Histone deacetylase inhibitors and paclitaxel cause synergistic effects on apoptosis and microtubule stabilization in papillary serous endometrial cancer cells

Sean C. Dowdy,1,2 Shujuan Jiang,3 X. Clare Zhou,1 Xiaonan Hou,2 Fan Jin,4 Karl C. Podratz,1,2 and Shi-Wen Jiang1,2

1Department of Obstetrics and Gynecology, 2Mayo Comprehensive Cancer Center, Mayo Clinic and Foundation, Rochester, Minnesota; 3Shandong Provincial Hospital, Jinan, China; and 4Women’s Hospital, Zhejiang University School of Medicine, Hangzhou, China

Abstract
The use of histone deacetylase (HDAC) inhibitors has shown promise for a variety of malignancies. In this investigation, we define the activity of this class of inhibitors in combination with traditional cytotoxic chemotherapy in endometrial cancer cells. Significant reductions in growth were observed in Ark2 and KLE endometrial cancer cells following treatment with paclitaxel, doxorubicin, carboplatin, or the HDAC inhibitor trichostatin A (TSA). However, only combined treatment with TSA/paclitaxel caused synergistic inhibition of cell growth. This combination also resulted in significant changes in cell morphology. Using cell cycle analysis, nuclear staining, and Western blot analysis for poly(ADP-ribose) polymerase and caspase-9 degradation products, TSA/paclitaxel showed the most dramatic activation of the apoptotic cascade. These effects were also observed when the HDAC inhibitors HDAC inhibitor-1 or oxamflatin were substituted for TSA. The anticancer properties of paclitaxel are known to result from paclitaxel stabilization of microtubules. Furthermore, using Western blot and immunohistochemical analysis, treatment with TSA/paclitaxel led to a significant increase in acetylated tubulin and microtubule stabilization. These effects were confirmed in a mouse xenograft model. Moreover, TSA/paclitaxel resulted in a 50% reduction in tumor weight compared with either agent alone. This study provides in vivo evidence of nonhistone protein acetylation as one possible mechanism by which HDAC inhibitors reduce cancer growth. The TSA/paclitaxel combination seems to hold promise for the treatment of serous endometrial carcinoma and other malignancies with limited sensitivity to paclitaxel. [Mol Cancer Ther 2006;5(11):2767–76]

Introduction
Endometrial cancer is the most common malignancy of the female reproductive tract. Most women are diagnosed at an early stage and enjoy excellent survival, but women with advanced disease have a poor prognosis. Five-year survival for this cohort is ~25% using current treatment regimens, which include the use of carboplatin, doxorubicin, and paclitaxel, alone or in combination (1–3). Papillary serous carcinoma, sometimes referred to as type II endometrial cancer, accounts for only 5% of cases but is estimated to be responsible for ~20% of deaths attributable to endometrial cancer (4, 5). In stark contrast to endometrioid carcinoma, type II endometrial carcinoma is heralded by frequent p53 mutations, an absence of hormone receptors, and a predilection for advanced-stage disease at presentation (6–8). To significantly reduce endometrial cancer-related deaths, new therapeutic options are needed for women with this particularly aggressive histologic subtype.

In recent years, the importance of epigenetics in cancer development has been appreciated (9). Changes in DNA methylation and histone acetylation have been linked to aberrant silencing of multiple tumor suppressor genes from a wide variety of tumors (10, 11). DNA methyltransferase and histone deacetylase (HDAC) inhibitors are currently being investigated in phase I/II trials for patients with hematologic and solid malignancies. Although these investigations are actively recruiting patients, to date these agents are well tolerated and efficacious (12). In regards to endometrial cancer, epigenetic defects have been linked to silencing of multiple genes, including hMLH1, progesterone receptor-B, and PTEN (13–15). Silencing of hMLH1 and/or MSH2 by methylation is associated with microsatellite instability, invasive growth, and acquired resistance to cisplatin (16–18). Treatment of hMLH1-deficient cells with demethylation reagent reactivates MLH1 gene expression and restores normal DNA repair function (15). Similarly, epigenetic silencing of progesterone receptor-B occurs commonly in high-grade endometrial carcinomas, making these
tumors recalcitrant to progestational therapy. We have shown that treatment with epigenetic modification reagents results in demethylation and reexpression of progesterone receptor-B in endometrial cancer cells (19, 20).

The antitumor effects of these drugs are thought to result from hyperacetylation of histones, demethylation of genomic DNA, and a resulting reactivation of genes that inhibit proliferation (21). In addition to these transcriptional effects, inhibitors of deacetylase have also been shown to have significant post-translational effects on nonhistone proteins, including tubulin (22, 23). The clinical significance of nonprotein acetylation is unknown. Furthermore, the use of these agents combined with traditional cytotoxic drugs has not been investigated.

