BH3 (Bcl-2 homology domain 3)-only proteins have an important role in the cisplatin resistance of cells. However, the effect of BH3-only proteins on cisplatin-resistant ovarian cancer cells has not been thoroughly elucidated. Our results from the present study indicate that Puma plays a critical role in the apoptosis of chemo-resistant ovarian cancer cells treated with BetA (betulinic acid). The reduction of Puma expression inhibits Bax activation and apoptosis. However, p53 gene silencing has little effect on Puma activation. Further experiments demonstrated that Akt-mediated FoxO3a (forkhead box O3a) nuclear translocation and the JNK (c-Jun N-terminal kinase)/c-Jun pathway only partially trigger Puma induction and apoptosis, whereas dominant-negative c-Jun expression with FoxO3a reduction completely inhibits Puma expression and cell death. Furthermore, our results suggest that JNK regulates the Akt/FoxO3a signalling pathway. Therefore, the dual effect of JNK can efficiently trigger Puma activation and apoptosis in chemoresistant cells. Taken together, our results demonstrate the role of Puma in BetA-induced apoptosis and the molecular mechanisms of Puma expression regulated by BetA during ovarian cancer cell apoptosis. Our findings suggest that the JNK-potentiated Akt/FoxO3a and JNK-mediated c-Jun pathways cooperatively trigger Puma expression, which determines the threshold for overcoming chemoresistance in ovarian cancer cells.

Key words: Akt dephosphorylation, cisplatin-resistant, c-Jun N-terminal kinase (JNK) activation, ovarian cancer, Puma.

INTRODUCTION

Ovarian cancer is the most common cause of cancer death from gynaecological tumours. Ovarian cancer patients initially respond to standard treatments such as cytoreductive surgery and subsequent cisplatin-based chemotherapy. However, there is a distinct problem with this therapy in that ovarian cancer cells are resistant to cisplatin, leading to a survival rate of less than 30% in patients with ovarian cancers [1]. Thus finding a new low-toxicity and efficient drug to overcome cisplatin resistance would be a major breakthrough.

BetA (betulinic acid) is a naturally occurring triterpenoid that was initially reported to specifically kill melanoma cells by inducing apoptosis [2] and was subsequently shown to have efficacy in neuroectoderm-derived tumours. The anticancer activity of BetA against other types of cancers, such as leukaemia, prostate, ovarian, breast, lung and colon cancer, has also been reported [3]. Moreover, there have been promising studies indicating that BetA not only has inherent single-agent tumoricidal activity against different tumour cells, but also functions co-operatively with other anticancer drugs to trigger apoptosis in chemoresistant neuroblastoma cells [4,5]. These results indicate that BetA may overcome some forms of drug resistance.

A previous study reported that the extrinsic death receptor pathway does not participate in BetA-induced apoptosis [6]. A decrease in the mitochondrial inner transmembrane potential is associated with BetA treatment, which suggests that the intrinsic mitochondrial pathway is involved in BetA-induced apoptosis. This apoptosis pathway is closely related to the Bcl-2 family of proteins. Anti-apoptotic members sequester multi-domain pro-apoptotic proteins, thereby inhibiting their active role in apoptosis. In contrast, BH3 (Bcl-2 homology domain 3)-only proteins that are considered stress sensors can dissociate Bax-like proteins from their anti-apoptotic sequestrators, thus leading to apoptosis [7].

The expression of Bcl-2 family proteins is regulated during carcinogenesis [7], and the expression of both the Bcl-2 and Bcl-xl anti-apoptotic proteins is associated with resistance to antitumour agents such as cisplatin [8], most notably in ovarian carcinoma [9,10]. In ovarian carcinoma, the inhibition of the protective function of anti-apoptotic Bcl-2 members can either restore the normal apoptotic process in cancer cells or circumvent resistance to chemotherapy. In this regard, enhanced expression of BH3-only proteins can effectively bind the anti-apoptotic members and prevent the function of these proteins.

Some reports suggest that the BH3-only protein Puma has important roles in p53-dependent and -independent apoptosis in human cancer cells and mediates cell death through the Bcl-2 family proteins Bax/Bak and the mitochondrial pathway [11,12]. Experiments have revealed that Puma expression is up-regulated in chemosensitive ovarian cells treated with cisplatin, but not in chemoresistant cells treated with cisplatin. Thus the elevation of Puma expression can overcome chemoresistance in ovarian cancer cells [13].

Abbreviations used: BetA, betulinic acid; BH3, Bcl-2 homology domain 3; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; FoxO3a, forkhead box O3a; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HA, haemagglutinin; JNK, c-Jun N-terminal kinase; Mcl-1, myeloid cell leukaemia sequence 1; MAPK, mitogen-activated protein kinase; MOI, multiplicity of infection; MT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PKB, protein kinase B; RT, reverse transcription; siRNA, small interfering RNA.

*Correspondence may be addressed to either of these authors (email zhuyuan1022@163.com or niec1022@scu.edu.cn).
In the present study, we showed for the first time that BetA can induce apoptosis in cisplatin-resistant ovarian cancer cells. Expression of the BH3-only proteins Puma and Noxa increased following cell death, whereas only Puma siRNA (small interfering RNA) could effectively inhibit Bax translocation, conformational change, oligomerization and the subsequent apoptosis in cisplatin-resistant cells. Moreover, our results demonstrated that Puma expression is independent of p53 regulation in apoptosis. Further experiments revealed that Akt-mediated FoxO3a (forkhead box O3a) nuclear translocation and the JNK (c-Jun N-terminal kinase)/c-Jun pathway co-operatively modulate Puma expression. Furthermore, we found that JNK mediates the Akt/FoxO3a pathway, indicating that JNK is an upstream event of BetA treatment-induced apoptosis. These results elucidated the mechanisms of Puma regulation in BetA-induced apoptosis and confirmed that Puma activation is a major molecular event in mediating BetA-induced apoptosis in chemoresistant ovarian cancer cells.

