positive correlation between reactive oxygen species (ROS) and apoptosis[18]. However, the relationship between ROS and ovarian cancer cisplatin resistance is still unclear. Although a large number of studies have shown that ROS accumulation is cytotoxic and conducive to the treatment of cancer cells, the potential of ROS in the treatment of drug resistance of cancer cells has not been systematically studied[19]. Therefore, the study of the effect of ROS on cisplatin induced apoptosis is of great significance to clarify the mechanism of ovarian cancer cisplatin resistance.

ROS is one of the important members of JNK signaling pathway and plays a very important role in physiological processes such as inflammatory response and apoptosis. c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) play a key role in cell apoptosis induced by various types of stress, such as reactive oxygen species (ROS) and calcium ion (Ca\(^{2+}\)) overload[20]. Moreover, some studies have confirmed that ABT-737 can enhance the activation of JNK pathway by inhibiting the effect of Bcl-2/Bcl-xL, and then induce apoptosis[21-24]. So, whether ABT-737 could induce the activation of JNK pathway by inducing the accumulation of reactive oxygen species, and then promote the sensitivity of ovarian cancer cisplatin resistant cells to cisplatin. We need to further study this question. Apoptosis signal regulated kinase 1 (ASK1) plays a bridge and link role in ROS mediated JNK activation signal pathway. The signal pathway activated by ASK1 is one of the important ways for ROS participate in JNK signal transduction[20]. Therefore, we speculate that ABT-737 may reverse cisplatin resistance of cisplatin-resistant ovarian cancer cells by regulating ROS-ASK1-JNK signaling pathway.
In the present study, we detected that the effect of ABT-737 on cisplatin sensitivity of cisplatin-resistant ovarian cancer A2780/DDP cells, and deeply explored the molecular mechanism of ABT-737 affecting cisplatin resistance of A2780/DDP cells. We confirmed that ABT-737 could significantly increase the sensitivity of cisplatin-resistant ovarian cancer A2780/DDP cells to cisplatin, which is mediated by ROS-dependent activation of the ASK1-JNK MAPK signaling pathway. In addition, Ca\(^{2+}\) overload is also involved in ABT-737 increases the sensitivity of A2780/DDP cells to cisplatin.

**Materials And Methods**

**Reagents and antibodies**

ABT-737 (a BH3 mimetic), NAC (ROS inhibitor), U0126 (ERK inhibitor), SB203580 (p38 inhibitor) SP600125 (JNK inhibitor), LY294002 (Akt inhibitor) and BAPTA-AM (calcium chelator) were purchased from Selleck Chemicals LLC (Houston, TX, USA). Antibodies against Bcl-xL (1:200, mouse) and Bcl-2 (1:200, rabbit) were purchased from BD Bioscience (Franklin Lakes, NJ, USA). Antibodies against Bax (1:1000, rabbit) and Bak (1:1000, rabbit) were purchased from Abcam (Cambridge, MA, USA). Antibodies against p-Ask1 (1:1000, rabbit), Ask1 (1:1000, rabbit), p-P38 (1:500, rabbit), P38 (1:500, rabbit), p-Akt (1:2000, rabbit) and Akt (1:2000, rabbit) were purchased from Proteintech Group Inc (Chicago, IL, USA). Antibodies against Cleaved-capase3 (1:1000, rabbit), p-JNK (1:2000, rabbit), JNK (1:2000, rabbit), p-ERK (1:1000, rabbit) and ERK (1:1000, rabbit) were purchased from Cell Signaling (Beverly, MA, USA). The antibody against β-actin (1:2000, mouse) was purchased from Beyotime Biotechnology Limited Company (Shanghai, China).

**Cell culture**

Human cisplatin-resistant ovarian cancer A2780/DDP cells were provided by the Department of Biochemistry and molecular biology, Basic Medical College, Shanxi Medical University, China. Cells were cultured in RPIM 1640 culture medium (Gibco, Carlsbad, CA) and supplemented with 10 % (V/V) fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) at 37 °C with 5 % CO\(_2\).

**Cell viability assays**

Human cisplatin-resistant ovarian cancer A2780/DDP cells were plated at 1.2×10^4 cells/well in 96-well plates (Corning Incorporated, Tewksbury, MA). After 24 h of incubation at 37 °C with 5 % CO\(_2\), cells were treated as indicated. Next, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (10 μL, 5 mg/mL in phosphate buffered saline (PBS); Sigma-Aldrich, St. Louis, MO) was added to each well and plates were incubated for 4-6 h. Formazan crystals were dissolved in 150 μL DMSO and wells were shaken 10-15 min using a tablet oscillation device. Absorbance was recorded at a wavelength of 490 nm (Bio-Rad, Hercules, CA, USA).

