·Basic Research·

Synergistic cytotoxicity effect of histone deacetylase inhibitor combined with paclitaxel on lung cancer cell lines and its mechanism

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[Abstract] Background and Objective: Histone deacetylase (HDAC) inhibitors could inhibit the function of cell-signaling networks through decreasing the expression of multiple genes and proteins, thus affecting cell proliferation, survival, and chemosensitivity. HDAC inhibitors combined with paclitaxel may enhance the inhibitory effect of drugs on lung cancer cells. This study investigated the mechanism of the HDAC inhibitor trichostatin A (TSA) and observed the synergistic antiproliferative effects of TSA and paclitaxel (TAX) on lung cancer cell lines H322 and H1299. **Methods:** H322 and H1299 cells were divided into control group, TAX group, TSA group, and combination (TF) group (that is, TSA followed by TAX). Cell proliferation was determined by the methyl-thiazolyl-tetrazolium (MTT) assay. Cell cycle and apoptosis were determined by flow cytometry. The protein expression levels of survivin, extracellular signal-regulated kinase-1 (ERK), and poly-(ADP-ribose) polymerase (PARP) were determined by Western blot analysis. **Results:** When combined with TSA, the half-maximal inhibitory concentration (IC_{50}) of TAX decreased from (48.07±26.12) nmol/L to (6.34±5.72) nmol/L in H322 cells and from (110.6±38.7) nmol/L to (63.7±11.8) nmol/L in H1299 cells, with significant differences ($P<0.05$). The apoptotic proportion of H322 in the TF group was more than that in the TAX group ($P<0.05$). There was more necrosis in the TF group of H1299 cells than in the other groups of H1299. Phosphorylated ERK (pERK) was up-regulated in the TAX group of the H322 cell line. The expression of survivin was up-regulated in the TAX group of both cell lines. The expressions of survivin and pERK were down-regulated in the TSA and TF groups of both cell lines. Cleaved PARP could be detected in the TAX and TF groups of H322, but significantly more in the TF group. Cleaved PARP could not be detected in each group of H1299. **Conclusions:** TSA had a synergistic cytotoxic effect on lung cancer cell lines H322 and H1299 with TAX when the cells were treated with TSA followed by TAX. The mechanism may be that TSA down-regulates the expression level of survivin after exposure to TAX and blocks pERK protein expression.

Key words: lung cancer, histone deacetylase, trichostatin A, paclitaxel, apoptosis, ERK

Histone deacetylase (HDAC) inhibitors are a new kind of agent that show inhibition on various kinds of tumors. They inhibit the function of the signal transduction network mediated by numerous genes and proteins, and thus affect cell proliferation and sensitivity to chemotherapy. The diverse functions of HDAC inhibitors provide new thought on the combination of these agents with other therapies. In the hope of providing evidence for their clinical application, we used HDAC inhibitor trichostatin A (TSA) in combinatoin with paclitaxel (TAX) and detected their synergistic inhibition on lung cancer cells.

Materials and methods

Materials

Human lung cancer cell lines H322 and H1299 were kindly offered by the Academy of Military Medical Science. Reagents included methyl-thiazolyl-tetrazolium (MTT) and dimethyl sulfoxide (DMSO, both from Sigma), rabbit anti-human t-ERK polyclonal antibody, mouse anti-human p-ERK polyclonal antibody, mouse anti-human poly-(ADP-ribose) polymerase (PARP) monoclonal antibody, mouse anti-human β-actin monoclonal antibody, and mouse anti-human survivin monoclonal antibody (Cell Signaling, US), bicinchoninic acid (BCA) protein detection reagent kit (Pierce, US), TAX (Sigma, US) and TSA

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Methods

Cell culture The H322 and H1299 cell lines were cultured in high-glucose Dulbecco's modified eagle medium (DMEM) (supplemented with 10% fetal bovine serum, 10 u/mL penicillin and 100 u/mL streptomycin), as is routine. Cultured cells were digested in a 0.25% trypsin buffer, processed, and then incubated at 37℃ in a humid chamber at 5% CO_2.

