PS-341 and Histone Deacetylase Inhibitor Synergistically Induce Apoptosis in Head and Neck Squamous Cell Carcinoma Cells

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Abstract
Proteasome inhibitor PS-341 (also known as bortezomib) and histone deacetylase (HDAC) inhibitors have emerged as novel therapeutic agents for a variety of malignancies. In this study, we examined whether PS-341 and the HDAC inhibitor trichostatin A (TSA) induced apoptosis in head and neck squamous cell carcinoma (HNSCC), a common and lethal malignancy. We found that, although TSA treatment alone did not induce apoptosis in HNSCC cells, it significantly enhanced PS-341–induced apoptosis in HNSCC cells in vitro. Consistently, TSA significantly improved PS-341–mediated inhibition of HNSCC tumor growth in nude mice. Mechanistically, we found that TSA increased PS-341–induced Noxa expression and caspase activation in HNSCC cells. The knockdown of Noxa significantly reduced apoptosis induced by cotreatment of PS-341 and TSA. Taken together, our results provide new insight into the mechanisms of synergistic antitumor activity of the PS-341 and HDAC inhibitor regimen, offering a new therapeutic strategy for HNSCC patients.

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Introduction
Head and neck squamous cell carcinoma (HNSCC) ranks among the 10 most common cancers worldwide, with more than 780,000 new cases diagnosed each year (1). Despite the latest innovations in basic science and clinical research, the overall survival rate for HNSCC remains low, and it is reported that 25% of HNSCC patients develop a second cancer within 5 years of diagnosis (2, 3). Thus, improvement in conventional therapy is urgently needed to effectively treat HNSCC. Recently, the proteasome inhibitor PS-341, also known as bortezomib or Velcade, has been proposed as an alternative approach for conventional cisplatin-based chemotherapy to overcome cisplatin resistance in head and neck malignancies (4). PS-341 is a dipeptidyl boronic acid derivative that specifically inhibits the function of the 26S proteasome (5). This ability distinguishes PS-341 from other proteasome inhibitors such as MG-132 that also inhibit thiol proteases such as cathepsin B and calpains as well as the 26S proteasome (5, 6). PS-341 potently induces apoptosis in a broad range of human cancer cell lines, including myeloma, prostate and breast cancers, and HNSCC (4, 5, 7). Activation of the antiapoptotic transcription factor NF-κB is dependent on the 26S proteasome. The inhibition of NF-κB by PS-341 has been found to induce apoptosis in several human cancer cells and is considered to be an important target of the PS-341 antitumor effect (4, 5). Very recently, we and others have shown that PS-341 activates the proapoptotic endoplasmic reticulum (ER) stress in numerous human cancer cells, in addition to the inhibition of the prosurvival NF-κB signaling pathway (4–8). We found that PS-341 induced ER stress and subsequently activated a coordinated cellular response, called unfolded protein response, in HNSCC cells (7). PS-341 induced activation of the ER transmembrane stress-sensing kinase PERK and subsequent attenuation of general protein synthesis in HNSCC cells. PS-341 robustly increased protein levels of the proapoptotic transcription factors activating transcription factor 4 (ATF4) and C/EBP homology protein (4, 8). Mechanistically, PS-341 induced the expression of the proapoptotic Bcl-2 homology domain 3 only (BH3-only) protein Noxa through ATF4 in HNSCC cells. The knockdown of Noxa significantly reduced PS-341–mediated apoptosis in HNSCC cells, suggesting that induction of new gene transcription is required for PS-341–mediated apoptosis (4). However, although PS-341 was able to induce apoptosis in HNSCC cell lines, clinical studies showed that the cytotoxicity of PS-341 remains limited when used as a single agent (9).