EXPERIMENTAL

Reagents

Cisplatin, BetA, LY294001, SP600125, PD98059, SB203580, Hoechst 33342, and the anti-Bax 6A7 monoclonal and anti-actin antibodies were obtained from Sigma. Antibodies against Lamin B1, Puma, Noxa, Bim, caspase-9, PARP (poly(ADP-ribose) polymerase), p53, phospho-Akt (Ser473), Akt, phospho-JNK (Thr183/Tyr185), phospho-c-Jun (Ser73), phospho-p38 (Thr180/Tyr182), phospho-ERK (extracellular-signal-regulated kinase) (Thr202/Tyr204) and cleaved caspase-3 were from Cell Signaling Technology. Antibodies against phospho-FoxO3a (Thr32) and FoxO3a were from Upstate Biotechnology. Antibodies against phospho-FoxO3a (Thr52) and FoxO3a were from Upstate Biotechnology.

Gene silencing with siRNAs and plasmids

siRNA oligonucleotides were created with the following sequences: Puma, 5'-UCUCAUCAUGGGACUUCCUG-3'; Noxa, 5'-CUUCCGCGAACAUCUCUG-3'; p53, 5'-CGCGCAUGAGCGAGGCCCAUA-3'; and FoxO3a, 5'-ACUCCGGGUCA-GCUCCAC-3' were purchased from Dharmacon. The constitutively active Akt1 construct [HA (haemagglutinin)–PKB (protein kinase B)-T308D/S473D] was described previously [14]. The dominant-negative c-Jun plasmid (pGFP-TAM67; where the JNK (c-Jun N-terminal kinase)/c-Jun pathway co-operatively mediated FoxO3a (forkhead box O3a) nuclear translocation and Bax were from Santa Cruz Biotechnology. Antibodies against phospho-FoxO3a (Thr52) and FoxO3a were from Upstate Biotechnology.

Cell viability and apoptosis assay

Ovarian cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyl-2H-tetrazolium bromide] assay in 12 replicates. This procedure has been described previously [17]. The following four methods were used to assess induced apoptotic cell death: detection of DNA fragmentation with the Cell Death Detection ELISA kit; Western blot analysis of caspase activation and PARP cleavage; measurement of apoptotic cells by flow cytometry (Annexin V); and cell staining with Hoechst 33342. The cell death detection ELISA was performed to quantify the apoptotic cells by detecting the histone-associated DNA fragments (mono- and oligo-nucleosomes) generated by apoptotic cells [14]. In brief, the ovarian cells were treated with the experimental compounds and collected to prepare the cytosolic fractions that contained the smaller fragments of DNA. Equal volumes of these cytosolic fractions were incubated in anti-histone antibody-coated wells (96-well plates), and the histones of the DNA fragments were allowed to bind to the anti-histone antibodies. The peroxidase-labelled mouse monoclonal DNA antibodies were used to localize and detect the bound fragmented DNA using photometric detection with 2,29-azino-di-(3-ethylbenzthiazoline sulfonate) as the substrate, according to the manufacturer’s instructions.

Cell fractionation

Mitochondrial and cytoplasmic cell fractions were obtained by differential centrifugation as described previously [18,19]. Briefly, cells were harvested and resuspended in 3 vol. of hypotonic buffer (210 mM sucrose, 70 mM mannitol, 10 mM Hepes, pH 7.4, and 1 mM EDTA) containing 1 mM PMSF, 50 mg/ml trypsin inhibitor, 10 mg/ml leupeptin, 5 mg/ml aprotinin and 10 mg/ml pepstatin. After gentle homogenization with a Dounce homogenizer, cell lysates were centrifuged at 1000 g for 5 min to remove unbroken cells and nuclei. The supernatant was collected and centrifuged at 10000 g to pellet the mitochondria-enriched heavy membrane fraction. The supernatant was further centrifuged at 100000 g to obtain a cytosolic fraction.

Nuclear and cytoplasmic lysates were prepared according to a protocol published previously [20]. Briefly, cells were lysed on ice for 20 min in a buffer containing 10 mmol/l Hepes, pH 7.4,
10 mmol/l KCl, 0.01 mmol/l EDTA, 0.1 mmol/l EGTA, 2 mmol/l dithiothreitol, 10 mmol/l NaF, 1 mmol/l sodium orthovanadate, 30 mmol/l sodium glycerophosphate and protease inhibitors. Nuclei were sedimented by centrifugation, and the supernatant (cytoplasmic fraction) was retained. Nuclei were lysed in the same buffer containing 1% (v/v) Nonidet P40 and 400 mmol/l NaCl.

**RNA isolation and RT (reverse transcription)—PCR**

Ovarian cancer cells were treated as described in the Figure legends, and total RNA was isolated with TRIzol®. The oligonucleotides used for amplification were: p53, 5′- CCT- ACCAGGGCGACCTACGG-3′ and 5′-GGAAGACTCCA-GTGTTAATC-3′; and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-GAAGGTGAAGGTGGAAGTCGTA-3′ and 5′-CCGACTCTTGTGCCCTTGCAGA-3′. Total RNA (1 μg) was used for RT–PCR with the Superscript One-step kit (Invitrogen) (20 cycles for p53 and GAPDH). The PCR products, which were 486 bp long for p53 and 260 bp long for GAPDH, were fractionated on a 1% agarose gel containing ethidium bromide and visualized under UV light.

**ChIP (chromatin immunoprecipitation) assay**

The ChIP assay was performed using the Chromatin Immunoprecipitation Assay kit (Upstate Biotechnology) according to the manufacturer’s protocol. ChIP was performed with 3 μg of anti-c-Jun polyclonal antibody (Pierce) or anti-FoxO3a antibody (Sigma) incubated with Protein G-coated magnetic beads overnight with rotation at 4°C. The DNA fragments that co-immunoprecipitated with the target proteins c-Jun or FoxO3a were subjected to quantitative real-time PCR analysis using various primer sets. The Ct (threshold cycle) value of each sample was normalized to the Ct value obtained from the PCR reaction using the corresponding input genomic DNA as a template. Primer sequences for Puma and FoxO3a binding were designed as described previously [21]. Primer sequences were: Puma FoxO3a, 5′- GCGGAGGGCTGGCTTCCTGC-3′ and 5′-AACAGCCGTTATGGCC-3′; and Puma and FoxO3a binding to 5′-GTGGTAA TC-3′ and 5′-GCCGCCACTGCAGTTAGAG-3′; and Puma c-Jun, 5′-GGAAGACTCCA-GTGTTAATC-3′; and Puma c-Jun, 5′-GGAAGACTCCA-GTGTTAATC-3′.

