

# Inhibition of paclitaxel resistance and apoptosis induction by cucurbitacin B in ovarian carcinoma cells

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**Abstract.** Ovarian cancer is the leading cause of mortality among all gynecological malignancies. Drug resistance is a cause of ovarian cancer recurrence and low rate of overall survival. There is a requirement for more effective treatment approaches. Cucurbitacin B (CuB) is an antineoplastic agent derived from traditional Chinese medicinal herbs. Its activity against paclitaxel-resistant human ovarian cancer cells has, however, not yet been established. The purpose of the present study was to investigate the effect and mechanism of CuB on human paclitaxel-resistant ovarian cancer A2780/Taxol cells. Cell viability was evaluated by a cell counting assay, while cell cycle arrest and apoptosis were assessed by microscopy and flow cytometry, and proteins associated with apoptotic pathways and drug resistance were evaluated by western blotting. The present results demonstrated that CuB exerts dose- and time-dependent cytotoxicity against the ovarian cancer A2780 cell line, with half-maximal inhibitory concentration ( $IC_{50}$ ) values 0.48, 0.25 and 0.21  $\mu$ M following 24, 48 and 72 h of incubation, respectively. Compared with its sensitive counterpart, A2780, paclitaxel-resistant A2780/Taxol cells had almost identical  $IC_{50}$  values. Cell cycle analysis demonstrated that treatment with CuB may induce cell cycle arrest at the G<sub>2</sub>/M phase of the cell cycle in the two cell lines. As revealed by Annexin V/propidium iodide-labeled flow cytometry and Hoechst 33258 staining, CuB-induced apoptosis was accompanied by activation of caspase-3 and downregulation of B-cell lymphoma-2. Western blotting demonstrated that CuB may enhance the expression of p53 and p21 in the two cell lines. CuB may also downregulate the expression of P-glycoprotein.

These results indicate that CuB may exert a therapeutic effect on paclitaxel-resistant human ovarian cancer.

## Introduction

Ovarian cancer is the fourth leading cause of cancer-associated mortality in women, with a 5-year survival rate of 25-35% in Western countries (1). Ovarian cancer often remains undetected until a late stage, and is commonly referred to as a silent killer (2-8). Currently, the standard therapy for advanced ovarian cancer includes surgery and systemic chemotherapy (5,9-11). Since 1990, combined platinum-based agents and paclitaxel (Taxol; Bristol-Myers Squibb, New York, NY, USA) have become the first-line chemotherapy for ovarian cancer (12,13). Despite a high initial sensitivity to chemotherapy and frequent complete clinical response, the majority of patients with advanced-stage tumors may relapse and gradually develop resistance to the majority of chemotherapeutic drugs (14-16). It has not yet been possible to overcome multidrug resistance (MDR) in the clinical settings.

Much effort has been focused on identifying natural compounds that may reverse the MDR phenotype of cancer cells and/or sensitize MDR cancer cells to chemotherapy without undesirable side effects (17-19). Traditional Chinese medicines are excellent starting materials, since they are not only a rich source for diverse chemicals, but have been used in humans for thousands of years (20-22). Recently, certain natural compounds have been shown to be capable of reversing the drug resistance of ovarian cancer (23). Abouzeid *et al* (24) and Sarisozen *et al* (25) reported that transferring-targeted combination micelles of curcumin and paclitaxel have a clear synergistic effect against paclitaxel-resistant SK-OV-3TR cells. Yang *et al* (26) demonstrated that tectorigenin may sensitize paclitaxel-resistant human ovarian cancer cells through downregulating the Akt and nuclear factor- $\kappa$ B signaling pathways. Li *et al* (27) revealed that emodin can sensitize paclitaxel-resistant human ovarian cancer cells to paclitaxel-induced apoptosis *in vitro*. Zhou *et al* (28) reported that silibinin is able to restore paclitaxel sensitivity in paclitaxel-resistant human ovarian carcinoma cells.

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Cucurbitacin B (CuB) is a particularly potent member of the triterpenoid family isolated from plants, and has shown anti-proliferative effects on various cancer cells *in vitro* and *in vivo* (29). CuB may induce cell cycle arrest and apoptosis in cancer cells (30-42).

In the present study, the inhibitory effect of CuB on the paclitaxel-resistant human ovarian cancer A2780/Taxol cell line and its parental A2780 cell line was assessed. To the best of our knowledge, the present study demonstrated for the first time that CuB inhibits the growth of these two types of cells through induction of cell cycle arrest and apoptosis, by a number of molecular mechanisms.