**TUNEL assays**
Apoptosis was detected using an In Situ Cell Death Detection kit (Roche, Indianapolis, IN) according to the manufacturer’s instructions. For flow cytometry, cells were harvested by trypsin (0.4 %, Solarbio life science, Beijing, China) and washed with PBS. Cells were fixed with 4% (w/v) paraformaldehyde (Solarbio life science, Beijing, China)/PBS for 20 min on ice and washed with PBS. Cells were fixed with 70% (w/v) ethanol and incubated for 4 h at -20 °C. The cells were centrifuged and washed with PBS, and then incubated with 0.1% TritonX-100 for 5 min at room temperature. After washing with PBS, cells were incubated with proteinase K (20 μg/mL) for 8-10 min at room temperature, washed with PBS, and incubated with 80 μL equilibration buffer for 5 min at room temperature. Cells were centrifuged at 3000g for 5 min and the pellet was incubated with 50 μL terminal deoxynucleotidyl transferase (TdT) mixture (1:9=TdT: equilibration buffer) for 1 h at 37 °C in a humidified atmosphere. Cells were washed with PBS and resuspended in 200-300 μL PBS to for flow cytometry.

For confocal microscopy, A2780/DDP cells were plated at 4×10^4 cells/well in 24-well plates (Corning Incorporated, Tewksbury, MA). After 24 h, cells were treated as indicated at 37 °C with 5 % CO₂. Cells were washed with 0.1 M PBS three times and fixed with 4% (w/v) paraformaldehyde/PBS for 20-30 min at room temperature. After washing with 0.1 M PBS three times, cells were incubated with TdT-mediated dUTP nick-end labeling (TUNEL) reaction mix containing 10 U of terminal deoxyriboinosine transferase, 10 mM dUTP biotin, and 2.5 mM cobalt chloride in 1× terminal transferase reaction buffer for 1 h at 37 °C in a humidified atmosphere. Apoptotic cells with characteristic nuclear fragmentation (staining green) were counted in six randomly chosen fields. The experiment was repeated three times.

**Detection of cell apoptosis**

Cells were cultured in 24-well plates (Corning Incorporated, Tewksbury, MA), and then treated with different indicated drugs for 24h. Cells were washed with PBS three times and fixed with 4% (w/v) paraformaldehyde/PBS for 20-30 min at room temperature. After washing with PBS three times, cells were incubated with Hoechst 33258/H₂O (2 μg/mL) for 5 min. And then washed three times with PBS. Cell were examined with fluorescence microscope (Olympus Corporation, Tokyo Japan).

A2780/DDP cells were exposed to indicated treatment for a certain time, and then flow cytometry was performed according to the manufacture's instructions of Annexin V-FITC/PI Apoptosis detection Kit (Dalian Meilun Biotechnology Co Ltd Dalian, China) to detect changes in apoptosis levels. Cells were harvested by trypsin (0.4 %, Solarbio life science, Beijing, China) and washed with PBS for two times. Add 1×binding buffer to the cell precipitation and resuspend cells, make the cell concentration reach to 1×10⁶ cells/ml. And then absorb 100 μL cell suspension (total number of cells is 1×10⁵ cells) into a new tube and add 5 μL annexin V-FITC and 5-10 μL PI, gently mix and incubate at room temperature in the dark for 15 min. After staining incubation, 400μL 1×binding buffer was added to each tube, was mixed and detected by flow cytometry.

**Measurement of ROS formation**
A2780/DDP cells were cultured in 24-well plate, and DCFH-DA (Beyotime Biotechnology Limited Company, Shanghai, China) was added at a final concentration of 5 μM after incubation with indicated treatment for 24 h. Cells were then incubated at 37°C for 20 min. The cells were washed three times with PBS to sufficiently remove any DCFH-DA that did not enter the cells. The fluorescence microscope (Olympus Corporation, Tokyo Japan) was used to detect the changes of intracellular ROS production in A2780/DDP cells.

**Measurement of intracellular Ca$^{2+}$ levels**

A2780/DDP cells were cultured in a 24-well plate and subjected to indicated treatment for 24 h. After the cell culture medium was discarded, a serum-free cell culture medium containing 5 mM of Fura-4 AM was added and incubated at 37°C for 30 min. Changes in intracellular calcium ion levels were detected by a fluorescence microscope (Olympus Corporation, Tokyo Japan).

**Western blot assays**

Cells were lysed in RIPA buffer by sonication. Protein concentrations were quantified using a protein assay kit (Bio-Rad). Equivalent amounts of proteins (30-40 μg) were separated by 12% SDS-poly-acrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, Bedford, MA). The membranes were blocked with 5% non-fat dry milk in PBST buffer (10 mM Tris-HCl pH 7.6, 100 mM NaCl and 0.1% Tween-20) for 90-120 min at room temperature and then incubated with the relevant primary antibody overnight at 4°C. The membranes were washed with PBST buffer for three times in 10 minues/time. The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) at a 1:1000 dilution for 90 min at room temperature and then washed with PBST buffer for three times in 10 minues/time. The immunoreactive bands were visualized by Enhanced Chemi Luminescent detecting agents (Thermo). The reactive bands were measured with an ECL image detecting system (Gene Company Limited) and protein levels were quantified by densitometry using Quantity One software (Bio-Rad Laboratories).

**Measurement of oxidative stress indices**

Cells were cultured in 6-well plates and cultured at 37°C. After the cells were grown to 80% confluence, they were treated with indicated treatment for 24 h. Intracellular levels of hydrogen peroxide, superoxide anion and hydroxyl radicals (Beyotime Biotechnology, China) were measured by a microplate reader using detection kits according to the manufacturer's protocols.

