Synergistic Efficacy in Human Ovarian Cancer Cells by Histone Deacetylase Inhibitor TSA and Proteasome Inhibitor PS-341

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Histone deacetylase inhibitors and proteasome inhibitor are all emerging as new classes of anticancer agents. We chose TSA and PS-341 to identify whether they have a synergistic efficacy on human ovarian cancer cells. After incubated with 500 nM TSA or/and 40 nM PS-341, we found that combined groups resulted in a striking increase of apoptosis and G2/M blocking rates, no matter in A2780, cisplatin-sensitive ovarian cancer cell line OV2008 or its resistant variant C13’. This demonstrated that TSA interacted synergistically with PS-341, which raised the possibility that combined the two drugs may represent a novel strategy in ovarian cancer.

Keywords: Histone deacetylase inhibitor; Proteasome inhibitor; Ovarian cancer; p21

INTRODUCTION

Despite advances in therapy, ovarian cancer remains the most lethal of the gynaecological cancers. The current standard therapy for patients with advanced ovarian cancer is cytoreductive surgery followed by the administration of systematic chemotherapy. At least 70% of ovarian cancers will respond to a combination of platinum- and taxane-based chemotherapy administered after surgery; unfortunately, the initial response is not durable and tumors become resistant (1). We ask whether we can use drugs whose mechanisms of action are different from those of Cisplatin or Paclitaxel to treat ovarian cancer.

Histone deacetylase inhibitors (HDACIs) are now emerging as a new class of anticancer agents with potent activity in a wide spectrum of tumors. They cause the accumulation of acetylated histones and other proteins regulating chromatin structure and transcription, thereby altering the transactivation and expression of specific genes that regulate cell growth arrest, differentiation, or apoptosis (2). Up to now, at least 12 HDACIs are under evaluation in over 100 clinical trials and have produced encouraging therapeutic responses with surprisingly good safety profiles (3, 4). Vorinostat, a histone deacetylase inhibitor, was recently approved by the US Food and Drug Administration for treatment of cutaneous T-cell lymphoma (5). TSA, one of the most extensively studied HDACIs, could inhibit cell proliferation and induced apoptosis in ovarian cancer cells in preclinical studies, raising the possibility that it may have a role to play in ovarian cancer treatment (6).

Proteasome inhibitors represent a diverse group of agents that target the 20S proteasome, a component of the ubiquitin-proteasome complex that is responsible for the degradation of unwanted cellular proteins (7). PS-341 is a typically small molecule inhibitor of the ubiquitin-proteasome pathway. Proteasome plays an essential role in the degradation of most intracellular proteins, including those that regulate the cell cycle, cell survival and apoptosis, cell adhesion and trafficking, and transcription factor activation (8, 9). Furthermore, PS-341 has been reported to alter the levels of p21, p27, Bcl-2, Bax, XIAP, survivin, and p53, leading to cell cycle arrest and apoptosis in several tumor types (10). PS-341 recently received Food and Drug Administration (FDA) approval for the treatment of multiple myeloma (11), and it is currently being evaluated for the treatment of solid tumors, including ovarian cancer (12).

A number of investigators have examined that combined with HDACIs and proteasome inhibitors did exert a synergistic effect in some malignant tumors, such as human multiple myeloma, non-small cell lung cancer, and head and neck squamous cell carcinomas (13–15). Such reports showed that coadministration of HDIs with proteasome inhibitors could result in a synergistic increase in mitochondrial injury, caspase activation, and apoptosis in association with multiple perturbations in signal transduction pathways, including inactivation of NF-κB signaling, downregulation of Bcl-XL, and disruption of MAP kinase pathway. Because of most ovarian cancer patients would typically experience resistance to cytotoxic drugs, whether similar interaction might occur in ovarian cancer cells has not been reported so far. Here we report that combined exposure of ovarian cancer cells to TSA and PS-341 resulted in a synergistic increase of apoptosis and G2/M blocking rates.
and PS-341 results in a synergistic increase in release of pro-apoptotic cytochrome c into the cytosol, activation of caspase, and blocking cells in G2/M phase, as well as inhibits AKT/mTOR pathway. Moreover, similar synergistic interactions occur both in cisplatin-sensitive ovarian cancer cell line OV2008 and its resistant variant C13*. Together, these findings suggest that a therapeutic strategy combining TSA and PS-341 should also be considered in ovarian cancer.

