Co-treatment with BEZ235 enhances chemosensitivity of A549/DDP cells to cisplatin via inhibition of PI3K/Akt/mTOR signaling and downregulation of ERCC1 expression

ANZHOU XIA[1*], HUANHUAN LI[1*], RAN LI[1], LINGHONG LU[1] and XIAOLI WU[2]

[1]Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, Xuzhou Medical University, Xuzhou, Jiangsu 221004; [2]Department of Pharmacy, Huai'an First People's Hospital, Nanjing Medical University, Huaian, Jiangsu 223300, P.R. China

Received April 30, 2018; Accepted July 17, 2018

DOI: 10.3892/or.2018.6583

Abstract. The activation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling and upregulation of excision repair cross complementation group 1 (ERCC1) are the two most important factors that confer resistance to cisplatin (DDP) therapy in non-small-cell lung cancer (NSCLC). Therefore, inhibition of the PI3K/Akt/mTOR signaling pathway and ERCC1 expression is a potential approach for the treatment of patients with advanced NSCLC. In the present study, whether combined treatment with DDP and BEZ235, a dual PI3K/mTOR inhibitor, could provide a synergistic antitumor effect in A549/DDP cells was investigated, and the possible mechanisms involved were explored. The half-maximal inhibitory concentration (IC_{50}) values were calculated in A549/DDP cells. Synergistic interaction of BEZ235 and DDP was evaluated by combination index (CI) analysis. The levels of phosphorylated Akt (p-Akt), phosphorylated mTOR (p-mTOR), apoptosis-related proteins and ERCC1 were detected by western blot analysis. Apoptotic cells were quantified by flow cytometry and Hoechst 33342 staining. The migration and invasion abilities of A549/DDP cells were evaluated by wound healing and Transwell assays, respectively. It was observed that the dose reduction index (DRI) of BEZ235 was 13.82 and for DDP it was 13.58, and the CI of combination was <1 over a wide range of doses. In addition, the levels of p-Akt, p-mTOR and ERCC1 were significantly elevated by DDP treatment, and were reduced by co-administration of BEZ235 and DDP. Furthermore, the combination treatment significantly induced apoptotic cell death, decreased migration and invasion abilities compared with those treated with either BEZ235 or DDP alone. In conclusion, the combination of BEZ235 with DDP had synergistic antitumor effects in A549/DDP cells as reflected by reduced proliferation, increased apoptosis, and suppression of the migration and invasion abilities of A549/DDP cells, and the mechanism mediating these effects may be associated with the inhibition of PI3K/Akt/mTOR signaling and downregulation of ERCC1 expression.

Correspondence to: Mrs. Xiaoli Wu, Department of Pharmacy, Huai'an First People's Hospital, Nanjing Medical University, 1 West Huanghe Road, Huaiyin, Huaian, Jiangsu 223300, P.R. China
E-mail: 15189597304@163.com

*Contributed equally

Key words: BEZ235, cisplatin, A549/DDP cells, ERCC1, resistance, apoptosis

Introduction

Lung cancer is one of the most common malignant tumors, with the highest rate of morbidity and mortality worldwide (1). Non-small cell lung cancer (NSCLC) accounts for ~80% of bronchogenic carcinomas, and ~65% of the patients with NSCLC present locally advanced or metastatic disease at the time of diagnosis (2,3). Cisplatin [cis-diamminedichloroplatinum (II); DDP] is one of the most effective anticancer drugs for the treatment of lung cancer and other tumors. Although the combination of DDP with third-generation anticancer drugs (gemcitabine, paclitaxel, vinorelbine and docetaxel) has improved the efficiency and overall survival in patients with NSCLC, the development of primary or acquired DDP resistance often reduces the therapeutic efficacy, even leading to treatment failure (4,5). Therefore, it is important to explore the mechanisms underlying DDP resistance, and to search for effective strategies that may avoid and overcome drug-resistance in NSCLC therapy.

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway is involved in the regulation of cell survival, proliferation, differentiation, angiogenesis and apoptosis (6,7). Sustained activation of the PI3K/Akt/mTOR signaling pathway promotes cancer cell proliferation, invasion and metastasis, and inhibits cell apoptosis in different types of cancer (8,9). In addition, excessive activation of the PI3K/Akt/mTOR pathway confers resistance to various cancer therapies and is often associated with a poor prognosis of various types of cancer, including NSCLC (10-12). Therefore, inhibition

of the PI3K/Akt/mTOR pathway is one of the more efficient strategies to potentiate the antitumor effects of DDP-based treatment for NSCLC.

Nucleotide excision repair (NER) is a universal and highly functionally conserved DNA repair pathway that repairs DNA lesions that alter the helical structure of the DNA molecule and interfere with DNA replication and transcription. Excision repair cross complementation group 1 (ERCC1) is one of the most important components of the NER enzyme family due to its involvement in the excision of DNA. During the DNA repair process, ERCC1 dimerizes with xeroderma pigmentosum complementation group F (XPF) forming an ERCC1-XPF complex that has a structure-specific endonuclease activity, which makes the incision 5' to the lesion site (13). ERCC1-XPF is essential for the repair of DDP-induced DNA adducts. When ERCCl is highly expressed, the efficiency of DNA repair is greatly increased and cancerous cells are able to continue to survive and grow, resulting in tumor resistance to DDP (14,15). Several studies have revealed that elevated expression of ERCC1 (and ERCC1-XPF complexes) is associated with DDP resistance and may act as a prognostic marker for poor survival in patients with various types of cancer treated with platinum-based chemotherapy, including NSCLC, breast, gastric and urothelial ovarian cancer (16-19). Conversely, suppression of ERCC1 expression can enhance or restore sensitivity to DDP in various types of cancer (20,21). In addition, negative ERCC1 expression or low ERCC1 expression was reported to be associated with increased survival in patients with advanced NSCLC treated with platinum-based chemotherapy (22). Therefore, agents targeting ERCC1 may enhance platinum activity and/or reverse resistance, although this strategy has to be further validated.

BEZ235, a novel dual PI3K/mTOR inhibitor, can reverse the hyperactivation of the PI3K/Akt/mTOR pathway, resulting in antitumor activity in cancer of various origins (23-25). Recently, studies have revealed that combined treatment with AZD6244, Trichostatin A and BEZ235 exerted synergistic antitumor effects on NSCLC cells (26,27). However, few studies have extensively examined the synergistic effect between DDP and BEZ235 in NSCLC, and the association between the level of ERCC1 expression and the activity of the PI3K/Akt/mTOR signaling pathway is not fully understood. Hence, in the present study, the following issues were investigated: i) whether combination of BEZ235 with DDP could enhance sensitivity of A549/DDP cells to DDP; and ii) if so, whether the mechanism underlying the synergistic effect of combination therapy was associated with downregulation of ERCC1 expression by BEZ235 in A549/DDP cells.

Materials and methods

Cell culture. The human lung adenocarcinoma cell line (A549) and DDP-resistant A549 cell line (A549/DDP; final concentration of 3.33 μM DDP to maintain drug resistance) were purchased from Guangzhou Biological Technology Co., Ltd. (Guangzhou, China) and the Central South University Advanced Research Center (Hunan, China), respectively. All the cell lines were cultured at 37˚C and 5% CO_2 in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; ExCell, Biology, Inc., Shanghai, China) and 1% penicillin/streptomycin (Beyotime Institute of Biotechnology, Haimen, China). A549/DDP cells were cultured in medium without DDP for 1 week prior to experiments. Exponentially growing cells were used in all experiments.