## Materials and methods

**Reagents.** CuB (with a purity of 98%) was obtained from Professor Yihui Deng's laboratory (Department of Pharmacy, Shenyang Pharmaceutical University, Shenyang, China). Primary antibodies directed against B-cell lymphoma-2 (Bcl-2; cat. no. sc-783), caspase-3 (cat. no. sc-271028), p53 (cat. no. sc-126), p21 (cat. no. sc-6246), P-glycoprotein (P-gp; cat. no. sc-8313) and  $\beta$ -actin (cat. no. sc-130300) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Secondary antibodies directed against rabbit (Peroxidase Conjugated AffiniPure Goat Anti-rabbit IgG (cat. no. ZB-2301), or mouse (Peroxidase Conjugated AffiniPure Goat anti-mouse IgG (cat. no. ZB-2305) were from ZSGB-BIO (Beijing, China). The ECL Western Blotting Detection reagent was purchased from GE Healthcare Life Sciences (Chalfont, UK). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit, propidium iodide (PI), Hoechst 33258, dimethyl sulfoxide (DMSO) and other reagents were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

**Cell cultures.** Ovarian carcinoma A2780 and paclitaxel-resistant A2780 (A2780/Taxol) ovarian carcinoma cell lines were purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). Cells were maintained in DMEM (A2780) or RPMI-1640 with 800 ng/ml paclitaxel (A2780/Taxol) supplemented with 10% FBS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. The cells were cultured in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C.

**Cell viability assay.** The viability of A2780 and A2780/Taxol cells was evaluated by counting the numbers of viable cells at 24, 48 and 72 h following the addition of 10  $\mu$ l CuB to achieve final concentrations of 0.0625, 0.125, 0.25, 0.5 and 1  $\mu$ M. The cells were stained with Trypan Blue and counted using a hemocytometer. The final concentration of 0.5% DMSO produced no inhibition of cell growth. At least three replicate experiments were performed with three wells per concentration.

**Cell cycle analysis.** A total of 8x10<sup>5</sup> A2780/Taxol cells and their parental A2780 cells were incubated with 0.0625, 0.125, 0.25, 0.5 or 1  $\mu$ M CuB or DMSO for 24 h. The cells were harvested, washed with iced-cold PBS and fixed in 70% ethanol at -20°C for 24 h. Subsequently, cells were washed with PBS and

resuspended in PBS containing 100  $\mu$ g/ml PI (Sigma-Aldrich; Merck KGaA), 50  $\mu$ g/ml ribonuclease A and 0.1% Triton X-100 in the dark for 30 min at 37°C. Flow cytometry analysis was performed on a FACSCalibur instrument (BD Biosciences, Franklin Lakes, NJ, USA) using the ModFit LT program (version 3.0; BD Biosciences).

**Annexin V/PI staining.** Apoptosis of the cells was detected with an Annexin V-FITC Apoptosis Detection kit (Sigma-Aldrich; Merck KGaA). Annexin V-FITC is a sensitive probe for identifying cells undergoing apoptosis, as phosphatidylserine exposure occurs early in the apoptotic process. PI is a non-specific DNA dye that is excluded from live cells with intact plasma membranes, but can be incorporated into non-viable cells. Upon detection, single-positive populations may present as early apoptotic (Annexin V<sup>+</sup>/PI<sup>-</sup>) or necrotic cells (Annexin V/PI<sup>+</sup>), whereas double-positive populations (Annexin V<sup>+</sup>/PI<sup>+</sup>) are indicative of late stage apoptosis (43).

**Hoechst 33258 staining.** A2780/Taxol and parental A2780 cells (3x10<sup>5</sup> cells) were grown on coverslips placed on 6-well plates at 37°C overnight. Following 0.25, 0.5 and 1  $\mu$ M CuB treatment, the cells were fixed with methanol and acetic acid (3/1 v/v) at 4°C for 15 min, and stained with Hoechst 33258 for 30 min in the dark at 37°C. Subsequent to washing in PBS 3 times, the cells were mounted with a medium containing 80% glycerol (cat. no. G5516; Sigma-Aldrich; Merck KGaA) in PBS. The processed cells were then observed under a fluorescence microscope with 3 fields of view (magnification, x40; Olympus Corporation, Tokyo, Japan).