**Measurement of total antioxidant capacity**

A2780/DDP cells were cultured in 6-well plates, and then treated with indicated treatment for 24 h. Changes in the levels of total antioxidant capacity (Beyotime Biotechnology, China) were detected using the corresponding kit according to the manufacturer's directions.

**Statistical analysis**
Statistical analysis of the data was analyzed by t test. $P<0.05$ and $P<0.01$ were considered to represent a statistically significant difference. Data are representative of three independent experiments performed in triplicate.

## Results

**ABT-737 and Cisplatin inhibit the growth of cisplatin-resistant ovarian cancer A2780/DDP cells in time and dose-dependent**

A2780/DDP cells were treated with different concentrations of ABT-737 (0, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0 μmol/L) and cisplatin (0, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0 μg/ml) for 24h and 48h, and cell survival rate was examined by CCK8 assays. The results showed that the cell viability decreased in a time and dose-dependent (Fig 1a and 1b). Based on CCK8 assay results, we selected 5 μmol/L as the optimum concentration of ABT-737 and selected 10 μg/mL as the optimum concentration of cisplatin.

**ABT-737 increases the sensitivity of cisplatin-resistant ovarian cancer A2780/DDP cells to cisplatin**

Next, in order to investigate whether ABT-737 would affect the anti-tumor effect of cisplatin, we treated cisplatin-resistant ovarian cancer A2780/DDP cells with different concentrations of ABT-737 (10.0, 20.0, 40.0 μmol/L) combined with cisplatin (5.0, 10.0, 20.0 μg/mL) for 24h, separately (Fig. 2a, 2b and 2c). Based on these above CCK8 assay results, we selected the optimum combination of treatment which is ABT-737 (5μmol/L) combined with cisplatin (10μg/mL) for 24 h in A2780/DDP cells (Fig. 2d). These results of CCK8 assays showed that ABT-737 increased cisplatin-induced growth inhibition.

In order to further confirm whether ABT-737 would increase apoptosis induced by cisplatin, we treated cells with cisplatin and/or ABT-737 for 24h and detected the expression of apoptotic-related proteins, including Cleaved-caspase 3, Bax, Bak, Bcl-2 and Bcl-xL. The results showed that cisplatin combination with ABT-737 would obviously increased the expression of Bax, Bak, cleaved-caspase3 and cleaved-PARP (Fig. 3a and 3b). In addition, we also evaluated apoptosis using TUNEL assays (Fig. 3c and 3d). On the basis of these results, we treated cells with cisplatin and/or ABT-737 for 24h and then examined apoptotic chromatin condensation with Hoechst 33258 staining. The results indicated that ABT-737 increased cisplatin-induced apoptotic chromatin condensation (Fig. 3e). At last, we also detected apoptosis with caspase 3 enzyme activity assays (Fig. 3f). These results showed that ABT-737 increased cisplatin-induced caspase 3 enzyme activity and DNA damage. Both of these results suggested that ABT-737 obviously increased apoptosis induced by cisplatin in cisplatin-resistant ovarian cancer A2780/DDP cells.

**ABT-737 increases the sensitivity of cisplatin-resistant ovarian cancer A2780/DDP cells to cisplatin through JNK pathway**

MAPK and PI3K/Akt are the main signal pathways regulating cell proliferation and apoptosis. Some studies have shown that ABT-737 can regulate different types of apoptosis through MAPK and PI3K/Akt
signaling pathways. However, it is unclear whether ABT-737 can increase the sensitivity of cisplatin-resistant ovarian cancer cells to cisplatin through MAPK and PI3K/Akt signaling pathways. We used western blotting to detect the activation of MAPK and PI3K/Akt signaling pathways induced by the combination of ABT-737 and cisplatin for 24 h. The results showed that the combination of ABT-737 and cisplatin could significantly increase the expression levels of p-JNK, p-ERK, p-p38 and p-Akt proteins (Fig. 3a, 3b, 3c, 3d and 3e). In addition, we used specific inhibitors of MAPK and PI3K/Akt signaling pathways to inhibit these two signaling pathways and found that SP600125 (JNK inhibitor) could significantly reverse the growth inhibition and apoptosis induced by the combination of ABT-737 and cisplatin. However, U0126 (ERK1/2 inhibitor), SB203580 (p38 inhibitor) and LY294002 (Akt inhibitor) have no inhibitory effect on growth inhibition and apoptosis induced by the combination of ABT-737 and cisplatin (Fig. 3f and 3g). The above results showed that ABT-737 can increase the sensitivity of ovarian cancer cisplatin resistant cells to cisplatin through the activation of JNK pathway.

