ABT737 reverses cisplatin resistance by regulating ER-mitochondria Ca\textsuperscript{2+} signal transduction in human ovarian cancer cells

QI XIE\textsuperscript{1}, JING SU\textsuperscript{1}, BINGXUAN JIAO\textsuperscript{2}, LUYAN SHEN\textsuperscript{1}, LIWEI MA\textsuperscript{1}, XIANZHI QU\textsuperscript{3}, CHUNYAN YU\textsuperscript{4}, XIANRUI JIANG\textsuperscript{1}, YE XU\textsuperscript{5} and LIANKUN SUN\textsuperscript{1}

\textsuperscript{1}Department of Pathophysiology, Basic College of Medicine, \textsuperscript{2}School of Public Health, Jilin University, Changchun, Jilin 130021; \textsuperscript{3}Department of Hepatobiliary and Pancreas Surgery, The China-Japan Union Hospital, Jilin University, Changchun, Jilin 130031; \textsuperscript{4}Department of Pathology, Basic Medical College, Beihua University; \textsuperscript{5}Department of Histology and Embryology, Basic College of Medicine, Jilin Medical University, Jilin, Jilin 132013, P.R. China

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Abstract. Bcl-2, which belongs to the Bcl-2 family, is frequently overexpressed in various types of cancer cells and contributes to drug resistance. However, the function of Bcl-2 in cisplatin resistance in human ovarian cancer cells is not fully understood. In this study, we found that the pharmacological inhibitor ABT737 or genetic knockdown of Bcl-2 increased cisplatin cytotoxicity in cisplatin-resistant ovarian cancer cells. Additionally, treatment with ABT737 or Bcl-2 siRNA increased cisplatin-induced free Ca\textsuperscript{2+} levels in the cytosol and mitochondria, which increased endoplasmic reticulum (ER)-associated and mitochondria-mediated apoptosis. In addition, ABT737 or Bcl-2 siRNA increased the ER-mitochondria contact sites induced by cisplatin in cisplatin-resistant SKOV3/DDP ovarian cancer cells. Consistently with the \textit{in vitro} results, ABT737 potently synergized with cisplatin in inhibiting the growth of human ovarian cancer xenografts in nude mice. Collectively, these results indicate that pharmacological inhibitors or genetic knockdown of Bcl-2 may be a potential strategy for improving cisplatin treatment of ovarian cancer.

Introduction

Cis-diamminedichloroplatinum (II), best known as cisplatin, is a classical chemotherapy drug that is widely used for treating advanced cancers, including ovarian cancer. The antitumor activity of cisplatin is due to its interaction with nuclear DNA, which results in the formation of DNA adducts and subsequent DNA damage-mediated apoptotic signaling (1,2). Cisplatin also has unrelated effects and accumulates in different organelles, including the endoplasmic reticulum (ER), lysosomes, and mitochondria, thus resulting in activation of apoptotic signaling pathways (3). The effect of cross-talk between different organelles on the antitumor efficacy of cisplatin has received substantial attention in recent years. The regulatory networks between the ER and mitochondria involve mitochondrial energy metabolism, lipid metabolism, Ca\textsuperscript{2+} signaling transmission and cell apoptosis (4). ER stress not only triggers a cascade of cellular events that lead to apoptosis but also amplifies the apoptotic signal. ER stress enhances the mitochondrial apoptosis pathway via highly efficient transport of Ca\textsuperscript{2+} from the ER to the mitochondria (5). Hence, studying the cellular mechanisms underlying the signaling between the ER and mitochondria should provide new insights into cisplatin resistance in ovarian cancer.

The ER is a central membrane-bound organelle that is the main Ca\textsuperscript{2+} storage compartment in most cell types and has an extremely important role in maintaining Ca\textsuperscript{2+} homeostasis. The large release of Ca\textsuperscript{2+} from the ER, an early event in cell apoptosis, results in cytosolic and mitochondrial Ca\textsuperscript{2+} overload (6). Calpains are a complex family of Ca\textsuperscript{2+}-dependent cysteine proteases that cleave various protein substrates. The ubiquitous calpain isoforms can be divided into calpain-1 (\(\mu\)-calpain) and calpain-2 (m-calpain) according to the cytosolic Ca\textsuperscript{2+} concentration (\(\mu\)M or mM range) required for their activation (7). Recent research has shown that calpain plays a key role in ER stress-mediated apoptosis and mitochondria-mediated...
apoptosis (8-10). Huang et al. have reported that the ER stress inducer thapsigargin (TG) increases intracellular Ca^{2+} levels and subsequently activates calpains; this activation is followed by the subsequent activation of caspase-3 and caspase-9 and the induction of CHOP. As expected, EGTA and BAPTA-AM, intracellular Ca^{2+} chelators, inhibit the TG-induced activation of the calpains, caspase-3, and caspase-9 and suppress the TG-induced CHOP protein in hepatic stellate cells (8). Interestingly, after silencing calpain-1 and calpain-2, researchers have demonstrated that calpain-1 (but not calpain-2) triggers ER stress in an *in vitro* model of hypoxia/reoxygenation (10). However, the relationship between calpains and ER-mitochondria interactions remains unclear. Ca^{2+} can also be taken up by the mitochondria through the mitochondrial calcium channel uniporter (MCU), which causes mitochondrial depolarization, thereby contributing to the opening of the permeability transition pore (PTP) and altering the permeability of the inner membrane of the mitochondria (IMM). The PTP can further lead to mitochondrial swelling and mitochondrial outer membrane permeabilization (MOMP), with a consequent release of Cyto C and caspase-activating factors and the induction of cell apoptosis (11). In our previous study, using the cytoplasmic Ca^{2+}-indicating fluorophore Fluo-4/AM and the mitochondrial Ca^{2+}-indicating fluorophore Rhod-2/AM, we found that cisplatin causes pro-apoptotic Ca^{2+} release from the ER to the cytosol and mitochondria, thus leading to cytosolic and mitochondrial Ca^{2+} overload, which contributes to ER-mediated apoptosis and mitochondria-mediated apoptosis in cisplatin-sensitive SKOV3 ovarian cancer cells (12). These results have confirmed that a large release of Ca^{2+} from the ER to the cytosol and mitochondria is very important for ER-mediated apoptosis and mitochondrial apoptosis. However, the exact mechanism by which the cisplatin-induced ER Ca^{2+} release enters the mitochondria is not known.

