

# Synergistic activity of the histone deacetylase inhibitor trichostatin A and the proteasome inhibitor PS-341 against taxane-resistant ovarian cancer cell lines

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**Abstract.** Although a combination of platinum- and taxane-based chemotherapy is recommended for at least 70% patients with ovarian cancer as treatment subsequent to surgery, the initial response to the chemotherapy is not durable and tumors become resistant. Histone deacetylase and proteasome inhibitors are novel therapeutic agents. However, the moderate antitumoral effect of the inhibitors has restricted their clinical use when used as single agents. The aim of the present study was to investigate the synergistic activity of trichostatin A (TSA) and PS-341 in ovarian cancer cells, along with the investigation of the molecular mechanisms of taxane resistance. The taxane-sensitive ovarian cancer A2780 cell line and its resistant variant, A2780T, were treated with taxane, TSA and PS-341 at various concentrations. An Annexin V assay was performed to determine the levels of cell viability and apoptosis, while flow cytometry and immunofluorescence staining for the mitotic phase-specific protein phosphorylated-histone H3 (Ser10) were used for cell cycle detection. The effects of combined TSA and PS-341 on cell cycle-associated proteins were tested by western blot analysis. Furthermore, the present study examined the apoptosis and cell cycle arrest induced by the 3 agents subsequent to overexpression or downregulation of cyclin B1 in A2780 and A2780T cells, respectively. It was found that TSA interacted synergistically with PS-341, resulting in a marked increase in apoptosis and the rate of G<sub>2</sub>/M arrest in A2780T cells. A lower

basal level of cyclin B1 expression and the incompetence of the upregulation of the cyclin may explain the taxane resistance found in A2780T cells. Collectively, the combination of TSA and PS-341 increased cyclin B1 expression level regardless of the basal expression level, resulting in the proliferation inhibition and apoptosis in A2780 and A2780T cells, which raised the possibility that a combination of the two drugs may represent a novel strategy for the treatment of ovarian cancer, particularly in taxane-resistant ovarian cancer.

## Introduction

Despite numerous therapeutic advances, epithelial ovarian cancer remains the most lethal type of gynecologic malignancy, as the 5-year survival rate is <25% for patients who are diagnosed with stage III-IV disease (1). Cytoreductive surgery followed by the administration of systemic chemotherapy remains an important treatment for advanced stage ovarian cancer. A combination of platinum- and taxane-based chemotherapy is recommended as the therapy subsequent to surgery for at least 70% of patients with ovarian cancer. However, the initial response to chemotherapy is not permanent, and the tumors become resistant (2). Novel treatment strategies are therefore required.

The targeting of histone deacetylase (HDAC) activity using pharmacological small molecule HDAC inhibitors (HDACi) has become a notable therapeutic strategy (3). HDACi cause changes in the acetylation status of chromatin and other non-histone proteins, resulting in changes in gene expression, the induction of apoptosis, cell cycle arrest and the inhibition of angiogenesis and metastasis (4). Currently, 7 structurally distinct classes of HDACi are known, and inhibitors of 4 different classes are now in clinical development (5). Vorinostat, a broad spectrum HDACi, which inhibits zinc-dependent HDAC (Class I, II and IV), was the first HDACi approved by the United States Food and Drug Administration (FDA) and has been demonstrated to be successful in the treatment of refractory cutaneous T cell lymphoma (6). Trichostatin A (TSA), 1 of the most extensively studied HDACi, has been shown to inhibit cell proliferation

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and induce apoptosis in ovarian cancer cells in preclinical studies. This raises the possibility that TSA may serve a role in the treatment of ovarian cancer (4,7).

Proteasome inhibitors are a novel group of therapeutic agents that are designed to restrict the degradation of proteins through the inhibition of proteasome activity. The inhibitors constrain cancer progression through the recovery of key protein functions in the regulation of apoptosis, cell cycle progression and angiogenesis (8). The anti-tumor effect of PS-341 (bortezomib/Velcade), the first FDA-approved proteasome inhibitor for the treatment of multiple myeloma and mantle cell lymphoma, has been extended to numerous other types of malignancy through the preferential induction of toxicity and cell death in tumor cells (9). In addition, the inhibitor is being evaluated for the treatment of solid tumors, including ovarian cancer (10). PS-341 exhibits a wide range of molecular effects, including the stabilization of cell cycle regulation proteins, the inhibition of nuclear factor- $\kappa$ B activation, the induction of apoptosis and the counterbalance of B-cell lymphoma 2 resistance and angiogenesis (11). Despite being the most widely used proteasome inhibitor, and the first to have been identified, the efficacy of PS-341 is limited when it is used as a single agent. The present study considers whether the anticancer effect of PS-341 is enhanced when combined with other therapeutic agents, such as TSA.

A growing number of studies in the present field provide evidence to support the hypothesis that the combined treatment of proteasome inhibitors and HDACi in malignancies exhibits a synergistic effect, including in the treatment of human multiple myeloma, recurrent glioblastoma, and renal, pancreatic and hepatocellular cancer (12-15). A previous study demonstrated that TSA significantly improved PS-341-mediated inhibition of head and neck squamous cell carcinoma both *in vitro* and *in vivo* (16). The present study examined the efficacy of the combination of HDAC inhibition by TSA, and proteasome inhibition by PS-341, in ovarian cancer cell lines *in vitro* with respect to their potential synergistic effect on levels of apoptosis and cell cycle arrest. The present study also sought to investigate the molecular mechanism of taxane resistance and the synergistic activity of TSA and PS-341 by the assessment of downstream effector pathways, to provide supporting data for a mechanism-based regimen for the treatment of ovarian cancer, particularly for taxane-resistant ovarian cancer.

