# TAT-fused IP3R-derived peptide enhances cisplatin sensitivity of ovarian cancer cells by increasing ER $Ca^{2+}$ release

QI XIE[1], YE XU[2], WEINAN GAO[3], YONG ZHANG[1], JING SU[1], YANAN LIU[1], YUTING GUO[2], MINGHAN DOU[2], KEBANG HU[4] and LIANKUN SUN[1]

[1]Department of Pathophysiology, Basic College of Medicine, Jilin University, Changchun, Jilin 130021; [2]Department of Histology and Embryology, Basic College of Medicine, Jilin Medical University, Jilin, Jilin 132013; [3]Department of Clinical Medicine, College of Clinical Medicine, Jilin University, Changchun, Jilin 130021; [4]Department of Urology, First Hospital of Jilin University, Changchun, Jilin 130031, P.R. China

Received August 12, 2016; Accepted October 25, 2017

DOI: 10.3892/ijmm.2017.3260

**Abstract.** Ovarian cancer is the most common gynecological malignancy. At present, cisplatin is used to treat ovarian cancer; however, the development of cisplatin resistance during therapy is a common obstacle to achieving favorable outcomes. Recently, the B-cell lymphoma 2 (Bcl-2) BH4 domain has been reported to mediate the prosurvival activity of Bcl-2 in cancer; however, the involvement of the BH4 domain of Bcl-2 in the cisplatin resistance of ovarian carcinoma cells is not entirely clear. In this study, we observed the cytoplasmic and mitochondrial levels of $Ca^{2+}$ by confocal laser microscopy. We also detected cell apoptosis using western blot analysis and flow cytometry. The present study demonstrated that TAT-fused inositol 1,4,5-trisphosphate receptor-derived peptide (TAT-$IDP^S$), which targets the BH4 domain of Bcl-2, increased cisplatin-induced $Ca^{2+}$ flux from the endoplasmic reticulum (ER) into the cytosol and mitochondria. In addition, TAT-$IDP^S$ increased cisplatin-induced expression of mitochondrial apoptosis-associated proteins and ER stress-associated proteins. These results indicated that TAT-$IDP^S$ may enhance the cytotoxicity of cisplatin toward ovarian carcinoma cells by increasing ER $Ca^{2+}$ release.

## Introduction

Ovarian cancer is the most common form of gynecological cancer and is a serious threat to women's health and survival worldwide. Cis-diammine-dichloroplatinum (II), also known as cisplatin, is a classic chemotherapy drug that is widely used for the treatment of advanced cancers, including ovarian cancer (1,2). Cisplatin exhibits a satisfactory therapeutic effect during the early stages of administration; however, patients often become resistant to cisplatin during long-term administration. It has previously been reported that overexpression of the anti-apoptotic B-cell lymphoma 2 (Bcl-2) protein facilitates the pathophysiology and cisplatin resistance of cancer by inhibiting apoptosis (3). Bcl-2 contains BH1, BH2, BH3 and BH4 domains. Bcl-2 has been reported to bind to Bcl-2-associated X protein (Bax)/Bcl-2 homologous antagonist killer via its BH3 domain and inhibit cytochrome *c* release from the mitochondria, thus maintaining mitochondrial integrity. Notably, BH3-mimetic Bcl-2 inhibitors have attracted much attention and have been extensively studied (4). The BH3-mimetics ABT737 and ABT-263 have been reported to enhance cisplatin sensitivity in cholangiocarcinoma and ovarian carcinoma cells (5,6). However, the function of the Bcl-2 BH4 domain in ovarian carcinoma cells remains unclear.

Bcl-2 has been reported to induce a reduction in endoplasmic reticulum (ER) $Ca^{2+}$ load and release via its BH4 domain (7). The ER is the main store of intracellular $Ca^{2+}$ and a crucial organelle in eukaryotic cells. A large release of $Ca^{2+}$ from the ER can activate calpain-1, which is a $Ca^{2+}$-dependent cysteine protease responsible for the transition from caspase-4 to cleaved caspase-4, which in turn activates ER stress-induced apoptosis (8). Furthermore, $Ca^{2+}$ release from the ER can enhance mitochondrial $Ca^{2+}$ uptake, which is accompanied by a release of cytochrome *c* and activation of the mitochondrial apoptotic pathway (9). In our previous study, it was demonstrated that the expression of Bcl-2 was higher in cisplatin-resistant SKOV3/DDP ovarian cancer cells compared with in cisplatin-sensitive SKOV3 ovarian cancer cells (10). A low dose of cisplatin induced apoptosis, which was accompanied by cytoplasmic and mitochondrial $Ca^{2+}$ overload in SKOV3 cells, but not in SKOV3/DDP cells (11). These results suggested that Bcl-2 contributes to cisplatin resistance in ovarian cancer by inhibiting ER $Ca^{2+}$ release.

Bcl-2 inhibits ER $Ca^{2+}$ release by directly binding and inhibiting the inositol 1,4,5-trisphosphate receptor (IP3R),

*Correspondence to:* Dr Kebang Hu, Department of Urology, First Hospital of Jilin University, 3302 Jilin Street, Changchun, Jilin 130031, P.R. China
E-mail: hukebang@126.com

Professor Liankun Sun, Department of Pathophysiology, Basic College of Medicine, Jilin University, 126 Xinmin Street, Changchun, Jilin 130021, P.R. China
E-mail: sunlk@jlu.edu.cn

*Key words:* cisplatin, ovarian cancer, calcium, endoplasmic reticulum, peptide

which is the main ER $Ca^{2+}$ release channel, via its BH4 domain. IP3R transmits signals between the cytoplasm and ER microenvironment, and can modulate the mitochondrial $Ca^{2+}$ signal (12). By deleting the BH4 domain, Liu *et al* demonstrated that the BH4 domain was important for the prosurvival activity of Bcl-2 (13). Stabilized TAT-fused IP3R-derived peptide (TAT-$IDP^S$) can specifically target the BH4 domain of Bcl-2 and reverse Bcl-2-mediated inhibition of IP3-induced $Ca^{2+}$ elevation, ultimately increasing $Ca^{2+}$-dependent cell apoptosis (14). TAT-$IDP^S$ has been reported to induce apoptosis of diffuse large B-cell lymphoma cells by promoting IP3-induced proapoptotic $Ca^{2+}$ signaling (14). These results indicated that targeting the BH4 domain may induce conversion of Bcl-2 from a prosurvival protein into a proapoptotic protein. Notably, Zhong *et al* indicated that TAT-$IDP^S$ sensitivity is positively correlated with Bcl-2 expression (15). Therefore, it may be hypothesized that TAT-$IDP^S$ increases the sensitivity of cells to cisplatin via IP3-dependent $Ca^{2+}$ release in SKOV3/DDP cells.

The present study aimed to evaluate the effects of TAT-$IDP^S$ on the cytotoxicity of cisplatin in SKOV3/DDP cells. The results demonstrated that TAT-$IDP^S$ increased cisplatin-induced apoptosis by increasing $Ca^{2+}$ release from the ER to the cytoplasm and mitochondria. These experimental results indicated that TAT-$IDP^S$, combined with chemotherapy, may be considered a viable treatment strategy for the prevention and treatment of ovarian cancer.