Recently, histone deacetylase (HDAC) inhibitors such as trichostatin A (TSA) have emerged as novel
therapeutic agents for human cancers (10–16). TSA, a classic potent inhibitor of HDAC, is a hydroxamic acid–derived compound from the metabolic product of streptomycin (10). There are at least 18 HDACs, which are subdivided into four classes: class I (HDAC 1, 2, 3, and 8), class IIa (HDAC 4, 5, 7, and 9), class IIb (HDAC 6 and 10), class III (SIRT 1, 2, 3, 4, 5, 6, and 7), and class IV (HDAC 11; refs. 10, 11). TSA is known to inhibit zinc–dependent deacetylases, including class I, II, and IV HDACs (10). It has been shown that HDACs are highly overexpressed in a variety of human cancers, suppressing transcription of tumor suppressor genes such as p21WAF1/CIP1 through chromatin structure modulation mediated by deacetylating lysine-4 residues of histone-H3 (12, 13). HDAC inhibitors can reverse this process by blocking HDAC activity and promoting acetylation of histone-H3 to reactivate transcription of these dormant tumor suppressor genes, thereby inducing cytotoxicity in cancer cells (10, 11, 16). Interestingly, there are several studies showing that HDAC inhibitors induce synergistic cytotoxicity in myeloma and pancreatic cancer cells when used simultaneously with PS-341 (17–24). Use of HDAC inhibitors, including TSA, has shown that inhibition of HDAC activity abrogates the formation of a cytoprotective structure termed “aggresome,” which promotes the degradation of the ubiquitin conjugated proteins in many cancer cells on PS-341 treatment (22). Studies proposed that the simultaneous treatment of a HDAC inhibitor such as TSA with proteasome inhibitor PS-341 restores or even enhances the diminished cytotoxicity of PS-341 through prevention of the protective response toward the accumulation of misfolded proteins on PS-341 treatment in many cancer cells (22–24). However, the possible effects and therapeutic mechanism of this combination in HNSCC remains undefined. In this report, we examined whether TSA enhanced PS-341–mediated apoptosis in HNSCC cells. Our results revealed that cotreatment of PS-341 and TSA in HNSCC cells enhanced apoptosis by increasing Noxa expression. The significantly enhanced antitumor activity that results from the combination of PS-341 and TSA offers promise as a novel treatment for HNSCC patients.

Materials and Methods

Cell culture and reagents

The HNSCC cell lines UMSCC1, UMSCC9, and UMSCC23 were obtained from Dr. Thomas Carey (University of Michigan, Ann Arbor, MI) and FaDu was purchased from the American Type Culture Collection. These cell lines were cultured in DMEM with 10% fetal bovine serum and penicillin-streptomycin (Invitrogen). Cells were maintained at 37°C with 5% carbon dioxide. PS-341 was purchased from L.L.C. Laboratories. PS-341 was dissolved in DMSO as a 10 mmol/L stock solution, aliquoted, stored at −20°C, and subsequently diluted with cell culture medium before use.

Trypan blue assay and DNA ladder

For cytotoxicity assay, UMSCC1, UMSCC9, UMSCC23, and FaDu cells were seeded on 12-well culture plates at 2 × 10^5 per well. Cells were then treated with PS-341 and/or TSA and incubated at 37°C with 5% CO2 for 24 hours. After 24 hours of exposure to these chemotherapeutic agents, viability of the cells was determined using trypan blue assay. The assays were done in triplicate samples, and the results are representative of three independent experiments.

For the DNA ladder assay, cells were seeded onto 6-cm tissue culture dishes at 7 × 10^5 per dish, then treated with PS-341 and/or TSA at 37°C with 5% CO2 for 24 hours. Both detached and attached cells were scraped, harvested, and lysed using DNA lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L NaCl, 10 mmol/L EDTA (pH 8.0), and 10% SDS] with 100 μg/mL proteinase K for 2 hours at 50°C. Genomic DNA was extracted with phenol-chloroform-isoamyl alcohol (Roche) at least twice according to the manufacturer’s protocol. DNA was precipitated with 3 mol/L sodium acetate and ethyl alcohol. Precipitated DNA was washed with 70% ethanol and resuspended in TE buffer with RNase (pH 8.0), and 5 μg of each DNA sample were resolved on a 1.5% agarose gel to visualize fragments.

Western blot and Northern blot analyses

Cells (2 × 10^6) were plated in 10-cm tissue culture dishes a day before PS-341, TSA, and PS-341 plus TSA treatment. Whole-cell extracts were prepared with whole-cell lysate buffer (Sigma-Aldrich) with protease inhibitors. Lysates (50 μg) were separated by 8–15% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane with a Bio-Rad semi-dry transfer apparatus. The membranes were blotted with 5% milk at room temperature for 1 hour and subsequently probed with primary antibodies at 4°C overnight. Primary antibodies were acquired from the following sources: ATF4, GADD34, and histone H3 from Santa Cruz; caspase-3, caspase-7, and caspase-9 from Cell Signaling; acetyl-histone 3 from BD Biosciences; and Noxa from Abcam, Inc. Secondary antibodies mouse anti-rabbit-IgG-HPR (Promega) and goat anti-mouse-IgG-horseradish peroxidase (Bio-Rad) were used to detect the primary antibodies. The signals were detected using enhanced chemiluminescence reagents (Pierce). For Northern blot, cells were treated with PS-341, TSA, or PS-341 plus TSA and total RNA was harvested using Trizol (Invitrogen) according to the manufacturer’s instructions. Five micrograms of total RNA were resolved on 1.5% agarose formaldehyde gels and transferred onto a Zeta-Probe (Bio-Rad) overnight. The membranes were hybridized with 32P-labeled Noxa cDNA probes and exposed to autoradiographic film as described previously (4).
siRNA transfection