**Western blot analysis.** Cells (1x10<sup>6</sup> cells/well) seeded onto 6-well plates were treated with 0.0625, 0.125, 0.25, 0.5 and 1  $\mu$ M CuB or DMSO. The medium was aspirated, and the cells were washed with cold PBS. They were then scraped, washed twice with cold PBS followed by centrifugation at 1,000 x g for 5 min at 4°C. The pellet was resuspended in lysis buffer supplemented with proteases and phosphatase inhibitors (cat. no. P8340; Sigma-Aldrich; Merck KGaA) and incubated for 1 h at 4°C. The lysate was collected by centrifugation at 14,000 x g for 40 min at 4°C, and the supernatant was stored at -20°C. For western blot analysis, 50  $\mu$ g proteins were resolved in 8-10% PAGE and transferred to polyvinylidene difluoride membranes. The blot was blocked with a blocking buffer (5% nonfat dry milk/0.1% Tween-20 in TBS) for 1 h at room temperature, and next incubated with appropriate primary antibodies directed against Bcl-2 (1:200), caspase-3 (1:200), p53 (1:1,000), p21 (1:1,000), P-gp (1:200) or  $\beta$ -actin (1:1,000) overnight at 4°C. The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:1,000-2,000) for 2 h at room temperature and detected with enhanced chemiluminescence, and protein levels were quantified using a Tanon 5200 Chemiluminescent Imaging system (version 1.02; Tanon Science and Technology Co., Ltd., Shanghai, China).  $\beta$ -actin was detected on the same membrane and used as loading control.

**Evaluation of multidrug resistance in ovarian cancer cells.** The multidrug resistance of ovarian cancer cells was evaluated by the cell count and P-gp expression. A2780/Taxol and A2780 cells at a density of 8x10<sup>5</sup> cells/well were cultured with