## Materials and methods

**Cell lines and cell culture.** The taxane-sensitive ovarian cancer A2780 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) (17). The taxane resistant variant ovarian cancer A2780T cell line was donated from the Biological Sciences Collegiate Division of Huazhong University of Science and Technology (Wuhan, China), and was cultured in RPMI-1640 medium supplemented with 10% FBS and 80 nM taxane (Bristol-Myers Squibb Caribbean Company, New York, NY, USA) at 37°C with 5% CO<sub>2</sub>. For time-course study of cyclin B1 expression, cells were trypsinized and transferred to a new 6-well plate. After 24 h of attachment, the culture medium was

replaced for fresh RPMI-1640 supplemented with 10% FBS, containing DMSO or 1  $\mu$ M taxane. Cells were harvested at the indicated time points and analyzed by western blot analysis.

**Chemicals and antibodies.** TSA was purchased from Sigma-Aldrich (Merck Millipore, St. Louis, MO, USA), and the specific proteasome inhibitor PS-341 was purchased from Millenium Pharmaceuticals (Takeda Pharmaceutical Company, Ltd., Cambridge, MA, USA). The stock solutions of TSA and PS-341 were reconstituted in dimethylsulfoxide (DMSO) at a concentration of 1 mM, stored at -20°C and diluted into the complete cell culture medium prior to use. Taxane was purchased from Bristol-Myers Squibb Caribbean Company (New York, NY, USA). Propidium iodide and DMSO were purchased from Sigma-Aldrich (Merck Millipore). Antibodies were obtained from the following commercial sources: Anti-cyclin A (cat. no., 611268; dilution, 1:500), anti-cyclin B1 (cat. no., 554179; dilution, 1:500), anti-cyclin D (cat. no., 554181; dilution, 1:500), anti-CDK1 (cat. no., 610037; dilution, 1:1,000), anti-CDK2 (cat. no., 610146; dilution, 1:1,000), anti-Rb (cat. no., 554145; dilution, 1:500) and anti-MAD2 (cat. no., 610679; dilution, 1:1,000) were obtained from BD Pharmingen (San Diego, CA, USA); anti-BUB1 (cat. no., sc-28257; dilution, 1:500), anti-phosphorylated-histone H3 (H3P; Ser10; cat. no., sc-8656-R; dilution, 1:1000) and anti- $\beta$ -actin (cat. no., sc-7210; dilution, 1:1,000) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

**Flow cytometry.** All experiments were performed in triplicate. A total of 1x10<sup>6</sup> cells were plated in 6-well plates. Subsequent to 24 h treatment with the chemotherapy drugs (1  $\mu$ M taxane, 500 nM TSA and 40 nM PS-341, individually or in combination) or DMSO as control, the cells were washed twice with PBS, stained with 5  $\mu$ l Annexin V-Phycoerythrin (PE) and 5  $\mu$ l 7-amino-actinomycin D (5  $\mu$ g/ml) in 1X binding buffer following the protocol of the manufacturer (Nanjing Keygen Biotech, Co., Ltd, Nanjing, China) and analyzed using fluorescence-activated cell sorting (FACS) analysis. With regard to cell cycle detection, the cells were harvested and washed twice prior to being fixed in ice-cold 70% ethanol and stored at -20°C overnight. The cells were then washed once in PBS and resuspended in a solution of 5 mg/ml propidium iodide and 0.5 mg/ml RNase A (Thermo Fisher Scientific, Inc.) in PBS for 30 min in the dark prior to being sorted by FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). The results were analyzed with Cell Quest version 3.3 software (BD Biosciences).

**Protein extraction and western blot analysis.** Subsequent to incubation, the cells were harvested and lysed in an ice-cold SDS lysis buffer (Beyotime Institute of Technology, Shanghai, China) for 30 min. The lysates were centrifuged at 12,000 x g at 4°C for 15 min, and the supernatant was denatured in SDS sample buffer at 100°C for 8 min, then stored at -20°C. Equal amounts of protein were separated by Tricine-SDS-PAGE and transferred to nitrocellulose membranes (18). The membrane was blocked in TBS with Tween 20 with 5% non-fat milk for 1 h at 37°C, then incubated with primary antibodies targeting genes investigated in the experiment (e.g., cyclin A, B1 and D) at 4°C overnight followed by incubation with the appropriate secondary antibodies against the species of the primary