**MATERIALS AND METHODS**

**Cell line and cell culture**

A cisplatin-sensitive ovarian cancer cell line (OV2008) and its resistant variant (C13*) were gifts from Dr. Rakesh Goel from the Ottawa Regional Cancer Center, Ottawa, Canada. A2780 ovarian cancer cell line was obtained from the American Type Culture Collection (Manassas, VA), and cells were all grown in RPMI 1640 supplemented with 10% FBS (16).

**Chemicals and antibodies**

Trichostatin A (TSA) was purchased from Sigma-Aldrich (St. Louis, MO) and the specific proteasome inhibitor PS-341 was purchased from Millenium Pharmaceuticals (Cambridge, MA). The stock solution for TSA and PS-341 was reconstituted in dimethyl sulfoxide (DMSO) at a concentration of 1 mM, stored at −20°C, and diluted into complete cell culture medium before use. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT), propidium iodide (PI), and 5-bromo-2′-deoxyuridine (BrdU) were purchased from Sigma Chemical Corporation, USA. Antibodies were obtained from the following commercial sources: anti-XIAP, anti-caspase-3, anti-caspase-9, anti-cytochrome c, anti-phospho-akt (Ser473), and anti-phospho-4EBP1 (Thr37/46) were obtained from Cell Signaling Technology (Beverly, MA); anti-bax and anti-caspase-8 were purchased from Newmarkers; and anti-Mcl-1, anti-p21 and anti-β-actin were obtained from Santa Cruz Biotechnology.

**Cell survival assays with MTT**

Cells (6 × 10^4 per well) were grown in 96-well plates for 24 hr before exposed to different concentrations of the drugs (TSA, PS-341, alone or combination of the two reagents). Cell proliferation and viability were measured daily for up to 3 consecutive days using MTT assay. To each well, 20 ul MTT (5 mg/mL) was added, and the cells were further incubated for 4 hr at 37°C. Finally, the absorbance of the dye was measured spectrophotometrically at 570 nm and 630 nm as a reference wavelength. Each experimental data point represented average values obtained from four replicates, and each experiment was performed in triplicate.

**Flow cytometry**

For detecting apoptosis, 1 × 10^6 cells were washed twice with phosphate-buffered saline (PBS) and stained with 5 µL Annexin-V-fluorescein isothiocyanate and 10 µL PI (5 µg/mL) in 1 × binding buffer (Nanjing Keygen Biotech, Co., Ltd.) for 15 min at room temperature in the dark. For detecting cell cycle, cells were harvested and fixed in ice-cold 70% ethanol and stored at −20°C overnight. Then, these were washed once in PBS and resuspended in a solution containing propidium iodide (5 mg/mL) and RNase A (0.5 mg/mL) in PBS for additional 30 min. Cells were sorted using a FACS (BD Biosciences, USA) and analyzed with CellQuest version 3.3 software. The apoptotic cells were calculated after FACS analysis.

**Protein extraction and western blot**

Cells were incubated with various agents for the indicated times. Thereafter, these were washed twice with PBS and lysed in an ice-cold SDS lysis buffer for 30 min. The lysates were centrifuged at 12,000 × g at 4°C for 15 min; the supernatant was denatured in SDS sample buffer at 100°C for 8 min and stored at −20°C. Equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membrane was blocked in TBST with 5% non-fat milk for 1 hr at 37°C, and then incubated with the various primary antibodies and secondary antibodies sequence. And then visualized with NBT/BCIP/buffer (1:1:500). Protein loading was assessed by blotting the same membrane with an antibody against β-actin.

