

## RESEARCH ARTICLE

# Histone Deacetylase Inhibitor Trichostatin A Enhances Anti-tumor Effects of Docetaxel or Erlotinib in A549 Cell Line

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### Abstract

**Background and Objective:** Histone deacetylase (HDAC) inhibitors represent a promising class of potential anticancer agents for treatment of human malignancies. In this study, we investigated the effect of trichostatin A (TSA), one such HDAC inhibitor, in combination with docetaxel (TXT), a cytotoxic chemotherapy agent or erlotinib, a novel molecular target therapy drug, on lung cancer A549 cells. **Methods:** A549 cells were treated with TXT, erlotinib alone or in combination with TSA, respectively. Cell viability, apoptosis, and cell cycle distribution were evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, Hoechst33258 staining and flow cytometry. Moreover, immunofluorescent staining and Western blot analysis were employed to examine alterations of  $\alpha$ -tubulin, heat shock protein 90 (hsp90), epidermal growth factor receptor (EGFR), and caspase-3 in response to the different exogenous stimuli. **Results:** Compared with single-agent treatment, co-treatment of A549 cells with TSA/TXT or TSA/erlotinib synergistically inhibited cell proliferation, induced apoptosis, and caused cell cycle delay at the G<sub>2</sub>/M transition. Treatment with TSA/TXT or TSA/erlotinib led to a significant increase of cleaved caspase-3 expression, also resulting in elevated acetylation of  $\alpha$ -tubulin or hsp90 and decreased expression of EGFR, which was negatively associated with the level of acetylated hsp90. **Conclusions:** Synergistic anti-tumor effects are observed between TXT or erlotinib and TSA on lung cancer cells. Such combinations may provide a more effective strategy for treating human lung cancer.

**Keywords:** TrichostatinA - docetaxel - erlotinib -  $\alpha$ -tubulin - hsp90 - epidermal growth factor receptor

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### Introduction

Lung cancer is the leading cause of cancer-related death in men and women, which results in more than one million deaths worldwide annually (Sun et al., 2007; Long et al., 2010). Despite the recent advances in diagnosis and multimodality therapies, the prognosis remains poor with 5-year survival rates of only 16% for all stages (Jemal et al., 2008). New therapeutic agents and strategies are desperately needed to improve the outcomes of lung cancer patients.

In recent years, the importance of epigenetic alterations has been appreciated in cancer development (Egger et al., 2004), including the role of abnormal DNA methylation and histone acetylation on aberrant silencing of multiple tumor suppressor genes in a diversity of human cancers (Esteller et al., 2002; Jones et al., 2002). Histone deacetylase (HDAC) inhibitors, which interfere with the function of histone deacetylase, are emerging as potent anticancer agents due to their effective anti-proliferative activity in a wide variety of tumors, mediated by mitotic defects through the aberrant acetylation of histone and non-histone proteins (Dowdy et al., 2006). It has been

reported that trichostatin A (TSA), a potent specific inhibitor of HDAC, is able to lead to cell growth arrest, differentiation, and/or apoptosis in a number of cancers (Kim et al., 2010; Sharma et al., 2010; Cheng et al., 2012). Evidence also suggests that TSA may have a promising therapeutic effect on cancer cells when combined with radiotherapy or chemotherapy (Ranganathan et al., 2005; Hajji et al., 2010).

Docetaxel, a semisynthetic derivative of paclitaxel originally derived from the yew tree, inhibits several types of tumor in vitro and in vivo (Labourey et al., 2007; Liu et al., 2011). Docetaxel inhibits cell mitotic division and promotes apoptosis through stabilizing microtubule assembly. Our previous research shows that TSA and paclitaxel synergistically inhibits the proliferation of papillary serous endometrial cancer cells (Jiang et al., 2007). One of the key observations is that TSA combined with paclitaxel causes a marked increase of acetylated tubulin and microtubule stabilization. The ability of TSA to potentiate the anticancer effects achieved by paclitaxel opens a new avenue to treat not only for women with endometrial cancer but also for patients with other malignancies with limited sensitivity to paclitaxel (Dowdy

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et al., 2006 ; Jiang et al., 2007).