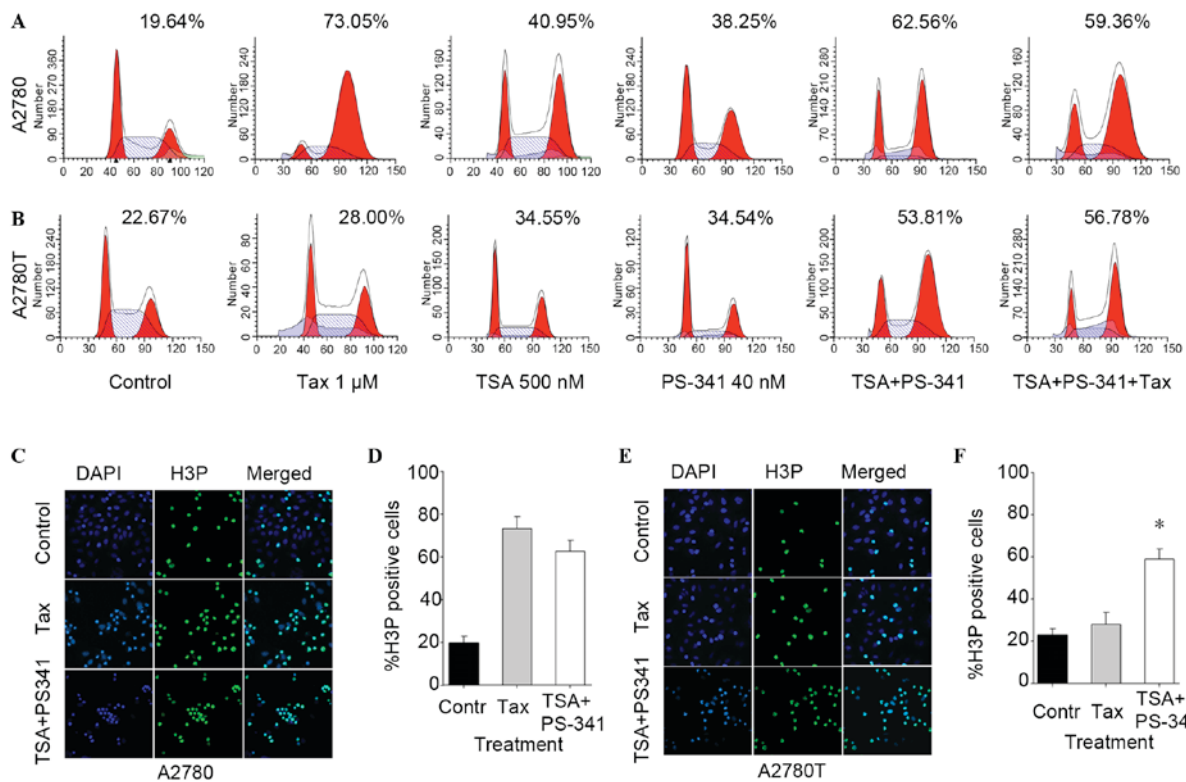


Figure 1. Induction of G<sub>2</sub>/M arrest in taxane-resistant ovarian cancer cell lines by TSA and PS-341. Ovarian cancer (A) A2780 and (B) A2780T cell lines were treated as indicated for 24 h, and G<sub>2</sub>/M arrest was detected by flow cytometry. Each data point represents the mean percentage of cells arrested in G<sub>2</sub>/M phase of 3 replicates. (C) Immunofluorescence staining of mitotic-phase specific protein H3P was performed subsequent to the treatment of A2780 cells with Tax or TSA+PS-341 for 24 h. Representative images are shown in figure C (magnification, x200) and (D) the histogram data indicate the mean  $\pm$  standard deviation of percentages of H3P positive cells of 3 independent experiments. The aforementioned treatments were performed in A2780T cells prior to the immunofluorescence staining of H3P. (E) Representative images are shown (magnification, x200) and (F) the percentages of H3P positive cells were counted for each treatment. Cells treated with the combination of TSA and PS-341 vs. taxane alone. \*P<0.05. Tax, taxane alone; TSA, trichostatin A; H3P, phosphorylated-histone H3.

antibody [alkaline phosphatase labeled goat anti-mouse IgG (cat. no., A0258; dilution, 1:1,000), alkaline phosphatase labeled goat anti-rabbit IgG (cat. no., A0239; dilution, 1:1,000; Beyotime Institute of Technology)] at 37°C for 1 h. The proteins were subsequently visualized with the BCIP/NBT Alkaline Phosphatase Color Development kit following the manufacturer's protocol (cat. no., C3206; Beyotime Institute of Technology) which included a nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt/buffer.  $\beta$ -actin was used as an internal reference.

#### Overexpression and knockdown of cyclin B1 by transfection.

The full-length coding sequence of the human cyclin B1 plasmid with a green fluorescent protein (GFP) tag (chloroxylenol/cyclin B1-GFP; lot no., 26061) was purchased from Addgene, Inc. (Cambridge, MA, USA). In total,  $1.5 \times 10^5$  ovarian cancer cells were plated per well in a 6-well plate on day 0. On day 1, the A2780 cells were transfected with 2  $\mu$ g plasmid in Opti-minimal essential medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). On day 2, the cells were treated with taxane alone or combined TSA and PS341 for an additional 24 h. The small interfering RNA (siRNA) corresponding to positions 776-796 of cyclin B1 (5'-CUCGUACAGCCUUGGAGACAtt-3') and the control siRNA targeting GFP were ordered from Invitrogen

(Thermo Fisher Scientific, Inc.). The A2780T cells were transfected with siRNA using Lipofectamine 2000 according to the protocol of the manufacturer (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 24 h subsequent to transfection, the cells were treated with taxane or combined TSA and PS341 for an additional 24 h.