**Analysis of cytosolic and mitochondria cytochrome**

Cells were lysed in mitochondria lysis buffer [210 mmol/L mannitol, 70 mmol/L sucrose, 10 mmol/L HEPES (pH 7.4), 1 mmol/L EDTA, and protease inhibitor cocktail] with a Dounce homogenizer and subjected to centrifugation at 1,000 g for 15 min at 4°C. The supernatant was washed once in PBS and then centrifuged to obtain the mitochondria-enriched heavy membrane fraction, and the resulting supernatant was further centrifuged to obtain the cytosolic fraction. For immunoblot analysis, equal cytosolic and mitochondria extract amounts of proteins (50 µg) were subjected to 10% SDS-PAGE. After transferring to nitrocellulose membrane, the membrane was probed by cytochrome c antibody.

**RNA isolation and reverse transcription PCR**

To determine p21 mRNA levels induced by drug treatment, genomic DNA-free total RNA were prepared at indicated time points from control and drug-treated ovarian cancer cells using a TRIzol reagent kit (Invitrogen Life Technologies). Quantitative PCR was performed using the SYBR Green Real Time PCR method. Each sample was tested in triplicate, and relative gene expression was analyzed using the 2^−ΔΔCT method (17), and the results expressed as fold induction compared with the untreated group. PCR was performed with a 5′ sense primer (5′-GCC GGC GTT CGA GTG GTA-3′) and a 3′ antisense primer (5′-GCC GGC GTT TGG AGT GTG A-3′) for p21. To amplify 18s RNA internal control, a 5′ sense primer (5′-AGT CCC CTT TGG ACA CA-3′) and a 3′ antisense primer (5′-GAT CCG AGG GCC TCA CTA AAC-3′) were used.

**RNA interference**

Annealed, purified, and desalted double-stranded Stealth siRNA p21 (CUU CGA CUU UGU CAC CGA G) and control siRNA were ordered from Invitrogen Corporation. Then, 1.5 × 10^6 ovarian cancer cells were plated per well in a 6-well
To determine the rational combination doses of TSA and PS-341, we treated cells with various concentrations of TSA (0 nM, 250 nM, 500 nM, 750 nM, and 1 uM), combined with the corresponding concentrations of PS-341 (0 nM, 20 nM, 40 nM, 60 nM, and 80 nM) using MTT assay. We then chose the lowest effective combination concentration of 500 nM TSA and 40 nM PS-341 to treat the ovarian cancer cells (data not shown). Then, the cells were exposed to 500 nM TSA with 40 nM PS-341 to determine the time-dependent change in survival rate for 12, 24, 36, 48, and 60 hr. Treatment of cells with TSA or PS-341 alone was minimally toxic to the ovarian cancer cells. Such as in OV2008, a cisplatin-sensitive cell line, the survival rate was about 73.7 ± 1.5% and 75.0 ± 3.2%, respectively, after treated with 500 nM TSA or 40 nM PS-341 for 48 hr. However, when they were combined, the survival rate was just about 51.1 ± 2.4% (p < .05). Similar effects were also detected in other ovarial cancer cell lines, A2780 and the cisplatin-resistant variant C13*, suggesting that there is no difference in the synergistic effect no matter whether the ovarian cancer cell was sensitive to cisplatin or not (Figure 1).

**RESULTS**

**TSA interacts synergistically with PS-341 to inhibit cell growth in ovarian cancer cells**

Next, we examined the ability of TSA and PS-341 regimen to induce apoptosis in these cell lines. Cells were incubated with drugs alone or combined for 48 h, and then were evaluated by flow cytometry. As shown in Figure 2, at a dose of 500 nM TSA or 40 nM PS-341, only a modest effect on apoptosis had been observed. However, when the drugs were added together, the Annexin V positive cells increased obviously in all three cell lines and the effect of apoptosis is more significant than single drug treatment (p < .01).