Noxa siRNA SMART pool (D-005275-05) and luciferase siRNA were purchased from Dharmacon. UMSCC1 or UMSCC23 cells (3.5 × 10⁵) were plated in 6-cm tissue culture dishes a day before transfection. Noxa siRNA or luciferase siRNA (100 µmol/L) was transfected into cells overnight with Oligofectamine diluted in Opti-Mem (Invitrogen) according to the manufacturer's protocol. Forty-eight hours following transfection, cells were treated with PS-341 or PS-341 plus TSA for 16 hours.

**In vivo tumor growth model**

All animal practices in this study were in accordance with the institutional animal welfare guidelines of the University of California Los Angeles. Eight-week-old nude mice were obtained from The Jackson Laboratory. UMSCC1 or UMSCC23 cells (2 × 10⁶) were injected s.c. into the right and left flank (day 0). One week after tumor cell inoculation, a group of mice (n = 5) were i.p. injected with PS-341 (200 µg/kg), TSA (1 mg/kg), PS-341 plus TSA, or vehicle control for 3 weeks. Tumor sizes were measured daily and calculated using the formula (length × width²)/2.

**Results**

**TSA enhances PS-341-mediated apoptosis in HNSCC cells**

To examine the consequence of combined exposure to PS-341 and TSA, UMSCC1 cells were treated with PS-341, TSA, or their combination for 24 hours. TSA treatment alone did not affect cell death in HNSCC cells. Whereas a low dose of PS-341 modestly induced cell death in UMSCC1 cells, the addition of TSA resulted in a markedly enhanced overall cell death in a dose-dependent manner (Fig. 1A). In addition, TSA significantly enhanced cell death induced by the low dose of PS-341 in other HNSCC cell lines, including UMSCC23, UMSCC9, and FaDu (Fig. 1B–D). To confirm that TSA enhanced PS-341-mediated apoptosis, we isolated genomic DNA and performed a DNA ladder assay on UMSCC1 and UMSCC23 cells. As shown in Fig. 1E, the induction of genomic DNA fragmentation in UMSCC1 and UMSCC23 cells was significantly enhanced by the cotreatment with PS-341 and TSA (Fig. 1E). To explore whether TSA could enhance PS-341-mediated inhibition of tumor growth in vivo, UMSCC1 cells were inoculated into nude mice and treated with PS-341, TSA, or their combination for 3 weeks. As shown in Fig. 1F, UMSCC1 tumor growth in nude mice was significantly inhibited by cotreatment of PS-341 and TSA compared with PS-341 or TSA treatment alone.

Previously, we found that PS-341–induced apoptosis in HNSCC cells involves activating intrinsic caspase cascades. Thus, we performed Western blot analysis to determine whether TSA promoted PS-341–induced caspase activation. As shown in Fig. 2A and B, TSA significantly enhanced the activation of the apical caspase-9 induced by PS-341 in both UMSCC1 and UMSCC23 cells. Consistently, the activation of executioner caspase-3 and caspase-7 was also increased by the cotreatment of PS-341 and TSA when compared with PS-341 treatment alone. For UMSCC1 and UMSCC23 cells, individual treatment with TSA did not trigger caspase activation. In conclusion, our results suggest that cotreatment with PS-341 and TSA synergistically promotes apoptosis in HNSCC cells.

**TSA does not modulate PS-341-induced ER stress in HNSCC cells**

Previously, we and others have shown that the cytotoxicity of PS-341 involves the regulation of expression of the
Figure 1. TSA enhances PS-341–mediated apoptosis in HNSCC cells. A, TSA enhanced cell death induced by PS-341 in UMSCC1 cells. Cell viability was determined by the trypan blue exclusion assay. The assays were done in triplicate samples, and the results are representative of three independent experiments (**, P < 0.01). Bars, SD. B to D, TSA enhanced PS-341–induced cell death in UMSCC23 (B), UMSCC9 (C), and FaDu (D) cells. **, P < 0.01.