MTT detection Cells at the logarithmic growth phase were obtained and injected to a 96-well plate at a cell density of 1×10^4 cells/well and incubated overnight. Cells of each line were assigned to three groups: TAX 96-hour group (TAX-MTT group), TSA 12-hour group (TSA-MTT group), and TSA 12-hour + TAX 84-hour group (TF-MTT group). The culture medium was replaced every 12 h. For the TF-MTT group, the concentration of TSA in all wells was 300 nmol/L, while the concentration gradients of TAX were 0.001, 0.01, 0.1, 1, 10, 100 and 1000 nmol/L. When switching from TSA to TAX, the culture medium was removed by pipette, the cells were washed with 200 μL of phosphate buffered saline (PBS) once, and TAX of a corresponding concentration was added. The concentration gradients of TSA for the TSA inhibition curve were 18.75, 37.5, 75, 150, 300, 600, and 1200 nmol/L at 12 h, and the TSA culture medium was replaced by a blank culture medium. Three wells were prepared for each group, with normal-control and blank-control wells. The cells were incubated for 96 hours in 96-well plates. As is routine, the MTT and lysis buffers were added sequentially; the mixture was oscillated and then measured for absorbance (*A* value). The cell-survival rate was calculated based on this formula: survival rate = (*A* value of treatment group/*A* value of blank control group) × 100% . The curve regression model was used to accommodate the dose-effect relationship, and the half-maximal inhibitory concentration (IC_{50}) of TSA at various time points was obtained. In total, three repeated experiments were performed.

Flow cytometry for the analysis of the cell cycle and apoptosis rate Cells at the log growth phase were obtained and injected into 6-well plates at the cell density of 1×10^4 cells/well. When the cells reached 60% confluence, they were assigned to normal control group, TAX 24-hour group (TAX-FCM group), TSA 12-hour group (TSA-FCM group), and TSA 12-hour + TAX 24-hour group (TF-FCM group). The culture medium was replaced every 12 h. TSA concentration was 300 nmol/L, and TAX concentration equaled 10 nmol/L. When ready for treatment, the cells were digested in 0.25% trypsin and the digested cells were collected, centrifuged at 1000 r/min for 10 min, and washed with PBS twice. Then the cells were resuspended in 0.6 mL of PBS (supplemented with 1% fetal bovine serum), and 1.4 mL of cold ethanol was added. The suspension was well blended and the cells were fixed and preserved at -20℃ . On the day of detection, the cells were washed with PBS twice and resuspended in 0.5 mL of PBS and 5 μL of ribonuclease (RNase) was added. The suspension was bathed in water at 37℃ for 30 min. With 50 μL of 1 mg/mL of propidium iodide (PI) added, the suspension was light-shielded and incubated for 20 min at room temperature and then underwent detection. A total of three repeated experiments were performed.

Fluorescent microscopic observation of the nuclear morphology of the cells Cells at the log growth phase were obtained and injected into 6-well plates, with 1×10^5 cells and 2 mL of culture medium in each well. The cells were grouped as described above. When ready for treatment, Hoechst 33342 was added to the resulting concentration of 1:1000, and the cells were incubated for 20 min in a humid chamber at 37℃ and 5% CO_2. Hoechst 33342 gave off a blue light when irradiated by ultraviolet light under a fluorescent microscope. Images were taken by a fluorescent microscope (× 20) and an optical microscope, respectively.

Western blot detection of protein expression The expression of total and phosphorylated extracellular signal regulated kinases (tERK and pERK), human PARP, and survivin was measured.

Cells were grouped as described above. When ready for treatment, total protein was extracted with detergent-based cell lysis and protein concentration was measured with the Bradford method. Equal amounts of protein were obtained and separated by vertical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% milk in an oscillator at room temperature, and then incubated with the primary antibody at 4℃ overnight. The membrane was washed at room temperature, a secondary antibody was added, and then the membrane was illuminated by ECL PLUS (Amersham).

Statistical analysis

Data processing was conducted by statistical software SPSS11.0. A one-way ANOVA was used to test the significance of the difference between the groups and was also used in the correlation analysis for some parameters. The test level was set to α=0.05. Based on the combination index (CI) in median-effect analysis,[1] CI<1 indicated synergism, CI>1 indicated antagonism, and CI=1 indicated effect additivity. If more than two points on the curve showed CI<1, synergism between the two agents was indicated.