## Materials and methods

*Antibodies and drugs*. Anti-caspase-3 (sc-7272), anti-caspase-4 (sc-56056) and anti-cleaved caspase-4 (sc-22173-R) antibodies (Abs) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-cleaved caspase-3 (ab2302), anti-protein disulfide isomerase (PDI; ab2792) and anti-CCAAT-enhancer-binding protein homologous protein (CHOP; ab11419) Abs were purchased from Abcam (Hong Kong) Ltd. (Hong Kong, China). Anti-β-actin (60008-1-Ig), anti-Bcl-2-associated X protein (Bax; 50599-2-Ig), anti-Bcl-2 (12789-1-AP), anti-cytochrome *c* (10993-1-AP), peroxidase-conjugated AffiniPure goat anti-mouse immunoglobulin (Ig)G (H+L) (SA00001-1) and peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L) (SA00001-2) Abs were purchased from Proteintech Group, Inc. (Chicago, IL, USA). The anti-calpain-1 catalytic subunit (#31038-1) Ab was purchased from Signalway Antibody LLC (College Park, MD, USA). Cisplatin was purchased from Sigma-Aldrich; Merck KgaA (Darmstadt, Germany) and was dissolved in normal saline for *in vitro* use. TAT-$IDP^S$ was synthesized and purified by ChinaPeptides Co., Ltd. (Shanghai, China) and was dissolved in $ddH_2O$. The sequence for TAT-$IDP^S$ is RKKRRQRRRGGNVYTEIKCNSLLPLDDIVRV. 2-Aminoethyl diphenylborinate (2-APB; ab120124) was purchased from Abcam (Hong Kong) Ltd.

*Cell lines*. Cisplatin-resistant ovarian cancer SKOV3/DDP cells were obtained from the Chinese Academy of Medical Sciences (Beijing, China) and Peking Union Medical College (Beijing, China). Cells were cultured at 37˚C in an atmosphere containing 5% $CO_2$ in RPMI-1640 culture medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). Cisplatin-resistant SKOV3/DDP cells were maintained in medium containing 1 μg/ml cisplatin (Sigma-Aldrich; Merck KGaA) for 24 h to maintain resistance.

*Cell viability assays*. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. SKOV3/DDP cells were seeded into 96-well culture plates in 100 μl medium at a density of $1x10^4$ cells/well. After 2 days, agents [cisplatin (15 μg/ml) and/or TAT-IDPS (25 μM) in the presence or absence of 50 μM 2-APB] were added to quadruplicate wells and incubated for 24 or 48 h at 37˚C. For the MTT assay (Beyotime Institute of Biotechnology, Shanghai, China), 20 μl/well MTT (5 mg/ml in PBS) was added to each well; after 4 h at 37˚C, dimethyl sulfoxide (150 μl/well; Beijing Chemical Industry Group Co., Beijing, China) was added and the plates were agitated at room temperature for 10 min. Absorbance was measured at a wavelength of 570 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

*Western blotting*. Whole-cell protein extracts were isolated from SKOV3/DDP cells using cell lysis buffer [50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM sodium-EDTA, 1 mM EDTA; 1% (v/v) Triton X-100; 2.5 mM sodium pyrophosphate; 1 mM β-glycerophosphate; 1 mM $Na_3VO_4$; 1 mM NaF; 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride] for western blotting. Protein concentration was quantified using the Quick Start Bradford protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For western blot analysis, lysates (30 μg) were separated by 10% (w/v) SDS-PAGE and were transferred onto immobilon-P transfer membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% (w/v) non-fat dry milk in buffer [10 mM Tris-HCl (pH 7.6), 100 mM NaCl, and 0.1% Tween-20] for 1 h at room temperature and were then incubated with the desired primary Abs [caspase-3 (dilution, 1:200); caspase-4 (dilution, 1:200); cleaved caspase-4 (dilution, 1:200); cleaved caspase-3 (dilution, 1:1,000); PDI (dilution, 1:1,000); CHOP (dilution, 1:1,000); β-actin (dilution, 1:2,000); Bax (dilution, 1:1,000); cytochrome *c* (dilution, 1:1,000); calpain-1 (dilution, 1:200)] overnight at 4˚C, followed by incubation with horseradish peroxidase-conjugated secondary Abs (dilution, 1:2,000) for 1.5 h at room temperature. Immunodetection was performed using enhanced chemiluminescence reagents (PE0010-b; Solarbio, New York, NY, USA) and images were captured using Syngene Bio Imaging (Syngene, Cambridge, UK). Protein expression levels were normalized to those of β-actin and the ratios of normalized protein to β-actin are presented as the mean ± standard deviation from three independent experiments. Protein expression levels were semi-quantified by densitometry using Quantity One software (version 4.6.9; Bio-Rad Laboratories, Inc.).

*Immunofluorescence staining and confocal laser microscopy*. Cells ($4x10^4$ cells/well) were cultured on coverslips overnight, following treatment with increasing doses of cisplatin (5, 10 and 15 μg/ml) for 24 h at 37˚C, and were fixed with 4% (w/v) paraformaldehyde and stained with nuclear

Hoechst 33342 (1 $\mu$g/ml; Sigma-Aldrich; Merck KGaA) for 5 min. Subsequently, the cells were washed with PBS and were examined under an Olympus FV1000 confocal laser microscope (Olympus Corporation, Tokyo, Japan) to reveal cell chromatin condensation. The expression of calpain-1 was examined by indirect immunofluorescence. Cells ($4x10^4$ cells/well) were cultured on coverslips overnight, and were then treated with the indicated dose of cisplatin with or without TAT-IDP$^S$ for 24 h at 37˚C, after which the cells were rinsed with PBS three times. Subsequently, cells were fixed with 4% (w/v) paraformaldehyde for 20 min, permeabilized with 0.1% (v/v) Triton X-100 for 5 min, blocked with bovine serum albumin (PA129262; Thermo Fisher Scientific, Inc.), and incubated with anti-calpain-1 (dilution, 1:100) overnight at 4˚C. Cells were then incubated with Alexa Fluor 488 donkey anti-rabbit IgG (#R37602, 1:400 dilution; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at room temperature, stained with Hoechst 33342 (1 $\mu$g/ml) for 5 min at room temperature, and washed with PBS three times. After mounting, cells were examined under an Olympus FV1000 confocal laser microscope (Olympus Corporation). The same parameters of illumination and detection were maintained digitally throughout the experiments.

*Calcium concentration analysis*. The $Ca^{2+}$-sensitive fluorescent dyes Fluo-4/AM (Molecular Probes; Thermo Fisher Scientific, Inc.) and Rhod-2/AM (AAT Bioquest, Sunnyvale, CA, USA) were used to measure $Ca^{2+}$ concentration according to the manufacturers' protocols. Prior to exposure to various 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.

*Flow cytometry*. Cell death was detected using the Muse® Annexin V Dead Cell kit (EMD Millipore). SKVO3/DDP cells were seeded in 6-well culture plates at a density of $2x10^5$ cells/well. Following treatment under different experimental conditions, cells were trypsinized and resuspended in RPMI-1640 medium supplemented with 10% FBS at a concentration of $1x10^6$ cells/ml. In the dark, cells were incubated with Annexin V at room temperature for 20 min. Finally, cells were measured using the Muse® Cell Analyzer (version 1.5.0.0; EMD Millipore). All experiments were performed in triplicate.