E, TSA enhanced PS-341–mediated apoptosis in HNSCC cells. UMSCC1 and UMSCC23 cells were treated with PS-341 alone, TSA alone, and PS-341 and TSA together for 24 h. After treatment, the detached and attached cells were pooled, and genomic DNA was extracted with phenol-chloroform-isomyl alcohol and genomic DNAs were separated on a 1.5% agarose gel. M, 1-kb DNA ladder. F, TSA enhanced PS-341–mediated inhibition of HNSCC tumor growth in vivo. UMSCC1 cells were s.c. inoculated into nude mice for 1 wk and then treated with vehicle control, TSA, PS-341, or PS-341 and TSA for 3 wk. Tumor size was daily measured for the indicated days. *, P < 0.05, PS-341 plus TSA versus PS-341 or TSA (n = 5).
proapoptotic molecule Noxa in many solid tumors including HNSCC (4, 29). Because our data revealed that TSA modulated acetylation of histone H3, we examined whether TSA enhanced PS-341-induced Noxa expression in SCC cell lines. As shown in Fig. 5, Western blot analysis revealed that TSA strongly enhanced PS-341 induced Noxa expression in both UMSCC1 and UMSCC23 cells in a time-dependent manner, whereas TSA treatment alone did not affect Noxa expression. In contrast, TSA did not modulate the expression of another BH3-only Bcl-2 family member, Puma, in UMSCC1 and UMSCC23 cells (Fig. 5A). Moreover, there was no notable change in Bax expression on cotreatment with PS-341 and TSA, thereby suggesting that Puma and Bax might not be critical proapoptotic proteins involved in enhanced apoptosis by PS-341 and TSA. Consistent with our Western blot analysis, TSA also enhanced mRNA expression of Noxa induced by PS-341 in UMSCC1 and UMSCC23 cells in a time-dependent manner compared with PS-341 treatment alone (Fig. 5B).

To further determine whether elevated expression of Noxa is the downstream factor responsible for increased apoptosis, we used siRNA to knock down Noxa expression in UMSCC1 and UMSCC23 cells. Western blot analysis showed that Noxa siRNA completely inhibited Noxa expression in UMSCC1 and UMSCC23 cells induced by cotreatment of TSA and PS-341 compared with cells transfected with luciferase siRNA (Fig. 6A). Cotreatment of PS-341 and TSA resulted in 38% of apoptosis in control UMSCC1 cells and 15% in Noxa-knockdown cells, showing a significant increase in cell viability on depletion of Noxa expression (Fig. 6B). Similarly, PS-341 and TSA cotreatment induced 44% of apoptosis in control UMSCC23 cells but only 22% in Noxa-knockdown cells, indicating that Noxa is a primary proapoptotic molecule involved in apoptosis induced by PS-341 alone as well as cotreatment with TSA and PS-341 in both UMSCC1 and UMSCC23 cells. In conclusion, our results suggest that the enhanced apoptosis induced by cotreatment of PS-341 and TSA is mediated by increasing Noxa expression.

Discussion

The proteasome inhibitor PS-341 has been offered as an alternative to conventional cancer therapy for various solid tumors (6, 29). In vitro and in vivo studies conducted by our laboratory and others have shown that PS-341 also has a promising antitumor activity in HNSCC cells (4, 5). However, a higher concentration of PS-341 is required to induce apoptosis in solid tumors including
HNSCC when compared with myeloma (4). Because we previously showed that PS-341–induced apoptosis requires the induction of the proapoptotic genes, in this study, we investigated whether the classic HDAC inhibitor TSA enhanced PS-341–induced apoptosis by epigenetic modification of histones. We revealed that a marked increase in cytotoxicity of PS-341 plus TSA treatment compared with PS-341 alone was associated with notable enhancement of DNA fragmentation caused by enhanced apoptosis in SCC cell lines. We showed that TSA strongly enhanced PS-341–induced activation of caspase-9, caspase-3, and caspase-7. Our results suggest that the synergy between PS-341 and TSA in HNSCC is accomplished by enhancing the intrinsic apoptotic pathway.