Reagents. BEZ235 was purchased from Selleck Chemicals (Houston, TX, USA) and dissolved in DMSO at 42˚C to obtain a stock concentration of 5 mM. DDP was purchased from Qilu Pharmaceutical Co., Ltd. (Shandong, China) and dissolved in PBS to obtain a stock concentration of 6.67 mM. BEZ235 and DDP were stored at -80˚C and -20˚C, respectively, and diluted to the desired final concentration in RPMI-1640 medium at the time of use.

Cell viability assay. The cytotoxicity of DDP and BEZ235 was determined using MTT. Cells were seeded into 96-well plates at a density of $5x10^3$ cells/well in 100 μl medium and cultured overnight. Subsequently, the cells were incubated with indicated treatments for 48 h, followed by the addition of 5 mg/ml MTT (Biosharp, Hefei, China). Following incubation at 37˚C for 4 h, the formazan crystals in the cells were dissolved in 150 μl DMSO and shaken for 10 min. The absorbance at 490 nm was determined using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). To determine whether the combination of drugs was synergistic, additive or antagonistic, the Chou-Talalay method (28) was used based on the median-effect equation. The formulas of dose reduction index (DRI) and combination index (CI) for two drugs were as follows: $DRI=D_x/D$ and $CI=(D)_1/(D_x)_1 + (D)_2/(D_x)_2$. D_x was the dose of the drug alone that inhibited x%; D was the portion of each drug in combination that also inhibited x%. CI<1, =1 and >1 indicated synergism, an additive effect and antagonism, respectively; DRI>1 indicated an enhanced cytotoxicity for the drug combination. CalcuSyn software (version 2.0; Biosoft, Cambridge, UK) was used to realize the aforementioned process.

Wound healing assay. A549/DDP cells were seeded in 6-well plates at a density of $5x10^5$ cells/well in 2 ml of medium overnight. Then the cell monolayer was scraped with a pipette tip to form a wound and washed gently with PBS. Cells were subsequently treated with indicated concentrations of DDP, BEZ235 or DDP combined with BEZ235. Wounds were imaged at 0 and 24 h following the scratch under a light microscope (magnification, x100; Leica Microsystems GmbH, Wetzlar, Germany).

Invasion assays. Cells in serum-free media ($5x10^5$ cells in 200 μl) were added to the upper chamber of an insert (8-μm pore size; Costar™; Corning Inc., Corning, NY, USA) coated with Matrigel (1:7 dilution; BD Biosciences, San Jose, CA, USA). Media (600 μl) containing 10% FBS were added to the lower chamber. Following 24 h, the non-invading cells were removed with a cotton wool. Cells that invaded through the Transwell filter were fixed with methylalcohol for 4 min, stained with 0.1% crystal violet for 7 min and imaged using a light microscope (Olympus Corp., Tokyo, Japan) at an x100 magnification. The number of cells in three random fields was counted.

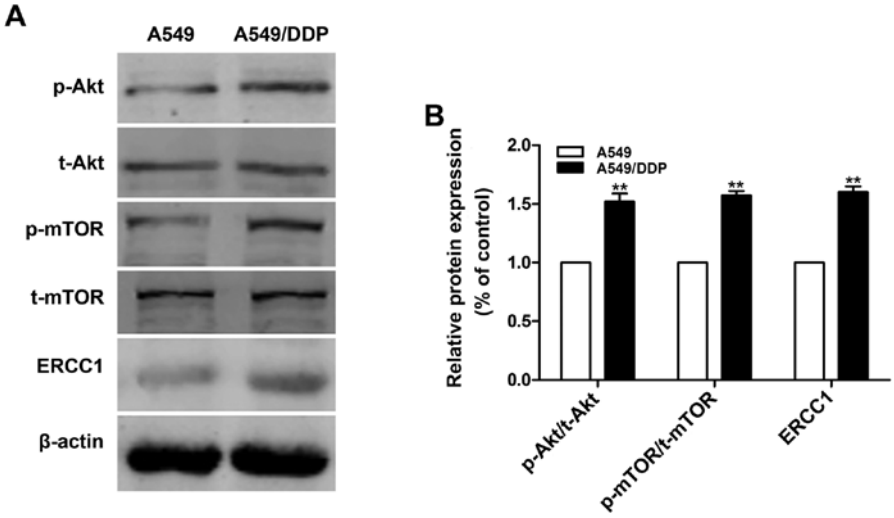

Figure 1. Activation of the PI3K/Akt/mTOR signaling pathway and high expression of ERCC1 may be involved in the drug resistance of A549/DDP cells to DDP. (A) Protein levels of t-Akt, p-Akt, t-mTOR, p-mTOR and ERCC1 were determined by western blotting. (B) Quantitative analysis of the protein levels of t-Akt, p-Akt, t-mTOR, p-mTOR and ERCC1. β-actin was used as a loading control. Data are presented as the mean ± standard error, n=3. **P<0.01 vs. the A549-cell group. DDP, cisplatin; T, total; p, phospho; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin; ERCC1, excision repair cross complementation group 1.

Hoechst 33342 staining. A549/DDP cells were cultured on glass coverslips and treated with indicated concentrations of DDP, BEZ235 or DDP combined with BEZ235 for 48 h. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature following treatment, followed by staining with 1 mg/ml Hoechst 33342 (Wanleibio Co., Ltd., Shanghai, China) at room temperature for 5 min. Following washing three times with PBS, the device was mounted with anti-fluorescence quenching agent (Beyotime Institute of Biotechnology) and cover-slipped. The cells were then immediately imaged using an Olympus BX43F fluorescent microscope (Olympus Corp.) at x400 magnification. The percentage of apoptosis rate was determined in each culture.

Flow cytometry. The apoptotic cells were quantified using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Briefly, A549/DDP cells were seeded in 6-well plates at a density of $4x10^5$ cells/well in 2 ml of medium overnight. Following treatment with BEZ235 or DDP alone or in combination, cells were detached with EDTA-free trypsin and washed twice with cooled PBS and re-suspended in 500 μl binding buffer. The cells were then treated with 5 μl Annexin V-FITC followed by treatment with 5 μl PI at room temperature for 15 min in the dark. Fluorescence was determined by flow cytometry. Flow cytometric data were analyzed using the MACSQuantify software (version 2.4; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Western blot analysis. Following treatment with the selected compounds, the cells were collected and lysed in RIPA buffer (Beyotime Institute of Biotechnology) containing protease and phosphatase inhibitors (Beyotime Institute of Biotechnology). Protein concentrations were quantified using the bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). Equal amounts of proteins (60 μg) were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes (ExCell Biology). Following blocking with PBS containing 3% bovine serum albumin (BSA; Vicmed Biotech Co., Ltd., Xuzhou, China) for 1 h at room temperature, the membranes were incubated with primary antibodies, including anti-ERCC1 (1:1,000; cat. no. 4801; AbSci, Vancouver, WA, USA), anti-Akt (1:1,000; cat. no. 4685), anti-phospho (p)-Akt (Ser473; 1:1,000; cat. no. 4060), anti-mTOR (1:1,000; cat. no. 2983), anti-p-mTOR (Ser2448; 1:1,000; cat. no. 5536), anti-pro caspase-3 (1:1,000; cat. no. 9665) and anti-cleaved caspase-3 (1:1,000; cat. no. 9665; Cell Signaling Technology, Inc., Danvers, MA, USA), overnight at 4˚C. Equal lane loading was confirmed using a monoclonal antibody against β-actin (1:1,000; cat. no. AP0060; Bioworld Technology, Inc., Minneapolis, MN, USA). The membranes were washed three times with PBS-Tween (PBS-T) buffer for 15 min and incubated with Near-infrared fluorescence-conjugated secondary antibodies (1:1,000; cat. no. V926-32211; Vicmed Biotech) for 1 h. Following washing with the PBS-T buffer, the membranes were scanned with the Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA). The intensity of the bands was analyzed using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Statistical analysis. All experiments were run in triplicate. All statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± standard error of the mean. The difference between the groups was analyzed using Student's t-test when only two groups were compared or one-way analysis of variance (ANOVA) followed by the least significant difference/Dunett-T3 tests when more than two groups were compared. P<0.05 was considered to indicate a statistically significant difference.