**Immunofluorescence assay.** The confluent cells grown on glass cover-slips were rinsed once with PBS, fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton-X in PBS for 5 min at room temperature. The cells were incubated with the primary antibody phospho-histone H3 (H3P; Ser10) at a dilution of 1:100 or PBS overnight at 4°C, followed by incubation with the fluorescein isothiocyanate-conjugated secondary antibody at 37°C for 1 h (cat. no., A0562; dilution, 1:200; Beyotime Institute of Technology). All controls were incubated with PBS. The nuclei were stained with DAPI (Millipore Sigma, St. Louis, Missouri, USA). All immunofluorescence images were captured with a Nikon Eclipse 80i Microscope (Nikon Corporation, Tokyo, Japan). With respect to drugs treatment, the cells culture medium were replaced for fresh containing 1  $\mu$ M taxane alone, 500 nM TSA combined with 40 nM PS-341, or equal volume percentage of DMSO as control. Each treatment lasted 24 h and was stopped by rinsing the cells at 3 time points with cold PBS.



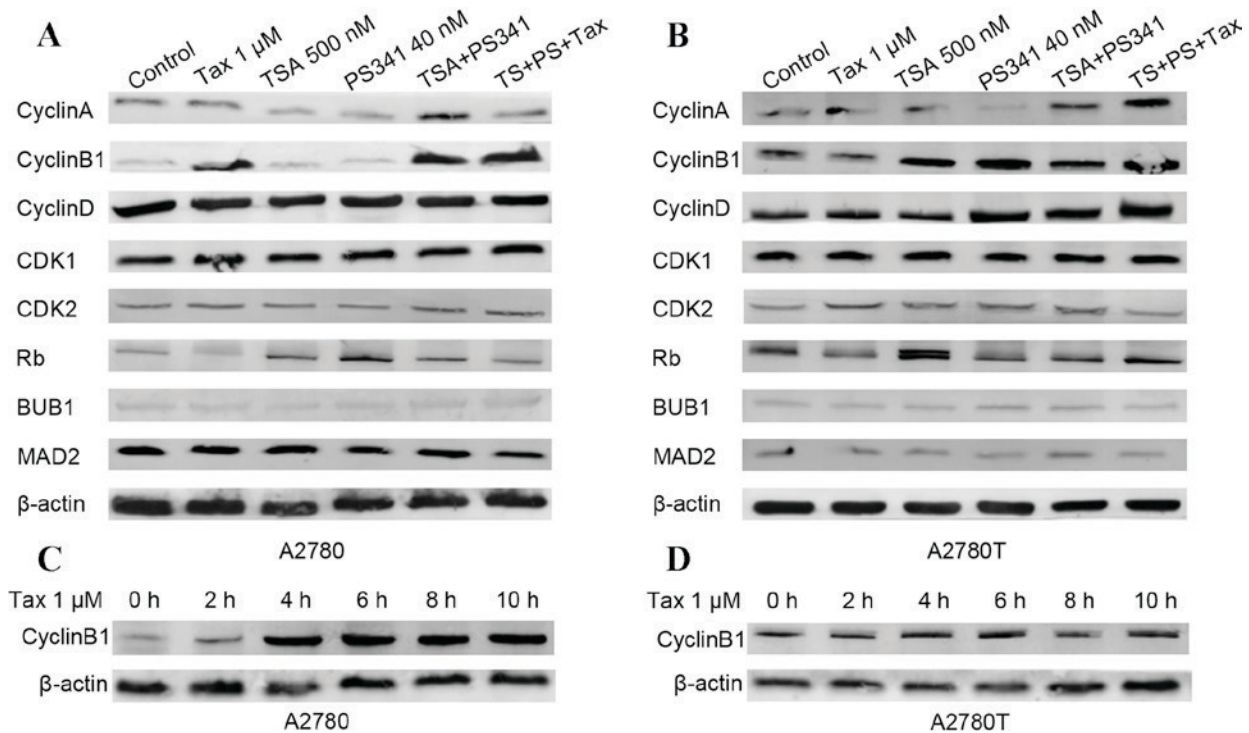


Figure 2. Effect of taxane and TSA in combination with PS-341 on the expression of cell cycle-associated proteins in ovarian cancer cells. (A) A2780 and (B) A2780T cells were incubated with chemical therapeutic drugs as indicated for 24 h, then western blot analysis was used to assess the expression level of cell cycle-associated proteins. (C) A2780 and (D) A2780T cells were incubated with 1  $\mu$ M taxane for the indicated time points and cyclin B1 expression was tested by western blot analysis. Tax, taxane alone; TSA, trichostatin.

**Colony formation assay.** Cell proliferation was determined by colony formation assay according to a published protocol (17). The cells were seeded at 200 cells per well in 6-well plates and treated with DMSO as control, taxane alone or TSA combined with PS-341 for 3 h. Subsequent to 10–14 days, colonies were fixed in 4% paraformaldehyde for 20 min and stained with 0.5% crystal violet in distilled water for 15 min at room temperature. The cells were then rinsed with tap water and allowed to air dry. The number of colonies formed was counted with a charge-coupled device camera at  $\times 4$  magnification on a DM-IRB inverted microscope (Leitz, Wetzlar, Germany).