Previously, we have shown that inhibition of the 26S proteasome by PS-341 results in ER stress, which subsequently stimulates a coordinated cellular response, called unfolded protein response, to induce apoptosis in HNSCC (4, 5, 7). Thus, we investigated whether TSA modulated PS-341–induced ER stress by examining the expression levels of two ER stress markers, ATF4 and its downstream factor GADD34, in HNSCC cells. Cotreatment of PS-341 and TSA induced a similar level of ATF4 and GADD34 when compared with PS-341 treatment alone, suggesting that TSA does not modulate PS-341–induced ER stress in HNSCC cells. Studies on the effect of HDAC inhibitors in many cancer cells suggested that the cytotoxicity of HDAC inhibitors is induced by epigenetic modulation on histone cores such as histone H3 (30–34). HDAC inhibitors including TSA induce cytotoxicity in many solid tumors by increasing acetylation of core histones to change the chromatin structure to transcriptionally reactivate dormant tumor suppressor genes (30, 31). Therefore, we explored whether TSA increases the acetylation of H3 in HNSCC. Our data displayed that hyperacetylation of histone H3 was observed in UMSCC1 and UMSCC23 cells that were treated with TSA alone and cotreated with PS-341 and TSA.

Figure 4. Promoting histone H3 acetylation in HNSCC cells by TSA. A and B, TSA induced hyperacetylation of histone H3 in HNSCC cells. UMSCC1 and UMSCC23 cells were treated with PS-341 alone (0.5 μmol/L), TSA alone (300 nmol/L), or PS-341 and TSA together for the indicated time periods. Aliquots (50 μg) of proteins were probed with acetyl-histone H3 antibodies (1:1,000). For a loading control, the membrane were stripped and reprobed with monoclonal antibodies against α-tubulin.

Figure 5. TSA enhances PS-341–induced Noxa expression. A, TSA enhanced PS-341–induced Noxa expression as determined by Western blot analysis. Cells were treated with PS-341 alone, TSA alone, or PS-341 and TSA together for the indicated time periods. Aliquots (50 μg) of cell lysates were probed with antibodies against Noxa, Puma, or Bax. α-Tubulin was used as an internal control. B, TSA enhanced Noxa mRNA expression induced by PS-341. Total RNA was isolated and determined by real-time PCR.
Individual treatment with PS-341 in both UMSCC1 and UMSCC23 cells showed no effect on acetylation of histone H3, suggesting that TSA may enhance PS-341–induced apoptosis by promoting gene expression. Previously, we found that PS-341 induced apoptosis through induction of Noxa (4). Intriguingly, cotreatment with PS-341 and TSA in both UMSCC1 and UMSCC23 cells notably upregulated Noxa compared with cells that were treated with PS-341 alone. Although it is likely that TSA promoted Noxa expression through epigenetic modification on histone H3, currently, the precise mechanism by which PS-341–induced Noxa is expression enhanced by TSA is not clear. Mechanistic association of hyperacetylation at histone H3 with Noxa expression on PS-341 and TSA cotreatment needs to be further elucidated. Moreover, it remains a possibility that TSA may also affect other molecules or signaling pathways to promote PS-341–mediated apoptosis in HNSCC cells.

The cytotoxicity of PS-341 depends on ER stress mediated by the accumulation of misfolded or unfolded proteins; nevertheless, cancer cells can attenuate the antitumor activity of PS-341 by preventing this accumulation of ubiquitin-conjugated proteins through the formation of a cytoprotective structure known as aggresome (22–24). Aggresome formation promotes the degradation of the ubiquitin-conjugated proteins on PS-341 treatment (10, 11), increasing the survival rate of a variety of malignancies including multiple myeloma (22) and ovarian cancer (24). Thus, it will be interesting to examine whether TSA enhances PS-341–induced apoptosis by inhibiting aggresome formation in HNSCC cells. In conclusion, our findings indicate that inhibition of the proteasome and HDACs with PS-341 and TSA, respectively, synergistically induces apoptosis in HNSCC cells by enhancing Noxa expression and promoting caspase activation. Our results provide an additional rationale for the use of the PS-341 and TSA combination in patients with HNSCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure 6. Noxa plays a critical role in TSA– and PS-341–induced apoptosis in HNSCC cells. A, knockdown of Noxa in UMSCC1 and UMSCC23 cells. SCC cells were transfected with Noxa siRNA or control luciferase siRNA using Fugene. Cell lysates were probed with antibodies against Noxa. α-Tubulin was used as an internal control. B, knockdown of Noxa inhibited PS-341– and TSA-induced apoptosis. Cells were transfected with siRNA as described in A. Forty-eight hours after transfection, cells were treated with PS-341 alone or PS-341 and TSA together for 16 h. Cell viability was determined by trypan blue exclusion assay. The results are average values from three independent experiments (**, P < 0.01).
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