**ASK1 is required for ABT-737-induced JNK activation and apoptosis of cisplatin-resistant ovarian cancer A2780/DDP cells**

It has been found that in many types of cells, ASK1 plays a key role in the activation of p38 and JNK induced by oxidative stress. However, the upstream signaling pathway that ABT-737 increases the sensitivity of cisplatin-resistant ovarian cancer cells to cisplatin by inducing the activation of JNK signaling pathway is still unclear. Here, we used western blotting to detect the activation of ASK1 protein induced by the combination of ABT-737 and cisplatin for 24 h. We found that ABT-737 combined with cisplatin could significantly increase the phosphorylation level of ASK1 protein (Fig. 5a and 5b). After treatment with ASK1 inhibitor GS-4997, the result of Hoechst 33258 nuclear staining showed that chromatin pyknosis and nuclear fragmentation were significantly reduced (Fig. 5c), the CCK8 assay showed that the inhibition of cell survival rate was significantly alleviated (Fig. 5d), and the result of Annexin V-FITC/PI double staining showed that the apoptosis rate of cells was significantly reduced (Fig. 4e). The above results suggested that ASK1 plays an important role in the apoptosis of ovarian-cancer cisplatin resistant cells induced by ABT-737. In addition, we detected the phosphorylation levels of ASK1 and JNK proteins after treatment with ASK1 inhibitor GS-4997 by western blotting. These results showed that inhibiting the expression of ASK1 could significantly inhibit the activation level of JNK in cells (Fig. 5f, 5g and 5h), indicating that ASK1 is necessary for JNK activation induced by ABT-737.

**ABT-737 enhances the imbalance of antioxidant system induced by cisplatin in cisplatin-resistant ovarian cancer A2780/DDP cells**

A large number of studies have shown that ABT-737 can induce cell apoptosis by increasing oxidative stress. Oxidative stress is one of the main anti-tumor mechanisms by which some chemotherapeutic drugs induce apoptosis. Reactive oxygen species (ROS) plays a vital role in maintaining cell redox balance and can induce cell apoptosis. Firstly, we detected the production of ROS in cells after exposure to the combination of ABT-737 and cisplatin for 24 h by fluorescence microscope. The results showed that ABT-737 combined with cisplatin could significantly increase the production of ROS in A2780/DDP
cells (Fig. 6a). In addition, we also found that the levels of oxidative stress indices, including hydrogen peroxide, superoxide anion, hydroxyl radicals, were significantly increased after exposure to ABT-737 combined with cisplatin for 24h (Fig. 6b, 6c, and 6d), while the level of T-AOC of A2780/DDP cells was significantly reduced after exposure to ABT-737 combined with cisplatin for 24h (Fig. 6e). Our results indicated that ABT-737 could obviously enhance the imbalance between oxidation and antioxidant status induced by cisplatin in A2780/DDP cells.

The ROS production induced by ABT-737 mediates JNK activation and apoptosis of cisplatin-resistant ovarian cancer A2780/DDP cells

In order to further clarify the role of oxidative stress in ABT-737 induced apoptosis of A2780/DDP cells. We used NAC (N-acety-L-cysteine, an antioxidant) pretreatment to detect the changes of ASK1 and JNK phosphorylation levels of A2780/DDP cells by the combined action of ABT-737 and cisplatin. The result showed that NAC could obviously inhibit the phosphorylation levels of ASK1 and JNK proteins (Fig. 7a, 7b, and 7c). It suggested that the ROS formation induced by ABT-737 could mediate JNK activation. In addition, the CCK8 assay showed that the inhibition of cell survival rate was significantly alleviated (Fig. 7d), the result of Hoechst 33258 nuclear staining showed that chromatin pyknosis and nuclear fragmentation were significantly reduced (Fig. 7e), and the result of Annexin V-FITC/PI double staining showed that the apoptosis rate of cells was significantly reduced (Fig. 7f). These results showed that the production of ROS induced by ABT-737 could obviously induce apoptosis of cisplatin-resistant ovarian cancer A2780/DDP cells.

Intracellular Ca\textsuperscript{2+} accumulation is involved in ABT-737-induced apoptosis of cisplatin-resistant ovarian cancer A2780/DDP cells

Intracellular calcium ions accumulation is another important mechanism of apoptosis. Therefore, we detected the changes of intracellular calcium ions concentration under the action of cisplatin, ABT-737, cisplatin+ABT-737 and cisplatin+ABT-737+BAPTA-AM with fluorescence microscope. The results showed that compared with the control group, the level of intracellular calcium ions is the highest under the combined action of cisplatin and ABT-737, indicating that ABT-737 could significantly increase the accumulation of intracellular calcium ions induced by cisplatin (Fig. 8a). In addition, both of the CCK8 assay results (Fig. 8b), Hoechst 33258 nuclear staining results (Fig. 8c) and Annexin V-FITC/PI double staining results of cell apoptosis (Fig. 8d) showed that calcium chelator (BAPTA-AM) could significantly reduce the inhibition of survival rate of cisplatin-resistant ovarian cancer A2780/DDP cells induced by ABT-737 and inhibit the apoptosis of A2780/DDP cells induced by ABT-737.

Discussion

Ovarian cancer is one of the three major malignant tumors of the female reproductive system. Because its early symptoms are not obvious, and the corresponding symptoms are mostly in the late stage of the disease, it is difficult to treat ovarian cancer and has a high mortality. Once the disease was confirmed,
surgical treatment is carried out in most cases, and several courses of paclitaxel combined with platinum chemotherapy were performed after operation to increase the survival rate of patients. However, the emergence of chemoresistance has seriously restricted the effect of this combination therapy, and even led to the failure of treatment, resulting in the recurrence of ovarian cancer[25, 26]. Therefore, exploring the molecular mechanism of chemotherapy resistance in ovarian cancer and giving symptomatic treatment has immeasurable significance for reversing chemotherapy resistance of ovarian cancer and improving the curative effect of malignant tumor treatment of ovarian cancer.