In eukaryotic cells, intracellular organelles determine cell fate and maintain cellular homeostasis through their physical interactions with one another; the functional or physical interaction between mitochondria and the ER (referred to as mitochondria-associated ER membranes, MAM) is a prime example (13,14). MAM serves to establish close communication between the mitochondria and ER, including efficient transportation of Ca^{2+} from the ER to the mitochondria. The subcellular compartment and function of MAM is under intense investigation because it is increasingly recognized as an important region controlling cell physiology. In the MAM, Ca^{2+} release has been proposed to occur through the ER Ca^{2+} channel inositol 1,4,5-trisphosphate receptor (IP3R, the major ER Ca^{2+} release channel) to voltage-dependent anion channel 1 (VDAC1, the main mitochondrial Ca^{2+} uptake pathway) on the outer mitochondrial membrane (OMM) (13). Glucose-regulated protein 75 (Grp75) and mitofusin 2 (Mfn2), known as molecular bridges, help to increase ER-mitochondria contact sites (15). Electron microscopy and fluorescence microscopy, two important technologies, have been widely used to observe the regions of close contacts between the ER and mitochondria (16). Moreover, Wieckowski et al. have provided a detailed introduction on the optimized protocols to isolate MAM fraction from tissues and cells (17). By adapting the protocol, Paillard at the Université de Montpellier and his colleagues have found that lethal hypoxia-reoxygenation contributes to H9c2 cell injury accompanied by a significant increase in Grp75 in the MAM, which indicates increased ER and mitochondria physical interactions. Using Rhod-2/AM loading, they have also shown that increased ER and mitochondrial interactions lead to lethal reperfusion injury through mitochondrial Ca^{2+} overload (18). Guo et al. have shown that overexpression of Mn2 enhances H2O2-induced vascular smooth muscle cell apoptosis, perhaps through excessive ER-mitochondria tethering and Ca^{2+} transfer (19). These results suggest that there is a positive correlation between MAM and cell apoptosis. However, it is unclear whether there is a link between MAM and cisplatin resistance in ovarian cancer. Although a number of indicators suggest that MAM may be useful in cancer treatment, the potential of MAM in cancer treatment has not been studied systematically. Hence, it is very important to explore the effects of MAM on cisplatin-induced cell apoptosis to clarify the mechanism of cisplatin resistance.

A systematic survey of the transcriptional profiles of various cancer cell types has indicated that the dysregulation of Bcl-2 is a key distinguishing factor between normal and cancer cells. Additionally, empirical evidence has shown a positive correlation between Bcl-2 expression and cisplatin resistance in cancer cells (20). In our previous studies, we have demonstrated that Bcl-2 expression in cisplatin-resistant SKOV3/DDP cells is significantly higher than that in cisplatin-sensitive SKOV3 cells (21). During ER stress, by interacting with the IP3R, Bcl-2 and Bcl-xL have been shown to decrease the ER Ca^{2+} load, thereby preventing excessive pro-apoptotic Ca^{2+} signals and mitochondrial Ca^{2+} overload, and finally to inhibit cell apoptosis (22). High expression of Bcl-2 also results in partial VDAC closure, which is accompanied by a marked decrease in mitochondrial Ca^{2+} levels (23). Interestingly, by analyzing MAM composition in Chinese hamster ovary cells, Meunier and Hayashi have demonstrated that Bcl-2 is enriched at the MAM (24). However, the exact role of Bcl-2 in cisplatin-induced SKOV3/DDP cell apoptosis remains unknown. ABT-737, a potent small-molecule inhibitor of Bcl-xL and Becl-2, can bind to the hydrophobic groove in Bcl-2 and Bcl-xL and consequently prevent them from sequestering proapoptotic BH3-only protein. ABT737 exhibits synergistic cytotoxicity and induced significant apoptosis in various cancer types, including ovarian, lung and bladder cancers (25). Moreover, ABT737 has been reported to enhance the cisplatin-induced apoptosis in cancer cells (25). Hence, we propose that ABT737 reverses cisplatin resistance by regulating ER-mitochondria Ca^{2+} signal transduction in human ovarian cancer cells.