**Statistical analysis.** The data are presented as the mean  $\pm$  standard deviation of at least 3 separate experiments. Comparisons between 2 groups were analyzed using a Student's *t*-test.  $P < 0.05$  was considered to indicate a statistically significant difference in all experiments.

## Results

**TSA interacts synergistically with PS-341 to induce  $G_2/M$  arrest in taxane-resistant ovarian cancer cells.** In order to explore the combined effect of TSA and PS-341 on the taxane-resistant A2780T cell line, the present study treated the taxane sensitive and resistant cell lines with combined 500 nM TSA and 40 nM PS-341, or with each as a single agent.

Furthermore, the present study compared the synergistic effect of TSA and PS-341 with the effect induced by taxane alone in ovarian cancer cells. The results of the flow cytometric analysis showed that treatment with TSA and PS-341 induced  $G_2/M$  arrest in the 2 ovarian cancer cell lines while taxane

treatment induced  $G_2/M$  arrest in A2780 but not A2780T cells (Fig. 1A and B). Compared with the percentage cell cycle arrest induced by TSA or PS-341 alone, a greater percentage of A2780 and A2780T cells arrested in the  $G_2/M$  phase was observed subsequent to the cells being treated with combined TSA and PS-341.

The expression of the mitotic phase-specific protein H3P (Ser10) was examined by immunofluorescence staining subsequent to the aforementioned treatment. A similar pattern of change was observed in A2780 and A2780T cells subsequent to TSA and PS-341 treatment (figures not shown). Consistent with the cell cycle arrest results, the combination of TSA and PS341 increased H3P (Ser10) expression in A2780T cells compared with taxane, which was not observed in A2780 cells (Fig. 1C–F). The increase of H3P (Ser10) indicated that the combination of TSA and PS-341 demonstrated synergistic effects in the induction of  $G_2/M$  arrest, particularly mitotic phase arrest in taxane-resistant ovarian cancer cells.

**Combination of TSA and PS-341 resulted in enhanced expression of cyclin B1 in taxane-sensitive and taxane-resistant cell lines.** Western blot analysis was subsequently used to assess the effect of the combination of TSA and PS-341 on the expression of various cell cycle-associated proteins in taxane-sensitive and taxane-resistant cell lines. As shown in Fig. 2A, the treatment of cells with taxane alone and the combination of TSA and PS-341 induced a marked increase in the expression of cyclin B1 protein in the A2780 cells. However, the upregulation of cyclin B1 expression was only detected subsequent to treatment with TSA and PS-341 in A2780T cells, not subsequent to taxane treatment alone, as shown in Fig. 2B. The difference in