Results

The inhibition of the proliferation in cultured cells in vitro by HDAC inhibitor TSA

As shown on the inhibition curves, TSA significantly improved the inhibition of TAX on H322 and H1299 cells when these two agents were used in combination. The IC_{50} of TAX in H322 cells was significantly reduced from (48.07±26.12) nmol/L to (6.34±5.72) nmol/L (P<0.05), while the IC_{50} of TAX in H1299 cells also significantly decreased from (110.6±38.7) nmol/L to (63.7±11.8) nmol/L (P<0.05). According to the CI analysis, in the inhibition curves of the TF-MTT groups for both cell lines, the CI value at each point was <1, indicating a synergic effect between TSA and TAX. The growth curves for the two groups of cells are shown in Figures 1 and 2.

The influence of TSA on the cell cycle and apoptosis of H322 and H1299 cells

When H322 and H1299 cells were treated with TSA for 12 h, no significant changes in the cell cycle were seen as compared to the control group. When treated with TAX for 24 h, the H322 cells at the G_2/M phase significantly increased as compared to control group (P<0.05), accompanied by cell apoptosis. The TF-FCM group had fewer G_0/G_1-phase cells than either the control or the TSA-FCM groups. When compared to cells in the TAX-FCM group, cells in the TF-FCM group were significantly arrested in the G_0/G_1 phase, with significantly increased apoptotic cells (P< 0.05) (Table 1, Fig. 3). With regard to the H1299 cell line, the TAX- and TF-FCM groups had significantly more G_0/G_1-phase cells and significantly fewer G_2/M- and S-phase cells than the control group (both P<0.05). All four groups had low rates of apoptosis (Table 2, Fig. 4).

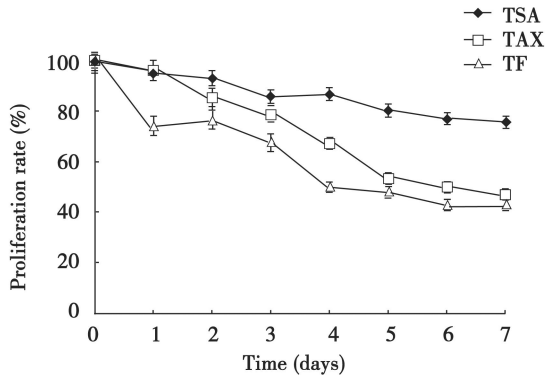

Figure 1 Proliferation curves of H322 cells treated with TSA at 12 h, TAX at 96 h, and combined action (TSA 12 h followed by TAX 84 h)

TSA, trichostatin A; TAX, paclitaxel; TF, TSA followed by TAX.

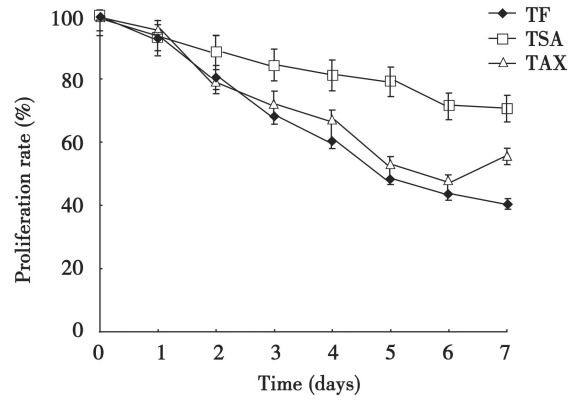

Figure 2 Proliferation curve of H1299 cells treated with TSA at 12 h, TAX at 96 h, and combined action (TSA 12 h followed by TAX 84 h)

Abbreviations as in Figure 1.