Results

Activation of the PI3K/Akt/mTOR pathway and ERCC1 upregulation are involved in the chemoresistance of A549/DDP

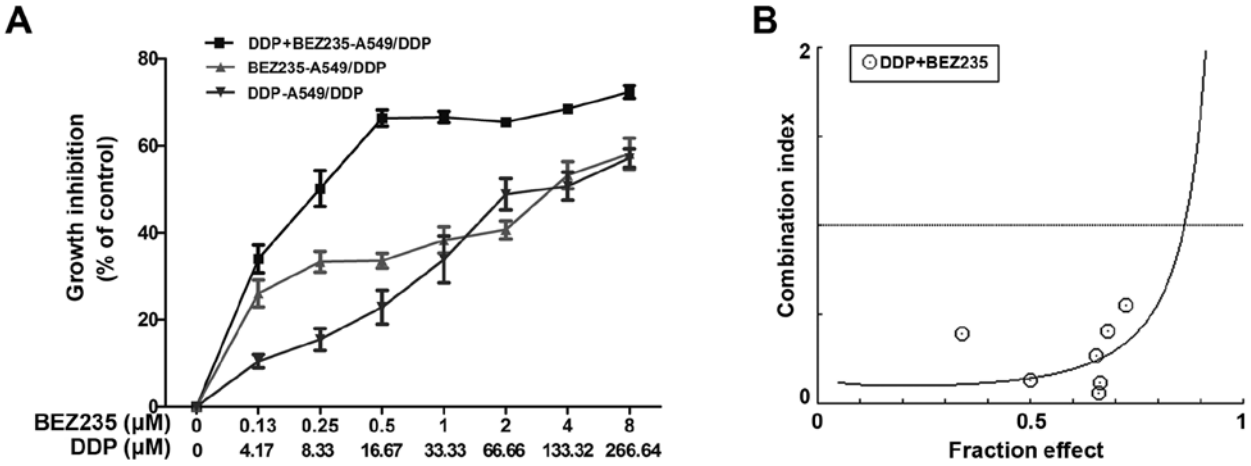

Figure 2. BEZ235 inhibits A549/DDP cell proliferation and enhances the chemotherapeutic effect of DDP. (A) A549/DDP cells were treated with the indicated concentrations of DDP and BEZ235 or DDP combined with BEZ235 for 48 h. Values were expressed in percentage of cell growth inhibition. (B) CI was calculated using CompuSyn software. CI values <1, =1 and >1 indicate synergism, an additive effect and antagonism, respectively. Data are presented as the mean ± standard error, n=3. DDP, cisplatin; CI, combination index.

cells. To determine the chemoresistance ability of A549/DDP cells to DDP, an MTT assay was performed. The half-maximal inhibitory concentration (IC_{50}) of DDP in A549/DDP cells was 9.21-fold higher than that of A549 cells when treated with DDP for 48 h. Thus, A549/DDP cells demonstrated great resistance to DDP compared with A549 cells (data not shown). To analyze whether the PI3K/Akt/mTOR pathway and ERCC1 were involved in the chemoresistance of A549/DDP cells, western blotting was performed. As displayed in Fig. 1, the activation of Akt and mTOR in A549/DDP cells was higher than that of A549 cells (P<0.01), and the levels of ERCC1 were significantly increased (P<0.01). The results demonstrated that the activation of the PI3K/Akt/mTOR pathway and ERCC1 upregulation were associated with A549/DDP cell drug resistance.

BEZ235 inhibits A549/DDP cell proliferation and enhances the chemotherapeutic effect of DDP. MTT assays revealed that DDP or BEZ235 treatment significantly reduced A549/DDP cell viability. The IC_{50} values of DDP and BEZ235 were 118.44 and 3.62 μM, respectively. To investigate whether BEZ235 sensitized and synergized DDP-induced lung cancer cell death, the A549/DDP cells were treated with BEZ235 combined with DDP at a 33:1 constant ratio according to their IC_{50} for 48 h. The IC_{50} values of BEZ235 and DDP were 0.26 μM and 8.72 μM, respectively, and the DRI of BEZ235 and DDP were 13.82 and 13.58, respectively. (Fig. 2A). Additionally, the CI values revealed synergistic effects between both drugs for almost all the concentrations tested. The lowest CI values was identified at 16.67 μM DDP and 0.5 μM BEZ235 (Fig. 2B). Thus, the indicated DDP and BEZ235 concentrations were used in the subsequent experiments. These results indicated that BEZ235 synergistically suppressed cell proliferation and sensitized A549/DDP cells to DDP.

BEZ235 enhances the DDP-induced apoptosis in A549/DDP cells. To determine whether the synergistic growth inhibition of DDP and BEZ235 resulted from apoptosis, A549/DDP cells were exposed to DDP and BEZ235, alone and in combination for 48 h. Evaluation of the apoptotic ratio was performed using AnnexinV-FITC/PI staining with flow cytometry. As displayed in Fig. 3A, compared with DDP or BEZ235 treatment alone, DDP and BEZ235 combination treatment induced an increase in the percentage of apoptotic cells: control, 5.68±0.59%; DDP, 15.04±1.36%; BEZ235, 10.58±0.68%; DDP and BEZ235 combination, 28.57±2.05%. To further confirm these findings, the effect of DDP and BEZ235 on caspase activity was examined by western blotting. The results revealed that, compared with the control group, treatment with DDP or BEZ235 induced cleaved-caspase-3 activation and decreased the pro-caspase-3 expression level (P<0.05 or P<0.01). Combination treatment significantly activated cleaved-caspase-3 and decreased the levels of pro-caspase-3 when compared with DDP or BEZ235 used alone (P<0.01; Fig. 3B). The changes of nuclear morphology of A549/DDP cells were also observed under a fluorescence microscope by Hoechst 33342 DNA staining. The results revealed that the nuclei were intact and staining was less bright in the control group. However, A549/DDP cells treated with DDP or BEZ235 exhibited severe chromatin condensation and apoptotic body formation of the nuclei, and the nuclei were much brighter than that of the control group. In addition, the combination group exhibited significantly more apoptotic cells with condensed or fragmented nuclei than those treated with either DDP or BEZ235 alone (Fig. 3C). These findings indicated that co-treatment with DDP and BEZ235 synergistically induced A549/DDP cell apoptosis.