Epidermal growth factor receptor (EGFR), is associated with neoplastic cell proliferation, migration, resistance to apoptosis, and angiogenesis. EGFR is expressed in a majority of non-small cell lung carcinomas (NSCLCs). Thus, EGFR has been an important target for the development of therapeutic agents relevant to NSCLCs (Hynes et al., 2005). Erlotinib, which possesses small-molecule EGFR tyrosine kinase activity and can in some instances inhibit tumor cell growth and induce tumor cell apoptosis, has been proved to be of therapeutic benefit in individuals with NSCLCs, especially in primary mutation of EGFR (Giaccone et al., 2005). However, tumors with the wild-type and a second mutation (T790M) of EGFR are refractory to erlotinib (Pao et al., 2005). The treatment of lung cancer cells with erlotinib and HDAC inhibitor LBH589 results in synergistic effects on cells dependent on EGFR (Edwards et al., 2007).

Combination therapy with multiple drugs is a common practice in the treatment of cancer in order to get a synergistic effect or to reduce the side toxicity effects. HDAC inhibitors have promoted a considerable interest in combining it with other antitumor agents, and the combination therapies have been reported with encouraging effects (Edwards et al., 2007; Giommarelli et al., 2010). To date, however, no study has been conducted in the evaluations of the combination effects of TSA and traditional cytotoxic agents (TXT) or molecular targeting therapy (erlotinib) against human lung cancer cells. Therefore, the present study was designed to investigate the antitumor activity of combining TSA with docetaxel or erlotinib on lung cancer A549 cells, with special emphasis on acetylated non-histone expressions relevant to the regulation of cell cycle and apoptotic pathway. Our results demonstrated that TSA in combination with docetaxel or erlotinib could be valuable in the treatment of lung cancer.

## Materials and Methods

### Materials

The human lung adenocarcinoma A549 cells were obtained from central laboratory of Provincial Hospital affiliated to Shandong University. TSA was purchased from Bio Vision, USA, and TXT was purchased from Qilu Pharmaceutical Co, Ltd, Shandong, China. Erlotinib was provided by Genentech. Hoechst33258 was purchased from Sigma, USA. Cell cycle kit was purchased from Beckman Coulter, USA. Anti-acetyl- $\alpha$ -tubulin and anti-acetyl lysine monoclonal antibodies were purchased from Cell Signaling technology, USA. EGFR-specific polyclonal antibody was purchased from Protein Tech Group, Inc. Hsp90 and caspase-3 antibodies were also purchased from Cell Signaling technology.

### Cell culture

A549 cell line was cultured in RPMI 1640 containing 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin and supplemented with 10% calf blood serum (Sijiqing Laboratories, Hangzhou, China) at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>.

### Cell growth assay

The effects of TSA, TXT, erlotinib, TSA/TXT, and TSA/erlotinib on A549 cell growth were measured by MTT assay. The cells were chose in log growth phase ( $6 \times 10^4$  cells/mL) and cultured overnight in 96-well plates (100  $\mu$ L/well). The cells were incubated with TXT (10  $\mu$ g/mL), erlotinib (10  $\mu$ mol/L), TSA (250 nmol/L), the combination of TXT (10  $\mu$ g/mL) with TSA (250 nmol/L), or erlotinib (10  $\mu$ mol/L) with TSA (250 nmol/L), and DMSO solvent as control for 6, 12, 24, 36, 48 or 72 hrs. Three replicate wells were conducted at each condition in all treatment groups. After the cells were cultured at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> for 24 hrs, MTT 20  $\mu$ L (5 mg/mL) was added for each well and then the cells were blended and incubated at 37 °C for 4 hrs. After removing the supernatant and adding the DMSO 150  $\mu$ L for each well. The absorbance value (A value) was quantified at 490 nm using a Spectra Max M2 and the average of each group was calculated. The inhibitory rate of cell growth was calculated as  $[1 - \text{Atest group} / \text{Acontrol group}] \times 100\%$ . The growth curve was drawn using time as abscissa and inhibition rate as ordinate. At least three replicate experiments were performed with three wells per concentration.