**Materials and methods**

*Antibodies and drugs.* Anti-caspase-3 (sc-7272), anti-caspase-4 (sc-56056), anti-cleaved caspase-4 (sc-22173-R), and anti-caspase-9 (sc-56073) antibodies (Abs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-cleaved caspase-3 (ab2302), anti-cleaved caspase-9 (ab2324), anti-PDI (ab2792), anti-VDAC1 (ab14734), anti-CHOP (ab1419), anti-IP3R (ab5804) Abs were purchased from Abcam Ltd. (Hong Kong, China). Anti-β-actin (60008-1-Ig), anti-Bax (50599-2-lg), anti-Bcl-2 (12789-1-AP), anti-cytochrome c (Cyto C) (10993-1-AP), anti-Grp78/BIP (11587-1-AP), anti-
Grp75 (14887-1-AP), anti-mitofusin 2 (Mfn2) (12186-1-AP), anti-β-tubulin (10068-1-AP), anti-calreticulin (10292-1-AP), peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L) (SA00001-1), peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L) (SA00002-1) and anti-IgG control (30000-0-AP) Abs were purchased from Proteintech Group, Inc. (Chicago, IL, USA). The anti-calpain-1 catalytic subunit (#31038-1) Ab was purchased from SAB (College Park, MD, USA). Cisplatin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in normal saline (NS) for "in vitro" use and animal studies. ABT-737 was provided by Abbott Laboratories (Abbott Park, IL, USA) and dissolved in dimethyl sulfoxide (DMSO) for "in vitro" use and animal studies. 2-aminoethyl diphenylborinate (2-APB) (ab120124) was purchased from Abcam Ltd.

**Cell culture.** The cisplatin-resistant clone SKOV3/DDP was obtained from the Peking Union Medical College (Beijing, China). COC1/DDP and A2780/DDP cells were purchased from the American Type Culture Collection (Manassas, VA, USA). SKOV3/DPP, COC1/DDP and A2780/DDP cells were maintained in IMDM (Gibco; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA), supplemented with 10% (v/v) fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), 100 mg/ml streptomycin and 100 U/ml penicillin (each from Genview, Carlsbad, CA, USA), supplemented with 10% (v/v) fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) and 100 mg/ml streptomycin and 100 U/ml penicillin (each from Genview, St. Louis, MO, USA). The cells were incubated at 37°C in an atmosphere containing 5% CO₂.

**Cell viability assay.** Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Boehringer Mannheim, Germany). The cisplatin-resistant ovarian cancer cells, during the exponential growth phase, were seeded into 96-well culture plates in 100 µl of IMDM at a density of 1.0x10⁴ cells/well. After a 24-h incubation, the indicated drugs were added for another 24 h in four parallel wells. The cytoplasmic Ca²⁺-sensitive fluorescent dye Fluo-4/AM (Molecular Probes) and the mitochondrial Ca²⁺-sensitive fluorescent dye Rhod-2/AM (AAT Bioquest, Sunnyvale, CA, USA) were used to measure the Ca²⁺ concentration according to the manufacturer's instructions. Before exposure to different experimental conditions, the cells were incubated with Fluo-4/AM or Rhod-2/AM for 30 min at 37°C. Cell samples were then analyzed by confocal laser microscopy. All experiments were performed in triplicate.

**Immunofluorescence staining and confocal laser microscopy.** Cells were seeded onto coverslips in 24-well plates at a density of 5x10⁴ cells/well and allowed to recover overnight. After treatment with the indicated drugs, cells were washed three times with cold 0.1 M PBS and fixed in 4% (w/v) paraformaldehyde/PBS for 20-30 min, stained with the nuclear stain Hoechst 33342/H₂O (2 µg/ml, Sigma) for 30 min, washed with 0.01 M PBS, and examined using Olympus FV1000 confocal laser microscope to reveal chromatin condensation. The co-localization of IP3R and VDAC1 and the expression of PDI were examined by the indirect immunofluorescence method. Cells were cultured on coverslips overnight, then treated with the indicated drugs, and rinsed with 0.1 M

**Annexin V and cell death assay.** The Muse™ Annexin V Dead Cell kit (Ref. MCH 100110, Merck Millipore) was used to monitor cell death. Exponentially growing cisplatin-resistant ovarian cancer cells were seeded into 6-well culture plates at a density of 2x10⁵ cells/well. After exposure to different experimental conditions, the cells were trypsinized and resuspended in IMDM with 10% FBS at a concentration of 1x10⁵ cells/ml. Cells were incubated with Annexin V and Dead Cell reagent in a dark room at room temperature for 20 min. Finally, the samples were measured by flow cytometry (Muse Cell Analyzer, Merck Millipore).
PBS three times. After incubation, the cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 5 min, washed three times with 0.01 M PBS, and then blocked for 30 min in 5% (w/v) non-immune animal serum (goat) (Beyotime Biotechnology, Shanghai, China) PBS, and incubated with primary antibody (IP3R, VDAC1, PDI) overnight at 4°C. The next day, the slides were incubated with the Alexa Fluor-488/546-conjugated secondary antibody (1:400 dilution; Invitrogen) for 1 h, then stained with Hoechst 33342 (2 µg/ml) for 2 min, and washed three times with PBS. After mounting, the cells were examined by Olympus FV1000 confocal laser microscopy.