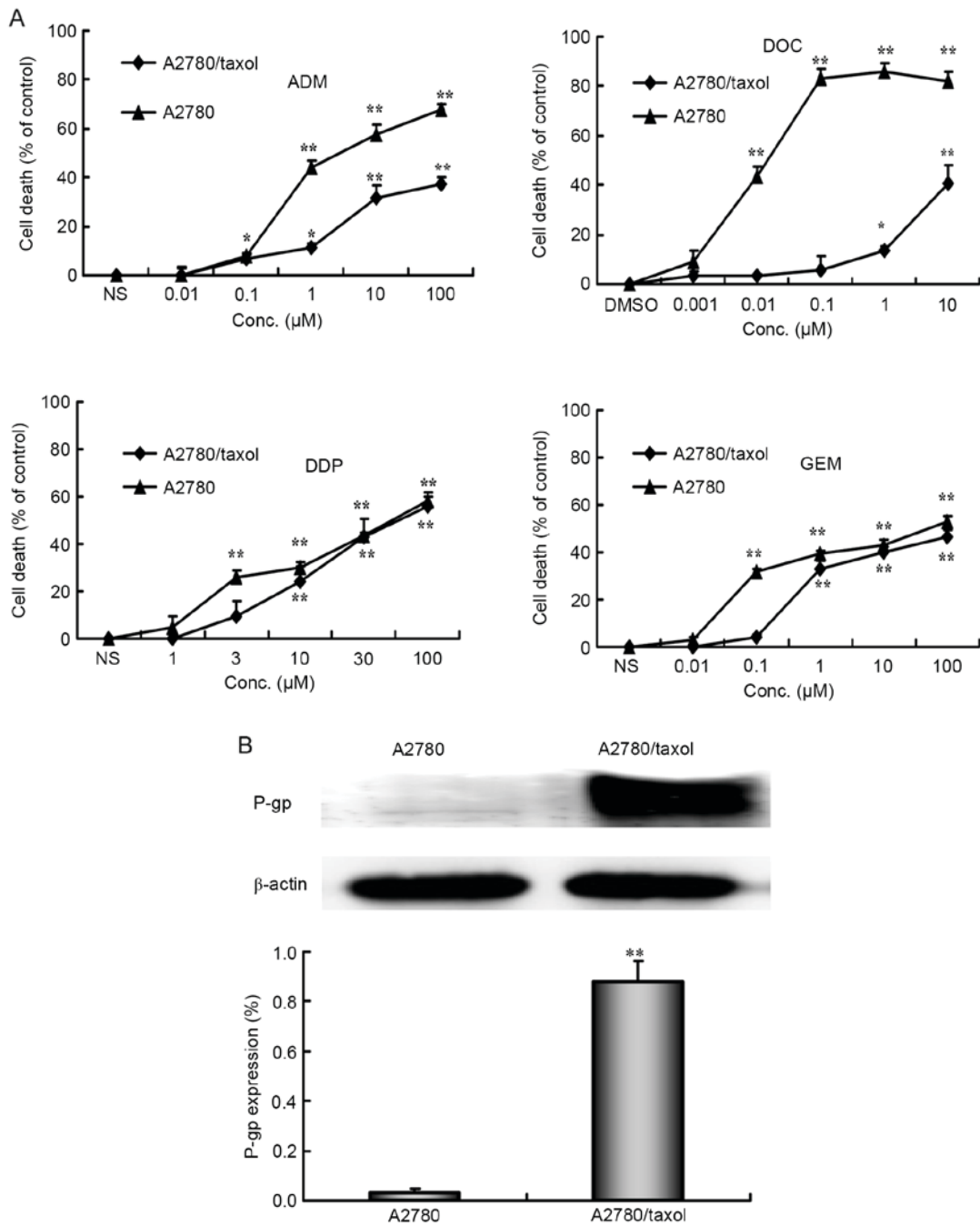


Figure 1. Evaluation of multidrug resistance in ovarian cancer cells. A2780/Taxol and A2780 cells at a density of  $8 \times 10^5$  cells/well were cultured with docetaxel (0.001, 0.01, 0.1, 1 and 10  $\mu\text{M}$ ), doxorubicin (0.01, 0.1, 1, 10 and 100  $\mu\text{M}$ ), cisplatin (1, 3, 10, 30 and 100  $\mu\text{M}$ ) and gemcitabine (0.01, 0.1, 1, 10 and 100  $\mu\text{M}$ ) for 24 h. (A) The viability of the cells was determined by a cell counting assay. Each point represents the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ . (B) P-gp expression in A2780/Taxol and A2780 cells. Each point represents the mean of three independent experiments. \*\* $P < 0.01$  compared with the control. DOC, docetaxel; GEM, gemcitabine; ADM, doxorubicin; DDP, cisplatin; P-gp, P-glycoprotein; Conc, concentration.

docetaxel (0.001, 0.01, 0.1, 1 and 10  $\mu\text{M}$ ), doxorubicin (0.01, 0.1, 1, 10 and 100  $\mu\text{M}$ ), cisplatin (1, 3, 10, 30 and 100  $\mu\text{M}$ ) and gemcitabine (0.01, 0.1, 1, 10 and 100  $\mu\text{M}$ ) for 24 h. The viability of the cells was determined using a cell counting assay.

**Statistical analysis.** All values are expressed as the mean  $\pm$  standard deviation. Comparison between groups was analyzed by one-way analysis of variance followed by Fisher's least significant difference test using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). Two-sided  $P < 0.05$  was considered to indicate a statistically significant difference.

**Results**

**Evaluation of MDR in A2780/Taxol cells.** The MDR of A2780/Taxol cells was evaluated by treating the A2780 and A2780/Taxol cells with docetaxel (0.001, 0.01, 0.1, 1 and 10  $\mu\text{M}$ ), doxorubicin (0.01, 0.1, 1, 10 and 100  $\mu\text{M}$ ), cisplatin (1, 3, 10, 30 and 100  $\mu\text{M}$ ) and gemcitabine (0.01, 0.1, 1, 10 and 100  $\mu\text{M}$ ). As shown in Fig. 1A, the A2780/Taxol cells were resistant not only to docetaxel [A2780/Taxol, half-maximal inhibitory concentration ( $\text{IC}_{50}$ )  $> 10 \mu\text{M}$  vs. A2780,  $\text{IC}_{50} = 0.11 \pm 0.22 \mu\text{M}$ ], but also to the common chemotherapeutic drug doxorubicin (A2780/Taxol,

$IC_{50} > 100 \mu M$  vs. A2780,  $IC_{50} = 8.30 \pm 2.50 \mu M$ ). However, such cells exhibited no resistance to cisplatin (A2780/Taxol,  $IC_{50} = 41.53 \pm 7.48 \mu M$  vs. A2780,  $IC_{50} = 42.52 \pm 14.47 \mu M$ ) or gemcitabine (A2780/Taxol,  $IC_{50} = 20.40 \pm 3.16 \mu M$  vs. A2780,  $IC_{50} = 18.19 \pm 2.47 \mu M$ ) at high concentrations. Compared with A2780 cells, A2780/Taxol cells displayed an increase of 90.91- and 12.05-fold resistance to docetaxel and doxorubicin, respectively. By western blot analysis, the paclitaxel-resistant A2780/Taxol cells also exhibited elevated P-gp protein expression compared with A2780 cells (Fig. 1B).