### Determination of apoptosis by Hoechst 33258 staining

The cells were cultured on axenic cover-slide in 6-well plates for overnight, making it up to fusion approximately 70%, then the cells were divided into six groups and treated with TSA (250 nmol/L),TXT (10  $\mu$ g/mL), TSA (250 nmol/L) plus TXT (10  $\mu$ g/mL), erlotinib (10  $\mu$ mol/L) and TSA (250 nmol/L) plus erlotinib (10  $\mu$ mol/L) respectively for 48 hrs.

After drug treatment as described above, cells were fixed with fixing solution for 5 min. After that, the cells were washed with PBS for three times and incubated in Hoechst 33258 solution (0.5 ml) for 5 min, then the cells were washed with PBS for three times again. Apoptosis was viewed under fluorescence microscopy.

### Flow cytometric analysis

Cell cycle analyses were carried out using Cell Cycle Kit according to the manufacture's protocol. A549 cells ( $6 \times 10^5$  cells/ml) were incubated for overnight and then divided into six groups treated with TSA250 (nmol/L),TXT (10  $\mu$ g/mL),TSA (250 nmol/L) plus TXT (10  $\mu$ g/mL), erlotinib (10  $\mu$ mol/L) and TSA (250 nmol/L) plus erlotinib (10  $\mu$ mol/L), respectively, for 24 hrs. The cells were harvested by trypsin and washed twice with PBS. Then the results were analyzed by Muticycle for windows software.

### Immunofluorescence analysis

The cells were cultured on cover-slide in 6-well plates overnight and then the culture media was changed. The cells were divided into four groups, and treated with TSA 250 (nmol/L), TXT (10  $\mu$ g/mL), and TSA (250 nmol/L) plus TXT (10  $\mu$ g/mL) respectively for 24 hrs. The cells were rinsed three times in PBS for 5 min each, fixed with 4% polyoxymethylene for 30min and then washed with

PBS for three times. The cells were treated with 0.3% Triton X-100 for 30 min and rinsed three times in PBS, blocked with goat serum for 30 min, aspirated blocking solution, applied diluted acetyl- $\alpha$ -tubulin primary antibody (1:800) and incubated overnight at 4 °C. The coverslips were rinsed three times in PBS and were incubated in fluorochrome-conjugated secondary antibody (1:400) for 1 hrs at room temperature in dark, rinsed three times in PBS for 5 min each. A drip of anti-fade reagent was added on the slides, acetyl- $\alpha$ -tubulin expression was detected under fluorescence microscopy.

#### Western blotting analysis

Cells in different groups were treated with TSA (250 nmol/L), TXT (10  $\mu$ g/mL), TSA (250 nmol/L) plus TXT (10  $\mu$ g/mL), erlotinib (10  $\mu$ mol/L), and TSA (250 nmol/L) plus erlotinib (10  $\mu$ mol/L) for 24 hrs. The whole protein was afterward extracted by Nuclear and Cytoplasmic Protein Extraction Kit and the concentration was analyzed in Bradford way. Equivalent cellular proteins were isolated and resolved in SDS PAGE and electro-transferred to PVDF membranes. The membranes were blocked in PBS buffer containing 10% nonfat dried milk, added the primary antibody solution and incubated overnight at 4 °C. Secondary antibody binding was carried out at room temperature for 1 h. The PVDF membranes were rinsed three times for 5 min each. Chemiluminescence detection was carried out with the ECL plus Western Blotting Detection System to detect the cleaved-caspase-3 expression, acetyl- $\alpha$ -tubulin, acetyl lysine, hsp90 and EGFR.