At present, a large number of studies have found that the high expression of anti-apoptotic proteins Bcl-2 and Bcl-xL is closely related to the chemoresistance in ovarian cancer. Targeting anti-apoptotic proteins with genetic knockdown of Bcl-2/Bcl-xL or with small molecule inhibitors of Bcl-2/Bcl-xL enhanced sensitivity to platinum or paclitaxel in ovarian cancer cell lines[27] and ex vivo patient samples[28, 29]. ABT-737 is a novel and potent inhibitor of B-cell lymphoma 2 (BCL-2) family proteins, which are critical for cell survival and overexpressed in many tumor cells, with high affinity towards Bcl-xL, Bcl-2, and Bcl-w but no affinity towards less homologous proteins, such as Bcl-B, Mcl-1, and A1. ABT-737 has shown that single-agent activity against lymphoma and small-cell lung cancer as well as substantial antmyeloma activity both in vitro and in vivo. In recent studies, acute myeloid leukemia blast, origenitor, and stem cells are effectively killed by ABT-737 with normal hematopoietic cells intact[30, 31]. Our results confirmed that ABT-737 is not only inhibit the cell survival rate of human cisplatin-resistant ovarian cancer A2780/DDP cells in a time and dose-dependent manner, but also increases the proliferation inhibition of A2780/DDP cells induced by cisplatin in a dose-dependent manner. At the same time, ABT-737 could significantly inhibit the expression of anti-apoptotic proteins Bcl-2 and Bcl-xL, increase the expression of apoptosis proteins Cleaved-caspase 3, Bax and Bak, and could enhance the activity of caspase 3, so as to increase cisplatin induced apoptosis in A2780/DDP cells. These results showed that ABT-737 could not only inhibit the survival rate of A2780/DDP cells alone, but also increases the sensitivity of A2780/DDP cells to cisplatin. Although it has been confirmed that ABT-737 can increase the sensitivity of cisplatin-resistant ovarian cancer cells to cisplatin, the potential mechanism of ABT-737 reversing cisplatin resistance in ovarian cancer still needs to be further explored.

Mitogen activated protein kinases (MAPKs) are a group of evolutionarily conserved serine-threonine kinases, which can be divided into four subfamilies: ERK, p38, JNK and ERK5, representing four classical MAPK pathways respectively. MAPK pathway is a cascade reaction of three kinases. The upstream kinase (MAPKKK) responds to various extracellular and intracellular signals and activates the intermediate kinase (MAPKK) through direct phosphorylation[32]. MAPKK specifically phosphorylated and activated MAPK, MAPK is the key signaling pathway that regulates cell promotion, apoptosis and drug resistance under the stimulation of different types of endogenous or existing factors[20, 32]. A large number of studies have confirmed that MAPK signaling pathway plays an important role in the proliferation and apoptosis of a variety of tumor cells induced by ABT-737[21, 33]. So, whether MAPK signaling pathway is involved in the process of ABT-737 reversing chemotherapy resistance of tumor, for example, whether it is involved in ABT-737 reversing cisplatin resistance of ovarian cancer is still unknown. In the present study, we found that ABT-737 could significantly increase the sensitivity of
A2780/DDP cells to cisplatin. In addition, we further examined the effect of ABT-737 combined with cisplatin on MAPK signaling pathway in A2780/DDP cells. The results showed that ABT-737 could significantly increase the phosphorylation levels of JNK, ERK and p38 in MAPK signaling pathway. However, after JNK, ERK and p38 in MAPK signaling pathway were inhibited by specific inhibitors, we found that only JNK inhibitor (SP600125) pretreatment could significantly inhibit the proliferation inhibition of A2780/DDP cells induced by ABT-737 combined with cisplatin, and reduce the apoptosis of A2780/DDP cells induced by ABT-737 combined with cisplatin. However, it has no effect that ERK and p38 MAPK signaling pathway were inhibited by ERK1/2 inhibitor (U0126) and p38 inhibitor (SB203580) on the cell proliferation inhibition and apoptosis induced by ABT-737 combined with cisplatin in A2780/DDP cells.

Although we also detected the activation of Akt protein when ABT-737 combined with cisplatin, the results showed that the phosphorylation level of Akt increased significantly when ABT-737 combined with cisplatin. However, pretreatment with LY294002 (Akt inhibitor) did not affect the proliferation inhibition and apoptosis of A2780/DDP cells induced by ABT-737 combined with cisplatin. The above results confirmed that ABT-737 increased sensitivity of A2780/DDP cells to cisplatin may be mediated by JNK-MAPK signaling pathway. Although the mechanism of ABT-737 increases the sensitivity of cisplatin-resistant ovarian cancer cells to cisplatin has been studied. To my knowledge, it should be firstly reported that ABT-737 increases the sensitivity of cisplatin-resistant ovarian cancer cells to cisplatin by inducing the activation of JNK pathway.