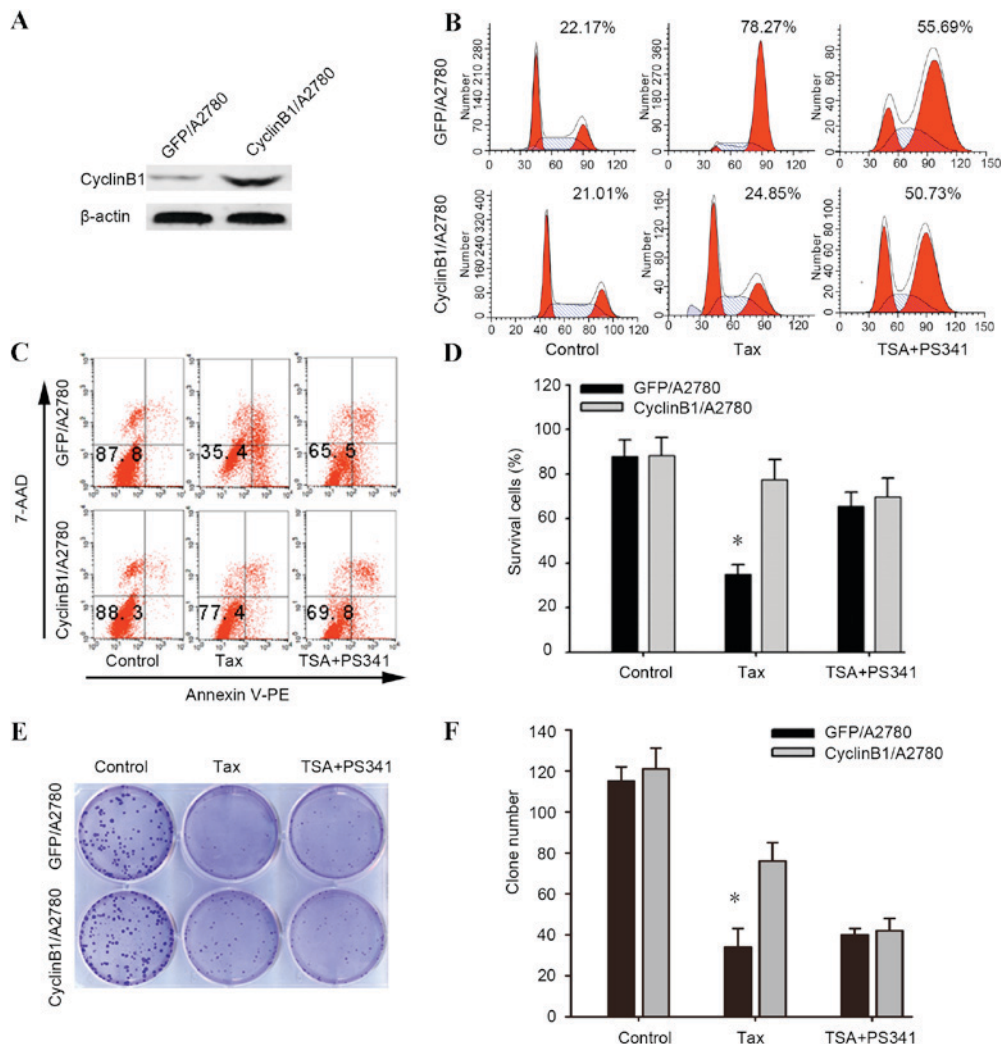


Figure 3. Drug resistance induced by the overexpression of cyclin B1 in A2780 cells was reversed by the synergistic effect of TSA and PS-341. (A) Expression level of cyclin B1 was detected by western blot analysis in A2780 cells subsequent to transfection with pCMX/cyclin B1-GFP plasmid or the control plasmid pCMX-GFP. (B) Cyclin B1-overexpressing A2780 and control cells were treated with 1  $\mu$ M taxane alone or 500 nM TSA plus 40 nM PS-341, and the cell cycle was analyzed by flow cytometry. The graphs represent the typical results of the findings with respect to G<sub>2</sub>/M arrest. (C) Cyclin B1-overexpressing A2780 and control cells were treated as described in (B), and apoptosis was analyzed by flow cytometry. (D) The graphs represent the results of cell apoptosis. The survival values represent the mean  $\pm$  SD of 3 replicates. Cyclin B1-overexpression A2780 cells treated with taxane vs. the control plasmid-transfected cells with the same treatment. Cyclin B1-overexpressed A2780 and control cells were treated with 1  $\mu$ M taxane, or with the combination of 500 nM TSA and 40 nM PS-341 for 3 h, then the cells were cultured in fresh culture medium for an additional 14 days. (E) Image representing the results of the colony formation assay. (F) Histogram data represents the mean  $\pm$  SD of triplicate results. Cyclin B1-overexpression A2780 cells vs. the controls of the same treatment with taxane. \*P<0.05. SD, standard deviation; Tax, taxane alone; TSA, trichostatin A; GFP, green fluorescent protein.

cyclin B1 expression in response to taxane treatment indicated that cyclin B1 may be involved in taxane resistance. Consistent with the previous result, the expression of cyclin B1 in A2780 cells increased in a time-dependent manner subsequent to treatment (Fig. 2C), yet no change of cyclin B1 protein was observed in the A2780T cells (Fig. 2D). In addition, the basal expression level of cyclin B1 in the A2780T cells was higher compared with the level in the A2780 cells. Collectively, the findings of the western blot analysis indicated that the induction of G<sub>2</sub>/M arrest by taxane in A2780 cells primarily depends on the increased expression of cyclin B1.

*Overexpression of cyclin B1 resisted the effect of taxane in A2780 cells but did not affect the synergistic effect of TSA and PS-341.* In order to investigate the biological consequence of the high basal expression of cyclin B1 and the upregulation of

cyclin B1 in response to taxane or to the coadministration of TSA and PS-341, the present study overexpressed cyclin B1 in the taxane-sensitive A2780 cell line via plasmid transfection. Firstly, the result of the western blot analysis confirmed that plasmid transfection increased the level of cyclin B1 protein expression (Fig. 3A). The transfected cells were then treated with 1  $\mu$ M taxane or the combination of 500 nM TSA and 40 nM PS-341 for 24 h.

The percentage cyclin B1-overexpressing cells in G<sub>2</sub>/M arrest subsequent to treatment with taxane was detected to be 24.85% by flow cytometry, while that of the control GFP-transfected cells was 78.27%. No significant difference was observed between the 2 groups of transfected cells subsequent to treatment with a combination of 500 nM TSA and 40 nM PS-341 (Fig. 3B) (P=0.378). The flow cytometry analysis with respect to apoptosis revealed that cyclin B1-transfected cells