**Coadministration of TSA and PS-341 in ovarian cancer cells resulted in enhanced activation of caspases-3,-8,-9, and release of cytochrome c into the cytosolic fraction**

Western analysis was subsequently used to assess the effects of combining TSA and PS-341 on various apoptosis-associated events in all three cell lines. As shown in Figure 3(a), treatment of cells with TSA, PS-341 alone minimally triggered cleavage activation of caspases 3, 8, and 9, as well as release of cytochrome c from mitochondrial into cytosolic. Coexposure of TSA with PS-341 led to obvious increase in cleaved caspase, and cytosolic release of cytochrome c. Similar results were obtained in all three cell lines, including the cisplatin-resistant variant cell line C13*. Furthermore, we performed western blot to detect the changes of anti-apoptotic proteins and pro-apoptotic proteins (Figure 3b). Administration of TSA and PS-341 individually or in combination (48 hr) exerted minimal effects on the expression of Bcl-2, XIAP, Bcl-xl, or Bax. Interestingly, both treatment of the cells with PS341 alone and coadministration of TSA resulted in an increase in Mcl-1 levels, presumably corresponding to the degradation of Mcl-1 which was blocked by proteasome inhibitors PS341. Thus, these findings indicated that the synergism of TSA and PS-341 inducing apoptosis might depend mainly on stimulus mitochondrial injury and caspase activation, not on changing the expression of Bcl-2 family members in ovarian cancer cells.
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Figure 3. Effects of TSA and PS-341 on apoptosis-related proteins in ovarian cancer cells. (a) OV2008, A2780, and C13∗ cells incubated with 500 nM TSA or/and 40 nM PS-341 for 48 hr. Thereafter, western analysis was used to assess release of cytochrome into S-100 cytosolic fractions, and total cellular extracts were monitored for expression of the cleaved fragment of caspases 3, 8, and 9. (b) Western analysis was also used to assess expression of BCL-2 family members of Bcl-2, Mcl-1, XIAP, Bcl-XL, and Bax.

TSA interacts synergistically with PS-341 to induce G2/M arrest in ovarian cancer cells

Previous reports indicated that cell cycle arrest could also be induced by TSA or PS-341. Flow cytometric analysis showed that all of the three ovarian cancer cell lines treated with TSA and PS-341 for 48 hr were obviously arrested at the G2/M phase, and these effects were much notable than single drug treatment (p < .05) (Figure 4a). Such as in A2780, TSA and PS-341 combination resulted in an obvious increase in the population of cells arrested at G2/M phase (58.25% vs. 19.56% control, p < .05). Similar results were also found in the other two cell lines (cisplatin-sensitive and cisplatin-resistant). Next, we detected several cell cycle regulatory proteins, such as p21, cyclin B1, Bub1, CDK1, and MAD2 (Figure 4b). Although some changes were observed in different cell lines, we noticed coincidence accumulation of p21 in three cell lines. The combination also resulted in a marked inhibition in the activity of AKT/mTOR pathway, as indicated by reduced phosphorylation at AKT Ser473 and 4EBP1 Thr37/46.

Knockdown of p21 expression enhanced the synergism effect induced by TSA and PS-341 combination

To determine the biological consequence of p21 upregulation in response to coadministration of TSA and PS-341, we knocked down p21 expression in cisplatin-resistant variant C13∗ cells with RNA interference. First, western blot and real time PCR analysis indicated that the p21 protein and mRNA expression increased in a time-dependent manner in cells treated with 500 nM TSA and 40 nM PS-341 at the indicated times (Figure 5a). Next, we designed synthetic siRNA for silencing p21 expression. Western blot confirmed that p21 siRNA sequence effectively suppressed the p21 protein level. Then, the transfected cells were treated with 500 nM TSA and 40 nM PS-341 combination for 24 hr, the survival rate detected by MTT assay was 57.3 ± 2.3% compared with the negative scrambled siRNA transfected cells 74.5 ± 1.8% (Figure 5b). At the same time, the flow cytometry analysis on the apoptosis and cell cycle revealed that 24 hr exposure of p21 siRNA transfected C13∗ cells to 500 nM TSA and 40 nM PS-341 combination resulted in 28.63 ± 2.1% annexin V positive cells and 79.04 ± 2.9% G2/M arrest cells, whereas scrambled siRNA transfected cells resulted in 19.92 ± 1.6% annexin V positive cells and 50.53 ± 2.3% G2/M arrest cells (Figure 5c–d). These results indicated that the induced p21 upregulation might play an important role on the TSA and PS-341 combination treatment.