*Mitochondrial membrane potential ($\Delta\Psi m$)*. Alterations in $\Delta\Psi m$ during the early stages of apoptosis were assessed using the Muse® MitoPotential assay (MCH 100110; EMD Millipore). SKOV3/DDP cells were treated with cisplatin alone or in combination with TAT-IDP$^S$. Subsequently, cells were centrifuged at 3,000 x g for 5 min at room temperature, and the cell pellet was suspended in assay buffer ($1x10^5$ cells/100 $\mu$l). MitoPotential dye working solution was added, and the cell suspension was incubated at 37˚C for 20 min. Following the addition of Muse® MitoPotential 7-aminoactinomycin D dye (propidium iodide) for 5 min, alterations in $\Delta\Psi m$ and in cellular plasma membrane permeabilization were assessed on the basis of the fluorescence intensities of both dyes, which were analyzed by flow cytometry (Muse® Cell Analyzer version 1.5.0.0; EMD Millipore).

*Statistical analysis*. Data are representative of three independent experiments performed in triplicate and are presented as the mean ± standard deviation. Data analysis was performed using one-way analysis of variance, and Tukey's post-hoc test was used to determine statistical significance of all pairwise comparisons of interest. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using the Complex Samples module in IBM SPSS Statistics 20.0 (SPSS, Inc., Chicago, IL, USA)

## Results

*TAT-IDP$^S$ enhances cisplatin-induced cytosolic $Ca^{2+}$ elevation and mitochondrial $Ca^{2+}$ overload in SKOV3/DDP cells*. TAT-IDP$^S$ can enhance IP3R-mediated $Ca^{2+}$ signals by disrupting the Bcl-2-IP3R interaction. The present study explored whether TAT-IDP$^S$ increased cisplatin-induced cytosolic and mitochondrial $Ca^{2+}$ levels in SKOV3/DDP cells. SKOV3/DDP cells were treated with cisplatin (15 $\mu$g/ml) and/or TAT-IDP$^S$ (25 $\mu$M) for 24 h and $Ca^{2+}$ levels were then examined in the mitochondria and cytoplasm. The $Ca^{2+}$-sensitive fluorescent dyes Fluo-4/AM and Rhod-2/AM were used to detect cytosolic and mitochondrial $Ca^{2+}$ levels by confocal microscopy, respectively. Compared with in the control (untreated) group, cisplatin induced an elevation in free $Ca^{2+}$ in the cytosol and mitochondria. Furthermore, cisplatin-induced $Ca^{2+}$ elevation in the cytosol and mitochondria was further increased in the cells treated with cisplatin and TAT-IDP$^S$ (Fig. 1).

*TAT-IDP$^S$ increases cisplatin-induced apoptosis of SKOV3/DDP cells*. To evaluate the effects of TAT-IDP$^S$ on cisplatin-induced growth inhibition and apoptosis, SKOV3/DDP cells were treated with cisplatin (15 $\mu$g/ml) and/or TAT-IDP$^S$ (25 $\mu$M) for 24 h and growth inhibition was examined using MTT assays. The results indicated that cisplatin induced growth inhibition in SKOV3/DDP cells. In addition, cotreatment with cisplatin and TAT-IDP$^S$ increased the growth inhibitory effects of cisplatin, whereas TAT-IDP$^S$ alone had no effect on cell viability compared with in the control group (Fig. 2A). Under an optical microscope, it was demonstrated that SKOV3/DDP cells treated with cisplatin became round and fragmented compared with the control cells and that TAT-IDP$^S$ enhanced this effect (Fig. 2B). Furthermore, flow cytometric analysis demonstrated that the rate of apoptosis in SKOV3/DDP cells treated with cisplatin and TAT-IDP$^S$ was higher than that in cells treated with cisplatin alone (Fig. 2C and D). These results suggested that TAT-IDP$^S$ increased cisplatin-induced growth inhibition and apoptosis of SKOV3/DDP cells.

*TAT-IDP$^S$ increases cisplatin-induced ER stress-associated apoptosis by increasing calpain-1 expression in SKOV3/DDP cells*. Since calpain-1, which is a ubiquitous protease, can be activated by cytoplasmic $Ca^{2+}$ elevation (16), the present study detected the expression of calpain-1 using confocal microscopy. The results indicated that cotreatment with cisplatin and TAT-IDP$^S$ for 24 h increased cisplatin-induced calpain-1 accumulation (Fig. 3A). Calpain-1 has been reported to enhance ER-associated apoptosis by cleaving its protein substrates (16). Subsequently, the present study investigated whether TAT-IDP$^S$ may sensitize SKOV3/DDP cells

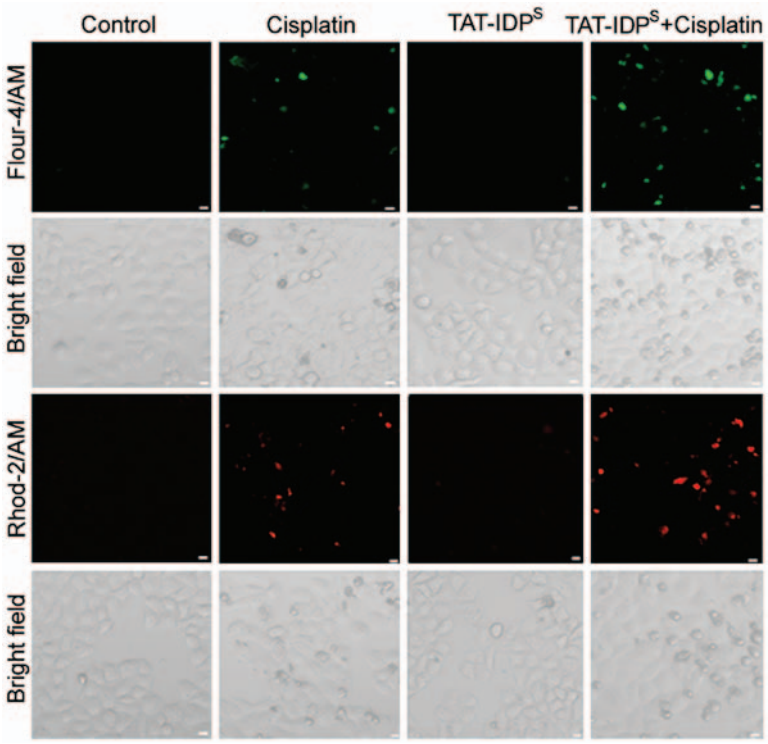


Figure 1. TAT-IDP$^S$ increases cisplatin-induced cytosolic $Ca^{2+}$ elevation and mitochondrial $Ca^{2+}$ overload in SKOV3/DDP cells. SKOV3/DDP cells were treated with cisplatin (15 $\mu$g/ml) and/or TAT-IDP$^S$ (25 $\mu$M) for 24 h and were then incubated with the fluorescent calcium indicators Fluo-4/AM and Rhod-2/AM. Cytosolic and mitochondrial $Ca^{2+}$ levels were observed by confocal microscopy (scale bar, 5 $\mu$m). TAT-IDP$^S$, TAT-fused IP3R-derived peptide.