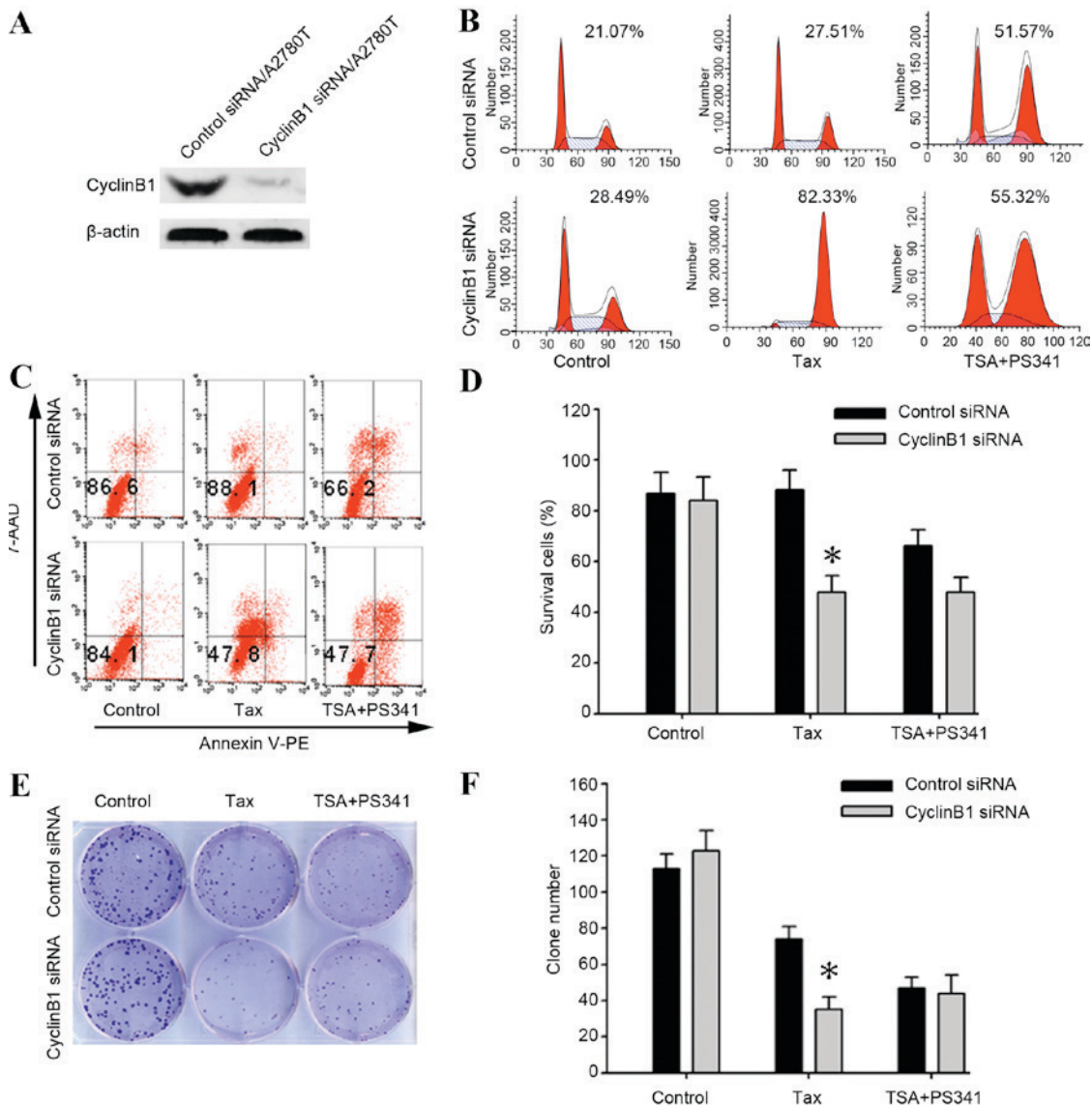


Figure 4. Knockdown of cyclin B1 expression reversed the resistance to taxane in A2780T cells and enhanced the synergistic effect induced by TSA and PS-341. (A) Expression of cyclin B1 in A2780T cells subsequent to transfection with cyclin B1 siRNA or non-targeting siRNA. (B) Following siRNA transfection, A2780T cells were treated with 1  $\mu$ M taxane alone, or 500 nM TSA plus 40 nM PS-341 for 24 h and cell cycle status was analyzed by flow cytometry. (C) A2780T cells were treated as in (B) and apoptosis was analyzed by flow cytometry. (D) The data shown are representative of 3 independent experiments. The cyclin B1 knock-down group vs. the control with the treatment of taxane or the combination of TSA and PS-341. Subsequent to the knockdown of cyclin B1, A2780T cells were treated with 1  $\mu$ M taxane, or a combination of 500 nM TSA and 40 nM PS-341 for 3 h, and a colony formation assay was used to detect the cell survival fraction. (E) The images shown represent the results of the colony formation assay. (F) The clone number represents the mean  $\pm$  standard deviation of the triplicate results of the cells with the cyclin B1 siRNA transfection vs. those with the non-targeting siRNA transfection. \* $P$ <0.05. siRNA, small interfering RNA; TSA, trichostatin A; 7-AAD, 7-amino-actinomycin D; Tax, taxane alone.

resulted in  $77.4 \pm 2.6\%$  Annexin V-PE and 7-amino-actinomycin (AA) D-negative cells subsequent to treatment of taxane. In contrast, the control GFP-transfected cells, treated with taxane, resulted in  $35.4 \pm 1.4\%$  annexin V-PE and 7-AAD-negative cells (Fig. 3C-D). Similar results were found in the colony formation assay (Fig. 3E-F). The aforementioned results indicated that the overexpression of cyclin B1 in A2780 cells enabled the cells to override the G<sub>2</sub> DNA damage checkpoint, thus reducing the anticancer effect of taxane. The effect of combined TSA and PS-341 was not affected by the upregulation of cyclin B1.