ASK1 is mitogen activated protein kinase 5 (MAP3K5), which is activated by a series of stimuli, including calcium overloaded, reactive oxygen species (ROS), endoplasmic reticulum stress (ERs), and extracellular inflammatory signals (LPS-lipopolysaccharides and TNF-tumor necrosis factor). The activation of ASK1 could phosphorylate and activate two MAP2K pathways, including MEE-4/7 and MKK-3/6, which hereby phosphorylates and activates c-Jun N-terminal kinase (JNK) and p38 MAPKs, respectively[34]. However, it is still unclear whether ASK1 as an upstream signaling pathway of JNK participate in the process of ABT-737 increasing cisplatin sensitivity of ovarian cancer. Therefore, we detected the phosphorylation level of ASK1 in A2780/DDP cells. The results showed that ABT-737 combined with cisplatin could significantly increase the phosphorylation level of ASK1 in A2780/DDP cells. In order to further confirm whether ASK1 is the upstream signal pathway of JNK in the process of ABT-737 increasing cisplatin sensitivity of ovarian cancer, we pretreated A2780/DDP cells with ASK1 inhibitor (GS-4997) and found that the JNK protein activation and apoptosis induced by ABT-737 combined with cisplatin were significantly reversed in A2780/DDP cells. These results suggested that ASK1/JNK signaling axis plays an important role in ABT-737 reversing ovarian cancer cisplatin resistance.

As the main mechanism of cell damage induced by exogenous chemicals, oxidative stress plays an important role in ABT-737-induced apoptosis of a variety of tumor cells, including ovarian cancer[35, 36]. Is there any involvement of oxidative stress in ABT-737 reversing ovarian cancer cisplatin resistance? Next, we detected the level of ROS in A2780/DDP cells by fluorescence microscope. The results showed that ABT-737 combined with cisplatin could significantly increase the level of ROS in A2780/DDP cells.
Moreover, the combined action of ABT-737 and cisplatin on A2780/DDP cells could significantly reduce the level of intracellular T-AOC (total antioxidant capacity). Our above results confirmed that ABT-737 may increase the sensitivity of A2780/DDP cells to cisplatin by increasing the oxidative stress and reducing the antioxidant capacity in A2780/DDP cells.

In addition, we found that ROS inhibitor (NAC) could significantly reduce the proliferation inhibition and apoptosis induced by ABT-737 combined with cisplatin in A2780/DDP cells. A large number of studies have shown that ABT-737 can induce oxidative stress in a variety of tumor cells[35, 37]. In the current study, we also found that ABT-737 induce the production of ROS and significantly increase the production of ROS induced by cisplatin alone in A2780/DDP cells. These results suggested that ABT-737 could increase the sensitivity of A2780/DDP cells to cisplatin by inducing oxidative stress. In addition, we found that NAC pretreatment could significantly reduce the phosphorylation level of ASK1 and JNK proteins induced by the combined action of ABT-737 and cisplatin, and inhibit the activation of ASK1 and JNK. From these data, we speculated that the activation of ASK1-JNK pathway dependent on oxidative stress is necessary for ABT-737 increase ovarian cancer cisplatin sensitivity.

Calcium is an important second messenger, which can participate in a large number of signal cascades reaction in organisms[20]. For a long time, it has been considered that calcium is closely related to the initiation and occurrence of cell death[38]. So does calcium signal participate in ABT-737 induced apoptosis of ovarian cancer cells? Or does calcium signaling involve in ABT-737 reverse ovarian cancer cisplatin resistance? At present, some studies have shown that using Bcl-2 inhibitor ABT-737 or reduce the expression of Bcl-2 with gene knockout technology can increase the accumulation of calcium ions in ovarian cancer cells. Overloaded calcium ions could further induce endoplasmic reticulum and mitochondria related cell apoptosis, and then increase the sensitivity of cisplatin-resistant ovarian cancer cells to cisplatin[10, 31]. Our previous research also detected the effect of ABT-737 on the calcium concentration in A2780/DDP cells. The results also showed that ABT-737 could significantly increase the calcium concentration in A2780/DDP cells induced by cisplatin. In addition, we found that the calcium chelator BAPTA-AM could significantly reverse the inhibition of cell proliferation induced by the combined action of ABT-737 and cisplatin, and also reduce the apoptosis of A2780/DDP cells induced by ABT-737 combined with cisplatin. At present, some studies have confirmed that ABT-737 can reverse cisplatin resistance of ovarian cancer by regulating endoplasmic reticulum mitochondrial calcium signal[10, 31]. That is, calcium signal can participate in regulating ovarian cancer cisplatin resistance. Therefore, according to our results, we speculate that ROS/Ca$^{2+}$-ASK1-JNK signal axis may be one of the main mechanisms by which ABT-737 increases the sensitivity of cisplatin-resistant ovarian cancer cells to cisplatin.

In conclusion, our study showed that ABT-737 could not only induce apoptosis of A2780/DDP cells in a time and dose-dependent, but also increase the sensitivity of A2780/DDP cells to cisplatin. We also found that ROS mediated activation of JNK signaling pathway is necessary for ABT-737 increase the sensitivity of A2780/DDP cells to cisplatin. In addition, calcium overload induced by ABT-737 is also involved in the process of ABT-737 increasing the sensitivity of A2780/DDP cells to cisplatin. Our research showed that
ABT-737, a BH3 simulant, plays a very important role in reversing ovarian cancer cisplatin resistance. In addition to the confirmed mechanism, ABT-737 can also increase the sensitivity of cisplatin-resistant ovarian cancer cells to cisplatin by regulating ROS/Ca^{2+}-ASK1-JNK signal axis, which provides an experimental and theoretical basis for effective clinical treatment of ovarian cancer and increasing the five-year survival rate of ovarian cancer patients.