**SDS/PAGE and immunoblotting**

SDS/PAGE and immunoblotting were performed as described previously [18]. Briefly, whole cells or the membrane fractions were resuspended in lysis buffer containing Nonidet P40 (10 mM Hepes, pH 7.4, 2 mM EGTA, 0.5% Nonidet P40, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM dithiothreitol, 50 μg/ml trypsin inhibitor, 10 μg/ml aprotinin and leupeptin) and placed on ice for 30 min. The lysates were centrifuged at 12 000 g for 12 min at 4°C, and the protein concentration was measured. Equivalent samples (30 μg of protein) were subjected to SDS/PAGE on 12% gels. The proteins were then transferred on to nitrocellulose membranes and probed with the indicated antibodies followed by the appropriate secondary antibodies conjugated to horseradish peroxidase (KPL). Immunoreactive bands were visualized using enhanced chemiluminescence (Pierce). The molecular sizes of the proteins detected were determined by comparison with pre-stained protein markers (Invitrogen).

**Bax conformational change and oligomerization**

Bax conformational change analysis was performed as described previously [18]. Cytosolic or mitochondrial fractions were lysed with CHAPS lysis buffer (10 mM Hepes, pH 7.4, 150 mM NaCl and 1% CHAPS) containing protease inhibitors as described previously [18]. The cell lysates were normalized for protein content, and 250 μg of total protein was incubated with 1 μg of anti-Bax 6A7 monoclonal antibody (Sigma) in 500 μl of CHAPS lysis buffer at 4°C for 3 h or overnight. Then, 25 μl of Protein G-agarose was added, and the reactions were incubated at 4°C for an additional 2 h. The beads were washed three times in CHAPS lysis buffer, boiled in loading buffer and the conformationally changed Bax protein in the immunoprecipitates was subjected to SDS/PAGE and immunoblot analysis with anti-Bax polyclonal antibody (Santa Cruz Biotechnology) as described above.

Bax oligomerization was detected as described previously [18]. Cells were washed with conjugating buffer containing 150 mM NaCl, 20 mM Hepes (pH 7.2), 1.5 mM MgCl2 and 10 mM glucose. Disuccinimidyl suberate in DMSO was added from a 10-fold stock solution to a final concentration of 2 mM [22]. The samples were incubated at room temperature (25°C) for 30 min with no reduced buffer, and the cross-linker was then quenched by the addition of 1 M Tris/HCl (pH 7.5) to a final concentration of 20 mM and incubation at room temperature for 15 min. The samples were then solubilized in 0.5% Nonidet P40 lysis buffer without a reducing agent and centrifuged at 12 000 g for 10 min. Bax was detected by Western blotting with an anti-Bax polyclonal antibody (Santa Cruz Biotechnology).

**Statistical analysis**

Statistical analysis of the differences between the experimental groups was performed using a Student’s t test. P < 0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

**Beta induces apoptosis in chemoresistant ovarian cancer cells**

We first examined the apoptotic effect of cisplatin in various cisplatin-sensitive (COC1 and A2780) and -resistant (A2780/CP, COC1/CP and OVCAR-3) human ovarian cancer cells. Apoptosis was detected by the Cell Death Detection ELISA kit with cisplatin treatment (5 μg/ml) at different periods of time. As shown in Figure 1(A), cisplatin caused apoptosis in chemosensitive ovarian cancer cells, but not in chemoresistant cells. We then investigated the antitumour effect of BetA at different concentrations on ovarian cancer cells. BetA caused a dose-dependent reduction in cell viability in cisplatin-sensitive and -resistant cells, showing that the IC₅₀ value was close to 10 μg/ml (Figure 1B). To examine BetA-induced apoptosis, the cells were treated with 10 μg/ml BetA and cell death was confirmed by a DNA fragmentation ELISA assay at various time points. BetA effectively induced apoptosis in all ovarian cancer cells, regardless of their differences in chemo-sensitivity (Figure 1C). Western blot analysis confirmed the cell death through the detection of caspase-3 cleavage in chemoresistant cells (Figure 1D). These results demonstrated that BetA could initiate cell death in cisplatin-resistant ovarian cancer cells. Thus our findings provided the first evidence that BetA can induce apoptosis in ovarian cancer cells regardless of their chemosensitivity. We also found that BetA enhanced the efficacy of cisplatin in chemoresistant ovarian cancer cells (Supplementary Figure S1 at http://www.BiochemJ.org/bj/444/bj4440291add.htm). These results suggest that, in chemotherapy-based regimens, BetA may be used as a ‘sensitizer’ that enhances the efficacy of anticancer agents in the inhibition of tumour growth [23].

As intrinsic mitochondrial pathways play an important role in apoptosis [18,22], we then determined whether mitochondria
After treatment with BetA for different periods of time, detected the cleaved caspase-3 fragments. Lysates were collected and immunoblotted with an antibody specific for caspase-3, which only and cell viability was assessed using the MTT assay, showing that the IC50 value was close to 10 μg/ml. The graph shows the results of quantitative analyses (n = 3, means ± S.D.). Dose-dependent effects of BetA in ovarian cell lines. Cells were exposed to BetA at the indicated concentrations in DMEM or RPMI 1640 medium with 10% (v/v) FBS in a 96-well plate for 48 h and cell viability was assessed using the MTT assay, showing that the IC50 value was close to 10 μg/ml. The graph shows the results of the MTT assay (n = 3, means ± S.D.). Cells were treated with BetA (10 μg/ml) for different periods of time and cell apoptosis was detected as described in (A). Detection of caspase-3 activation. After BetA (10 μg/ml) treatment, cell lysates were collected and immunoblotted with an antibody specific for caspase-3, which only detected the cleaved caspase-3 fragments. β-Actin was used as a protein loading control. Analysis of cytochrome c (Cyt c) release. After treatment with BetA for different periods of time, cells were subjected to subcellular fractionation. The cytosolic or mitochondrial fractions were immunoblotted (30 μg of protein/lane) with an antibody specific for cytochrome c. β-Actin and COX IV were used as a protein loading control. The results are representative of at least three independent experiments.