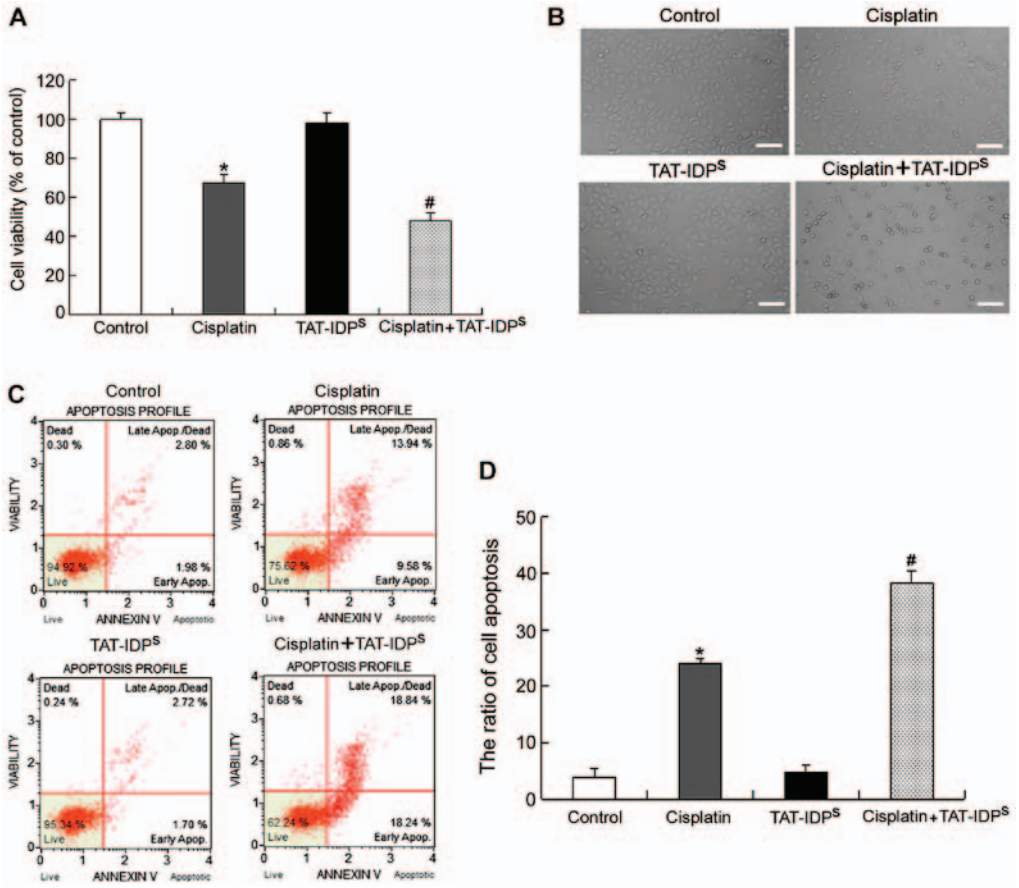


Figure 2. TAT-IDP$^S$ increases cisplatin-induced apoptosis of SKOV3/DDP cells. (A) SKOV3/DDP cells were treated with cisplatin (15 $\mu$g/ml) and/or TAT-IDP$^S$ (25 $\mu$M) for 24 h, and cell viability was determined using MTT assays. Data are presented as the mean ± standard deviation. (B) Optical microscopy images of SKOV3/DDP cells treated with cisplatin (15 $\mu$g/ml) and/or TAT-IDP$^S$ (25 $\mu$M) for 24 h (scale bar, 50 $\mu$m). (C) SKOV3/DDP cells were treated with cisplatin (15 $\mu$g/ml) and/or TAT-IDP$^S$ (25 $\mu$M) for 24 h, and were stained with Annexin V. (D) Quantification of apoptosis of SKOV3/DDP cells exposed to cisplatin (15 $\mu$g/ml) and/or TAT-IDP$^S$ (25 $\mu$M) for 24 h. Data are presented as the mean ± standard deviation, n=3. $^*$P<0.05 vs. the control group; $^\#$P<0.05 vs. the cisplatin group. TAT-IDP$^S$, TAT-fused IP3R-derived peptide.