**Inhibition of the viability of A2780/Taxol and A2780 cells by CuB.** A2780/Taxol and A2780 cells were treated with 0.0625–1  $\mu M$  of CuB for 24–72 h, and cell proliferation was measured by counting the number of viable cells. As shown in Fig. 2, cell proliferation was inhibited by CuB in a dose- and time-dependent manner. Similar  $IC_{50}$  values for CuB in the two cell lines were derived ( $IC_{50} = 0.27 \mu M$  after 72 h or 0.35  $\mu M$  after 48 h for A2780/Taxol cells, and  $IC_{50} = 0.21 \mu M$  after 72 h or 0.25  $\mu M$  after 48 h for A2780 cells). These results indicated that CuB has a potent anti-proliferative effect on ovarian cancer cells, regardless of whether they had resistance to paclitaxel (Fig. 2).

**Effect of CuB on the cell cycle.** A2780/Taxol and A2780 cells were treated with various concentration of CuB for 24 h. Following PI staining, the population in various phases of the cell cycle was determined by fluorescence-activated cell sorting (FACS) analysis. As shown in Fig. 3A, the percentage of  $G_2/M$  phase cells had increased from  $8.49 \pm 0.95\%$  in the control cells to  $55.22 \pm 2.10\%$  (1  $\mu M$  CuB) in A2780 cells, and from  $12.34 \pm 1.66\%$  in DMSO-treated to  $43.47 \pm 2.61\%$  (1  $\mu M$  CuB) in A2780/Taxol cells.

**Effect of CuB on cell apoptosis.** As shown by FACS, there appeared to be a dose-dependent increase in apoptotic cells in CuB-treated samples compared with the controls. The proportion of apoptotic cells increased from  $2.03 \pm 0.23$  to  $11.57 \pm 2.03\%$ , and from  $1.37 \pm 0.44$  to  $8.77 \pm 1.24\%$  in A2780 and A2780/Taxol cells, respectively ( $P < 0.01$ ; Fig. 3B). In addition, marked morphological changes suggestive of apoptosis, including chromatin condensation, nuclear fragmentation and apoptotic bodies, were observed by Hoechst 33258 staining (Fig. 3C).

**Effect of CuB on the expression of p53 and p21.** The present study investigated whether CuB modulates the expression of p53 and p21 in ovarian cancer cells. As shown in Fig. 4A, CuB treatment could upregulate the expression of p53 and p21 in a dose-dependent manner. Treatment of A2780/Taxol and A2780 cells with 0.25  $\mu M$  CuB for 24 h significantly upregulated the expression of the p21 ( $P < 0.05$ ), while CuB significantly upregulated the expression of the p53 at 0.5  $\mu M$  ( $P < 0.05$ ; Fig. 4A).

**Effect of CuB on the expression of Bcl-2 and caspase-3.** Bcl-2 is an important member of the Bcl-2 family of proteins, which regulate cell apoptosis (44). Bcl-2 is considered an important anti-apoptotic protein and is classified as an oncogene (45). As shown in Fig. 4A, Bcl-2 is expressed in A2780/Taxol