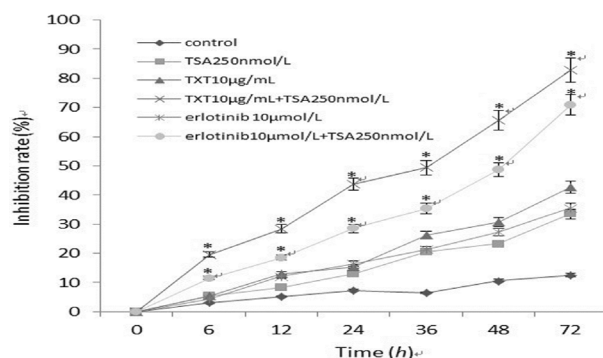
#### Data analysis

Data are presented as  $\bar{x} \pm s$  and were analyzed using the Student's t-test,  $\chi^2$  test and analysis of variance by SPSS 13.0 software.  $P < 0.05$  was considered to be statistically significant.

## Results

### TSA in combination with TXT or erlotinib produced synergistic inhibition on A549 cells

In this work, the inhibitory effects of TSA, TXT,



**Figure 1. TSA, TXT, Erlotinib and TSA in Combination with TXT or Erlotinib Inhibited the Growth of Lung Cancer Cell Line A549 in A Time-dependent Manner as Evidenced by MTT Assay.** A significant difference was observed between the combination treatment group and the control group or a single drug treatment group \* $P < 0.05$

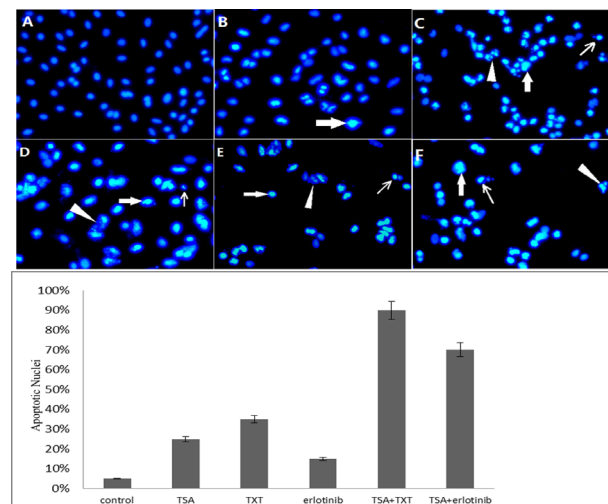
erlotinib alone and combination of TXT or erlotinib with TSA were determined over times by MTT assay, respectively. As illustrated in Figure 1, all three agents significantly inhibited cell proliferation in a time-dependent manner. Notably, TSA in combination with TXT or erlotinib caused a greater inhibitory effect on A549 cells than single-drug therapy ( $P < 0.05$ ). These data suggest that TAS-TXT or, TAS-erlotinib combination treatment yielded synergistic inhibition on A549 cells.