In this study, we examine the antiproliferative effects of HDAC inhibitors on endometrial cancer cells when combined with cytotoxic agents traditionally used to treat this disease, such as paclitaxel, doxorubicin, or carboplatin. We found that trichostatin A (TSA) and paclitaxel synergistically inhibit the proliferation of serous endometrial cancer cells. We also observed dramatic activation of the apoptosis cascade with this combination. One of the most important mechanisms by which paclitaxel inhibits tumor growth is by microtubule stabilization, which occurs through its action on tubulin acetylation. Importantly, we show that, when added to paclitaxel, TSA causes a marked increase in microtubule stabilization, indicating that these two agents work in a cooperative fashion in endometrial cancer cells. The effects of the TSA/paclitaxel combination on apoptosis and tubulin acetylation were subsequently confirmed in a mouse xenograft model. Furthermore, there was a statistically significant reduction in tumor weight in those mice treated with the drug combination compared to either agent alone. The ability of TSA to potentiate the anticancer effects achieved by paclitaxel may have important implications for the treatment of women with endometrial cancer and for patients harboring other malignancies with limited sensitivity to paclitaxel.

Materials and Methods

Cell Lines and Reagents

The Ark2 cell line was established from primary cultures of advanced stage of human uterine serous papillary carcinoma. These cells were generously provided by Dr. Alessandro Santi (University of Arkansas, Little Rock, AR). Poorly differentiated human endometrioid cancer KLE and AN3 cells were obtained from the American Type Culture Collection (Rockville, MD). Ark2, KLE and AN3 cells were plated at 20% confluence in 10-cm dishes 1 day before treatment and used to determine baseline cell levels. The cells were treated with TSA (25 nmol/L), paclitaxel (1.5 nmol/L), carboplatin (50 nmol/L), or doxorubicin (5 nmol/L) alone or in combination as indicated in figure legends. Cells were counted once daily thereafter for 4 consecutive days. Floating cells were washed away, and only the living cells were detached from dishes by trypsin digestion and counted. Growth curves were constructed for each experimental group. Average and SE at each time point were calculated based on three or more parallel experiments.

Assessment of Apoptosis

The Annexin V-FITC kit (BD Biosciences, San Diego, CA) was used to label apoptotic cells. Drug-treated cells were washed with cold PBS and diluted in 1× Annexin-binding buffer at a concentration of 1× 10⁶/mL. Cells (1× 10⁶) were mixed with 5 µL of Annexin V-FITC stock solution, and binding was carried out at room temperature for 15 minutes in the dark. The samples were diluted to 400 µL and immediately analyzed by flow cytometry for apoptotic cells. For nuclear staining, cells were washed with cold PBS and fixed with 4% paraformaldehyde. Fixed cells were washed with cold PBS and stained for 5 minutes with Hoechst dye (2 µg/mL in 0.1% Triton X-100). The stained cells were washed twice with 0.1% Triton X-100 and 1× PBS and visualized using fluorescent microscopy. Apoptotic cells with condensed or fragmented nuclei were counted. The results were presented as a percentage of apoptotic cells against total cells examined.

Western Blot Analyses

Ark2 and KLE cells were treated with paclitaxel and TSA as indicated in figure legends. Cells were harvested using a lysis buffer containing 20 mmol/L HEPES (pH 7.2), 25% glycerol, 0.42 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, and 0.5 mmol/L phenylmethylsulfonyl fluoride. Insoluble debris was discarded following centrifugation at 14,000 rpm for 15 minutes at 4°C. Protein concentrations were determined using Coomassie protein assay reagent (Pierce, Rockford, IL). Cellular proteins were resolved in SDS-PAGE (5–10% gradient; Bio-Rad Laboratories, Hercules, CA) and electrotransferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membranes were blocked for 2 hours in PBS buffer containing 0.1% Tween 20 and 10% nonfat dried milk. Antibodies against PARP and caspase-9 were applied following the manufacturer’s recommendations. Primary antibody binding was done at 4°C overnight with constant shaking. The anti-rabbit or thoxycarbonyl)aminomethyl|benzamide MS-275] and oxamflatin [(2E)-5-[3-(phenylsulfonylamino)phenyl]pent-2-en-4-yno hydroxamic acid] are products of Calbiochem (La Jolla, CA). Antibodies against poly(ADP-ribose) polymerase (PARP) and caspase-9 were purchased from Roche (Basel, Switzerland). Rabbit polyclonal antibody for β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Growth Assay