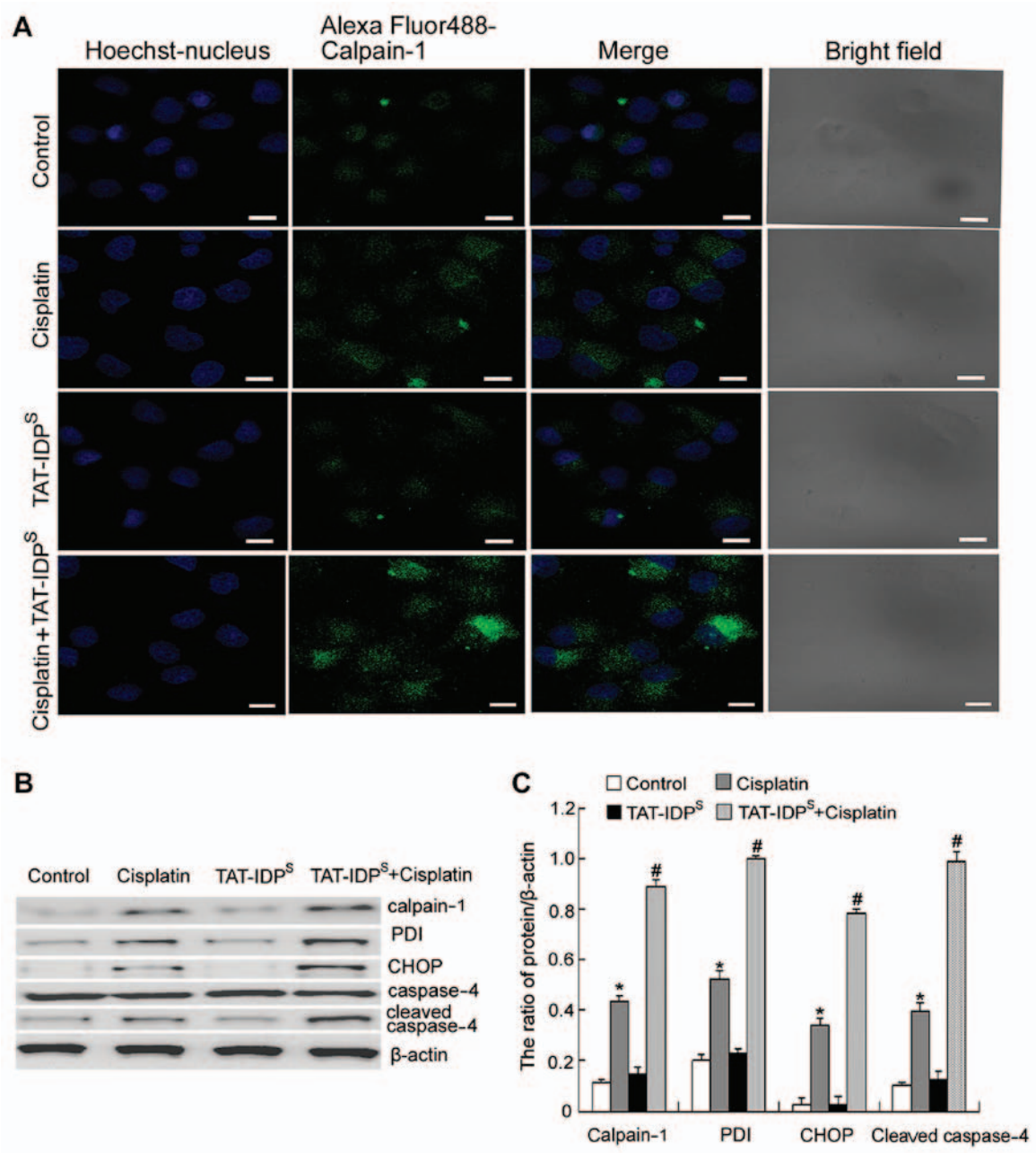


Figure 3. TAT-IDP$^S$ increases cisplatin-induced endoplasmic reticulum stress-associated apoptosis by increasing calpain-1 expression in SKOV3/DDP cells. (A) Detection of calpain-1 in the cytoplasm of SKOV3/DDP cells exposed to cisplatin (15 $\mu$g/ml) and/or TAT-IDP$^S$ (25 $\mu$M) for 24 h, under a confocal microscope (scale bar, 10 $\mu$m). (B) Western blot analysis was performed to detect the expression levels of calpain-1, PDI, CHOP and cleaved caspase-4 in SKOV3/DDP cells following treatment with cisplatin (15 $\mu$g/ml) and/or TAT-IDPS (25 $\mu$M) for 24 h. (C) Semi-quantification of calpain-1, PDI, CHOP and cleaved caspase-4 expression. Data are presented as the mean ± standard deviation, n=3. $^*$P<0.05 vs. the control group; $^\#$P<0.05 vs. the cisplatin group. CHOP, CCAAT-enhancer-binding protein homologous protein; PDI, protein disulfide isomerase; TAT-IDP$^S$, TAT-fused IP3R-derived peptide.

to cisplatin-induced ER stress-associated apoptosis. The expression levels of the following ER stress-associated apoptotic proteins: Calpain-1, PDI, CHOP and cleaved caspase-4, were detected by western blot analysis. Cisplatin induced the expression of all four proteins; furthermore, combined treatment with TAT-IDP$^S$ enhanced these effects (Fig. 3B and C). These results suggested that TAT-IDP$^S$ may increase cisplatin-induced ER stress-associated apoptosis of SKOV3/DDP cells by increasing calpain-1 expression.

*TAT-IDP$^S$ increases cisplatin-induced mitochondria-mediated apoptosis of SKOV3/DDP cells.* Mitochondrial $Ca^{2+}$ overload can lead to opening of the mitochondrial inner membrane permeability transition pore (MPTP) and can subsequently induce aberrant dissipation of $\Delta\Psi$m, thus resulting in cell apoptosis. Since an alteration in $\Delta\Psi$m is considered an early event in apoptosis (17), the present study monitored the effects of TAT-IDP$^S$ and/or cisplatin on $\Delta\Psi$m at 6 h. SKOV3/DDP cells were treated with cisplatin (15 $\mu$g/ml) and/or TAT-IDP$^S$ (25 $\mu$M) for 6 h, and alterations in $\Delta\Psi$m were determined using flow cytometry. TAT-IDP$^S$ enhanced the cisplatin-induced decrease in $\Delta\Psi$m (Fig. 4A and B). Finally, the expression levels of mitochondrial apoptosis-associated proteins (cytochrome *c*, Bcl-2, Bax and cleaved caspase-3)