BEZ235 restores the sensitivity of A549/DDP cells to DDP through downregulation of the PI3K/Akt/mTOR pathway and ERCC1 levels. To investigate the molecular mechanism underlying the synergistic effect of DDP and BEZ235 in A549/DDP cells, western blotting was performed to analyze the relevant targets of the PI3K/Akt/mTOR signaling pathway and ERCC1 expression levels. Cells were treated with DDP and BEZ235 alone or in combination for 48 h, followed by western blot analysis. As displayed in Fig. 4A, DDP upregulated the level of p-Akt, p-mTOR and ERCC1 (P<0.01), compared with the control group, while BEZ235 significantly downregulated the phosphorylation level of Akt and mTOR, and reduced

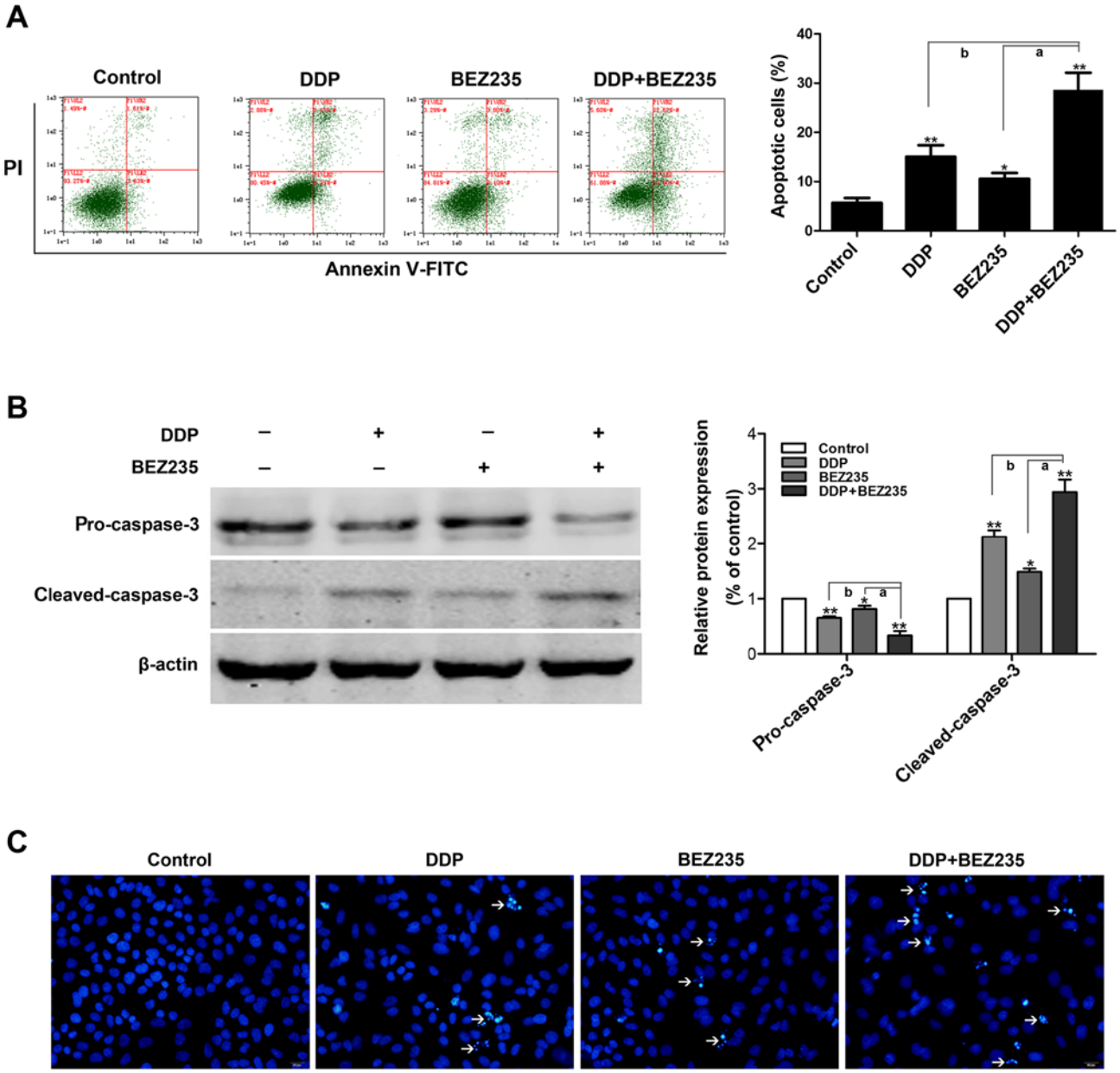

Figure 3. BEZ235 enhances DDP-induced apoptosis. A549/DDP cells were exposed to the indicated concentrations of DDP and/or BEZ235 for 48 h. (A) Apoptotic cells were detected by flow cytometry with Annexin V-FITC/PI apoptosis detection kit. Early apoptotic cells were identified by AnnexinV-FITC positive and PI negative staining in the bottom right quadrant; late apoptotic cells were identified by Annexin V-FITC positive and PI positive staining in the top right quadrant. Percentages of apoptotic cells (right). (B) Protein levels of pro-caspase-3 and cleaved-caspase-3 were determined by western blotting. The semi-quantitative analysis results of the protein levels of pro-caspase-3 and cleaved-caspase-3 in A549/DDP cells (right). (C) Hoechst 33342 staining of A549/DDP cells was detected by fluorescent microscopy. Arrows identify apoptotic nuclei. Magnification, x400; scale bar, 20 μm. Data are presented as the mean ± standard error, n=3. *P<0.05, **P<0.01 vs. the control group; ^{a}P<0.01 vs. BEZ235 alone; ^{b}P<0.01 vs. DDP alone. DDP, cisplatin; FITC, fluorescein isothiocyanate; PI, propidium iodide.

the expression of ERCC1 (P<0.01). Furthermore, compared with treatment with DDP alone, the combination of DDP and BEZ235 downregulated the level of p-Akt, p-mTOR and ERCC1 further (P<0.01). The levels of p-Akt/p-mTOR and ERCC1 in NSCLC were positively correlated, as determined by Pearson's correlation analysis (Fig. 4B).

BEZ235 and DDP synergistically inhibit the migration and invasion abilities of A549/DDP cells. Activation of Akt is associated with NSCLC invasion and migration, and our study demonstrated that Akt phosphorylation was upregulated in A549/DDP cells when treated with DDP. Several functional studies were performed to investigate whether BEZ235 enhanced the ability of DDP, and inhibited the migration and invasion of A549/DDP cells by downregulating Akt phosphorylation. A wound healing assay revealed that the rate of wound closure decreased significantly when treated with BEZ235 or DDP, and that compared with BEZ235 or DDP treatment, the combined treatment with BEZ235 and DDP inhibited the migration ability of A549/DDP cells further (P<0.01; Fig. 5A). Similarly, Transwell assays indicated that BEZ235 or DDP treatment effectively reduced the invasion ability of