Subcellular fractionation. The purification of the cytoplasmic fraction, ER fraction, pure mitochondrial fraction, crude mitochondrial fraction and the MAM fraction were performed as previously described (17). The above fractions were lysed in RIPA buffer [1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 10 mM Tris (pH 8.0), 0.14 M NaCl] for immunoblotting with antibodies against IP3R, VDAC1, Bcl-2, calreticulin, Cyto C, Mn2, Grp75 and tubulin. For co-immunoprecipitation analysis, the MAM lysates were pre-cleared with protein G agarose beads (Proteintech Group, Inc.) for 1 h at 4°C. Then, equal amounts of sample lysates were incubated with either 2 µg of IgG or anti-IP3R antibody for 20 h at 4°C, and this was followed by precipitation with protein G agarose beads. Immunoprecipitated proteins from MAM lysates and total MAM lysates were subjected to immunoblot analysis with the anti-IP3R antibody and anti-VDAC1 antibody. Total MAM lysates were used as the input control. The results are representative of three independent experiments.

Electron microscopy. Electron microscopy and morphometric analysis were performed as described previously (26). Cells were fixed for 30 min with ice-cold 2.5% glutaraldehyde in 0.1 M cacodylate buffer, embedded in Epon, and processed for transmission electron microscopy by standard procedures. Representative areas were chosen for ultra-thin sectioning and examined on a transmission electron microscope at a magnification of x20,000.

Bcl-2 knockdown by small interfering RNA. Small interfering RNA (siRNA) sequences targeting human Bcl-2 and a non-target sequence were constructed by Genechem (Shanghai, China). The Bcl-2 siRNA sequence was CCG-CAT-TTA-ATT-CAT-GGT-ATT and that of the non-target siRNA (Scramble) was TTC-TCC-GAA-CGT-GTC-ACG-T. Transfections with the siRNAs were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Briefly, cisplatin-resistant SKOV3/DDP cells were placed into 6-well plates and transfected the next day with 4 µg of Bcl-2 siRNA or siScramble, using 10 µl of Lipofectamine 2000 (at 1 µg/µl). Cells were harvested 2 days after transfection; whole cell lysates were isolated for western blots. For MTT assays, transfected cells were treated with cisplatin for 24 h, and then subjected to the MTT assay to determine cell viability.

Human tumor xenografts. BALB/c nude mice (4-6 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd., China. Animals were maintained in specific pathogen-free conditions and a controlled light and humidity environment. Animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory animals. SKOV3/DDP cells (5x10⁶) were subcutaneously injected into the right flank of each mouse. Tumor volume (mm³) was measured every 4 days using a Vernier caliper and calculated as 0.4 x (short length)² x long length. Treatment was initiated when tumors reached a volume of 45-55 mm³ (day 16). The mice were randomly divided into 4 groups (5 animals/group) and received NS (IP, daily), 4 mg/kg cisplatin (IP, every other day), 50 mg/kg ABT737 (IP, every other day), or a combination treatment for 20 days. The mice were sacrificed, and the experiment was terminated at the end of 36 days. Tumors were isolated, weighed, and imaged.

Immunohistochemistry. Immunohistochemical staining for cleaved caspase-3 was performed on 5-µm thick sections embedded in paraffin after formalin fixation. The sections were de-paraffinized in xylene and rehydrated in graded ethanol solutions (reducing concentration from 95 to 70%). The sections were incubated in H2O2 solution (3% H2O2 in PBS buffer) for 30 min to block endogenous peroxidase activity. Antigen retrieval was performed in retrieval buffer (pH 9.0, 20 mM Tris, 0.05 mM EDTA, 0.05% Tween-20 buffer) in a 99°C bath for 20 min. The sections were subsequently incubated at 4°C overnight with anti-cleaved caspase-3. After rinsing with PBS buffer, the secondary antibody (MaxVision™ HRP-Polymer Anti-Rabbit IHC kit, Maixin Bio, China) was applied for 15 min at RT. The DAB (Maixin Bio, China) solution was used as the chromogen. Finally, the sections were counterstained with hematoxylin (Sigma) to identify the nuclei. The images were observed and analyzed using a microscope (Leica DM 4000B) with Image-Pro Plus 6.0 software.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). Apoptosis analysis was performed using an In Situ Cell Death Detection kit (Roche, Indianapolis, IN, USA) to identify DNA breaks according to the manufacturer's instructions. Sections were incubated with TUNEL reaction mix containing 10 U of terminal deoxyribonuclease, 10 mM dUTP biotin, and 2.5 mM cobalt chloride in 1X terminal transferase reaction buffer for 1 h at 37°C in a humidified atmosphere. The DAB (Maixin Bio, China) solution was used as the chromogen. Finally, the sections were counterstained with hematoxylin (Sigma) to identify the nuclei. The images were observed and analyzed using a microscope (Leica DM 4000B) with Image-Pro Plus 6.0 software.