Ark2, KLE, and AN3 cells were plated at 20% confluence in 10-cm dishes 1 day before treatment and used to determine baseline cell levels. The cells were treated with TSA (25 nmol/L), paclitaxel (1.5 nmol/L), carboplatin (50 nmol/L), or doxorubicin (5 nmol/L) alone or in combination as indicated in figure legends. Cells were counted once daily thereafter for 4 consecutive days. Floating cells were washed away, and only the living cells were detached from dishes by trypsin digestion and counted. Growth curves were constructed for each experimental group. Average and SE at each time point were calculated based on three or more parallel experiments.
anti-mouse antibodies labeled with horseradish peroxidase (Amersham Corp., Arlington Heights, IL) were used at 1:5,000 dilution. Secondary antibody binding was carried out at room temperature for 1 hour. Chemiluminescence detection was carried out with the ECL Plus Western Blotting Detection System (Amersham). The blots were reprobed with β-actin antibody, and the results were used as loading controls. Tubulin acetylation levels were measured the same way as described above, except that anti-acetylated tubulin (1:2,000; Sigma) was used as primary antibody.

**Measurement of Mitochondrial Membrane Potentials**

Changes in mitochondrial membrane potentials (MMP) were measured by flow cytometry using cell-permeable mitochondrial-sensitive dye MitoTracker Red CMX (CMXRos, Molecular Probes, Eugene, OR). Cells (2 × 10⁶) were washed twice with cold PBS and stained in 1 mL of 25 nmol/L CMXRos diluted in serum-free medium. The staining was done at 37°C for 30 minutes. The cells were collected by centrifugation and washed thrice, each with 2 mL cold PBS. The cells were resuspended in PBS and subjected to flow cytometry (Becton Dickinson, San Jose, CA) measurement on FL3 (emission, 599 nm). The data were analyzed by FACScan program, and the results were presented as the percentage of cells with mitochondrial membrane permeability transition.

**Immunofluorescent Studies**

Ark2 and KLE cells were grown to 90% confluence on glass coverslips. The cells were treated with 1.0 nmol/L paclitaxel, 10 nmol/L TSA, or 1.0 nmol/L paclitaxel plus 10 nmol/L TSA for 12 hours. The cells were washed twice with cold PBS, fixed with methanol at −20°C for 10 minutes, rinsed with acetone (−20°C), and rehydrated in PBS for 30 minutes. The cells were incubated with anti-tubulin-FITC (Sigma) at room temperature for 60 minutes. The antibody was diluted to 1:25 in 1% bovine serum albumin. Following three cycles of washing with PBS, the cells were counterstained with 4,6-diamidino-2-phenylindole and the coverslips were mounted on glass slides. The immunofluorescent signals were observed and documented with confocal fluorescence microscopy.

**Establishment and Treatment of Mouse Xenografts**

Six-week-old female immunodeficient athymic, NCR nu/nu mice were purchased from the National Cancer Institute/NIH (Bethesda, MD) and maintained under pathogen-free conditions with irradiated chow. Ark2 cells (1 × 10⁶) resuspended in 0.2 mL PBS were injected s.c. into the right flank of each mouse, and tumor growth was monitored every day. Three weeks after tumor cell injection, 20 mice with confirmed tumor growth were randomly divided into four groups. The treatment groups received TSA (1 mg/kg), paclitaxel (5 mg/kg), or a combination of TSA and paclitaxel dissolved in 50 µL DMSO. The control group received solvent only. Drugs were delivered i.p. every 2 days for five cycles. Animals were sacrificed by carbon dioxide asphyxiation 2 hours after the i.p. injection of bromodeoxyuridine labeling reagent (Zymed Laboratories, San Francisco, CA). Tumors were carefully dissected from each mouse, and tumor weights were measured. Half of the tumor tissue was fixed with formalin and used for immunohistochemical studies, whereas the other half was snap frozen in liquid nitrogen and stored at −80°C. These tissues were used to extract protein for tubulin acetylation analysis. A “short-term” experiment was done to further evaluate tumor apoptosis in response to treatments. Each mouse was injected with 5 × 10⁶ Ark2 cells, and the treatments were initiated after 29 days of tumor growth. All other details of the experiment remained the same as the long-term study, except that the mice received a single administration of TSA (2 mg/kg), paclitaxel (10 mg/kg), or combination of TSA and paclitaxel, and the mice were sacrificed 2 days following drug administration.