**Abbreviations**

Bcl B cell lymphoma

ROS Reactive oxygen species

ASK1 Apoptosis signal regulating kinase 1

JNK c-Jun N-terminal kinase

MAPK Mitogen-activated protein kinase

**Declarations**

**Acknowledgements**

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**Author Contributions Statement**

Xiaoning Li designed and performed most of the experiments with assistance from Zihan Xing, Tao Gong, Lijun Yang, Tao Yang, Bingmei Chang and Xiaoxia Wang. Zihan Xing and Tao Gong sorted the data of the manuscript. Lijun Yang, Tao Yang, Bingmei Chang and Xiaoxia Wang provided ABT-737 and other inhibitors and performed related drug treatment. Baofeng Yu and Rui Guo designed the experiments and supervised the study. Xiaoning Li wrote the manuscript. All authors commented on the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**Data Availability Statements**

All data generated or analysed during this study are included in this published article.

**Open Access**
References


Figures
ABT-737 and Cisplatin inhibit the growth of A2780/DDP cells. a A2780/DDP cells were treated with ABT-737 (0, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0 μmol/L) for 24 and 48 h, and cell viability was detected by CCK8 assay. Data are presented as mean±SD, n=6, *P < 0.05 and **P<0.01 (24 h), #P < 0.05 and ##P<0.01 (48 h) versus control group. b A2780/DDP cells were treated with Cisplatin (0, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0 μmol/L) for 24 and 48 h, and cell viability was detected by CCK8 assay. Data are presented as mean±SD, n=6, *P < 0.05 and **P<0.01 (24 h), #P < 0.05 and ##P<0.01 (48 h) versus control group.
μmol/L) for 24 and 48 h, and cell viability was detected by CCK8 assay. Data are presented as mean±SD, n=6, *P < 0.05 and **P<0.01 (24 h), #P < 0.05 and ##P<0.01 (48 h) versus control group.

**Figure 2**

ABT-737 increases cisplatin-induced growth inhibition in A2780/DDP cells. A2780/DDP cells were treated with cisplatin (5μg/mL) alone or combined with ABT-737 (10.0, 20.0, 40.0 μmol/L) for 24h and cell viability was detected by CCK8 assay. Data are presented as mean±SD, n=6, *P < 0.05 versus control group, # P < 0.05 versus cisplatin group. A2780/DDP cells were treated with cisplatin (10μg/mL) alone or combined with ABT-737 (10.0, 20.0, 40.0 μmol/L) for 24h and cell viability was detected by CCK8 assay. Data are presented as mean±SD, n=6, *P < 0.05 versus control group, # P < 0.05 versus cisplatin group. A2780/DDP cells were treated with cisplatin (10μg/mL) alone or combined with ABT-737 (10.0, 20.0, 40.0 μmol/L) for 24h and cell viability was detected by CCK8 assay. Data are presented as mean±SD, n=6, *P < 0.05 versus control group, # P < 0.05 versus cisplatin group. A2780/DDP cells were treated with cisplatin (5μg/mL) alone or combined with ABT-737 (10.0 μmol/L) for 24h and cell viability was detected by CCK8 assay. Data are presented as mean±SD, n=6, *P < 0.05 versus control group, # P < 0.05 versus cisplatin group.
Figure 3

ABT-737 increases A2780/DDP cells apoptosis induced by cisplatin. Cisplatin (5.0 μg/mL) alone or combined with ABT-737 (10.0 μmol/L) for 24h. **a** Western blot analysis for the expression of Bcl-2, Bcl-xL, Bax, Bak and Cleaved-caspase 3. **b** Quantitative analysis of Bcl-2, Bcl-xL, Bax, Bak, and Cleaved-caspase 3 protein levels from **a**. Data are presented as mean±SD, n=3, *P < 0.05 versus cisplatin group. **c** TUNEL assays to detect apoptosis in A2780/DDP cells by flow cytometry. **d** TUNEL assays to detect apoptosis in...
A2780/DDP cells by confocal microscopy (scale bar = 10 μm). e Hoechst staining analysis of nuclear morphology (scale bar = 10 μm). f Caspase 3 enzyme activity assay in A2780/DDP cells. Data are presented as mean±SD, n=3, *P< 0.05 versus control group, #P<0.05 versus cisplatin group.