Statistical analyses. All values are presented as mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test with the software GraphPad Prism version 6.00 for Mac (GraphPad Software, La Jolla, CA, USA). A value of P<0.05 was considered statistically significant.

Results

ABT737 increases cisplatin-induced growth inhibition and apoptosis in cisplatin-resistant ovarian cancer cells. We treated cisplatin-resistant ovarian cancer cells (SKOV3/DDP,
COC1/DDP and A2780/DDP cells) with ABT737 in combination with cisplatin to investigate whether ABT737 would enhance the antitumor effects of cisplatin. On the basis of our previous studies (unpublished data), the non-toxic dose of
ABT737 in SKOV3/DDP, COC1/DDP and A2780/DDP cells for 24 h is 5 µM. Thus, we treated SKOV3/DDP, COC1/DDP and A2780/DDP cells with 15 µg/ml cisplatin and/or 5 µM ABT737 for 24 h. MTT assays indicated that ABT737 increased cisplatin-induced growth inhibition (Fig. 1A-C).

On the basis of these results, we treated cells with cisplatin and/or ABT737 for 24 h and then examined apoptotic chromatin condensation with Hoechst 33342 staining. These results showed that ABT737 increased cisplatin-induced apoptotic chromatin condensation (Fig. 1D-F). Additionally, flow cytometry analysis revealed that the apoptosis rate was clearly higher in SKOV3/DDP, COC1/DDP and A2780/DDP cells exposed to cisplatin and ABT737 for 24 h compared with cells treated with cisplatin alone (Fig. 1G-I). These results indicate that ABT737 restores sensitivity to cisplatin in cisplatin-resistant ovarian cancer cells.

ABT737 and cisplatin have a synergistic effect in the treatment of human ovarian cancer xenograft mouse models. After the in vitro experiments, in vivo experiments were performed to determine whether ABT737 contributes to suppressing tumor growth in combination with cisplatin. SKOV3/DDP cells were subcutaneously inoculated into BALB/c nude mice to establish a subcutaneous tumor model. The tumor growth curve over time was recorded (Fig. 2A). As predicted, ABT737 plus cisplatin caused marked tumor growth inhibi-
tion compared with treatment with cisplatin-only (Fig. 2B). The final tumor weights were 1.01, 0.39, 0.97 and 0.19 g for BALB/c nude mice injected i.p. with NS, cisplatin, ABT737 or a combination treatment, respectively (Fig. 2C). Next, we

Figure 3. ABT737 enhances cisplatin-induced mitochondrial apoptosis and ER-mediated apoptosis in SKOV3/DDP cells. (A) SKOV3/DDP cells were treated with 15 µg/ml cisplatin and/or 5 µM ABT737 for 6 h. ∆Ψm was assessed by staining for MitoPotential dye and 7-AAD, and analysed by Muse cell analyser. (B and C) SKOV3/DDP cells were treated with 15 µg/ml cisplatin and/or 5 µM ABT737 for 24 h. Western blot analysis for the expression of calpain-1, Cyto C, Bax, Bcl-2, cleaved caspase-9 and cleaved caspase-3 after normalization to β-actin and quantification. Data are presented as mean ± SD, n=3. *P<0.05 vs. control, #P<0.05 vs. cisplatin. (D) SKOV3/DDP cells were treated with 15 µg/ml cisplatin and/or 5 µM ABT737 for 24 h. Distribution of PDI was observed by confocal microscopy (bar, 10 µm). (E and F) SKOV3/DDP cells were treated with 15 µg/ml cisplatin and/or 5 µM ABT737 for 24 h. Western blot analysis for the expression of PDI, Grp78, CHOP and cleaved caspase-4 after normalization to β-actin and quantification. Data are presented as mean ± SD, n=3. *P<0.05 vs. control, #P<0.05 vs. cisplatin.
used immunohistochemical methods to detect the expression of the apoptosis-related protein cleaved caspase-3 in mice exposed to cisplatin and/or ABT737 for 20 days. As shown in Fig. 2D, ABT737 combined with cisplatin treatment led to the upregulation of cleaved caspase-3 compared with treatment with cisplatin only. Furthermore, TUNEL assays were used to determine whether ABT737 had a synergistic effect with cisplatin in inducing cell apoptosis in mouse models of ovarian cancer. These results showed that the number of apoptotic cells in the cisplatin plus ABT737 group was significantly greater than that of the cisplatin group (Fig. 2E and F). Together, these data suggest that ABT737 and cisplatin have a synergistic effect in the treatment of human ovarian cancer xenograft mouse models.