**Immunohistochemistry**

Tumors from xenografts were fixed with 10% neutral-buffered formalin and embedded in paraffin. The 6-µm sections were stained by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method using the ApopTag Peroxidase In Situ Apoptosis Detection kit (Chemicon International, Temecula, CA) based on the manufacturer’s instructions. Briefly, the sections were deparaffinized, rehydrated, and treated with 20 µg/mL proteinase K for 30 minutes at room temperature. Sections were washed with two changes of H2O for 2 minutes each. Endogenous peroxidases were blocked with 3% H2O2 in PBS for 5 minutes and washed with three changes of PBS. Equilibration buffer containing digoxigenin-conjugated nucleotides was placed directly onto the section for 10 seconds. Sections were incubated with terminal deoxynucleotidyl transferase in a humidified chamber at room temperature for 1 hour. Sections were then incubated for 10 minutes at room temperature in stop-wash buffer, rinsed in three changes of PBS for 1 minute each, and incubated with anti-digoxigenin conjugate for 30 minutes at room temperature. The color development was attained by exposing the sections to the peroxidase substrate solution and accessed with a light microscope. The sections were lightly counterstained with hematoxylin. For positive controls, the sections were treated with DNase I (10 units/mL) for 10 minutes at room temperature before incubation with the TUNEL reaction mixture. The TUNEL-positive and total number of cells examined were counted, and the data were expressed as a percentage of positive cells.

**Data Analysis**

All quantitative data were subject to ANOVA to determine if there were significant differences between groups. For data groups satisfying ANOVA criteria (P < 0.05), individual comparisons were done with the use of post hoc Bonferroni t tests with the assumption of two-tail distribution and equal variance. Statistical significance (P < 0.05) is marked by an asterisk in figures. For quantitative data from three or more repeated experiments, average values and SE were calculated and graphically presented.
Analysis of Drug Interaction for Synergy
Each individual and combination response was corrected by the level of the control within each experiment. The sum of the corrected individual treatments was calculated, and the pooled within-treatment variance from these values was used for comparison with the combination response. A one-way classification ANOVA, equivalent to a simple t test in these cases, was used to test the null hypothesis that the sum of the individual treatments was the same as the combination treatment. A synergistic interaction was considered significant if \( P < 0.05 \).

Results
TSA in Combination with Paclitaxel Induces Synergistic Cell Death
Clinical trials using HDAC inhibitors have shown promising results in hematologic and pulmonary malignancies. Although preliminary investigations indicate that TSA may be useful for endometrial cancer treatment, their effects in combination with available cytotoxic chemotherapy are unknown. We assessed three commonly used chemotherapeutic agents in women with endometrial cancer: doxorubicin, carboplatin, and paclitaxel. We first examined the growth-inhibitory effects of these drugs alone and in combination with TSA on serous endometrial cancer (Ark2) as well as endometrioid cancer (KLE and AN3) cell lines. As shown in Fig. 1, all four agents had significant growth-inhibitory effects on Ark2 cells. Although all combination treatments resulted in a greater reduction in cell growth than single-agent therapy, the paclitaxel-TSA combination showed the strongest effect, reducing cell counts by >90%. In contrast, when TSA and paclitaxel were given individually, cell counts were reduced by 55% and 70%, respectively. Only the paclitaxel-TSA drug combination showed synergistic effects on reducing cell growth (\( P < 0.05 \)). Whereas the growth curves in Fig. 1 are suppressed with single-agent treatment, the paclitaxel-TSA combination causes a considerable decrease in cell counts below baseline. These data suggest that the TSA-paclitaxel combination may be truly cytotoxic rather than simply cytostatic. Similar findings were observed for the endometrioid cell lines (data not shown). Although a consistent increase in response was seen with the TSA-paclitaxel combination, significance for synergy was not reached in the endometrioid carcinoma subtype.

Administration of TSA and Paclitaxel Results in Significant Morphologic Changes
Further illustrating the effects of this combination on cell death and apoptosis, Fig. 2 shows the morphologic changes following paclitaxel and TSA administration, alone and in combination. Reduced cell density and an abundance of floating dead cells are evident after treatment with TSA or paclitaxel in Ark2 and KLE cells. Compared with controls, paclitaxel-treated cells became polygonal and enlarged. TSA-treated cells became enlarged, whereas others formed characteristic, long digitiform processes. When given in combination, however, TSA and paclitaxel induced

Figure 1. Inhibitory effects of TSA and chemotherapeutic agents on cell proliferation. Ark2 cells were treated with paclitaxel (1.5 nmol/L; Taxol; A), carboplatin (50 nmol/L; Carbo; B), or doxorubicin (5 nmol/L; Dox; C), alone and in combination with TSA (25 nmol/L) for 4 d. Cells were counted daily. Points, mean growth curves of three parallel experiments; bars, SE. A strong synergism was observed between TSA and paclitaxel in Ark2 cells.
dramatic cell death as seen in Fig. 2D and H. Together, these data suggest that TSA may reduce the growth of both type I and type II endometrial cancers when used in combination with paclitaxel.