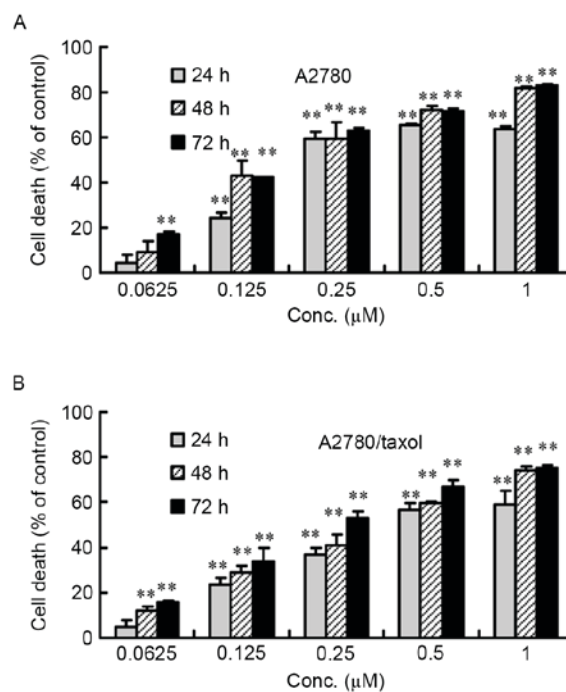


Figure 2. Cytotoxic effect of CuB on paclitaxel-resistant ovarian cancer A2780/Taxol cells and their parental A2780 cells. (A) A2780 and (B) A2780/Taxol cells at a density of  $8 \times 10^5$  cells/well were cultured with various concentrations of CuB for 24, 48 and 72 h. The viability of the cells was determined daily by a cell counting assay. Each point represents the mean  $\pm$  standard deviation of three independent experiments.  $^{**}P < 0.01$  compared with the control. CuB, cucurbitacin B; Conc, concentration.

and A2780 cells, and CuB could suppress Bcl-2 expression in a dose-dependent manner. In addition, CuB treatment significantly decreased the intensity of pro-caspase-3 expression (Fig. 4A).

**Effect of CuB on P-gp expression.** P-gp is an important protein of the cell membrane, which pumps various foreign substances out of the cell (19). P-gp is responsible for decreased drug accumulation in MDR cells, and can mediate the development of resistance to anticancer drugs (46). The present study determined whether CuB downregulates the expression of P-gp in A2780/Taxol cells. As confirmed by western blot analysis, CuB treatment suppressed the expression of P-gp in the A2780/Taxol cell line in a dose-dependent manner (Fig. 4B).

## Discussion

The high mortality of ovarian cancer is usually due to the failure of early detection and lack of effective therapies for late-stage cancers (3,5). The standard therapy for advanced ovarian cancer consists of extensive surgical resection followed by combination chemotherapy with paclitaxel/carboplatin (47). However, such drugs frequently induce resistance (48). Recently, natural compounds with a high potential and relatively low toxicity have been explored for use as single agents or in combination with a conventional chemotherapeutic agent (24). For instance, curcumin isolated from curry spice has been demonstrated to potentiate the antitumor activity of various chemotherapeutic agents in a