Figure 1 BetA induces apoptosis in cisplatin-resistant human ovarian cancer cells

(A) Analysis of cell apoptosis treated with cisplatin (CP). Cells were treated with cisplatin (5 μg/ml) for the indicated times, and then collected to examine apoptosis. Cell apoptosis was quantitatively detected by the Cell Death Detection ELISA kit as described in the Experimental section. The graph shows the results of quantitative analyses (n = 3, means ± S.D.). (B) Dose-dependent effects of BetA in ovarian cell lines. Cells were exposed to BetA at the indicated concentrations in DMEM or RPMI 1640 medium with 10% (v/v) FBS in a 96-well plate for 48 h and cell viability was assessed using the MTT assay, showing that the IC50 value was close to 10 μg/ml. The graph shows the results of quantitative analyses (n = 3, means ± S.D.). (C) Cells were treated with BetA (10 μg/ml) for different periods of time and cell apoptosis was detected as described in (A). (D) Detection of caspase-3 activation. After BetA (10 μg/ml) treatment, cell lysates were collected and immunoblotted with an antibody specific for caspase-3, which only detected the cleaved caspase-3 fragments. β-Actin was used as a protein loading control. (E) Analysis of cytochrome c (Cyt c) release. After treatment with BetA for different periods of time, cells were subjected to subcellular fractionation. The cytosolic or mitochondrial fractions were immunoblotted (30 μg of protein/lane) with an antibody specific for cytochrome c. β-Actin and COX IV were used as a protein loading control. The results are representative of at least three independent experiments.

Figure 2 BetA induces changes in Bcl-2 family proteins in ovarian cancer cells

(A) Time-dependent analysis of Bcl-2 family member’s expression levels in A2780/CP cells treated with BetA (10 μg/ml) for the indicated times. (B) Detection of Bcl-2 family protein levels in cells treated with or without BetA (10 μg/ml) for 24 h. Cells were treated and collected, then lysed in Nonidet P40 buffer for Western blot detection. β-Actin was used as a protein loading control. (C) Cells were treated with BetA as described in (B) and lysed for Western blot detection. Panel a, to detect Bax translocation, cells were subjected to subcellular fractionation. The cytosolic (Cyto) or mitochondrial (Mito) fractions were immunoblotted (30 μg of protein/lane) with an antibody specific for Bax. Panel b, to detect Bax conformational change, cells were cultured in the presence or absence of BetA for 24 h and then lysed in CHAPS buffer and subjected to immunoprecipitation (IP) with the anti-Bax 6A7 antibody (Sigma), and then immunoprecipitated cell lysates were detected with anti-Bax polyclonal antibody. A cell lysate obtained using the Nonidet P40 lysis buffer, and Bax was detected by Western blotting with an anti-Bax polyclonal antibody. Panel c, to detect Bax conformational change, cells were cultured in the presence or absence of BetA for 24 h and then lysed in CHAPS buffer and subjected to immunoprecipitation (IP) with the anti-Bax 6A7 antibody (Sigma), and then immunoprecipitated cell lysates were detected with anti-Bax polyclonal antibody. A cell lysate obtained using the Nonidet P40 lysis buffer, and Bax was detected by Western blotting with an anti-Bax polyclonal antibody (Santa Cruz). All results are representative of three independent experiments. Molecular mass is given in kDa on the right-hand side. Ab, antibody.

Expression changes of Bcl-2 family proteins are correlated with the induction of cell death

Mitochondrial dysfunction plays an important role in ovarian cell apoptosis, and the function of Bcl-2 proteins is related to the mitochondrial apoptotic pathway. Previous studies reported that Bcl-2 family proteins are associated with mitochondrial dysfunction and the chemoresistance of cells [24,25], and that the BH3-only proteins are important for chemoresistance in ovarian cancer cells [26].

Several reports have also showed that BetA can modulate the expression levels of Bcl-2 family proteins. For example, BetA treatment resulted in the high expression of the pro-apoptotic Bcl-2 family protein Bax in neuroblastoma, glioblastoma and melanoma cells [27]. Additionally, BetA triggered the up-regulation of Mcl-1, another anti-apoptotic Bcl-2 family protein, in melanoma cells, whereas no changes in Mcl-1 levels were detected in squamous cell carcinoma cells [28,29]. These results suggest that Bcl-2 family proteins have an important role in BetA-induced apoptosis. However, the function of BH3 proteins in ovarian cancer cells treated with BetA is unclear. Therefore, we examined the expression of Bcl-2 family proteins in cisplatin-sensitive and -resistant cells after BetA treatment.

We used A2780 and A2780/CP cells as models to study the function of BetA. As shown in Figures 2(A) and 2(B),
pro-apoptotic proteins, such as Bax and Bim, were not significantly changed in all cells treated with BetA at different time points. Anti-apoptotic Mcl-1, Bcl-2 and Bcl-xL proteins also exhibited no major alterations. However, in contrast with other proteins, the expression levels of Puma and Noxa markedly increased after BetA treatment. Meanwhile, Puma and Noxa expression levels were up-regulated in COC1 and COC1/CP cells after BetA treatment (Supplementary Figure S2 at http://www.BiochemJ.org/bj/444/bj4440291add.htm).

At the same time, we observed a Bax conformational change, translocation and oligomerization in ovarian cancer cells after BetA treatment (Figure 2C), providing further evidence that Bcl-2 family proteins are involved in BetA-induced apoptosis in ovarian cancer cells. Moreover, our experiments also suggest that Puma and Noxa play important roles in BetA-induced apoptosis in ovarian cancer cells.