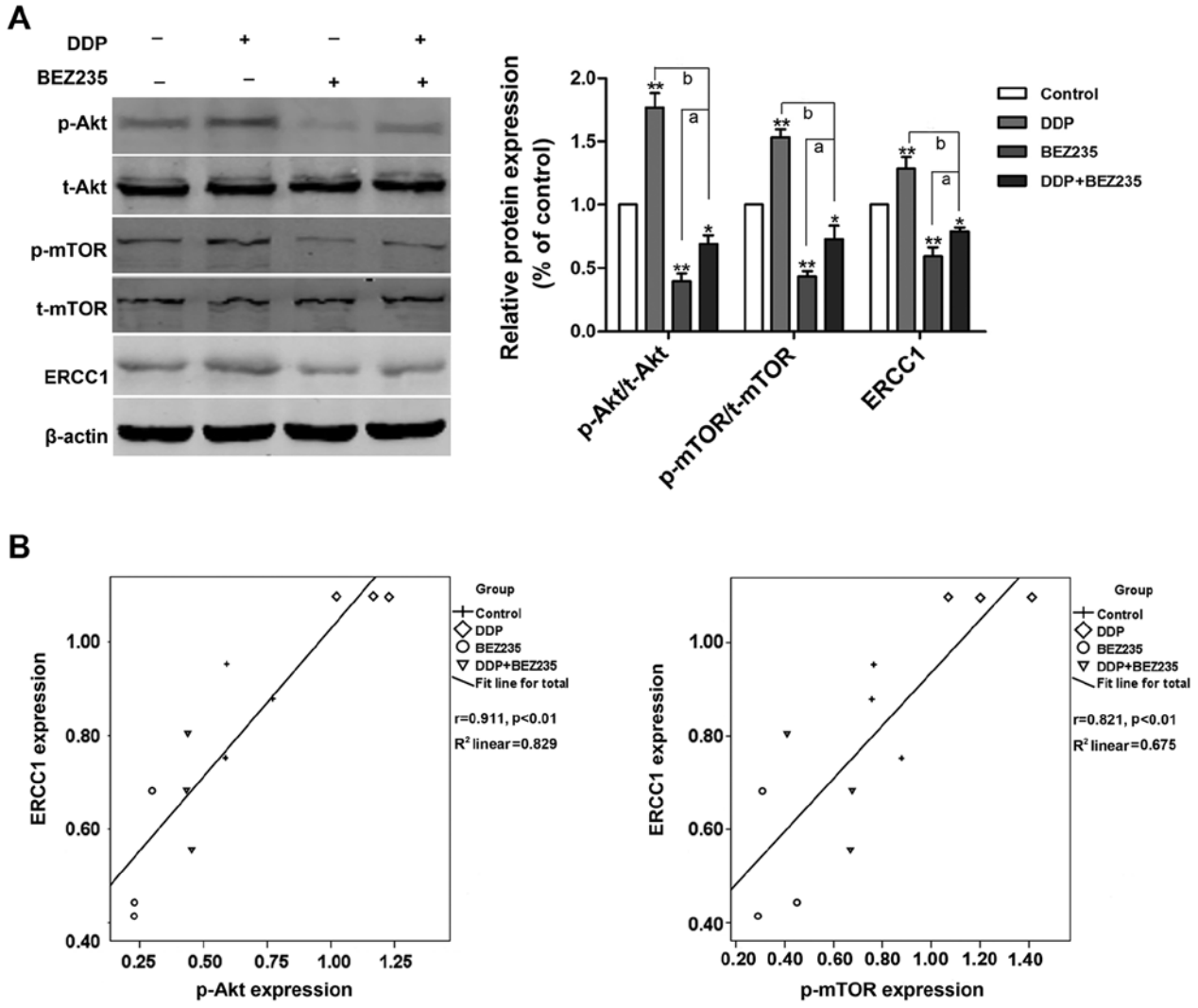

Figure 4. BEZ235 restores A549/DDP cell sensitivity to DDP, and downregulates PI3K/Akt/mTOR signaling and ERCC1 levels. A549/DDP cells were treated with the indicated concentrations of DDP and/or BEZ235 for 48 h. (A) Western blot analyses were performed for t-Akt, p-Akt, t-mTOR, p-mTOR and ERCC1. The semi-quantitative analysis results of relative protein expression in A549/DDP cells (right). (B) Correlation between ERCC1 and p-Akt expression, and p-mTOR expression in A549/DDP cells was determined using Pearson's correlation analysis. Data are presented as the mean ± standard error, n=3. *P<0.05, **P<0.01 vs. the control group; ^{a}P<0.05 vs. BEZ235 alone; ^{b}P<0.01 vs. DDP alone. DDP, cisplatin; T, total; p, phospho; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin; ERCC1, excision repair cross complementation group 1.

A549/DDP cells, and the combination treatment exhibited a stronger effect (P<0.01; Fig. 5B). These results demonstrated that BEZ235 and DDP have synergistic inhibitory effects on NSCLC *in vitro*.

Discussion

DDP has been extensively used in the treatment of various tumor types, including lung carcinoma. However, inherent and acquired drug resistance continues to be a major clinical problem in lung cancer management. Therefore, developing new therapeutic strategies to overcome drug-resistance is a key priority. In the present study, combination of BEZ235 with DDP enhanced the antitumor effects, and increased the sensitivity of drug-resistant lung cancer cell line A549/DDP to DDP.

BEZ235 is a synthetic low-molecular-mass imidazoquinoline compound that potently and reversibly inhibits PI3K and mTOR kinase activity by binding to the ATP-binding cleft of these enzymes (29). Accumulating preclinical studies have demonstrated that NVP-BEZ235 has beneficial pharmaceutical properties as an anticancer agent. It was previously reported that NVP-BEZ235 exhibited promising therapeutic activity in various types of cancer when used alone or combined with agents other than DDP (25,30). In the present study, BEZ235 also demonstrated strong antitumor activity in MTT assays. The combination of the two drugs significantly inhibited cell viability of the A549/DDP lung cancer cell line. Consistent with its anti-proliferative effects, co-treatment of BEZ235 with DDP was more effective in inhibiting cell migration and invasion than DDP or BEZ235 administered alone. In addition, the CI value was <1 for almost all the concentrations tested. These results indicated that the PI3K/mTOR dual inhibitor BEZ235 synergistically potentiated the antitumor effects of DDP in A549/DDP cells.

DDP is a platinum drug. The mechanism of action of DDP is thought to be the platinum interstrand and intrastrand crosslinks that form DDP-DNA adducts, which interfere