ABT737 enhances cisplatin-induced mitochondrial apoptosis and ER-mediated apoptosis in SKOV3/DDP cells. First, we investigated whether ABT737 would enhance cisplatin-induced mitochondrial apoptosis. To determine the effect of cisplatin and/or ABT737 on mitochondrial function, we measured ΔΨm by using flow cytometry after 6 h. As shown in Fig. 3A, the combination treatment further reduced the cisplatin-induced decrease of ΔΨm. Moreover, western blot analysis showed that the combination treatment increased the ratio of Bax/Bcl-2 and enhanced the expression of mitochondrial apoptosis-related proteins (calpain-1, Cyto C, cleaved caspase-9 and cleaved caspase-3), as compared with the cisplatin treatment (Fig. 3B and C).

A growing body of research suggests that cisplatin triggers ER stress and induces ER stress-mediated apoptotic events (2). Hence, we further investigated whether ABT737 enhanced cisplatin-induced ER stress-mediated apoptosis in SKOV3/DDP cells. Using confocal microscopy, we found that ABT737 increased the expression of PDI induced by cisplatin (Fig. 3D). We assessed the expression of ER stress-related proteins (PDI, Grp78, CHOP and cleaved caspase-4) by western blot analysis. These results showed that the combination treatment enhanced the expression of PDI, Grp78, CHOP and cleaved caspase-4, as compared with the cisplatin treatment (Fig. 3E and F). Thus, we demonstrated that the intrinsic mitochondrial apoptotic pathway and ER-mediated apoptosis are actively involved in the ABT737-mediated increase in the cytotoxic effects of cisplatin on SKOV3/DDP cells.

ABT737 increases cisplatin-induced Ca2+ transfer from the ER to the cytosol and mitochondria. Ca2+ is not only a key regulator of cell survival but also triggers cell apoptosis in response to various physiological and pathological conditions. A growing body of literature shows that cytoplasmic and mitochondrial Ca2+ overload can contribute to ER-mediated apoptosis and mitochondrial apoptosis (22,27). As described in Introduction, the ER is the most important Ca2+ storage compartment in eukaryotic cells. Therefore, we determined whether mitochondrial and cytoplasmic Ca2+ overload is a result of Ca2+ release from the ER. We used 2-APB, an IP3R antagonist that inhibits ER Ca2+ efflux (27). The fluorescent dyes Fluo-4/AM and Rhod-2/AM were used to detect cytoplasmic and mitochondrial Ca2+ levels in SKOV3/DDP cells, respectively. As shown in Fig. 4, the cytoplasmic and mitochondrial Ca2+ elevation induced by cisplatin or/and ABT737 in SKOV3/DDP cells was markedly reduced in the presence of 2-APB. These results strongly suggest that ABT737 increases cisplatin-induced Ca2+ transfer from the ER to the cytosol and mitochondria.
whether the increased mitochondrial Ca\(^{2+}\) levels were mediated by increasing the ER-mitochondria contact sites. IP3R and VDAC1 localize to the mitochondria and ER, respectively (13-15,28). We used confocal microscopy to analyze the co-localization of IP3R and VDAC1. The yellow areas represent co-localization between IP3R and VDAC1, and these results demonstrated that ABT737 enhanced cisplatin-induced ER-mitochondria contact sites (Fig. 5A).
Figure 6. Bcl-2 siRNA increases sensitivity to cisplatin by increasing cytoplasmic and mitochondrial Ca\textsuperscript{2+} levels. SiBcl-2 increases sensitivity to cisplatin by decreasing Bcl-2 expression at ER and mitochondria. (A) Western blotting analysed the knockdown efficiency of Bcl-2, siScramble was used as negative control. (B) Cell viability was determined by MTT assay. Data are presented as mean ± SD, n=3. *P<0.05 vs. control, #P<0.05 vs. cisplatin plus siScramble group. (C) Apoptosis was assessed by staining for Annexin V and 7-AAD, and analysed by Muse cell analyser. (D) The quantification of apoptosis in SKOV3/DDP cells exposed to different treatment for 24 h. Data are presented as mean ± SD, n=3. *P<0.05 vs. control, †P<0.05 vs. cisplatin plus siScramble group. (E) Confocal microscopy was used to detect cytoplasmic and mitochondrial Ca\textsuperscript{2+} in cells exposed to different treatment for 24 h (scale bar, 10 µm). (F) Representative transmission electron microscopy photomicrographs of SKOV3/DDP cells exposed to different treatment for 24 h (bar, 200 nm). White arrowheads indicate ER-mitochondria contacts. (G) Quantification of ER-mitochondria contacts of electron microscopy images. Data are presented as mean ± SD, n=3. *P<0.05 vs. control, †P<0.05 vs. cisplatin plus siScramble group.
Next, we detected the changes in the ER-mitochondria contact sites by western blotting and immunoprecipitation. Before studying the changes in ER-mitochondria interactions, we assessed the quality of each fraction by immunoblotting for subcellular organelle marker proteins: IP3R and calreticulin for the ER and MAM; VDAC1 for the mitochondria and MAM; Bcl-2 which enriches mitochondria and ER and is also present in MAM; Cyto C for the mitochondria; tubulin for the cytoplasm (17,24,28). As expected, the data showed that we obtained highly pure fractions, and there was no cross-contamination (Fig. 5B). By analyzing MAM composition, we found that ABT737 enhanced the cisplatin-induced interactions of VDAC1 with Grp75 and Mfn2 (Fig. 5C and E), thus suggesting that ER-mitochondria contact sites induced by cisplatin were enhanced in the combination treatment. Then, we treated cells with cisplatin and/or ABT737 for 24 h. The MAM fraction was immunoprecipitated using the anti-IP3R antibody followed by western blotting using the anti-VDAC1 antibody. These results showed that ABT737 enhanced the cisplatin-induced IP3R-VDAC1 interactions (Fig. 5D and F).