**TSA and Paclitaxel Induce Apoptosis in Endometrial Cancer Cells**

Previous studies in a variety of cancer cells have shown that the anticancer properties of both TSA and paclitaxel are caused in part by initiation of the apoptotic cascade. Our cell growth and morphologic studies strongly suggest the involvement of the cell death pathway. We did flow cytometry to measure the fraction of apoptotic cells following administration of either a single chemotherapeutic agent or combination treatment with TSA (Fig. 3A). Consistent with their effects on the cell growth curve, all three combinations of TSA with paclitaxel, doxorubicin, or carboplatin induced more cell death than any of the four agents alone in Ark2 cells. However, the TSA-paclitaxel combination resulted in the greatest effect with apoptosis present in 45% of cells. To confirm this result, we investigated cellular apoptosis by nuclear staining, an alternative cell apoptosis assay. As shown in Fig. 3B, a modest increase in the number of apoptotic nuclei was observed following administration of either TSA or paclitaxel alone. On day 4, 6% and 10% of cells showed apoptotic nuclei following treatment with TSA or paclitaxel, respectively, compared with 2.5% in controls. Following administration of both agents, however, apoptotic nuclei were present in 22% of cells, a much more significant result than that observed with either agent alone.

**Figure 2.** Morphologic changes and cell death induced by TSA and paclitaxel.Ark2 and KLE cells were treated with 1.5 nmol/L paclitaxel (B and F), 25 nmol/L TSA (C and G), or with both drugs (D and H) for 2 d. Compared with controls (A and E), many TSA-treated cells became enlarged or formed long digitiform processes (arrowheads). Solid arrows, paclitaxel-treated cells became polygonal and enlarged; open arrows, floating dead cells can be seen in cultures treated with TSA or paclitaxel. Reduced cell density and a marked increase in the number of floating dead cells were observed in cultures treated with both TSA and paclitaxel.

**Figure 3.** Apoptotic effects of combined drug treatment in Ark2 cells. A, cells were treated with paclitaxel (1.5 nmol/L), carboplatin (50 nmol/L), or doxorubicin (5 nmol/L), alone or in combination with TSA (25 nmol/L) for 3 d. Annexin V–positive cells were analyzed by flow cytometry. Whereas TSA/carboplatin and TSA/doxorubicin combinations induced more apoptotic events compared with treatment with a single reagent, the TSA/paclitaxel combination exhibited synergistic effects. B, time-response curves of TSA, paclitaxel, and TSA/paclitaxel treatment. Cells were treated with paclitaxel (1.0 nmol/L), TSA (15 nmol/L), or TSA/paclitaxel. Nuclei were visualized by Hoechst staining. The condensed and fragmented nuclei were counted at different time points. The strongest effect was observed in cells treated with TSA/paclitaxel.
The above results were obtained from the serous endometrial cancer cell line Ark2. To investigate the effects of these reagents on endometrioid endometrial cancer, paclitaxel and TSA were administered alone and in combination to KLE and AN3 cells (Fig. 4A). Flow cytometry indicated that combination treatment resulted in a significant increase in the proportion of apoptotic cells. However, a synergistic effect on apoptosis ($P < 0.05$) could only be shown in the Ark2 cell line (Fig. 4A).

It is possible that the increase in apoptosis and cell death seen with the use of TSA in the previous experiments was due to general cytotoxic effects rather than a HDAC-specific action. Therefore, we used alternative HDAC inhibitors oxamflatin and HDAC inhibitor-1 to test this possibility (Fig. 4B). A significant effect on apoptosis was again seen when these inhibitors were used in combination with paclitaxel, although oxamflatin seemed to be more effective. Thus, various HDAC inhibitors seem to be able to cooperate with paclitaxel in inducing apoptosis of endometrial cancer cells, suggesting the involvement of a common, HDAC-related mechanism.

Apoptotic Effects of the TSA/Paclitaxel Combination Result from Activation of the Intrinsic Mitochondria-Dependent Pathway

We next investigated whether treatment with TSA and paclitaxel increased the cleavage of proteins known to be involved in the apoptotic cascade. We used Western blot analysis to probe for intact and cleaved PARP and caspase-9 after treatment with the TSA and paclitaxel drug combinations used in the previous experiments (Fig. 5A and B). Whereas administration of paclitaxel or TSA alone caused little cleavage of PARP or caspase-9, a much stronger band was evident after the use of both reagents, indicating a significant activation of the apoptotic cascade with this combination. This effect was observed in all three endometrial cancer cell lines tested, although the strongest interaction was evident in Ark2 cells. The preferential results in the type II endometrial cancer cell line are consistent with our previously presented data (Fig. 1) and suggest that the TSA/paclitaxel combination activates the caspase-9-mediated, intrinsic cell death pathway.