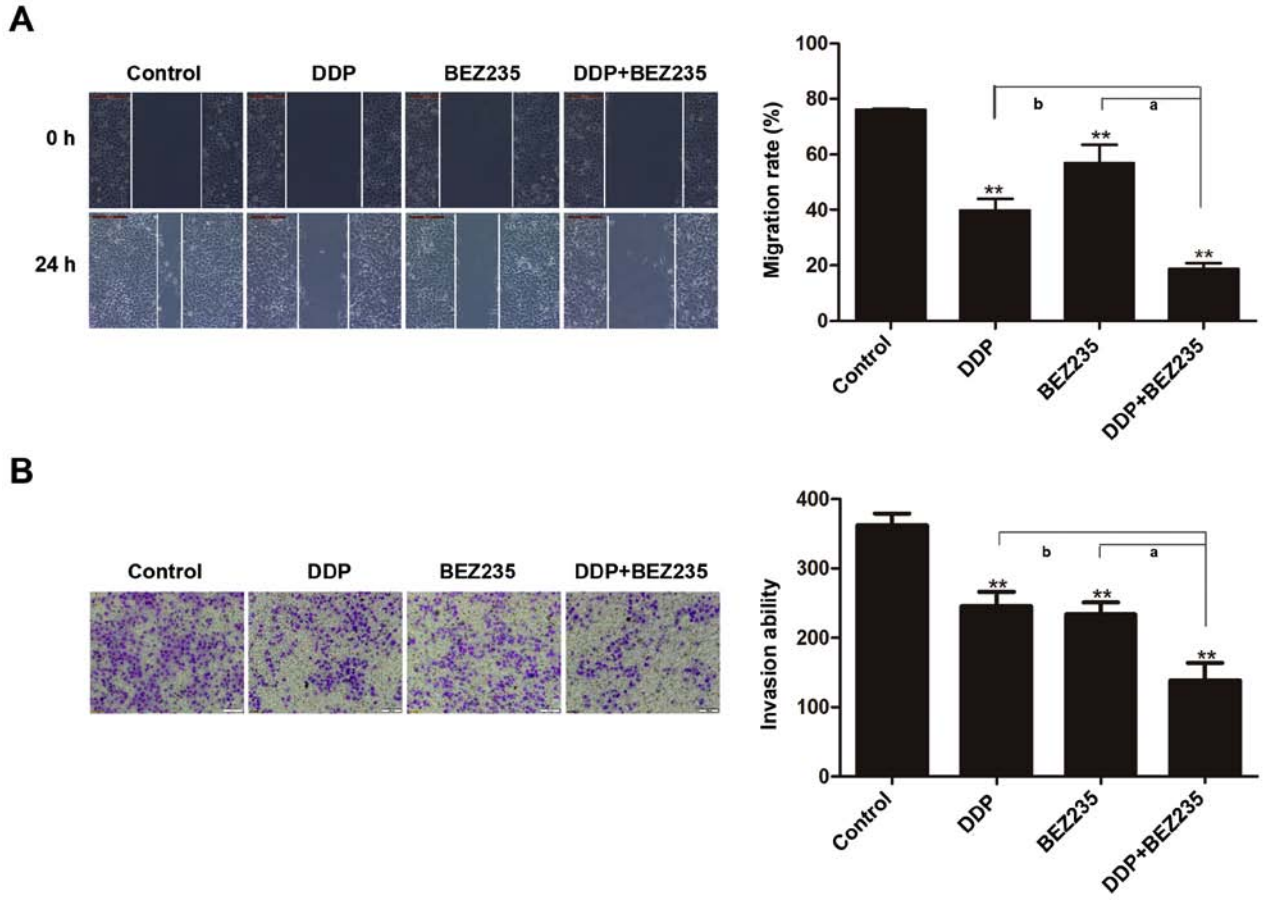

Figure 5. Co-treatment with BEZ235 and DDP synergistically inhibits the migration and invasion ability of A549/DDP cells. A549/DDP cells were treated with the indicated concentrations of BEZ235 and/or DDP for 48 h. (A) The migration ability was evaluated by wound healing assay. Images were captured at 0 and 24 h after the wound was made. Quantification of the migration rate (top right). (B) The number of invasive cells that passed through the membrane was evaluated by crystal violet staining. Images are representative of three independent experiments. Quantification of the invaded cells (bottom right). Data are presented as the mean ± standard error, n=3. **P<0.01 vs. the control group; ^{a}P<0.01 vs. BEZ235 alone; ^{b}P<0.01 vs. DDP alone. DDP, cisplatin.

with DNA replication and transcription, resulting in DNA fragmentation and error coding, eventually triggering cell death (31). Although the mechanisms of platinum resistance are not clearly understood, NER appears to play an important role in mediating platinum resistance or sensitivity to platinum chemotherapeutic agents in cancer treatment. ERCC1, an important component required for NER, is involved in repair of the intra and inter-strand DNA crosslink caused by platinum-based treatment. In addition, it has been reported that ERCC1 expression was significantly elevated by DDP treatment in several tumor types, but data on the role of ERCC1 in NSCLC are still limited and are contradictory (32,33). In the present study, the results revealed that the ERCC1 expression level was significantly higher in A549/DDP cells treated with DDP than that of the control group, whereas BEZ235 or co-administration of BEZ235 and DDP significantly decreased the level of ERCC1 expression compared with DDP treatment alone, indicating that BEZ235 blocked DDP-induced ERCC1 protein expression. Therefore, this indicated that the enhancement of DDP sensitivity by BEZ235 was at least, partly attributed to downregulation of ERCC1 expression in A549/DDP cells, thus leading to the reduction in the proficiency to repair DDP-induced DNA damage.

To explore the potential relationship between the PI3K/Akt/mTOR pathway and ERCC1 expression, the expression of PI3K/Akt/mTOR pathway proteins was analyzed by western blotting. The findings revealed that the levels of p-Akt and p-mTOR in A549/DDP cells were much higher than that of A549 cells, indicating that the PI3K/Akt/mTOR pathway is activated in A549/DDP cell lines. As A549/DDP cells were treated with BEZ235 and DDP alone or in combination for 48 h, the phosphorylation levels of Akt and mTOR were upregulated when treated with DDP alone. However, treatment with BEZ235 alone or in combination with DDP significantly decreased the phosphorylation levels of Akt and mTOR in A549/DDP cells compared with DDP treatment alone. Notably, the combination of DDP and BEZ235 did not further reduce the phosphorylation levels of Akt and mTOR in A549/DDP cells. In addition, the phosphorylation levels of Akt and mTOR and ERCC1 in A549/DDP cells were positively correlated as determined by Pearson's correlation coefficient analysis. The current findings have not determined the mechanism regarding the effects of BEZ235, however there are two possibilities: i) downregulation of ERCC1 protein expression may be an important event subsequent to the inhibition of the Akt/mTOR signaling pathway by BEZ235 in A549/DDP cells; and ii) BEZ235 may decrease the ERCC1 protein expression by other or unknown mechanisms. Based on these observations, inhibition of the PI3K/Akt/mTOR signaling pathway by BEZ235 may result in a downregulation of ERCC1 expression

that enhances the chemosensitivity of A549/DDP cells, and overcome resistance to DDP, but the detailed relationships involved warrant further elucidation.