Finally, we used electron microscopy to examine the ultrastructural changes in MAM in the cisplatin and/or ABT737 group for 24 h. White arrowheads indicate the ER-mitochondria interactions. The contact points between the two organelles were significantly increased in the combination-treatment group compared with the control group (Fig. 5G and H). In summary, we concluded that ABT737 enhances cisplatin-induced mitochondrial Ca2+ levels by increasing ER-mitochondria contact sites.

Bcl-2 siRNA increases sensitivity to cisplatin by increasing cytoplasmic and mitochondrial Ca2+ levels. To better clarify the role of Bcl-2 in cisplatin resistance in SKOV3/DDP cells, we depleted the endogenous Bcl-2 using a specific siRNA in SKOV3/DDP cells and observed that the Bcl-2 protein expression was clearly downregulated (Fig. 6A). MTT assays showed that Bcl-2 siRNA combined with cisplatin for 24 h markedly decreased cell viability compared with that in the cisplatin plus siScramble group (Fig. 6B). Additionally, flow cytometry analysis revealed that the apoptosis rate was substantially higher in SKOV3/DDP cells exposed to cisplatin combined with Bcl-2 siRNA for 24 h compared with cells treated with cisplatin plus siScramble (Fig. 6C and D). Consistently with the results in Fig. 4, Bcl-2 siRNA also enhanced the cisplatin-induced cytoplasmic and mitochondrial Ca2+ elevations (Fig. 6E). Finally, we used electron microscopy to examine the ultrastructural changes of MAM after treatment with cisplatin and/or Bcl-2 siRNA. White arrowheads indicate the ER-mitochondria interactions. The contact points between the two organelles were significantly increased after the cisplatin plus Bcl-2 siRNA treatment for 24 h compared with the cisplatin plus siScramble treatment (Fig. 6F and G). Together, these findings suggest that Bcl-2 siRNA improves the sensitivity of ovarian cancer cells to cisplatin by increasing cytoplasmic and mitochondrial Ca2+ levels.

Discussion

Bcl-2 overexpression is recognized as one of the key mechanisms underlying tumor cell apoptosis escape and intrinsic or acquired cisplatin resistance (20,29,30). Hence, Bcl-2 has attracted considerable attention as a possible target for the treatment of cancer, including ovarian cancer. ABT-737, a small molecule compound, inhibits Bcl-2 by mimicking the function of the BH3-only protein (31-33). Using a xenograft model of hepatoblastoma (HB), Lieber et al have reported that ABT737 restores the cisplatin sensitivity of cisplatin-resistant HB cells and significantly decreases tumor growth, as compared with that observed after cisplatin monotherapy (34). Additionally, our previous results have shown that ABT737 enhances cisplatin-induced Bcl-2 downregulation and cisplatin-induced apoptosis through the regulation of mitochondrial dynamics in cholangiocarcinoma cells (25). In this study, ABT737 enhanced cisplatin-induced apoptotic chromatin condensation and cell apoptosis in cisplatin-resistant ovarian cancer cells (Fig. 1). To evaluate the effect of cisplatin combined with ABT737 on antitumor activity in vivo, a BALB/c nude mouse subcutaneous transplant tumor model was established using SKOV3/DDP cells. The results indicated that cisplatin combined with ABT737 had an additive inhibitory effect on tumorigenesis (Fig. 2).

An increasing number of studies have found that cisplatin is actively involved in mitochondria-mediated and ER-mediated apoptosis (35,36). Interestingly, Bcl-2 is predominantly located in the mitochondria and ER (30). Therefore, this finding suggests that ABT737 may increase the cytotoxic effects of cisplatin via the above two pathways. In this study, we found that ABT737 enhanced cisplatin-induced Δψm collapse and Cyto C release and increased the cisplatin-induced elevated Bax/Bcl-2 ratio and caspase-9, cleaved caspase-3 expression. Additionally, ABT737 increased Grp78, CHOP and cleaved caspase-4 induced by cisplatin (Fig. 3). All of our results demonstrate that ABT737 improved the response of SKOV3/DDP cells to cisplatin by mitochondria-mediated and ER-mediated apoptosis.