### TSA in combination with TXT or erlotinib induced apoptosis of A549 cells

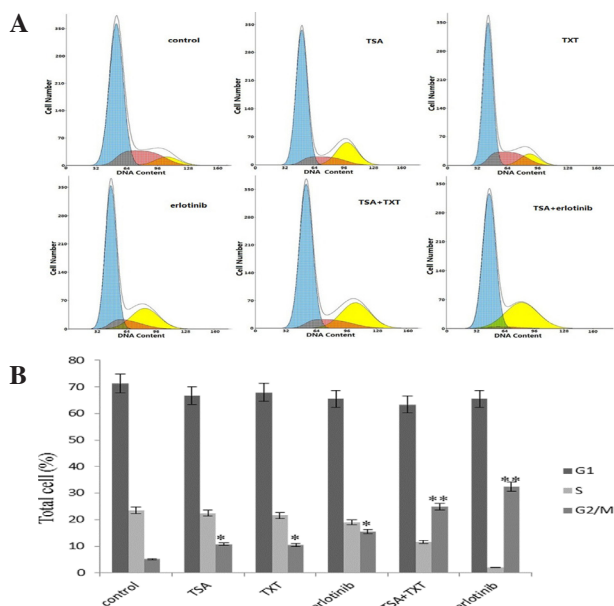
To examine the morphological changes of A549 cells, Hoechst 33258 staining was employed after the administration of drugs for 48 hrs. As shown in Figure 2, TSA, TXT, erlotinib alone and in combination with TSA led to marked morphological changes such as chromatin condensation, nuclear fragmentation and apoptotic bodies, which were in accordance with typical morphological characteristics of cells undergoing apoptosis (Figure 2A). Compared with single treatment, the two combinations of TSA with TXT or erlotinib elicited much more evident occurrence of apoptosis (Figure 2B). These findings suggest these agents exerted their inhibitory effects on A549 cells, mainly via triggering the apoptotic pathway.

### TSA in combination with TXT or erlotinib induced a delay at $G_2/M$ transition

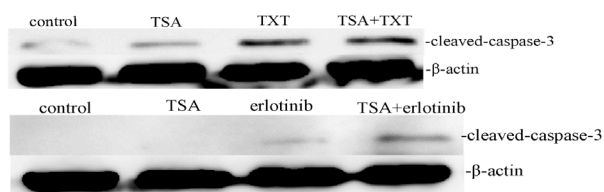
To further analyze the effects of these drugs alone and the combination with TSA, cell cycle distribution was tested by flow cytometry. As exhibited in Figure 3, TSA induced a delay in the  $G_2/M$  transition and, meanwhile, the  $G_2/M$  enrichment was accompanied by a reduction in S phase cells (Figure 3). TSA in combination with TXT or erlotinib significantly increased the delay in the  $G_2/M$  transition compared with TXT ( $24.9 \pm 2.1\%$ ) vs ( $10.5 \pm 1.2\%$ )



**Figure 2. Cell Apoptosis was Assessed by Hoechst 33258 Staining ( $\times 200$ ).** (A) Cells treated with DMSO as a control; (B) A549 cells treated with 250 nmol/L TSA; (C) with 10  $\mu$ g/mL docetaxel; (D) with 10  $\mu$ mol/L erlotinib; (E) with TSA in combination with docetaxel; and (F) with TSA in combination with erlotinib for 48 hrs. Chromatin condensation was observed in many treated cells (open arrows). Nuclear fragmentation appeared in some treated cells (arrowheads). apoptotic body presented in certain treated cells (solid arrows)



**Figure 3. Combined Effects of TSA/TXT or TSA/erlotinib on Cell-cycle Distribution.** (A) Histograms showed a number of cells per channel (vertical axis) vs DNA content (horizontal axis). (B) Percentage of cells at the G2/M transition after the treatment with drugs \*P<0.05 and \*\*P<0.01 compared with the control or compared between a single treatment and combination treatment



**Figure 4. Western Blot Analysis of Caspase-3 Expression in A549 Cells Treated with 250 nmol/L TSA, 10 µg/mL TXT, 10 µmol/L Erlotinib, and Their Combination for 24 hrs.** The cleaved caspase-3 was detected and β-actin was used as loading control. Western blots were representative of three independent experiments

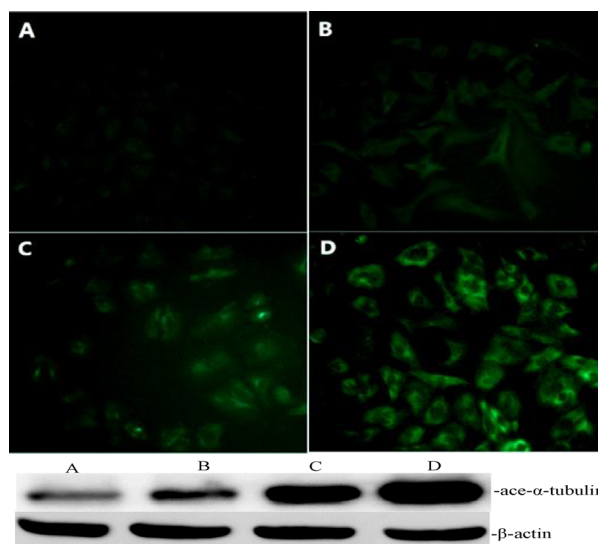
or erlotinib (34.5±3.1) vs (20.5±2.6) alone (P<0.05).