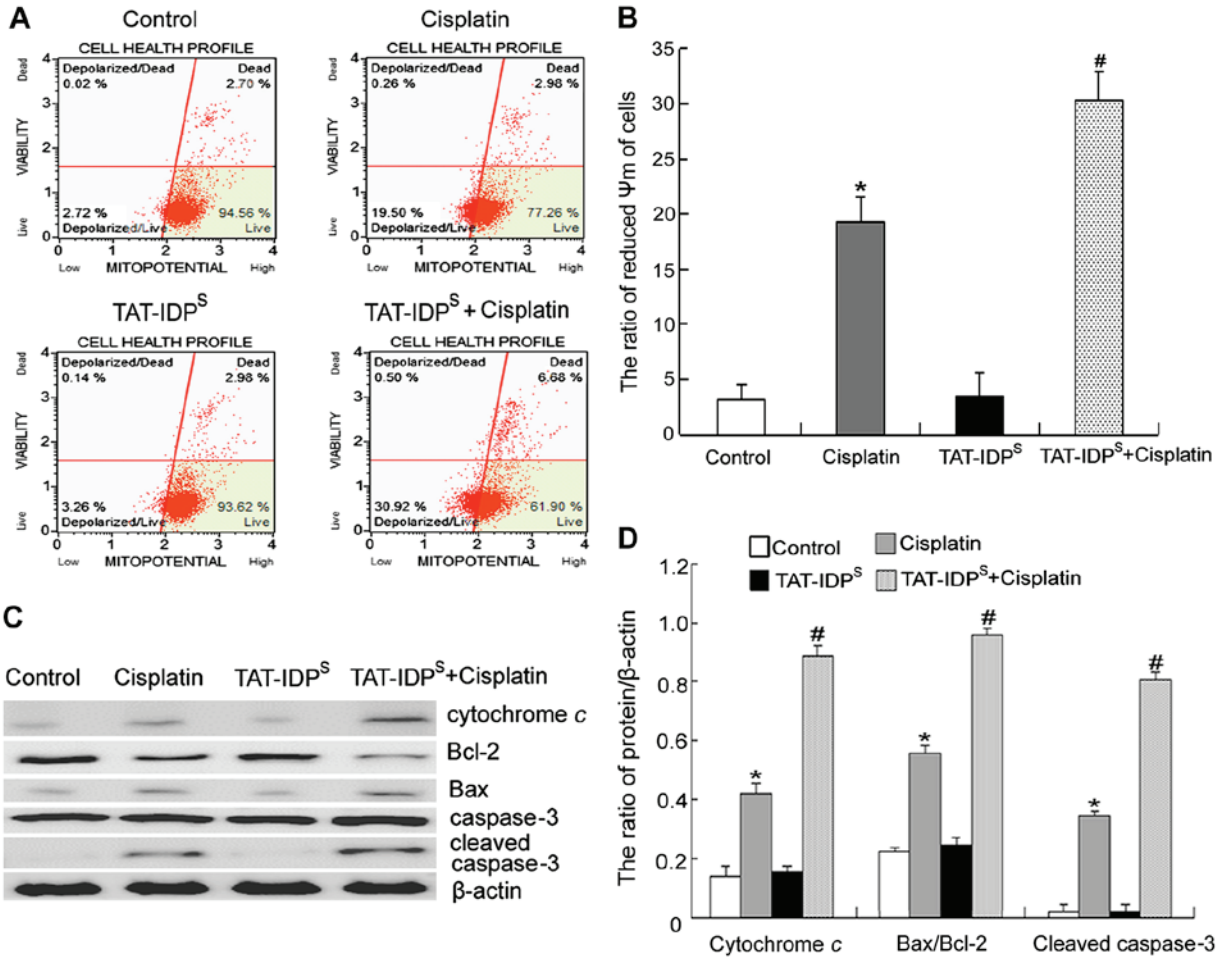


Figure 4. TAT-IDP$^S$ increases cisplatin-induced mitochondria-mediated apoptosis of SKOV3/DDP cells. (A) SKOV3/DDP cells were treated with cisplatin (15 $\mu$g/ml) and/or TAT-IDP$^S$ (25 $\mu$M) for 6 h. ΔΨm was assessed by staining with MitoPotential dye and 7-aminoactinomycin D, and was analyzed using the Muse® Cell Analyzer. (B) Quantification of ΔΨm. Data are presented as the mean ± standard deviation, n=3. (C) Western blot analysis of the expression of cytochrome *c*, Bcl-2, Bax and cleaved caspase-3 in SKOV3/DDP cells following treatment with cisplatin (15 $\mu$g/ml) and/or TAT-IDP$^S$ (25 $\mu$M) for 24 h. (D) Semi-quantification of cytochrome *c*, Bcl-2, Bax, and cleaved caspase-3 expression. Data are presented as the mean ± standard deviation, n=3. $^*$P<0.05 vs. the control group; $^\#$P<0.05 vs. the cisplatin group. ΔΨm, mitochondrial membrane potential; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; TAT-IDP$^S$, TAT-fused IP3R-derived peptide.

were detected using western blotting. The results indicated that cotreatment with cisplatin and TAT-IDP$^S$ for 24 h increased the cisplatin-induced Bax/Bcl-2 ratio, and the cisplatin-induced expression of cytochrome *c* and cleaved caspase-3 (Fig. 4C and D). These results suggested that TAT-IDP$^S$ may further reduce the decrease in ΔΨm induced by cisplatin, which may subsequently contribute to mitochondria-mediated apoptosis of SKOV3/DDP cells.

*2-APB reduces the growth inhibition and apoptosis induced by cotreatment with TAT-IDP$^S$ and cisplatin*. To further confirm that mitochondrial $Ca^{2+}$ overload caused by cotreatment with TAT-IDP$^S$ and cisplatin derives from the ER, cells were treated with 2-APB, an IP3R antagonist (18). Cisplatin and TAT-IDP$^S$-induced mitochondrial $Ca^{2+}$ elevation in SKOV3/DDP cells was markedly reduced in the presence of 2-APB (Fig. 5A). In addition, 2-APB reduced the decrease in ΔΨm induced by cisplatin and TAT-IDP$^S$ (Fig. 5B and C). Similarly, cisplatin and TAT-IDP$^S$-induced growth inhibition and apoptosis of SKOV3/DDP cells was markedly reduced in the presence of 2-APB (Fig. 5D-F). Taken together, these results strongly indicated that mitochondrial $Ca^{2+}$ overload caused by cotreatment with TAT-IDP$^S$ and cisplatin was derived from the ER.

## Discussion

Bcl-2 is an anti-apoptotic protein that promotes cell survival predominantly by inhibiting the release of cytochrome *c* from the mitochondria via its BH3 domain. In addition, Bcl-2 inhibits cell apoptosis by reducing $Ca^{2+}$ release from the ER via its BH4 domain. Recently, targeting the BH3 and BH4 domains to generate Bcl-2 inhibitors for cancer therapy has attracted attention. Obatoclax is a BH3 mimetic that can induce apoptosis of numerous types of cancer cell by interacting with the Bcl-2 BH3 domain; this agent has been tested in a phase II clinical trial in patients (19). Furthermore, Deng *et al* reported that BDA-366, which is an antagonist of the Bcl-2 BH4 domain, can induce apoptosis of human myeloma U266 cells (20). The results of the present study demonstrated that by targeting BH4, TAT-IDP$^S$ increases cisplatin-induced apoptosis of SKOV3/DDP cells (Fig. 2).