**Puma down-regulation by siRNA prevents apoptosis**

To identify further the functions of Puma and Noxa, we decided to knock down these proteins using siRNAs in A2780/CP cell lines (Figure 3A). Puma siRNA was able to inhibit Puma protein expression, but did not lead to cell death on its own. As described above, a 12 h exposure to BetA induced an obvious apoptotic response in A2780/CP cells. Whereas control siRNA did not modify the response to BetA treatment, we observed a strong decrease in the apoptotic response in cell lines when BetA treatment was combined with Puma siRNA (Figure 3B). Similar results were observed in A2780 cells (results not shown). Meanwhile, Puma siRNA treatment decreased cell death in COC1/CP cells, showing the same results observed in A2780/CP cells (Supplementary Figure S3 at http://www.BiochemJ.org/bj/444/bj4440291add.htm). In contrast, Noxa siRNA markedly induced the expression of Noxa, but had little or no effect on the levels of apoptosis induced by BetA in either cell line (Figures 3C and 3D, and Supplementary Figure S3). These results demonstrate that Noxa is dispensable for BetA-induced apoptosis. The results are in agreement with some previous studies that demonstrated that Noxa has an unimportant role in apoptosis. These studies revealed that forced expression of Noxa was sufficient to trigger neuronal cell apoptosis. Moreover, an experiment using Noxa-deficient mice also indicated that it plays a minor role in the apoptotic pathway [30,31]. In contrast, several studies revealed that Puma induction strongly correlated with apoptosis, and the ectopic expression of Puma was sufficient to induce apoptosis [30,31].

As previous studies revealed that BH3 proteins can directly or indirectly induce Bax activation in apoptosis [7,11,12], we also determined whether the reduction of Puma and Noxa affected Bax activation in apoptosis. Gene silencing of Puma obviously inhibited Bax conformational change, translocation and oligomerization, whereas knockdown of Noxa could not prevent Bax activation (Figures 3E and 3F). These results further demonstrated that Noxa was not involved in regulating Bax activation and apoptosis. Moreover, these results revealed that Puma mediates Bax activation and the subsequent mitochondrial-dependent apoptotic pathway in BetA-treated ovarian cancer cells.

**p53 is not involved in Puma activation**

We then examined which factor mediates Puma expression. As a previous report indicated that p53 expression is enhanced and p53 transcriptionally triggers Puma expression after stress induction [32], we wanted to assay whether PUMA is induced by BetA through a p53-dependent mechanism. However, A2780 cells express wild-type p53, whereas A2780/CP cells have mutant p53 [33,34]. We have detected a single pair missense mutation (G to T) at codon 172 in A2780/CP p53 by PCR amplification and sequence analysis (results not shown). This mutation may lead to a loss of protein function and thereby contribute to cisplatin resistance. Therefore we first detected the status of p53 in A2780 and A2780/CP cells with cisplatin treatment. Our results demonstrated that the expression levels of p53 in A2780 cells increased with cisplatin treatment, whereas p53 induction was unchanged in A2780/CP cells after treatment (Figure 4A, panels a and b), as described previously [33]. Moreover, cisplatin significantly induced apoptosis in A2780 cells, but not in A2780/CP cells (Figure 1A). However, overexpression of wild-type p53 in A2780/CP cells resulted in a modest increase in cisplatin-induced apoptosis (Figure 4B, panel a), whereas Lacz expression in A2780/CP cells produced little change in cisplatin-induced apoptosis (Figure 4B, panel a). Western blot analysis also showed that p53 expression increased in wild-type p53-infected cells (Figure 4B, panel b). Thus the p53 mutation in A2780/CP cells did contribute to cisplatin resistance in the present study.

We then detected p53 expression in cells after treatment with BetA. Western blotting showed that the expression level of p53 had minor changes in all cells regardless of their chemosensitivity (Figure 4C). Meanwhile, RT–PCR also revealed that the mRNA levels of p53 changed minimally at different periods of time after BetA treatment (Figure 4D).

To further confirm the effect of p53 in Puma expression, we then knocked down p53 with siRNA (p53 siRNA) in ovarian cancer cells. The siRNA was able to inhibit p53 expression, but did not induce caspase-3 cleavage or cleavage on its own (Figures 4E and 4F, and Supplementary Figure S4 at http://www.BiochemJ.org/bj/444/bj4440291add.htm). We observed that BetA combined with p53 siRNA could not prevent caspase-3 activation in ovarian cancer cells. Importantly, up-regulated Puma expression was invariant in p53 siRNA-transfected chemoresistant cells treated with BetA (Figure 4F and Supplementary Figure S4). These results demonstrate that p53 is not involved in regulating Puma expression even in A2780 cells, which express the wild-type p53. Our results also indicate that BetA-induced apoptosis is independent of p53 activation. In fact, these results are as expected. First, the p53 mutant may lead to inactivity of p53 in apoptosis. Secondly, BetA induced-apoptosis is not associated with the accumulation of wild-type p53 protein [27]. Thus there are other mechanisms that function in modulating Puma expression in ovarian cancer cells.

**The Akt pathway does not completely regulate Puma expression**

A previous report revealed that Akt dephosphorylation can mediate Puma expression through FoxO3a transcriptional regulation [35]. Moreover, BetA induces Akt dephosphorylation in melanoma cell apoptosis [36], we speculate that Akt is probably involved in regulating Puma expression in ovarian cancer cells.

Therefore, we measured whether Akt was involved in BetA-induced apoptosis in ovarian cancer cells. As shown in Figure 5(A), BetA induced the down-regulation of phospho-Akt at Ser473 in cisplatin-sensitive A2780 cells in a time-dependent manner, whereas the expression level of total Akt protein experienced little or no change. Similarly, BetA induced a decrease in phospho-Akt in other cells despite their differences in chemosensitivity.