Apoptosis is an ordered and orchestrated cellular process that occurs in physiological and pathological conditions. Numerous studies have illustrated that disordered apoptosis has been associated with the development of many types of solid cancer, including lung, breast, prostate, bladder and ovarian cancer (34-36). Besides enhancement of the repair of DDP-caused DNA damage by ERCC1, impaired DDP-induced apoptosis has also been implicated in the development of the DDP-resistance in the treatment of solid malignancies (37,38). Therefore, apoptosis induction has become a popular target of many treatment strategies in a wide variety of tumor cells (39,40). To determine whether the synergistic growth inhibition of BEZ235 and DDP resulted from apoptosis, A549/DDP cells were exposed to BEZ235 and DDP, alone and in combination for 48 h, and apoptosis was evaluated by flow cytometric analysis and Hoechst 33342 DNA staining. Consistent with its anti-proliferative effects, co-administration of BEZ235 with DDP significantly increased the apoptosis of A549/DDP cells compared with cells treated with either BEZ235 or DDP alone, indicating that combination of BEZ235 and DDP was more effective in inducing apoptosis of A549/DDP cells. It is generally accepted that synergy is achieved through a cooperation of two agents functioning via distinct mechanisms. The activation of the PI3K/Akt/mTOR pathway in A549/DDP cells promoted cell growth, survival and proliferation, and inhibited cell apoptosis. BEZ235 treatment induced A549/DDP cell apoptosis via inhibition of the PI3K/Akt/mTOR pathway. Notably, in the present study DDP treatment activated the Akt/mTOR signaling pathway as reflected by the increased levels of Akt and mTOR phosphorylation in A549/DDP cells, and the combination of DDP and BEZ235 did not further reduce the phosphorylation levels of Akt and mTOR when compared with BEZ235 treatment alone, indicating that inhibition of the Akt/mTOR signaling pathway by BEZ235 is not the only mechanism involved in promoting cell apoptosis. DDP is known to induce apoptosis following DNA damage by propagation of DNA damage recognition signals to downstream pathways, including nuclear factor-κB, p53, p73, MAPK and mitochondria-related apoptosis signaling pathways (38,41-42). Therefore, treatment with BEZ235 and DDP may synergistically induce apoptosis via the activation of complex signaling cascades in A549/DDP cells, and the exact mechanism of this synergy requires further investigation.

Caspases are central to the mechanism of apoptosis as they are both initiators and executioners. Caspase-3 is involved in both the intrinsic and extrinsic apoptotic pathways. It cleaves the inhibitor of the caspase-activated deoxyribonuclease, which is responsible for nuclear apoptosis (43). To further investigate the mechanism of co-treatment in the promotion of apoptosis of A549/DDP cells, the activity of caspase-3 was examined by western blotting. The results of the present study revealed that the combination of BEZ235 and DDP significantly enhanced cleaved-caspase-3 activation and decreased pro-caspase-3 expression level when compared with DDP or BEZ235 used alone. Consistent with this observation, Yu *et al* (44) reported that BEZ235 can increase the expression of caspase-3 in human glioma stem cells. Bhende *et al* (45) reported that BEZ235-treated follicular lymphoma cells had a 1.6-2-fold increase in caspase-3 activation following 24 h of incubation with the drug, compared with cells treated with the vehicle alone. The results of the present study supported this notion that BEZ235 synergistically induced apoptosis via a caspase-3-dependent pathway in A549/DDP cells. In addition, the results in the present study revealed that A549/DDP cells, which have a high level of ERCC1 expression, exhibited decreased apoptosis, and that A549/DDP cells co-treated with BEZ235 and DDP, in which the level of ERCC1 expression was reduced, exhibited an increased susceptibility to apoptosis. This observation indicated that the level of ERCC1 expression may be associated with the antitumor effects of DDP chemotherapy.

In summary, the findings of the present study demonstrated that BEZ235 increased the chemosensitivity to DDP in DDP-resistant lung cancer cell line A549/DDP, and BEZ235 combined with DDP may be a promising NSCLC therapy. The results may help in overcoming resistance to DDP and developing novel therapeutic strategies for patients with advanced NSCLC.

Acknowledgements

Not applicable.

Funding

XW received research grants from the Pharmaceutical research project Aosai Kang Hospital Grant, Jiangsu Province Pharmaceutical Association (grant no. 201516) and from the Project of Science and Technology Development fund of Nanjing Medical University (grant no. 2016NJMU139).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