The intrinsic pathway is initiated by dissipation of the MMP, leading to the release of cytochrome $c$ and other downstream apoptotic events. Figure 5C shows that treatment with TSA or paclitaxel alone resulted in the loss of MMP. There was a synergistic increase ($P < 0.05$) in the percentage of Ark2 cells with compromised MMP in cultures treated with the combination of TSA and paclitaxel, with 16% of cells showing loss of MMP after 24 hours of treatment. The effect is least pronounced in the KLE cell line, and although the combination resulted in increased loss of MMP in both endometrioid cell lines, the interaction was not synergistic. Given that these results are based on a single concentration of drug at a single point in time, we cannot exclude the possibility that the loss of MMP had already peaked with paclitaxel alone for the KLE cell line at the given concentration. Despite this, these results, together with those from Western blot analysis, lend further support that some of the effects of the TSA/paclitaxel combination on apoptosis may be mediated by the mitochondria-dependent mechanism.

The Combination of TSA and Paclitaxel Stabilizes Microtubules

To elucidate the mechanism for the synergism between TSA and paclitaxel, we examined their known anticancer properties. In contrast to other microtubule poisons, such as the Vinca alkaloids that inhibit microtubule polymerization, paclitaxel interferes with microtubule depolymerization (24, 25). The end result is the accumulation of acetylated $\alpha$-tubulin and stabilized microtubule structures, which disrupt the alignment of chromosomes during mitosis and lead to apoptosis. Interestingly, $\alpha$-tubulin has been found to be a nonhistone substrate for HDAC enzymes (26). At least one member of the HDAC family, HDAC-6, is able to remove the acetyl group from...
tubulin (27). Because tubulin deacetylation is associated with microtubule depolymerization, accumulation of acetylated tubulin following treatment with HDAC inhibitors leads to microtubule stabilization. To investigate the possibility that the combination of TSA and paclitaxel causes cell death by cooperating to stabilize microtubules, we did Western blot analysis on acetylated tubulin. When administered as single agents, TSA and paclitaxel caused a modest increase in acetylated tubulin (Fig. 6A). However, a marked additional increase in acetylated tubulin was observed when TSA and paclitaxel were given simultaneously. We next examined microtubule structure using immunostaining. As shown in Fig. 6B, altered microtubule structure accompanies the changes in tubulin acetylation. In control cells, microtubules were seen as fine, diffuse, and evenly distributed cytoplasmic networks. In contrast, cells treated with TSA or paclitaxel showed relatively thicker microtubule bundles organized in parallel. Microtubules became even more prominent in cells treated with combination therapy, appearing as bright cords peripheral to the nuclei. This effect was seen in both type I and type II endometrial cancer cells. These findings provide evidence that the synergistic growth-inhibitory effects of the TSA/paclitaxel combination may be mediated by a corresponding effect on microtubule stabilization.

**In vivo Studies Using a Mouse Xenograft Model**

We established an endometrial cancer mouse xenograft model to examine the *in vivo* effects of the TSA/paclitaxel combination. As described in Materials and Methods, athymic mice were treated with paclitaxel, TSA, or the TSA/paclitaxel combination 3 weeks after s.c. injection of Ark2 cells. Following five cycles of drug treatment, tumors were dissected and weighed. Whereas there was no difference in tumor weight between the control group and the groups treated with single-agent therapy (Fig. 7A and B), a significant reduction in tumor weight was found between the control and TSA/paclitaxel groups (>50% reduction). This effect was of borderline significance when analyzed for synergy (*P* = 0.05).