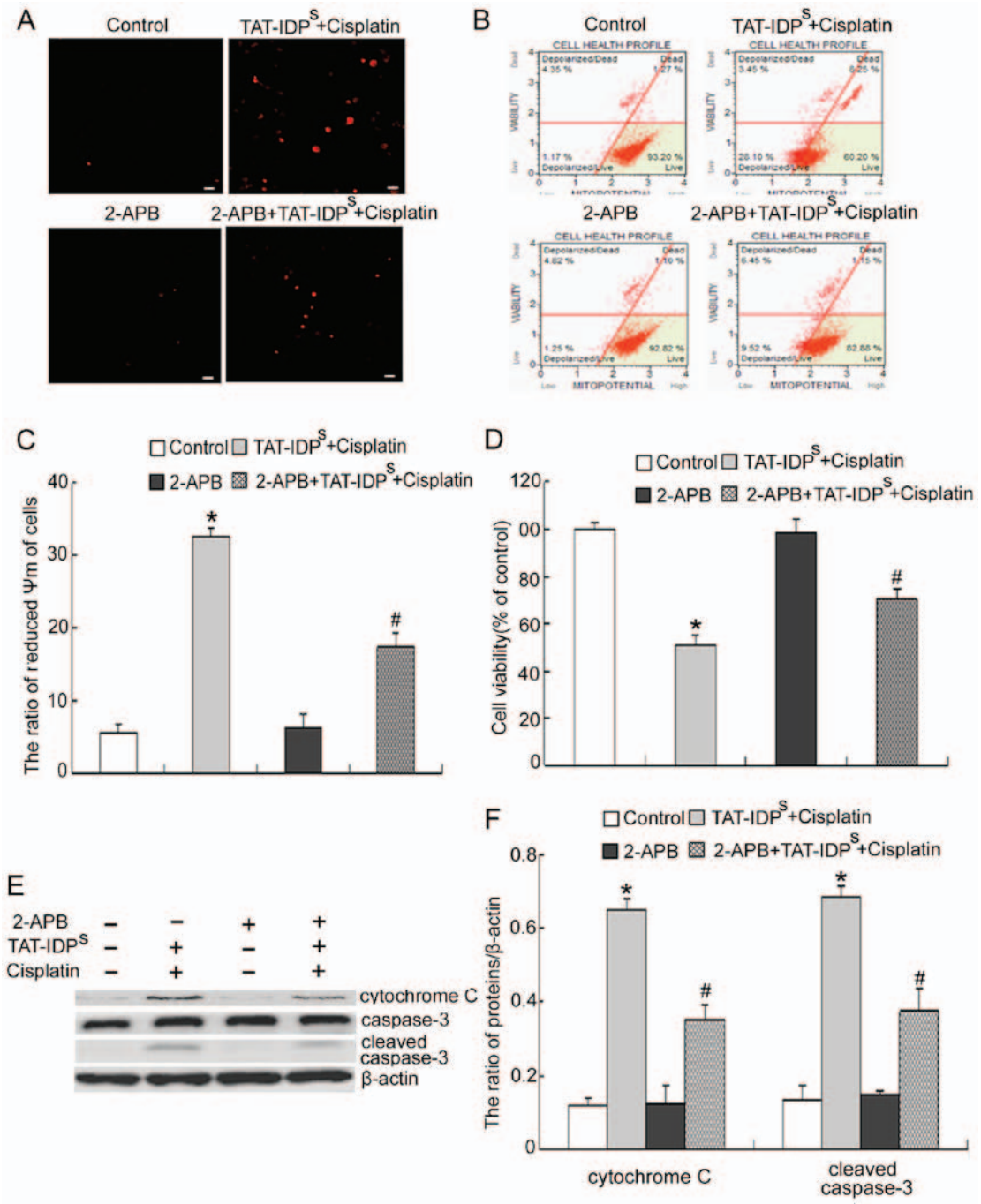


Figure 5. 2-APB reduces the growth inhibition and apoptosis induced by TAT-IDP$^S$ and cisplatin. SKOV3/DDP cells were treated with 15 $\mu$g/ml cisplatin and 25 $\mu$M TAT-IDP$^S$ in the presence or absence of 50 $\mu$M 2-APB for 24 h. (A) Cells were incubated with the fluorescent calcium indicator Rhod-2/AM and mitochondrial $Ca^{2+}$ levels were observed by confocal microscopy (scale bar, 40 $\mu$m). (B) ΔΨm was assessed by staining with MitoPotential dye and 7-aminoactinomycin D, and was analyzed using the Muse® Cell Analyzer. (C) Quantification of ΔΨm. Data are presented as the mean ± standard deviation, n=3. (D) Cell viability was determined using an MTT assay. Data are presented as the mean ± standard deviation. (E) Western blot analysis of the expression of cytochrome *c* and cleaved caspase-3 in SKOV3/DDP cells. (F) Semi-quantification of cytochrome *c* and cleaved caspase-3 expression. Data are presented as the mean ± standard deviation, n=3. $^*$P<0.05 vs. the control group; $^\#$P<0.05 vs. the cisplatin + TAT-IDP$^S$ group. ΔΨm, mitochondrial membrane potential; 2-APB, 2-aminoethyl diphenylborinate; TAT-IDP$^S$, TAT-fused IP3R-derived peptide.

As a major intracellular $Ca^{2+}$ store, the ER serves a central role in intracellular $Ca^{2+}$ signal transduction, and controls cell death and survival. The release of large amounts of $Ca^{2+}$ from the ER promotes cell apoptosis by affecting cytoplasmic and mitochondrial $Ca^{2+}$ homeostasis. There is strong evidence to suggest that Bcl-2 directly inhibits proapoptotic $Ca^{2+}$ release from IP3R at the ER into the cytoplasm and mitochondria via its BH4 domain (21-23). TAT-IDP$^S$ can reverse the Bcl-2-mediated inhibition of IP3-dependent $Ca^{2+}$ elevation by targeting the BH4 domain, subsequently triggering $Ca^{2+}$-dependent apoptosis or increasing sensitivity toward proapoptotic stimuli (15). The present study clearly demonstrated that TAT-IDP$^S$ increased cisplatin-induced cytoplasmic and mitochondrial $Ca^{2+}$ elevation (Fig. 1).

Elevation of cytoplasmic $Ca^{2+}$ levels can lead to activation of a series of $Ca^{2+}$-sensitive enzymes. Calpain-1 is the target of

cytoplasmic $Ca^{2+}$, which was originally termed $\mu$-calpain based on the $Ca^{2+}$ concentration ($\mu$M range) required for its optimal activity (24). Calpain-1 also participates in the apoptotic pathway triggered by ER stress. Matsuzaki *et al* confirmed that $Ca^{2+}$-dependent calpain activation promoted neural cell death by inducing the processing and activation of caspase-4 (25). According to the results of confocal microscopy and western blot analyses, the present study revealed that TAT-$IDP^S$ increased cisplatin-induced calpain-1 expression (Fig. 3) and increased cisplatin-induced ER stress-associated apoptosis of SKOV3/DDP cells.

Mitochondria are exposed to high $Ca^{2+}$ concentrations following the release of $Ca^{2+}$ from the ER. Accumulation of $Ca^{2+}$ in the mitochondrial matrix can lead to opening of the MPTP, which in turn causes mitochondrial swelling and rupture, accompanied by dissipation of $\Delta\Psi m$ (26-28). The $\Delta\Psi m$ collapse further induces rupture of the outer membrane and subsequent release of mitochondrial apoptotic factors, including cytochrome *c*, into the cytosol, finally leading to cell apoptosis (29,30). Our previous study demonstrated that mitochondrial $Ca^{2+}$ levels are closely associated with cisplatin resistance in ovarian carcinoma cells (11). The present study demonstrated that TAT-$IDP^S$ increased cisplatin-induced $\Delta\Psi m$ collapse and mitochondrial-mediated apoptosis of SKOV3/DDP cells. Using 2-APB to effectively block ER $Ca^{2+}$ release, the present study further confirmed that TAT-$IDP^S$ and cisplatin-induced mitochondrial $Ca^{2+}$ overload is derived from the ER (Fig. 5A).

In conclusion, the results of the present study indicated that TAT-$IDP^S$ increased the cisplatin-induced elevation of cytoplasmic and mitochondrial $Ca^{2+}$, which further increased cisplatin-induced ER stress-mediated apoptosis by increasing calpain-1 expression and activating the mitochondrial apoptotic pathway. These results suggested that $Ca^{2+}$ release from the ER may regulate cisplatin-induced SKOV3/DDP cell apoptosis, providing a rationale for drugs that specifically disrupt the Bcl-2-IP3R interaction as a novel tool for the treatment of ovarian cancer.

## Acknowledgements

The present study was supported by the National Nature and Science Foundation of China (NSFC grant nos. 81372793, 81472419 and 81202552) and the Department of Education of Jilin Province Project (grant no. 2016237). We thank Liwen Bianji for editing the English in this manuscript.