AX, XW and HL conceived and designed the study. HL performed the experiments. AX and HL wrote the manuscript. RL and LL analyzed the data of experiments. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136: E359-E386, 2015.
2. Devesa SS, Bray F, Vizcaino AP and Parkin DM: International lung cancer trends by histologic type: male:female differences diminishing and adenocarcinoma rates rising. Int J Cancer 117: 294-299, 2005.
3. Morgensztern D, Ng SH, Gao F and Govindan R: Trends in stage distribution for patients with non-small cell lung cancer: A National Cancer Database survey. J Thorac Oncol 5: 29-33, 2010.
4. Ma D, Wang J, Hao X, Wang Y, Hu X, Xing P and Li J: Gemcitabine combined with cisplatin as adjuvant chemotherapy for non-small cell lung cancer: A retrospective analysis. Thorac Cancer 8: 482-488, 2017.
5. Jia Y, Zhou D, Jia Q, Ying Y and Chen S: Synergistic and attenuated effect of HSS in combination treatment with docetaxel plus cisplatin in human non-small-cell lung SPC-A-1 tumor xenograft. Biomed Pharmacother 79: 27-34, 2016.
6. Müller A, Zang C, Chumduri C, Dörken B, Daniel PT and Scholz CW: Concurrent inhibition of PI3K and mTORC1/mTORC2 overcomes resistance to rapamycin induced apoptosis by down-regulation of Mcl-1 in mantle cell lymphoma. Int J Cancer 133: 1813-1824, 2013.
7. Sasore T, Reynolds AL and Kennedy BN: Targeting the PI3K/Akt/mTOR pathway in ocular neovascularization. Adv Exp Med Biol 801: 805-811, 2014.
8. Li H, Zhang B, Liu Y and Yin C: EBP50 inhibits the migration and invasion of human breast cancer cells via LIMK/cofilin and the PI3K/Akt/mTOR/MMP signaling pathway. Med Oncol 31: 162, 2014.
9. Ma J, Sawai H, Matsuo Y, Ochi N, Yasuda A, Takahashi H, Wakasugi T, Funahashi H, Sato M and Takeyama H: IGF-1 mediates PTEN suppression and enhances cell invasion and proliferation via activation of the IGF-1/PI3K/Akt signaling pathway in pancreatic cancer cells. J Surg Res 160: 90-101, 2010.
10. Burris HA III: Overcoming acquired resistance to anticancer therapy: Focus on the PI3K/AKT/mTOR pathway. Cancer Chemother Pharmacol 71: 829-842, 2013.
11. Xu JL, Wang ZW, Hu LM, Yin ZQ, Huang MD, Hu ZB, Shen HB and Shu YQ: Genetic variants in the PI3K/PTEN/AKT/mTOR pathway predict platinum-based chemotherapy response of advanced non-small cell lung cancers in a Chinese population. Asian Pac J Cancer Prev 13: 2157-2162, 2012.
12. Reungwetwattana T, Weroha SJ and Molina JR: Oncogenic pathways, molecularly targeted therapies, and highlighted clinical trials in non-small-cell lung cancer (NSCLC). Clin Lung Cancer 13: 252-266, 2012.
13. Tsodikov OV, Enzlin JH, Schärer OD and Ellenberger T: Crystal structure and DNA binding functions of ERCC1, a subunit of the DNA structure-specific endonuclease XPF-ERCC1. Proc Natl Acad Sci USA 102: 11236-11241, 2005.
14. Cai Y, Yan X, Zhang G, Zhao W and Jiao S: The predictive value of ERCC1 and p53 for the effect of panobinostat and cisplatin combination treatment in NSCLC. Oncotarget 6: 18997-19005, 2015.
15. Lee HW, Choi YW, Han JH, Kim JH, Jung JH, Jeong SH, Kang SY, Choi JH, Oh YT, Park KJ, *et al*: Expression of excision repair cross-complementation group 1 protein predicts poor outcome in advanced non-small cell lung cancer patients treated with platinum-based doublet chemotherapy. Lung Cancer 65: 377-382, 2009.
16. Palomba G, Atzori F, Budroni M, Ombra M, Cossu A, Sini M, Pusceddu V, Massidda B, Frau B, Notari F, *et al*: ERCC1 polymorphisms as prognostic markers in T4 breast cancer patients treated with platinum-based chemotherapy. J Transl Med 12: 272, 2014.
17. Yun J, Kim KM, Kim ST, Kim JH, Kim JA, Kong JH, Lee SH, Won YW, Sun JM, Lee J, *et al*: Predictive value of the ERCC1 expression for treatment response and survival in advanced gastric cancer patients receiving cisplatin-based first-line chemotherapy. Cancer Res Treat 42: 101-106, 2010.
18. Wang S, Pan H, Liu D, Mao N, Zuo C, Li L, Xie T, Huang D, Huang Y, Pan Q, *et al*: Excision repair cross complementation group 1 is a chemotherapy-tolerating gene in cisplatin-based treatment for non-small cell lung cancer. Int J Oncol 46: 809-817, 2015.
19. Song W and Ma H: The expression of ERCC1 and BRCA1 predicts prognosis of platinum-based chemotherapy in urothelial cancer. Onco Targets Ther 9: 3465-3471, 2016.
20. Usanova S, Piée-Staffa A, Sied U, Thomale J, Schneider A, Kaina B and Köberle B: Cisplatin sensitivity of testis tumour cells is due to deficiency in interstrand-crosslink repair and low ERCC1-XPF expression. Mol Cancer 9: 248, 2010.
21. Chang IY, Kim MH, Kim HB, Lee DY, Kim SH, Kim HY and You HJ: Small interfering RNA-induced suppression of ERCC1 enhances sensitivity of human cancer cells to cisplatin. Biochem Biophys Res Commun 327: 225-233, 2005.
22. Qin X, Yao W, Li W, Feng X, Huo X, Yang S, Zhao H and Gu X: ERCC1 and BRCA1 mRNA expressions are associated with clinical outcome of non-small cell lung cancer treated with platinum-based chemotherapy. Tumour Biol 35: 4697-4704, 2014.
23. Herrera VA, Zeindl-Eberhart E, Jung A, Huber RM and Bergner A: The dual PI3K/mTOR inhibitor BEZ235 is effective in lung cancer cell lines. Anticancer Res 31: 849-854, 2011.
24. Chen L, Jin T, Zhu K, Piao Y, Quan T, Quan C and Lin Z: PI3K/mTOR dual inhibitor BEZ235 and histone deacetylase inhibitor Trichostatin A synergistically exert anti-tumor activity in breast cancer. Oncotarget 8: 11937-11949, 2017.
25. Xie G, Wang Z, Chen Y, Zhang S, Feng L, Meng F and Yu Z: Dual blocking of PI3K and mTOR signaling by NVP-BEZ235 inhibits proliferation in cervical carcinoma cells and enhances therapeutic response. Cancer Lett 388: 12-20, 2017.
26. Qu Y, Wu X, Yin Y, Yang Y, Ma D and Li H: Antitumor activity of selective MEK1/2 inhibitor AZD6244 in combination with PI3K/mTOR inhibitor BEZ235 in gefitinib-resistant NSCLC xenograft models. J Exp Clin Cancer Res 33: 52, 2014.
27. Piao J, Chen L, Quan T, Li L, Quan C, Piao Y, Jin T and Lin Z: Superior efficacy of co-treatment with the dual PI3K/mTOR inhibitor BEZ235 and histone deacetylase inhibitor Trichostatin A against NSCLC. Oncotarget 7: 60169-60180, 2016.
28. Chou TC: Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 58: 621-681, 2006.
29. Maira SM, Stauffer F, Brueggen J, Furet P, Schnell C, Fritsch C, Brachmann S, Chène P, De Pover A, Schoemaker K, *et al*: Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. Mol Cancer Ther 7: 1851-1863, 2008.
30. Torki S, Soltani A, Shirzad H, Esmaeil N and Ghatrehsamani M: Synergistic antitumor effect of NVP-BEZ235 and CAPE on MDA-MB-231 breast cancer cells. Biomed Pharmacother 92: 39-45, 2017.
31. Eastman A: The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. Pharmacol Ther 34: 155-166, 1987.
32. Torii Y, Kato R, Minami Y, Hasegawa K, Fujii T and Udagawa Y: ERCC1 expression and chemosensitivity in uterine cervical adenocarcinoma cells. Anticancer Res 34: 107-115, 2014.
33. Li Q, Tsang B, Bostick-Bruton F and Reed E: Modulation of excision repair cross complementation group 1 (ERCC-1) mRNA expression by pharmacological agents in human ovarian carcinoma cells. Biochem Pharmacol 57: 347-353, 1999.
34. Fong MY and Kakar SS: The role of cancer stem cells and the side population in epithelial ovarian cancer. Histol Histopathol 25: 113-120, 2010.
35. Yang H and Dou QP: Targeting apoptosis pathway with natural terpenoids: Implications for treatment of breast and prostate cancer. Curr Drug Targets 11: 733-744, 2010.
36. de Souza PM and Lindsay MA: Apoptosis as a therapeutic target for the treatment of lung disease. Curr Opin Pharmacol 5: 232-237, 2005.
37. Li QQ, Lee RX, Liang H, Zhong Y and Reed E: Enhancement of cisplatin-induced apoptosis by β-elemene in resistant human ovarian cancer cells. Med Oncol 30: 424, 2013.
38. Siddik ZH: Cisplatin: Mode of cytotoxic action and molecular basis of resistance. Oncogene 22: 7265-7279, 2003.
39. Sun A, Chia JS, Chiang CP, Hsuen SP, Du JL, Wu CW and Wang WB: The chinese herbal medicine Tien-Hsien liquid inhibits cell growth and induces apoptosis in a wide variety of human cancer cells. J Altern Complement Med 11: 245-256, 2005.
40. Neuzil J, Tomasetti M, Mellick AS, Alleva R, Salvatore BA, Birringer M and Fariss MW: Vitamin E analogues: A new class of inducers of apoptosis with selective anti-cancer effects. Curr Cancer Drug Targets 4: 355-372, 2004.

41. Yang Z, Schumaker LM, Egorin MJ, Zuhowski EG, Guo Z and Cullen KJ: Cisplatin preferentially binds mitochondrial DNA and voltage-dependent anion channel protein in the mitochondrial membrane of head and neck squamous cell carcinoma: Possible role in apoptosis. Clin Cancer Res 12: 5817-5825, 2006.
42. Yan M, Ni J, Song D, Ding M and Huang J: Activation of unfolded protein response protects osteosarcoma cells from cisplatin-induced apoptosis through NF-κB pathway. Int J Clin Exp Pathol 8: 10204-10215, 2015.
43. Wolf BB, Schuler M, Echeverri F and Green DR: Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated DNase inactivation. J Biol Chem 274: 30651-30656, 1999.
44. Yu Y, Yu X, Ma J, Tong Y and Yao J: Effects of NVP-BEZ235 on the proliferation, migration, apoptosis and autophagy in HT-29 human colorectal adenocarcinoma cells. Int J Oncol 49: 285-293, 2016.
45. Bhende PM, Park SI, Lim MS, Dittmer DP and Damania B: The dual PI3K/mTOR inhibitor, NVP-BEZ235, is efficacious against follicular lymphoma. Leukemia 24: 1781-1784, 2010.