The decrease in phospho-Akt leads to dephosphorylation and activation of Akt signaling [37]. Active FoxO3a can initiate Puma expression through transcriptional regulation [35]. FoxO3a...
Figure 3  Puma is major molecular determinant in BetA-treated cell apoptosis

Legend can be found on page 297.
phosphorylation decreased during the time course with BetA induction, whereas the expression level of total FoxO3α was unchanged (Figure 5B). We then determined whether FoxO3α regulates Puma expression in apoptosis. We used the ChIP assay to detect the interactions between FoxO3α and the Puma promoter, as described previously [35]. Our results revealed that FoxO3α can act on the Puma promoter.

Down-regulation of Puma by siRNA decreases apoptosis in A2780/CP cells. (A) Cells were transfected with either control siRNA or Puma siRNA for 48 h and then treated with BetA (10 μg/ml) for 12 h. Cells were collected and lysed for immunoblotting with an antibody specific for Puma. β-Actin was used as a protein loading control. (B) Detection of cell apoptosis. DNA content, nuclear morphology and viability were assessed after a 12 h exposure to BetA in transfected A2780/CP cells. Nuclear morphology was detected by microscopy. Arrows indicate the condensed fragmented brightly stained nuclei, which are the hallmark of apoptosis. DNA content and cell viability was examined by flow cytometry with Annexin V staining. (C) Down-regulation of Noxa by siRNA does not decrease apoptosis in cisplatin-resistant cells. Cells were transfected with either control siRNA or Noxa siRNA for 48 h, and then transfected cells were detected by Western blotting in the presence of BetA for 12 h. Representative results of three experiments with consistent results are shown. (D) Graphs showing results of quantitative analyses of transfected apoptosis in A2780 and A2780/CP cells. Cells were transfected as described in (C) and then treated with BetA for 12 h. Cell apoptosis was examined as described in the Experimental section. (n = 3, means ± S.D., *P < 0.01). (E) Cells were transfected with the control or Puma siRNA vector for 48 h, and then treated with BetA for 24 h. Cells were collected and lysed for Western blot detection. Bax translocation, conformational change and oligomerization were examined as described in the Experimental section. β-Actin was used as a protein loading control. (F) Cells were transfected with control or Noxa siRNA for 48 h, and then treated and detected as described in (E). All data are representative of three independent experiments. CF, cleaved fragments; Ctrl, control; IP, immunoprecipitation.

Figure 4 p53 activation is not involved in Puma up-regulation

(A) Panel a, Western blotting to detect p53 expression in cells. A2780 or A2780/CP cells were treated with cisplatin (CP) (5 μM/ml) for the indicated times, and then collected to detect p53 expression. β-Actin was used as a protein loading control. Panel b, grey scanning of p53 levels. Panel c, p53 levels in cells were measured by densitometric analysis of the Western blots and compared with actin levels in cells. The relative amount of p53 from untreated cells was considered as 1. (B) A2780/CP cells were infected with adenoviral LacZ or p53 (MOI 0, 5, 10 or 20; 72 h), and then cells were treated with cisplatin (5 μg/ml) for 24 h. Apoptosis is shown in panel a. Panel b shows representative Western blots for p53. (C) Western blotting to detect p53 expression in cells treated with BetA (10 μg/ml). A2780 and A2780/CP cells were treated at indicated time and lysed. β-Actin was used as a protein loading control. (D) As described in (C), cells were treated and lysed to detect p53 RNA levels with RT–PCR. GAPDH was used as a protein loading control. (E) Cells were transfected with p53 siRNA or control (Ctrl) siRNA for 48 h and then treated with BetA for 24 h. Cells were lysed and determined by immunoblot analysis. (F) As described in (E), cells were transfected for 48 h and treated with BetA for 24 h. Western blotting was performed to detect caspase-3 cleavage and Puma expression. Representative results of three experiments with consistent results are shown.

Figure 5 Akt partially regulates Puma expression

(A) Western blot analysis of total Akt and phospho-Akt (p-Akt) (Ser473) levels in ovarian cells treated with BetA (10 μg/ml). A2780 and A2780/CP cells were treated with BetA for different periods of time, then lysed to detect total Akt and phospho-Akt levels. β-Actin was used as a protein loading control. (B) Detection of FoxO3α and phospho-FoxO3α (p-FoxO3α) levels in cells treated with BetA. Cells were treated as described in (A) and subjected to immunoprecipitation with anti-FoxO3α or anti-phospho-FoxO3α (Thr32) antibodies. (C) Cells were transfected with control (Ctrl) or FoxO3α siRNA for 48 h, and then treated with BetA for 24 h. Panel a, cells were lysed to detect protein level using Western blotting analysis. Panel b, treated cells were used to detect apoptosis with the Cell Death Detection ELISA kit as described in the Experimental section (n = 3, means ± S.D., *P < 0.05). (D) Panel a, A2780/CP cells were treated with or without BetA (10 μg/ml) and/or LY294002 (25 μM) for 24 h, and then lysed to detect protein expression. Panel b, A2780/CP cells were transfected with control and Akt1 vector for 48 h and treated with BetA for 24 h. Treated cells were lysed for detection. For FoxO3α nuclear translocation analysis, cells were incubated with BetA and subjected to subcellular fractionation as described in the Experimental section. C-FoxO3α, cytosolic FoxO3α; N-FoxO3α, nuclear FoxO3α. Lamin B1 was used as a nuclear marker. Representative results of three experiments with consistent results are shown.
promoter after BetA treatment (Supplementary Figure S5 at http://www.BiochemJ.org/bj/444/bj4440291add.htm) and indicated that FoxO3a can regulate Puma expression. To confirm the function of FoxO3a in Puma expression, gene silencing of FoxO3a by siRNA was performed. Our experiment demonstrated that Puma expression is up-regulated in control siRNA cells, whereas ablation of FoxO3a decreases Puma induction (Figure 5C, panel a, and Supplementary Figure S6A at http://www.BiochemJ.org/bj/444/bj4440291add.htm). Simultaneously, apoptosis also decreased with FoxO3a siRNA transfection. However, significantly, the reduction of FoxO3a could not completely inhibit Puma expression and apoptosis (Figure 5C, panel b, and Supplementary Figure S6B). These results suggest that FoxO3a may not be the sole transcription initiation factor responsible for Puma expression.