To evaluate apoptotic effects within the tumor cells, samples isolated from mice xenograft models were fixed and examined with the TUNEL assay (Fig. 7C). The percentage of apoptotic cells was determined by cell counting (Fig. 7D). Although there seemed to be a trend of increased apoptotic events following combined treatment with TSA and paclitaxel, the difference in the number of apoptotic cells did not reach statistical significance. The reason for this finding may be due to the time at which the tumors were harvested. By the 11th day of treatment, many drug-sensitive cancer cells may have already died and so would not be detected by the TUNEL assay. This possibility is supported by the low density of tumor cells present after 11 days of treatment. Furthermore, adjacent to tumor cells there were large amounts of connective tissue (Fig. 7C) with strong eosin staining (data not shown), suggesting that cancer cells had been replaced with connective tissue during drug treatment. The experiment was therefore repeated, and cells were examined for apoptosis 2 days after treatment. Figure 7C and D (bottom) shows that both TSA and paclitaxel alone induced tumor cell apoptosis, and this effect was potentiated when the TSA/paclitaxel combination was used. Interestingly, close examination of the tissue sections indicated a distinct distribution pattern of apoptotic cells in tumors treated with TSA/paclitaxel. The apoptotic cells appeared to highly concentrate into individual "death centers" located within the periphery but not within the central regions of the cancer tissues. Although the reason for this phenomenon is unclear, uneven drug concentrations (e.g., higher concentrations in the peripheral regions) and cell-cell communication of death signals may contribute to the formation of death loci in the periphery. An alternative explanation is that the central regions of the tumors may be arrested in the cell cycle and therefore resistant to this drug combination.

We wondered if the *in vivo* synergism between TSA and paclitaxel may be in part related to their effects on tubulin acetylation as observed in our *in vitro* studies. Western blot
Analysis was done on total proteins extracted from the tumor cells of mouse xenografts treated with solvent, TSA, paclitaxel, or TSA and paclitaxel. Whereas single-agent treatment with either TSA or paclitaxel alone had little effect on tubulin acetylation, combined treatment led to a dramatic increase in tubulin acetylation (Fig. 7E). These results are consistent with our cell line experiments and suggest that tubulin acetylation and microtubule reorganization may play an important role in the effectiveness of the TSA/paclitaxel combination.

**Discussion**

Uterine papillary serous carcinomas present difficult therapeutic challenges for clinicians. Their aggressiveness is readily shown by the fact that 67% of patients have advanced-stage disease at presentation, and at least 35% of patients with disease clinically confined to the uterus will show extraterine metastasis following definitive surgical staging (4, 28, 29). Unfortunately, new approaches for the treatment of women with papillary serous endometrial carcinoma have been slow to materialize. Treatment with single-agent paclitaxel results in median progression-free intervals of 7.3 months (30). Even when given in the adjuvant setting, median progression-free survival after administration of carboplatin and paclitaxel is only 30 months (31). In this report, we found TSA and paclitaxel to be the most effective combination of those tested, resulting in a synergistic activation of the apoptotic cascade in uterine papillary serous carcinoma cell lines. This effect was confirmed using a papillary serous carcinoma mouse xenograft. Administration of TSA/paclitaxel resulted in a >50% reduction in tumor weight in this model. To our knowledge, this is the first report suggesting a benefit to combining a HDAC inhibitor with paclitaxel. The effectiveness of this combination may be due in part to a relatively poorly studied aspect of HDAC action. Until recently, the effects of HDAC inhibitors have been investigated primarily in the context of chromatin structure and gene expression. Histone acetylation is known to result in the opening of condensed chromatin, which is in turn associated with transcriptional activation. It has been hypothesized that the antitumor effects of HDAC inhibitors result from reactivation of silenced tumor suppressor genes. However, it has become clear that a variety of nonhistone proteins are subject to acetylation and deacetylation modifications. The full range of nontranscriptional effects for this class of enzymes remains to be discovered. One nonhistone target for HDAC is tubulin. It has been recognized that disturbances in either microtubule assembly or disassembly have destructive effects on cellular functions, ultimately leading to cell death. Zhang et al. (27) recently reported that HDAC-6 is capable of interacting with purified tubulin and microtubules in vitro. They showed that HDAC-6 colocalizes with microtubules in NIH-3T3 cells and that inhibition of HDAC by TSA leads to increased tubulin acetylation. Knockout cells completely lacking HDAC-6 showed an elevation in α-tubulin acetylation. The authors conclude that HDAC-6 serves as a tubulin deacetylase (27). Similarly, Blagosklonny et al. (26) showed that acetylation of Lys 40 of α-tubulin occurs soon after TSA administration and parallels induction of apoptosis. Paclitaxel has also been shown to bind to and stabilize microtubules, eventually precipitating apoptosis (24, 25). Our data show that the TSA/paclitaxel combination has the most pronounced effects on microtubule stabilization, cell apoptosis, and inhibition of tumor growth in a mouse xenograft model. Moreover, we observed a significant increase in tubulin acetylation, suggesting that the anticancer activity of TSA/paclitaxel may be attributed in part to cooperative effects on microtubule stabilization. These two agents may stabilize microtubules via distinct mechanisms: direct binding for paclitaxel and acetylation for TSA.