*TSA in combination with TXT or erlotinib increased the expression of cleaved-caspase-3*

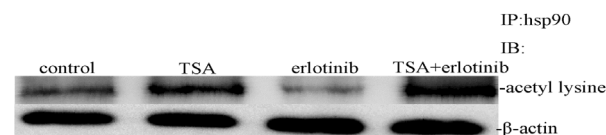
To investigate whether the treatment with docetaxel, erlotinib and their combination with TSA increased cleavage of caspase-3 known to be involved in the apoptotic cascade, Western blot analysis was used to examine the expression of caspase-3 in A549 cells following these interventions. The results showed that either single treatment or combination treatment resulted in activation of caspase-3. The level of cleaved caspase-3 protein in combination treatment cells was higher than that in single treatment cells (Figure 4). These findings indicated that the occurrence of apoptosis in response to these stimuli was through a caspase-dependent signal pathway.

*TSA in combination with TXT increased acetylation of α-tubulin*

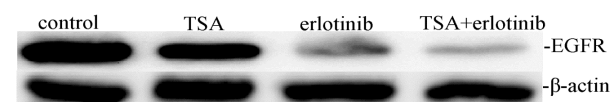
In order to understand the relationship between the combined activity of TSA and TXT and the mechanism



**Figure 5. Immunofluorescence and Western Blot Analysis in A549 Cells Treated with 250 nmol/L TSA, 10 µg/mL TXT, and Their Combination for 24 hrs.** Then the acetyl-α-tubulin was detected by immunofluorescence (×200) and Western blot



**Figure 6. TSA in Combination with Erlotinib Increased Acetylation of Hsp90 and Reduced the Expression of EGFR.** A549 cells were treated with 250 nmol/L TSA, 10 µmol/L erlotinib, and their combination for 24 hrs. Following this treatment, immunoprecipitates of hsp90 were immunoblotted with anti-acetyl lysine or anti-hsp90 antibody



**Figure 7. TSA in Combination with Erlotinib Reduced the Expression of EGFR.** A549 cells were treated with 250nmol/L TSA, 10µmol/L erlotinib, and their combination for 24 hrs. Following this treatment, the cell lysates were immunoblotted with anti-EGFR antibody. The levels of β-actin were served as loading control

responsible for cell apoptosis and cell cycle distribution on A549 cells, we next investigated the expression of acetyl-α-tubulin using Immunofluorescence and western blot analysis. As shown in Figure 5, compared with control group, treatment with either TSA or TXT alone for 24 hrs increased acetyl-α-tubulin expression. When cells were treated with TSA and TXT combination, the expression of acetyl-α-tubulin increased significantly.

*TSA in combination with erlotinib increased acetylation of hsp90 and reduced the expression of EGFR*

Lastly, we assessed the level of acetylated hsp90 in A549 cells treated with TSA, erlotinib and their combination for 24 hrs. As shown in Figure 6, TSA increased the expression of acetylated hsp90 compared with control group, whereas erlotinib did not. The combination treatment, however, significantly increased the expression of acetylated hsp90 compared with single agent. Because hsp90 play an important role in

stabilization of several proteins (including EGFR, raf-1, Akt and so on) implicated in the control of the cell growth and apoptosis (Powers et al., 2007), we assessed the expression of EGFR after the treatment with TSA, erlotinib and the combination for 24 hrs. We found that treatment of cells with TSA and erlotinib resulted in synergistic effects on depleting EGFR associated with acetylated hsp90 (Figure 7).