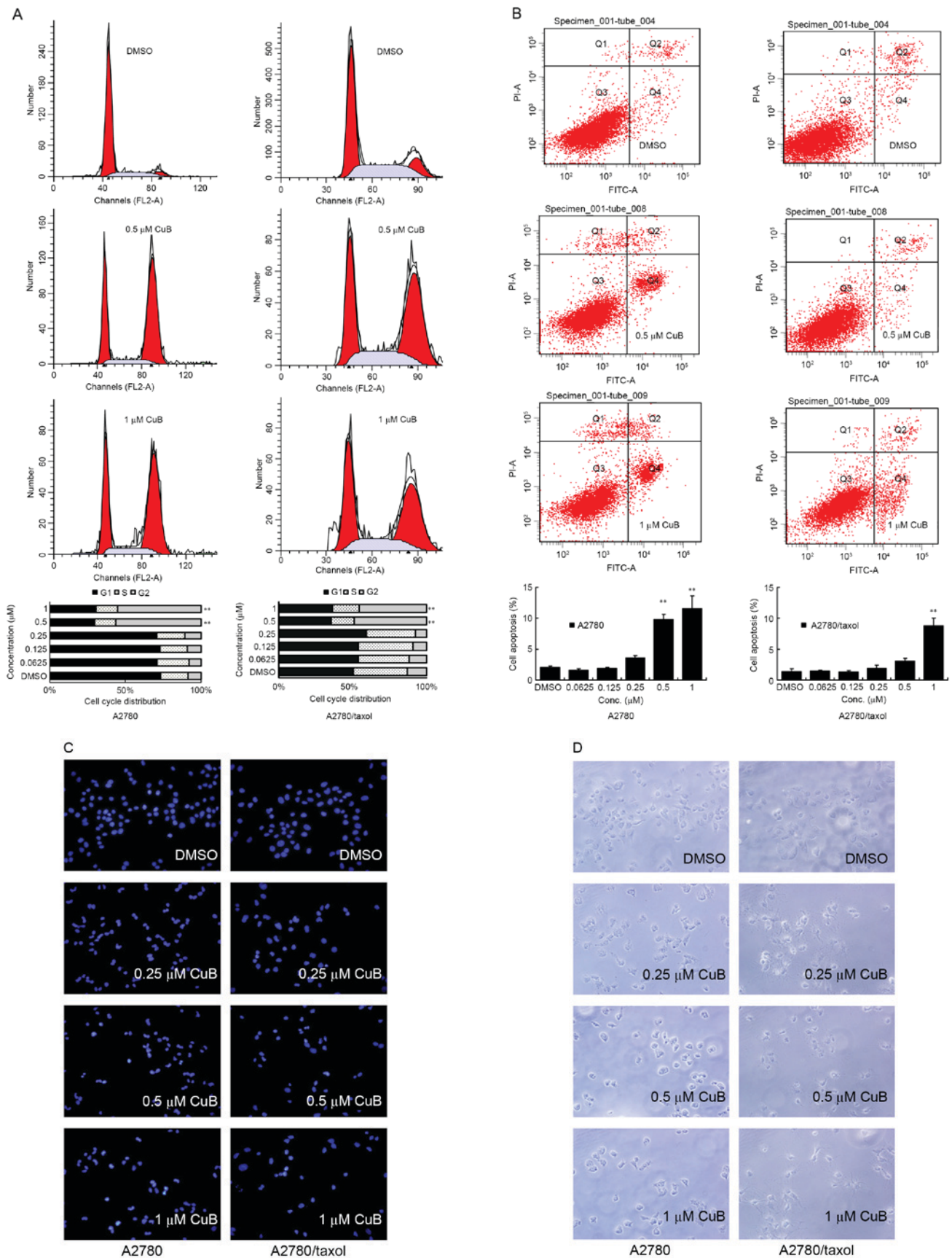


Figure 3. Effect of CuB on cell cycle distribution and apoptosis induction. A2780/Taxol and A2780 cells ( $1 \times 10^6$ ) were treated with various concentrations of CuB for 24 h. (A) Cell cycle analysis by flow cytometry. The percentage of G2/M phase cells increased from  $8.49 \pm 0.95\%$  in the control DMSO group to  $55.22 \pm 2.10\%$  in A2780 cells treated with  $1 \mu\text{M}$  CuB, and from  $12.34 \pm 1.66\%$  in the control DMSO group to  $43.47 \pm 2.61\%$  in A2780/Taxol cells treated with  $1 \mu\text{M}$  CuB.  $^{**}P < 0.01$  compared with the control. (B) Apoptosis analysis by flow cytometry. As shown by FACS, there appeared to be a dose-dependent increase in apoptotic cells in CuB-treated samples compared with the controls. The proportion of apoptotic cells increased from  $2.03 \pm 0.23$  to  $11.57 \pm 2.03\%$ , and from  $1.37 \pm 0.44$  to  $8.77 \pm 1.24\%$  in A2780 and A2780/Taxol cells, respectively.  $^{**}P < 0.01$  compared with the control. (C) Cells with Hoechst 33258 staining (magnification, x40). (D) Cell morphology under light microscopy (magnification, x40). CuB, cucurbitacin; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; PI, propidium iodide; Conc, concentration.