## References

1. Huang J, Liu K, Song D, Ding M, Wang J, Jin Q and Ni J: Krüppel-like factor 4 promotes high-mobility group box 1-induced chemotherapy resistance in osteosarcoma cells. Cancer Sci 107: 242-249, 2016.
2. Chen XX, Xie FF, Zhu XJ, Lin F, Pan SS, Gong LH, Qiu JG, Zhang WJ, Jiang QW, Mei XL, *et al*: Cyclin-dependent kinase inhibitor dinaciclib potently synergizes with cisplatin in preclinical models of ovarian cancer. Oncotarget 6: 14926-14939, 2015.
3. Siddiqui WA, Ahad A and Ahsan H: The mystery of BCL2 family: Bcl-2 proteins and apoptosis: an update. Arch Toxicol 89: 289-317, 2015.
4. Lutz RJ: Role of the BH3 (Bcl-2 homology 3) domain in the regulation of apoptosis and Bcl-2-related proteins. Biochem Soc Trans 28: 51-56, 2000.
5. Fan Z, Yu H, Cui N, Kong X, Liu X, Chang Y, Wu Y, Sun L and Wang G: ABT737 enhances cholangiocarcinoma sensitivity to cisplatin through regulation of mitochondrial dynamics. Exp Cell Res 335: 68-81, 2015.
6. Wong M, Tan N, Zha J, Peale FV, Yue P, Fairbrother WJ and Belmont LD: Navitoclax (ABT-263) reduces Bcl-x(L)-mediated chemoresistance in ovarian cancer models. Mol Cancer Ther 11: 1026-1035, 2012.
7. Han B, Park D, Li R, Xie M, Owonikoko TK, Zhang G, Sica GL, Ding C, Zhou J, Magis AT, *et al*: Small-molecule Bcl2 BH4 antagonist for lung cancer therapy. Cancer Cell 27: 852-863, 2015.
8. Nakajima A, Tsuji M, Inagaki M, Tamura Y, Kato M, Niiya A, Usui Y and Oguchi K: Neuroprotective effects of propofol on ER stress-mediated apoptosis in neuroblastoma SH-SY5Y cells. Eur J Pharmacol 725: 47-54, 2014.
9. Krebs J, Agellon LB and Michalak M: Ca(2+) homeostasis and endoplasmic reticulum (ER) stress: An integrated view of calcium signaling. Biochem Biophys Res Commun 460: 114-121, 2015.
10. Liu N, Xu Y, Sun JT, Su J, Xiang XY, Yi HW, Zhang ZC and Sun LK: The BH3 mimetic S1 induces endoplasmic reticulum stress-associated apoptosis in cisplatin-resistant human ovarian cancer cells although it activates autophagy. Oncol Rep 30: 2677-2684, 2013.
11. Xu Y, Wang C, Su J, Xie Q, Ma L, Zeng L, Yu Y, Liu S, Li S, Li Z, *et al*: Tolerance to endoplasmic reticulum stress mediates cisplatin resistance in human ovarian cancer cells by maintaining endoplasmic reticulum and mitochondrial homeostasis. Oncol Rep 34: 3051-3060, 2015.
12. Bonder DE and McCarthy KD: Astrocytic Gq-GPCR-linked IP3R-dependent $Ca^{2+}$ signaling does not mediate neurovascular coupling in mouse visual cortex in vivo. J Neurosci 34: 13139-13150, 2014.
13. Liu Z, Wild C, Ding Y, Ye N, Chen H, Wold EA and Zhou J: BH4 domain of Bcl-2 as a novel target for cancer therapy. Drug Discov Today 21: 989-996, 2016.
14. Akl H, Monaco G, La Rovere R, Welkenhuyzen K, Kiviluoto S, Vervliet T, Molgó J, Distelhorst CW, Missiaen L, Mikoshiba K, *et al*: IP3R2 levels dictate the apoptotic sensitivity of diffuse large B-cell lymphoma cells to an IP3R-derived peptide targeting the BH4 domain of Bcl-2. Cell Death Dis 4: e632, 2013.
15. Zhong F, Harr MW, Bultynck G, Monaco G, Parys JB, De Smedt H, Rong YP, Molitoris JK, Lam M, Ryder C, *et al*: Induction of $Ca^{2+}$-driven apoptosis in chronic lymphocytic leukemia cells by peptide-mediated disruption of Bcl-2-IP3 receptor interaction. Blood 117: 2924-2934, 2011.
16. Baudry M and Bi X: Calpain-1 and Calpain-2: The Yin and Yang of Synaptic Plasticity and Neurodegeneration. Trends Neurosci 39: 235-245, 2016.
17. Sun X, Xu H, Shen J, Guo S, Shi S, Dan J, Tian F, Tian Y and Tian Y: Real-time detection of intracellular reactive oxygen species and mitochondrial membrane potential in THP-1 macrophages during ultrasonic irradiation for optimal sonodynamic therapy. Ultrason Sonochem 22: 7-14, 2015.
18. Peppiatt CM, Collins TJ, Mackenzie L, Conway SJ, Holmes AB, Bootman MD, Berridge MJ, Seo JT and Roderick HL: 2-Aminoethoxydiphenyl borate (2-APB) antagonises inositol 1,4,5-trisphosphate-induced calcium release, inhibits calcium pumps and has a use-dependent and slowly reversible action on store-operated calcium entry channels. Cell Calcium 34: 97-108, 2003.
19. Schwartz-Roberts JL, Shajahan AN, Cook KL, Warri A, Abu-Asab M and Clarke R: GX15-070 (obatoclax) induces apoptosis and inhibits cathepsin D- and L-mediated autophagosomal lysis in antiestrogen-resistant breast cancer cells. Mol Cancer Ther 12: 448-459, 2013.
20. Deng J, Park D, Wang M, Nooka A, Deng Q, Matulis S, Kaufman J, Lonial S, Boise LH, Galipeau J, *et al*: BCL2-BH4 antagonist BDA-366 suppresses human myeloma growth. Oncotarget 7: 27753-27763, 2016.
21. Akl H, La Rovere RM, Janssens A, Vandenberghe P, Parys JB and Bultynck G: HA14-1 potentiates apoptosis in B-cell cancer cells sensitive to a peptide disrupting IP3 receptor/Bcl-2 complexes. Int J Dev Biol 59: 391-398, 2015.
22. Parys JB: The IP3 receptor as a hub for Bcl-2 family proteins in cell death control and beyond. Sci Signal 7: pe4, 2014.
23. Akl H, Vervloessem T, Kiviluoto S, Bittremieux M, Parys JB, De Smedt H and Bultynck G: A dual role for the anti-apoptotic Bcl-2 protein in cancer: Mitochondria versus endoplasmic reticulum. Biochim Biophys Acta 1843: 2240-2252, 2014.

24. Pu X, Storr SJ, Ahmad NS, Chan SY, Moseley PM, Televantou D, Cresti N, Boddy A, Ellis IO and Martin SG: Calpain-1 is associated with adverse relapse free survival in breast cancer: A confirmatory study. Histopathology 68: 1021-1029, 2016.
25. Matsuzaki S, Hiratsuka T, Kuwahara R, Katayama T and Tohyama M: Caspase-4 is partially cleaved by calpain via the impairment of $Ca^{2+}$ homeostasis under the ER stress. Neurochem Int 56: 352-356, 2010.
26. Rutter GA and Rizzuto R: Regulation of mitochondrial metabolism by ER $Ca^{2+}$ release: An intimate connection. Trends Biochem Sci 25: 215-221, 2000.
27. von Stockum S, Giorgio V, Trevisan E, Lippe G, Glick GD, Forte MA, Da-Rè C, Checchetto V, Mazzotta G, Costa R, *et al*: F-ATPase of *Drosophila* melanogaster forms 53-picosiemen (53-pS) channels responsible for mitochondrial $Ca^{2+}$-induced $Ca^{2+}$ release. J Biol Chem 290: 4537-4544, 2015.
28. Fülöp L, Rajki A, Maka E, Molnár MJ and Spät A: Mitochondrial $Ca^{2+}$ uptake correlates with the severity of the symptoms in autosomal dominant optic atrophy. Cell Calcium 57: 49-55, 2015.
29. Hajnóczky G, Csordás G, Das S, Garcia-Perez C, Saotome M, Sinha Roy S and Yi M: Mitochondrial calcium signalling and cell death: Approaches for assessing the role of mitochondrial $Ca^{2+}$ uptake in apoptosis. Cell Calcium 40: 553-560, 2006.
30. Giorgi C, Baldassari F, Bononi A, Bonora M, De Marchi E, Marchi S, Missiroli S, Patergnani S, Rimessi A, Suski JM, *et al*: Mitochondrial Ca(2+) and apoptosis. Cell Calcium 52: 36-43, 2012.