*Knockdown of cyclin B1 expression reversed the resistance to taxane in A2780T cells and enhanced the synergistic effect induced by TSA and PS-341.* To additionally explore

the role of cyclin B1 in taxane resistance, the present study designed synthetic siRNA in order to silence cyclin B1 expression. The results of western blot analysis confirmed that the cyclin B1 siRNA suppressed the cyclin B1 protein expression in taxane-resistant A2780T cells (Fig. 4A). The flow cytometry results revealed that 24 h exposure of the cyclin B1-siRNA transfected A2780T cells to 1  $\mu$ M taxane resulted in  $82.33 \pm 2.8\%$  cells arrested in the G<sub>2</sub>/M phase and  $47.8 \pm 2.2\%$  Annexin V-PE and 7-AAD-negative cells. By contrast, the aforementioned treatment of control cells resulted in  $27.51 \pm 2.6\%$  cells arrested in the G<sub>2</sub>/M phase and  $88.1 \pm 2.1\%$  Annexin V-PE and 7-AAD-negative cells (Fig. 4B-D). For proliferation investigation, the siRNA transfected cells were subsequently treated with 1  $\mu$ M taxane or a combination of



500 nM TSA and 40 nM PS-341 for 3 h. The surviving fraction of cells were detected by crystal violet staining. It was revealed that the clone number was significantly reduced subsequent to treatment with taxane in the cyclin B1 siRNA-transfected cells compared with the negative scrambled siRNA-transfected cells ( $P=0.011$ ). By contrast, similar clone numbers were observed in the negative scrambled siRNA- and cyclin B1 siRNA-transfected cells subsequent to treatment with TSA plus PS-341 ( $P=0.133$ ; Fig. 4E and F).

## Discussion

Encouraging *in vitro* and *in vivo* results have resulted in several clinical trials with HDAC or proteasome inhibitors alone. Although the efficacy of the inhibitors is limited when used as single agent therapies, previous studies have shown that the coadministration of a HDAC and proteasome inhibitor may exert greater therapeutic efficacy in numerous tumor cell lines and patients with lymphoma (19,20). Additionally, it was reported that the combined molecular targeting of HDAC and proteasomes synergistically induced apoptosis in non-small cell lung cancer (21). Another study demonstrated that PS-341 increased paclitaxel sensitivity in ovarian cancer cells (22). Thus, the present authors hypothesized that the HDACi TSA and the proteasome inhibitor PS-341 in combination may overcome taxane resistance and induce cell cycle arrest and apoptosis in taxane-resistant ovarian cancer cells. Indeed, the present results indicated that the combination of TSA and PS-341 resulted in the synergistic effect of  $G_2/M$  arrest in the taxane-sensitive and the taxane-resistant ovarian cancer cell lines (Fig. 1).

Taxane blocks the progression of the cell cycle through interference with the assembly and dynamics of microtubule spindles, thus preventing their attachment to kinetochores (23). The therapeutic efficacy of taxane depends on whether or not it can induce persistent cell cycle arrest and apoptosis. The molecular mechanism that underlies the regulation of the cell cycle and apoptosis, and that determines susceptibility to taxane in tumor cells, is not presently known. In addition, the molecular targets by which TSA and PS-341 induce  $G_2/M$  arrest and inhibit tumor cell proliferation is not fully understood.

Uncontrolled cell proliferation, which is associated with the loss of proper cell cycle control, is a prominent feature of ovarian cancer. The cell cycle is controlled by a highly conserved family of cyclin-dependent kinases (Cdks) and their regulatory subunits, known as cyclins. A number of studies have reported that several Cdks and cyclins are involved in the prognosis of various types of cancer (24-26). The present study found that the most marked difference between the response of A2780 and A2780T cells to taxane was the protein expression level of cyclin B1 (Fig. 2). The present study also found that the baseline expression level of cyclin B1 cells was higher in A2780T compared with A2780 cells that exhibit more sensitivity to taxane treatment. Cyclin B1 is indispensable for the transition between the  $G_2$  phase and mitosis, and the upregulation of the protein is closely associated with a poor prognosis in various types of cancer (27,28). Additionally, the overexpression of cyclin B1 is involved in resistance to radiotherapy in pancreatic cancer (29). Nuclear cyclin B1-positive types of breast carcinoma are resistant to adjuvant therapy (26). A

previous clinical study indicated that chemotherapy resistance in non-small cell lung cancer may also be enhanced with the increased expression of cyclin B1, and cyclin B1 inhibitors may increase the efficacy of chemotherapy (30). In addition, previous studies suggest that any blockade of the exit from mitosis, such as degradation-resistant cyclin B1 expression, exerts a lethal effect on cancer cells (31,32). Consistent with the aforementioned findings, the knockdown of cyclin B1 expression in A2780T cells increased the level of cancer cell apoptosis and reversed the resistance to taxane in the present study (Fig. 4).

It is also noteworthy that the combination of TSA and PS-341 induced a marked increase in the expression level of cyclin B1 in A2780 and A2780T cells, regardless of the basal expression level of cyclin B1 (Fig. 2). Additionally, the combination of TSA and PS341 generated a synergistic effect in terms of inducing  $G_2/M$  arrest and inhibiting cell proliferation in A2780 cells subsequent to the overexpression of cyclin B1 (Fig. 3), which indicates that the molecular mechanism underlining the synergistic effect of TSA and PS-341 differs from the mechanism of taxane.

In summary, the present study revealed that TSA and PS-341 synergistically enhanced the level of  $G_2/M$  arrest and the cytotoxic potential in ovarian cancer cells. An additional benefit of the combination of TSA and PS-341 is the potential inhibition of the growth of taxane-resistant ovarian cancer, which would increase the overall therapeutic effect. The present results regarding the synergistic efficacy of TSA and PS-341 exposes a novel perspective in terms of the therapeutic strategy for the treatment of ovarian cancer, and potentially other solid tumors.

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