To confirm that FoxO3a is a mediator of Akt-dependent Puma-induced apoptosis, it was necessary to detect the nuclear translocation of FoxO3a in chemoresistant cells. Our results showed that FoxO3a translocated from the cytosol to the nucleus and was accompanied by a decrease in phospho-Akt and an increase in Puma expression after BetA treatment. LY294002, the PI3K (phosphoinositide 3-kinase) inhibitor, obviously enhanced nuclear translocation of FoxO3a in cells treated with BetA (Figure 5D, panel a). Further experiments demonstrated that overexpression of Akt1 increased the level of Akt and phospho-Akt (Figure 5D, panel b, and Supplementary Figure S6C), but inhibited nuclear FoxO3a translocation (Figure 5D, panel b). These results indicated that FoxO3a is a mediator of Akt-dependent Puma expression. It should be noted that the increased protein level of Akt only partially prevented Puma induction. Combined with the results displayed in Figure 5(C), we speculate that other factors also participate in regulating Puma induction.

**JNK potentiates the Akt pathway to regulate Puma expression**

To determine which factors within the Akt/FoxO3a pathway mediate Puma expression, we first assayed the activation of some important protein kinases, such as JNK, p38-MAPK (mitogen-activated protein kinase) and ERK, which belong to the MAPK family. These MAPK family proteins have been reported to mediate the Akt/FoxO3a pathway [37,38]. As shown in Figure 6(A), the level of phospho-JNK1/2 increased at different periods after BetA treatment with Puma overexpression and dephosphorylation of FoxO3a. The levels of activated p38 (phospho-p38) were also elevated after BetA treatment throughout the time course. A similar profile was found for activated ERK1/2. Western blotting results also indicated that the levels of total JNK, p38 and ERK exhibited no change (results not shown). These results suggest that these kinases are involved in regulating Puma expression and apoptosis after BetA treatment.

To determine which of the kinases activated by BetA affects ovarian cancer cell survival, we treated cells with SP600125, SB203580 and PD98059, which are inhibitors of JNK1/2, p38 and MEK1 (MAP/ERK kinase 1; the upstream activator of ERK1/2) respectively. As shown in Figure 6(B), treatment of cells with the p38-MAPK inhibitor SB203580 or ERK1 inhibitor PD98059 alone did not eliclt cell death at 24 h. Furthermore, PD98059 and SB203580 failed to protect the cells against BetA-induced apoptosis. However, when cells were incubated with SP600125 and BetA, the percentage of cells undergoing apoptosis was dramatically lower than those incubated with BetA alone, indicating that inhibition of JNK1/2 activation protects against the cytotoxic effects of BetA.

Next, we investigated the consequences of JNK inhibition on Puma expression and FoxO3a activation. Western blotting results revealed that the activation of JNK (phospho-JNK) and its downstream target (phospho-c-Jun) upon BetA treatment was abolished in the presence of SP600125. Furthermore, SP600125 markedly attenuated the drop in phospho-FoxO3a levels (Figure 6C, panel a) and Puma expression (Figure 6C, panel a, and Supplementary Figure S7 at http://www.BiochemJ.org/bj/444/bj4440291add.htm) upon 24 h of treatment with BetA. Meanwhile, nuclear FoxO3a translocation decreased following SP600125 and BetA treatment. The phosphorylation status of Akt mirrored that of FoxO3a, and SP600125 obviously abolished the decline in the phospho-Akt levels upon BetA treatment (Figure 6C, panel a). However, the p38-MAPK inhibitor SB203580 and the ERK1 inhibitor PD98059 had little effect on Puma expression and FoxO3a activation (Figure 6C, panels b and c). These results confirm that JNK can regulate the Akt/FoxO3a pathway and induce Puma expression through the Akt/FoxO3a pathway.

Our results indicated that JNK and Akt co-operatively regulate FoxO3a transcriptional activation. However, FoxO3a was obviously not the sole transcription factor to regulate Puma expression. Therefore, another transcriptional factor must participate in triggering the induction of Puma. The JNK downstream target c-Jun is a transcriptional factor that was shown to trigger induction of Puma RNA levels [21]. Thus c-Jun might induce Puma expression by transcriptional regulation in ovarian cancer cells treated with BetA. Our results indeed indicated that c-Jun can act on the Puma promoter as described previously (Supplementary Figure S8 at http://www.BiochemJ.org/bj/444/bj4440291add.htm) [21]. To further confirm that the JNK/c-Jun pathway also mediates Puma activation and subsequent apoptosis, we utilized a dominant-negative mutant of c-Jun, TAM67, which was cloned into the pGFP-C2 plasmid. An immunoblot assay demonstrated that the location of GFP–TAM67 was in the nucleus of transfected cells (Figure 6D, panel a), consistent with the location of c-Jun in cells, as described previously [39]. Transfection of GFP–TAM67 indeed reduced Puma expression and caspase cleavage in cells treated with BetA (Figure 6D, panel b). Nevertheless, the reduction of c-Jun activity also partially prevented Puma expression. The inhibition of FoxO3a and c-Jun activation prevented Puma expression and apoptosis in cells treated with BetA (Figure 6D, panels b and c). Therefore our results suggest that c-Jun (regulated by JNK) and FoxO3a (regulated by Akt and JNK) co-ordinately mediate Puma expression and apoptosis in ovarian cancer cells treated with BetA.

At the same time, we also determined whether FoxO3a and c-Jun can act on the Noxa promoter in our system. We knocked down FoxO3a or c-Jun with siRNA to examine Noxa expression. The reduction in FoxO3a and c-Jun had little effect on Noxa expression (results not shown). These results are in agreement with previous reports [40–42]. Valis et al. [41] demonstrated that FoxO3a has little effect on Noxa expression. Another previous study revealed that Noxa expression is also independent of c-Jun [42]. Eferl et al. [40] even suggested that c-Jun antagonizes Noxa expression. Of course, we still need to perform further experiments to study the mechanism of Noxa up-regulation and the reasons why Noxa expression is not involved in apoptosis induced by BetA treatment.