Ca2+, a ubiquitous intracellular messenger, is a key determinant of cell function and survival. Altered Ca2+ homeostasis has an adverse effect on proliferating cells (37,38). A membrane-permeant inhibitor of IP3R, 2-APB, specifically inhibits IP3R-mediated Ca2+ release (39,40). Using 2-APB, Spletstoesser et al revealed that cisplatin-induced ER Ca2+ release results in programmed cell death due to activation of calpain (41). Calpain-1, a Ca2+-dependent cysteine endopeptidase, can be activated by a small increase in cytoplasmic Ca2+ levels (10,42). Studies by Zheng et al and Altznauer et al and have shown that Ca2+-induced calpain-1 triggers mitochondria-mediated and ER-mediated apoptosis (10,43). Recently, we showed that cisplatin increases cytosolic and mitochondrial Ca2+ levels, activates calpain-1, and eventually leads to mitochondria- and ER-mediated apoptosis (27). However, the effects of cisplatin plus ABT737 on intracellular Ca2+ signals have not been reported in the literature. In this study, to investigate the effect of cisplatin plus ABT737 on cytosolic and mitochondrial Ca2+ levels, we used the fluorescent dyes Fluo-4/AM and Rhod-2/AM to detect cytoplasmic and mitochondrial Ca2+ levels in SKOV3/DDP cells. Our experimental results showed that ABT737 increased cytoplasmic and mitochondrial Ca2+ levels induced by cisplatin and enhanced cisplatin-mediated calpain-1 activation, eventually leading to apoptosis (Figs. 3B and 4). These results indicate...
that cytoplasmic and mitochondrial Ca\(^{2+}\) overload increased SKOV3/DDP cell sensitivity to cisplatin-induced cytotoxicity, thus possibly providing a new approach for treating cisplatin-resistant ovarian cancer.

The signaling mechanisms linking MAM and cancer cell apoptosis have attracted substantial interest (44-47). The phosphorun acidic cluster sorting protein-2 (PACS-2), a cytosolic sorting protein, is required for the integrity of MAM (48). Using siRNA depletion of PACS-2, Simmen et al. have demonstrated that low expression of PACS-2 blocks the apoptosis of STS-mediated human melanoma A7 cells, possibly through decreased transfer of Ca\(^{2+}\) from the ER to the mitochondria (48). Similarly, using fluorescence labeling of PDI (the marker protein of ER) and VDAC1 (the marker protein of mitochondria), our previous studies showed that cisplatin increases ER-mitochondria contacts, and this is followed by highly efficient transportation of Ca\(^{2+}\) from the ER to the mitochondria, ultimately resulting in mitochondrial Ca\(^{2+}\) overload and apoptosis in SKOV3 cells (12). Next, we determined whether the increase in mitochondrial Ca\(^{2+}\) levels induced by cisplatin and ABT737 is achieved through the increase in ER-mitochondrial cross-talk in SKOV3/DDP cells. In our study, through the fluorescence labeling of IP3R and VDAC1, we showed that ABT737 increased cisplatin-induced ER-mitochondria contacts. We analyzed MAM composition and found that ABT737 increased cisplatin-induced Grp75 and Mfn2 expression at the MAM interface. These results indicate that ABT737 increases cisplatin-induced ER-mitochondria coupling leads to mitochondrial Ca\(^{2+}\) overload and subsequent apoptosis by favoring Ca\(^{2+}\) transfer from the ER to the mitochondria. Interestingly, it has been reported that ER close to the mitochondria may sense local cytoplasmic Ca\(^{2+}\) increases, and the mitochondrial Ca\(^{2+}\) increase is closely coupled to the increase in cytoplasmic Ca\(^{2+}\) levels (49). Cytoplasmic Ca\(^{2+}\)-dependent calpain-1 activation is an important element of mitochondria-mediated and ER-mediated apoptosis. We suggest that increased ER-mitochondria contact sites allow for calpain-1-mediated apoptotic effects. However, further investigations are required to test this possibility.

To further explore whether the downregulation of Bcl-2 can increase the cytotoxic effects of cisplatin, we inhibited Bcl-2 expression by using siRNA transfection in SKOV3/DDP cells. Our study revealed that Bcl-2 siRNA enhanced the inhibitory effect of cisplatin on SKOV3/DDP cell proliferation and also enhanced cisplatin-induced apoptosis (Fig. 6B-D). Moreover, Bcl-2 knockdown significantly enhanced cisplatin-induced cytoplasmic and mitochondrial Ca\(^{2+}\) elevation and cisplatin-induced ER-mitochondria cross-talk in SKOV3/DDP cells (Fig. 6E-G). These results further indicated that increasing MAM formation plays a fundamental role in cell apoptosis by driving higher Ca\(^{2+}\) accumulation in the mitochondria.

In conclusion, in a departure from conventional therapeutic strategies for overcoming cisplatin resistance, we explored an alternative therapeutic strategy linking cisplatin resistance to Bcl-2-regulated Ca\(^{2+}\) signals in ovarian cancer cells. The pharmacological inhibition of Bcl-2 or Bcl-2 siRNA reversed the cisplatin resistance of SKOV3/DDP cells by increasing cisplatin-induced cytoplasmic and mitochondrial Ca\(^{2+}\) levels. MAM not only promotes higher calcium transfer from the ER to the mitochondria, thus leading to mitochondrial Ca\(^{2+}\) overload, but also may allow for calpain-1-mediated apoptosis. Our experimental results elucidate the antitumor mechanism of Bcl-2 via MAM, suggesting a possibility for individualized treatment of ovarian cancer patients.

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