Interestingly, evidence exists for the convergence of the effects of TSA and paclitaxel on another family of proteins involved in both apoptosis and microtubule stabilization, the antiapoptotic bcl-2 family. In an analysis of B-cell

![Figure 6. Effects of TSA and paclitaxel on tubulin acetylation and microtubule stabilization.](image-url)
lymphomas with the t(14:18) translocation, which results in bcl-2 overexpression and resistance to apoptosis, Duan et al. (32) found that the HDAC inhibitors TSA and sodium butyrate down-regulated bcl-2 expression. This down-regulation is mediated by histone H3 deacetylation at bcl-2 promoters and by acetylation of SP1 and CAAT/ enhancer binding protein α, which reduced their binding to the bcl-2 promoter. Furthermore, overexpression of bcl-2 inhibited TSA-induced apoptosis. This finding bears significance in the context of the investigation by Nuydens et al. (33) of the effects of paclitaxel on neuronal morphology and microtubule stability. These investigators found that bcl-2 overexpression attenuated the increased ratio of acetylated tubulin to total tubulin induced by paclitaxel treatment. Thus, it is possible that inhibition of bcl-2 by TSA both relaxes repression of apoptosis and potentiates acetylation of tubulin by paclitaxel, explaining the massive cell death that occurs with this drug combination. Further studies are required to evaluate the involvement of bcl-2 in TSA-mediated cell death in endometrial cancer cells.

Induction of p21 is thought to be an important mechanism by which taxanes induce apoptosis (34). Similarly, HDAC inhibitors are known to induce p21, which interacts with Rho to promote the accumulation of stabilized, detyrosinated microtubules (35, 36). In an analysis of the antitumor activity of various HDAC inhibitors, however, Blagosklonny et al. (26) were unable to correlate cytotoxicity with p21 induction. Thus, the significance of p21 induction within the context of TSA/paclitaxel synergy is unclear. Although our results show that acetylation of α-tubulin is important for microtubule stabilization, this is not likely to be the only pathway leading to apoptosis. It is also important to point out that paclitaxel is known to

**Figure 7.** In vivo xenograft studies. The mouse xenograft models were established and treated as described in Materials and Methods. The tumors were dissected, weighed, and examined by the TUNEL method. A, representative mouse xenografts. B, mean tumor weights from mice treated with DMSO solvent (control), paclitaxel, TSA, or paclitaxel/TSA. Compared with controls, no effect on tumor growth was observed in the TSA or paclitaxel group. However, a significant (P < 0.05) reduction in tumor weight was observed in the paclitaxel/TSA group, although only borderline synergy was shown (P = 0.05). C, TUNEL detection of apoptosis in tumors following drug treatment. Arrows, apoptotic cells stain dark brown. D, results of apoptotic cell counting following the TUNEL assay. In the long-term treatment group (11 d), relatively few apoptotic cells were detected. In the short-term treatment group (2 d), however, the TSA/paclitaxel combination resulted in a significant increase in apoptosis. E, assessment of tubulin acetylation. A moderate increase in tubulin acetylation was found in tumors treated with paclitaxel or TSA. In contrast, a marked increase in tubulin acetylation was observed in xenografts treated with both paclitaxel and TSA.
cause cell cycle blockade; similar effects on cell proliferation have also been reported with TSA (37, 38). It would not be surprising to see cooperative inhibition by these two reagents on cell cycle progression, and our preliminary analysis indeed suggests that this is the case (data not shown). Although our initial efforts at characterizing the TSA/paclitaxel combination have focused on an analysis of apoptosis, further studies on cell cycle regulation are required to further explore the inhibitory mechanism for this drug combination in endometrial cancer. Nevertheless, our results show that HDAC inhibitors are a promising class of agents that may be useful in combination with traditional cytotoxic agents, particularly paclitaxel, against endometrial cancer. As we continue to learn more about the effects of HDAC inhibitors, these agents may prove to not only be more efficacious but also show reduced side effects when used in combination with current treatment regimens.

Acknowledgments
We thank Ying Zhao for her technical support and Dr. John Attewell for his assistance with statistical analysis.

References
Histone deacetylase inhibitors and paclitaxel cause synergistic effects on apoptosis and microtubule stabilization in papillary serous endometrial cancer cells

Sean C. Dowdy, Shujuan Jiang, X. Clare Zhou, et al.

Mol Cancer Ther 2006;5:2767-2776.

Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/5/11/2767

Cited articles
This article cites 38 articles, 13 of which you can access for free at:
http://mct.aacrjournals.org/content/5/11/2767.full.html#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
/content/5/11/2767.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.