## Discussion

In the present study, we demonstrated that the combination of TSA with TXT or TSA with erlotinib synergistically reduced the cell viability in a time-dependent fashion using MTT assay, implying that the TSA can potentiate anti-tumor effects of docetaxel or erlotinib on A549 cells. In addition, this work exhibited that the A549 cells treated with the combined regimens significantly led to the appearance of chromatin condensation, nuclear fragmentation and apoptotic bodies in co-treatment groups compared with single drug treatment group as evidenced by Hochst33258 staining. These morphological changes are consistent with typical characteristics of cells undergoing apoptosis (Liu et al., 2008). Moreover, the cell cycle delay was induced at the G<sub>2</sub>/M transition. These findings indicate that the growth inhibition of A549 cells by the co-treatment of TSA with TXT or erlotinib is mainly due to the induction of apoptosis as well as the cell cycle arrest in G<sub>2</sub>/M stage. Our data are similar, in part, to the previous report that TSA provides a barrier to cell cycle progression for anti-proliferation and promotes escape from mitotic catastrophe and cell apoptosis, by inhibiting an HDAC-mediated transcriptional action (Noh et al., 2009). To further characterize the specific apoptotic pathway activated by these agents, the caspase-3 expression was examined. We demonstrated that treatment of cells with TSA in combination with TXT or erlotinib significantly increased the cleaved caspase-3 compared with a single agent, suggesting that the apoptotic process occurred in response to such stresses is via a caspase-dependent pathway.

Next, to probe the possible mechanism by which TSA enhances the cytotoxic effects of docetaxel and erlotinib on A549 cells, we firstly examined the alteration of tubulin in this process. It is known that tubulin, which mainly consists of  $\alpha$ -tubulin and  $\beta$ -tubulin, is necessary to keep cell morphology and motility as well as participate in substance transport. Blagosklonny et al. found that  $\alpha$ -tubulin is a non-histone substrate for histone deacetylase enzymes (Blagosklonny et al., 2002). Our previous studies also found that  $\alpha$ -tubulin acetylation is associated with microtubule depolymerization, and accumulation of acetylated tubulin following treatment with HDAC inhibitors increases microtubules stabilization, leading to cell apoptosis of papillary serous endometrial cancer cells (Jiang et al., 2006). In this work, we demonstrated that the administration of TSA led to a significant increase in acetylated  $\alpha$ -tubulin. This indicates that the possible mechanism underlying the enhancement effect of TSA on A549 cells may be via increasing acetylated  $\alpha$ -tubulin,

which, in turn, induces cell cycle arrest, or apoptosis.

Recently, Lysine K294 in the middle domain of hsp90 has been identified as an important acetylation site (Scroggins et al., 2007). In this investigation, the treatment of A549 cells with TSA and erlotinib resulted in a significant increase in acetylated hsp90. This suggests that TSA acts as a deacetylase not only for  $\alpha$ -tubulin but for hsp90 as well. It has been known that EGFR, a client protein, plays an important role in the ERK or PI3K-Akt signaling pathway (Ozaki K et al., 2010), which associates with the function of hsp90. We found that co-treatment of cells with TSA and erlotinib produced a synergistic effect on reducing the expression of EGFR. Our finding indicates that TSA enhanced cell apoptosis in combination with erlotinib may be related to the increase in acetylated hsp90 and reduction of EGFR activities.

In conclusion, our study provides strong in vitro evidence that TSA, a HDAC inhibitor, inhibits cancer growth by triggering an apoptotic pathway, which is via a caspase-dependent manner. One possible mechanism underlying the action of TSA may be non-histone protein acetylation. The TSA/docetaxel or TSA/erlotinib combination seems to hold a promise for the treatment of advanced lung cancer with limited sensitivity to docetaxel or erlotinib.

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