**Concluding remarks**

Our findings can explain how BetA can induce apoptosis in chemoresistant ovarian cancer cells. Our previous study showed
that JNK is an upstream event of apoptosis [22]. The experiments described in the present study demonstrate that JNK is also an early response to BetA treatment. JNK can not only mediate the Akt/FoxO3a pathway, but also activate c-Jun in apoptosis. This dual effect of JNK can efficiently trigger Puma activation and subsequent apoptosis in chemoresistant cells (Figure 7). The present study also indicates further the importance of BH3-only proteins in chemoresistance, as our and other studies revealed that Bim is a major determinant of cisplatin resistance in cancer cells [22,43].

In conclusion, we have elucidated the molecular mechanisms of BetA-induced apoptosis in cisplatin-resistant ovarian cancer cells and demonstrated that Puma plays a critical role in cisplatin resistance. We provide the first evidence that c-Jun (regulated by JNK) and FoxO3a (regulated by Akt and JNK) co-ordinately mediate Puma expression and apoptosis in chemoresistant ovarian
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SUPPLEMENTARY ONLINE DATA

JNK- and Akt-mediated Puma expression in the apoptosis of cisplatin-resistant ovarian cancer cells

Zhiwei ZHAO*, Jingjing WANG*, Jingsheng TANG†, Xinyu LIU*, Qian ZHONG‡, Fang WANG*, Wenbin HU*, Zhu YUAN*1, Chunlai NIE*1 and Yuquan WEI*1

*The State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, 4# Keyuan Road, Hitech District, Sichuan University, Chengdu 610041, China, †ChongQing NewFine Biology Technology, 8# Lingfang Road, Banqiao District, Chongqing 402460, China, and ‡Department of Gynecology and Obstetrics, West China Second Hospital, Chengdu, Sichuan University, Chengdu 610041, China

Figure S1 Effect of BetA plus cisplatin on apoptotic death in cisplatin-resistant ovarian cancer cells

We used A2780/CP and COC1/CP cells as a model of cisplatin-resistant ovarian cells. Cells were cultured in the presence or absence of BetA (10 μg/ml) and cisplatin (5 μg/ml) over different periods of time. The graph shows the results of quantitative analyses of cell apoptosis in cisplatin-resistant cells (n = 3, means ± S.D.).

Figure S2 Effect of BetA on the expression of the Bcl-2 family of proteins in COC1 and COC1/CP cells

Cells were treated with or without BetA (10 μg/ml) for 24 h and collected, then lysed in Nonidet P40 buffer for Western blot detection. β-Actin was used as a protein loading control. All results are representative of three independent experiments.

Figure S3 The effect of Puma and Noxa down-regulation on apoptosis

(A) Cells were transfected with either control (Ctrl) siRNA or Puma siRNA for 48 h and then treated with BetA (10 μg/ml) for 12 h. Cells were collected and lysed for immunoblotting with an antibody specific for Puma. β-Actin was used as a protein loading control. (B) Cells were transfected with either control siRNA or Noxa siRNA for 48 h, and then transfected cells were detected by Western blotting in the presence of BetA for 12 h. Representative results of three experiments with consistent results are shown. (C) Graph showing the results of quantitative analysis of transfected apoptosis in COC1 and COC1/CP cells. Cells were transfected as described in (B), then treated with BetA for 12 h. Cell apoptosis was examined as described in the Experimental section of the main text. (n = 3, means ± S.D., **P < 0.01).
Cells were transfected with p53 siRNA or control siRNA for 48 h and then treated with BetA for 24 h. Cells were lysed and analysed using Western blotting to detect caspase-3 cleavage and Puma expression. Representative results of three experiments with consistent results are shown.

**Figure S5  Quantification of FoxO3a association with the Puma promoter**

ChIP was performed with anti-FoxO3a polyclonal antibody incubated with Protein G-coated magnetic beads at 4 °C overnight with rotation. DNA fragments co-immunoprecipitated with the target protein FoxO3a were subjected to quantitative real-time PCR. Quantitative real-time PCR assays were conducted from cells that were either left untreated (con) or treated with BetA. Numbers on the y axis represent the levels of FoxO3a association with the Puma promoter region after normalizing to C values from input samples. The results shown are the means ± S.D. from three independent experiments.

**Figure S6  Effect of Akt-FoxO3a pathway on Puma expression and apoptosis**

(A) Cells were transfected with control (Ctrl) or FoxO3a siRNA (RNAi) for 48 h, and then treated with BetA for 24 h. Treated cells were lysed to detect protein levels and analysed using Western blotting. Representative results of three experiments with consistent results are shown. (B) Cells were treated as described in (C) and then treated cells were used to detect apoptosis using a Cell Death Detection ELISA kit as described in the Experimental section of the main text (n = 3, means ± S.D., **P < 0.01). (C) Cells were transfected with control or Akt1 vector for 48 h, and treated with BetA for 24 h. Treated cells were lysed for detection of proteins using Western blotting. β-Actin was used as a protein loading control.
Figure S7  Effect of JNK pathway on Puma expression

Cells were treated with BetA (10 μg/ml) with or without 20 μmol/l SP600125, and then cells were collected at 24 h after treatment for detection by Western blotting. β-Actin was used as a protein loading control. p-, phospho-.

Figure S8  Quantification of c-Jun association with the Puma promoter

ChIP was performed with anti-c-Jun polyclonal antibody incubated with Protein G-coated magnetic beads at 4°C overnight with rotation. DNA fragments co-immunoprecipitated with the target protein c-Jun were subjected to quantitative real-time PCR. Quantitative real-time PCR assays were conducted from cells that were either left untreated (con) or treated with BetA. Numbers on the y axis represent the levels of c-Jun association with the Puma promoter region after normalizing to C_T values from input samples. The results shown are the means ± S.D. from three independent experiments.

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