DISCUSSION

Significant advances involving the use of HDACis have reported that some kinds of HDAC inhibitors are active against ovarian cancer cells (18, 19), suggesting a possible role for such agents in the therapeutic armamentarium against this disease. It also has been reported PS-341 had activity against tumor spheroids, and could trigger obvious apoptosis; these studies could be informative with respect to the use of PS-341 as anticancer agents for ovarian cancer (20, 21). Together, these results indicate that coadministration the
In this study, we showed that coadministration of HDAC inhibitor TSA with proteasome inhibitor PS-341 facilitated apoptosis and cell cycle arrest not only in cisplatin-sensitive cell line OV2008 but also in cisplatin-resistant variant cell line C13°. We also demonstrated that there was no difference in apoptosis and cell cycle arrest in the three ovarian cancer cell lines.

The mechanisms involved in the HDAC inhibitor and proteasome inhibitor are complex and differ among different cell types. In our study, we found that the combination of the two drugs induced mitochondrial permeability transition with a subsequent release of pro-apoptotic cytochrome c into the cytosol, resulting in the activation of caspase-9, caspase-8, and caspase-3, accompanied by minimal changes on the expression of Bcl-2, XIAP, Bcl-xl, or Bax. These results suggested that TSA and PS-341 combination inducing apoptosis might mainly depend on stimulus mitochondrial injury and caspase activation, which were similar to other studies (22–24).

To further characterize the growth inhibitory activity of TSA and PS-341 combination, we performed cell cycle analysis. We observed that cells incubated with the two drugs alone or combination were all blocked at the G2/M phase, and the effect was more obvious in combination group than in single-handed group. Prior investigations revealed that HDACi mediate G2/M-phase arrest was a much rarer event than HDACi-induced G1 arrest. And the blocked cells treated with HDACi eventually moved through the defective checkpoint and underwent apoptosis, although the underlying mechanisms responsible for HDACi-mediated G2/M arrest are poorly understood (25). Here we observed that TSA and PS-341 combination could promote p21 accumulation obviously in all three cell lines. However, other cell cycle regulatory proteins, such as cyclin B1, Bub1, CDK1, and MAD2, exerted variable changes in different cell lines. It is well known that p21 is a negative regulator of cell cycle progression and a modulator of apoptosis (26, 27). Some studies have shown that both HDAC inhibitor and proteasome inhibitor could induce p21 expression (28). To examine the potent meanings of p21 upregulation, we used RNA interference to knockdown of p21 expression in C13° cells. The p21 siRNA and negative siRNA transfected cells were treated by 500 nM TSA and 40 nM PS-341 combination for 24 hr, then MTT analysis revealed that the survival rate of p21 siRNA transfected cells was much lower than negative siRNA transfected cells. This suggested that p21 upregulated expression might play a negative role in the TSA and PS-341 synergism. The flow cytometry analysis revealed that knockdown of p21 expression enhanced the synergism effects on apoptosis and G2/M phase arrest. Previous studies have indicated that p21 inhibits apoptosis which might account for its regulation of gene transcription or inhibition of the activity of proteins, including procaspase 3, caspase 8, caspase 10 (29, 30). Paradoxically, p21 might also promote apoptosis depending on both p53-dependent and p53-independent upregulation of the pro-apoptotic protein BAX (31). Exactly how induced p21 upregulation inhibited apoptosis and cell cycle arrest needs further investigation. In addition, combined treatment with TSA and PS-341 also resulted in marked inhibition in the activity of AKT/mTOR pathway, which played a pivotal role in the genesis of diseases including cancer.

In summary, our study provided evidence that the TSA combined with PS-341 potently inhibited the growth of human ovarian cancer cells by inducing apoptosis, accumulation of cells in G2-M phase. Our results regarding the synergistic efficacy of TSA and PS-341 may open new and interesting perspectives in the therapeutic strategy for the treatment of human ovarian cancer.

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DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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