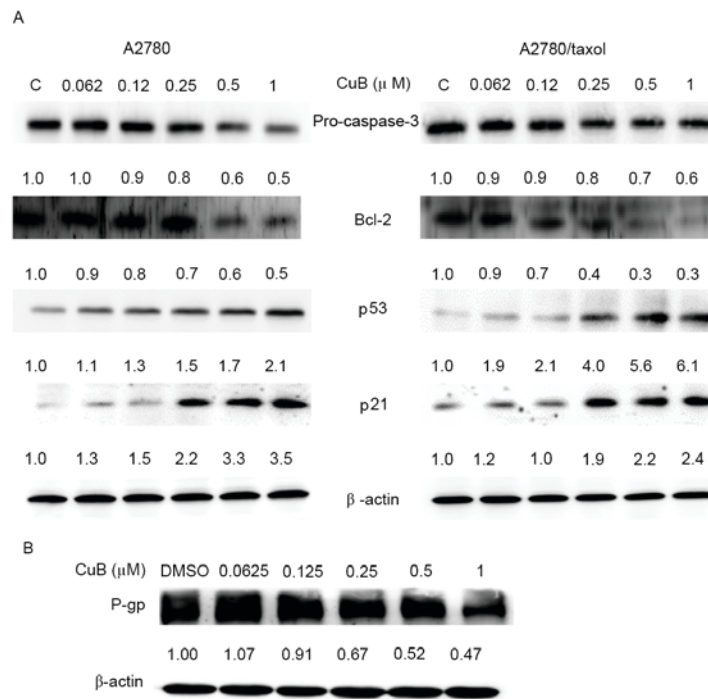


Figure 4. Western blot analysis of Bcl-2, p53, p21, caspase-3 and P-gp protein expression. (A) A2780 and A2780/Taxol cells were treated with various concentrations of CuB for 24 h, and the protein expression of Bcl-2, p53, p21 and caspase-3 was analyzed. (B) A2780/Taxol cells were treated with various concentrations of CuB for 24 h, and the protein expression of P-gp was analyzed. Upon CuB treatment, the cells were harvested and processed for western blotting.  $\beta$ -actin was used as an internal control. Immunoblots are representative of three independent experiments. Bcl-2, B-cell lymphoma 2; CuB, cucurbitacin; C, DMSO; P-gp, P-glycoprotein.

wide range of cancer cells, including drug-resistant ovarian cancer (24,25,49,50).

CuBs are isolated from various plants, which have been used as folk medicines in countries such as China and India (32). In recent years, their anti-proliferative effect has been demonstrated on a variety of cancer cells by *in vivo* and *in vitro* experiments (32,35,42,51). The present study confirmed that CuB also has an inhibitory effect on the growth of paclitaxel-resistant A2780/Taxol cells and their parental A2780 cells in a dose- and time-dependent manner. Through a series of experiments, it was demonstrated that CuB treatment resulted in the accumulation of cells at the G<sub>2</sub>/M phase of the cell cycle. CuB could also induce cell apoptosis in a dose-dependent manner. These results indicated that the inhibitory effect of CuB on paclitaxel-resistant A2780/Taxol cells was due to the induction of cell cycle arrest as well as apoptosis.

MDR is the major cause of failure in anticancer treatment (19). The mechanisms associated with MDR may be classified into two major categories: Pump (P-gp) resistance and non-pump (apoptosis) resistance (52). The former is mainly caused by overexpression of the P-gp protein, which is capable of reducing the intracellular accumulation of drugs by expelling the drugs outside of the cell (53). This may lead to resistance to a wide range of anticancer drugs, including doxorubicin, vinblastine, dactinomycin and paclitaxel (54-56). Approximately 50% of human cancers overexpress P-gp at levels sufficient to confer MDR (56-59). Through the present study, it was demonstrated that the MDR of A2780/Taxol cells may be in part attributed to overexpression of the P-gp protein, while CuB may significantly inhibit P-gp expression.

The non-pump resistance mechanism is the result of an anti-cell death effect, which mainly improves survival and suppresses apoptosis (60). Usually, resistance to anticancer drugs is associated with a low propensity to apoptosis (61). Increased expression of anti-apoptotic proteins, including Bcl-2, has been noted in MDR cells (42,62,63). Therefore, inhibition of Bcl-2 may be a strategy to overcome MDR. The present results demonstrated that CuB induced the apoptosis of A2780/Taxol cells in a dose-dependent manner, which was accompanied by decreased expression of pro-caspase-3 and Bcl-2.

p53 is commonly considered as a tumor-suppressor gene, which can regulate genes involved in apoptosis and may modulate mitochondrial proteins, including Bcl-2, Bcl-2-associated X protein, Bcl-2 homologous antagonist/killer and Bcl-extra large, to promote the release of cytochrome *c* and apoptosis (64). It may also induce cell cycle arrest and/or apoptosis in response to cellular stresses, including ionizing radiation, ultraviolet light, growth factor deprivation, reactive oxygen species and DNA damage induced by various cytotoxic agents (65). There has been accumulating evidence that the status of p53 has a significant impact on drug sensitivity (66-68). As confirmed by the present study, CuB treatment increases the protein levels of p53 and p21 in a dose-dependent manner.

In summary, to the best of our knowledge, the present study demonstrated for the first time that CuB can inhibit the growth of paclitaxel-resistant A2780/Taxol cells and induce cell apoptosis through upregulation of p53 and p21, downregulation of Bcl-2, activation of caspase-3 and suppression of P-gp. This compound may therefore provide an adjuvant therapy for the treatment of paclitaxel-resistant ovarian cancer.

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