Figure 4
ABT-737 increases the sensitivity of A2780/DDP cells to cisplatin through JNK pathway. **a** Cisplatin (5.0 μg/mL) alone or combined with ABT-737 (10.0 μmol/L) for 24h. Western blot analysis for the expression of p-JNK, JNK, p-ERK1/2, ERK1/2, p-p38, p38, p-AKT and AKT. **b. c. d. e** Quantitative analysis of p-ERK1/2, p-JNK/JNK, p-p38/p38 and p-AKT/AKT protein levels from **a**. Data are presented as mean±SD, n=3, *P < 0.05 versus cisplatin group. **f** Cisplatin (5.0 μg/mL) combined with ABT-737 (10.0 μmol/L) or combined with ABT-737 (10.0 μmol/L) and U0126 (5.0 μmol/L), SB203580 (5.0 μmol/L), SP600125 (5.0 μmol/L) and LY294002 (5.0 μmol/L) for 24h and cell viability was detected by CCK8 assay. Data are presented as mean±SD, n=6, *P < 0.05 versus control group, #P < 0.05 versus cisplatin+ABT-737 group. **g** Cisplatin (5.0 μg/mL) combined with ABT-737 (10.0 μmol/L) or combined with ABT-737 (10.0 μmol/L) and U0126 (5.0 μmol/L), SB203580 (5.0 μmol/L), SP600125 (5.0 μmol/L) and LY294002 (5.0 μmol/L) for 24h and cell apoptosis was detected by flow cytometry.
Figure 5

ASK1 is required for ABT-737-induced JNK activation and apoptosis of A2780/DDP cells. a Cisplatin (5.0 μg/mL) alone or combined with ABT-737 (10.0 μmol/L) for 24h. Western blot analysis for the expression of p-ASK1 and ASK1. b Quantitative analysis of p-ASK1/ASK1 protein levels from a. Data are presented as mean±SD, n=3, *P< 0.05 versus control group, #P< 0.05 versus cisplatin group. Cisplatin (5.0 μg/mL) combined with ABT-737 (10.0 μmol/L) or combined with ABT-737 (10.0 μmol/L) and GS-4997 (5.0 μmol/L).
μmol/L) for 24h. c Hoechst staining analysis of nuclear morphology (scale bar = 10 μm). d The cell viability was detected by CCK8 assay. Data are presented as mean±SD, n=6, *P < 0.05 versus control group, #P < 0.05 versus cisplatin+ABT-737 group. e The cell apoptosis was detected by flow cytometry. f Western blot analysis for the expression of p-ASK1, ASK1, p-JNK and JNK. g.h Quantitative analysis of p-ASK1/ASK1 and p-JNK/JNK protein levels from f. Data are presented as mean±SD, n=3, *P < 0.05 versus control group, #P < 0.05 versus cisplatin+ABT-737 group.

Figure 6
ABT-737 enhances the imbalance of antioxidant system induced by cisplatin in A2780/DDP cells. Cisplatin (5.0 μg/mL) alone or combined with ABT-737 (10.0 μmol/L) for 24h. **a** Fluorescence microscopy was used to observe the formation of intracellular ROS in A2780/DDP cells (scale bar = 10 μm). **b, c, d** The oxidative stress indices (including hydroxyl radicals, superoxide anions and hydrogen peroxide) were detected by assay kits. Data are presented as mean±SD, n=3, *P< 0.05 versus cisplatin group. **e** The total antioxidant capacity (T-AOC) was detected by assay kit. Data are presented as mean±SD, n=3, *P< 0.05 versus cisplatin group.

**Figure 7**
The ROS production induced by ABT-737 mediates JNK activation and apoptosis of A2780/DDP cells. Cisplatin (5.0 μg/mL) combined with ABT-737 (10.0 μmol/L) or combined with ABT-737 (10.0 μmol/L) and NAC (100.0 μmol/L) for 24h. a Western blot analysis for the expression of p-ASK1, ASK1, p-JNK and JNK. b, c Quantitative analysis of p-ASK1/ASK1 and p-JNK/JNK protein levels from a. Data are presented as mean±SD, n=3, *P < 0.05 versus control group, #P < 0.05 versus cisplatin+ABT-737 group. d The cell viability was detected by CCK8 assay. Data are presented as mean±SD, n=6, *P < 0.05 versus control group, #P < 0.05 versus cisplatin+ABT-737 group. e. Hoechst staining analysis of nuclear morphology (scale bar = 10 μm). f The cell apoptosis was detected by flow cytometry.

Figure 8
Intracellular Ca$^{2+}$ accumulation is involved in ABT-737-induced apoptosis of A2780/DDP cells. a Cisplatin (5.0 μg/mL) alone, or combined with ABT-737 (10.0 μmol/L), or combined with ABT-737 (10.0 μmol/L) and BAPTA (10.0 μmol/L) for 24h and then fluorescence microscopy was used to detect the concentration of intracellular Ca$^{2+}$ (scale bar = 10 μm). b Cisplatin (5.0μg/mL) combined with ABT-737 (10.0 μmol/L), or combined with ABT-737 (10.0 μmol/L) and BAPTA-AM (10.0 μmol/L) for 24h and CCK8 assay was used to detect cell viability. Data are presented as mean±SD, n=6, *P < 0.05 versus control group, #P < 0.05 versus cisplatin+ABT-737 group. c Cisplatin (5.0 μg/mL) combined with ABT-737 (10.0 μmol/L), or combined with ABT-737 (10.0 μmol/L) and BAPTA-AM (10.0 μmol/L) for 24h and then the nuclear morphology was detected by hoechst staining assay (scale bar = 10 μm). d Cisplatin (5μg/mL) combined with ABT-737 (10.0 μmol/L), or combined with ABT-737 (10.0 μmol/L) and BAPTA-AM (10.0 μmol/L) for 24h and then the cell apoptosis was detected by flow cytometry.