Table 1 Cell cycle and apoptosis changes of H322 cells in each group (%, $x\pm s$)

Group	Cell proportion			Apoptosis rate
	G_0/G_1 phase	S phase	G_2/M phase	
Control	55.80±4.43	31.25±2.06	12.95±2.17	0.91±0.76
TSA	53.47±4.81	31.64±2.85	14.89±2.23	1.47±0.54
TAX	11.75±1.12	29.84±2.36	58.41±5.37	12.33±1.78
TF	42.70±3.96	38.49±3.35	18.81±2.14	30.00±4.56[a]

[a]Apoptosis rate of H322 cells is significantly higher in combined group (TF) than in other groups (P<0.05).

TSA, trichostatin A; TAX, paclitaxel; TF, TSA followed by TAX.

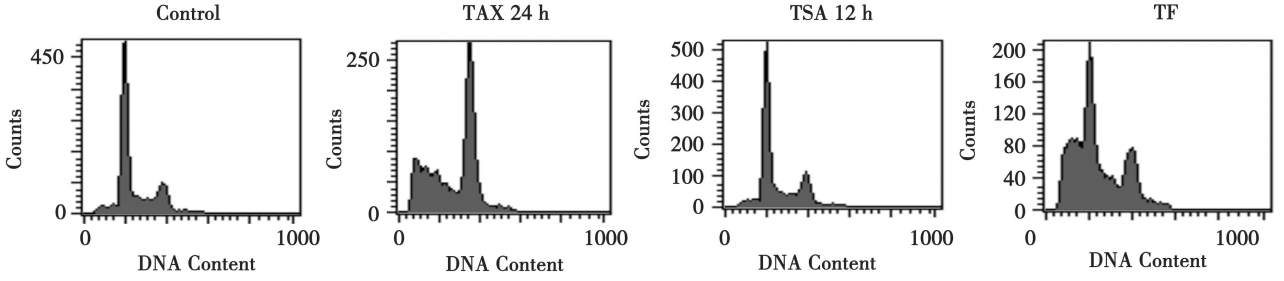

Figure 3 Flow cytometric analysis of each group of H322 cells

In the control group, H322 and H1299 cells were seen as round, oval, or occasionally semi-lunar. When treated by TSA, TAX, or the combination, H1299 cells showed frequent eccentric and horseshoe-like nuclei, together with necrotic changes, such as polynuclear and vacuolar degeneration, and minimal apoptotic bodies. The TF-FCM group had more cell death than any other group. When stained with Hoechst 33342, H322 cells in the TAX- and the TF-FCM groups showed more cell debris and apoptotic bodies. A small amount of apoptotic bodies could be found in the TSA-FCM group (Fig. 5).

The influence of TSA on the expression of ERK and survivin proteins

When comparing pERK expression levels of the normal control group and the TAX group to the tERK expression levels of the corresponding groups, pERK/tERK was 1.26 in H322 cells of the normal control group and 1.42 in the TAX group, suggesting

Table 2 Cell cycle and apoptosis changes of H1299 cells in each group (%, $x\pm s$)

Group	Cell proportion			Apoptosis rate
	G_0/G_1 phase	S phase	G_2/M phase	
Control	53.25±4.56	30.46±3.14	16.29±2.12	0.03±0.05
TSA	58.51±5.54	28.23±3.07	13.26±1.81	0.02±0.05
TAX	69.92±5.63[a]	18.80±2.32[b]	11.29±2.09[b]	0.33±0.28
TF	78.82±6.38[a]	12.26±1.84[b]	8.91±1.17[b]	1.47±0.89

[a]$P<0.05$, comparing with control group with significant increase; [b]$P<0.05$, comparing with control group with significant decrease.

Abbreviations as in Table 1.

that the expression of the pERK protein in H322 cells increased slightly with the use of TAX. In H1299 cells, pERK/tERK was 0.45 in the normal control group and 0.23 in the TAX group. That is, the expression of the pERK protein decreased. When treated with TSA alone, both H322 and H1299 cells expressed less pERK protein. In the TF group, the combination significantly inhibited the expression of the pERK protein. Both H322 and H1299 cells of the TF group expressed less protein than those of the TAX group (Fig. 6).

When treated with TAX for 24 h, both H322 and H129 cells expressed more survivin, which was 1.71 and 1.61 times, respectively, of that in the normal control group. TSA downregulated the expression levels of survivin to 78% and 46%,

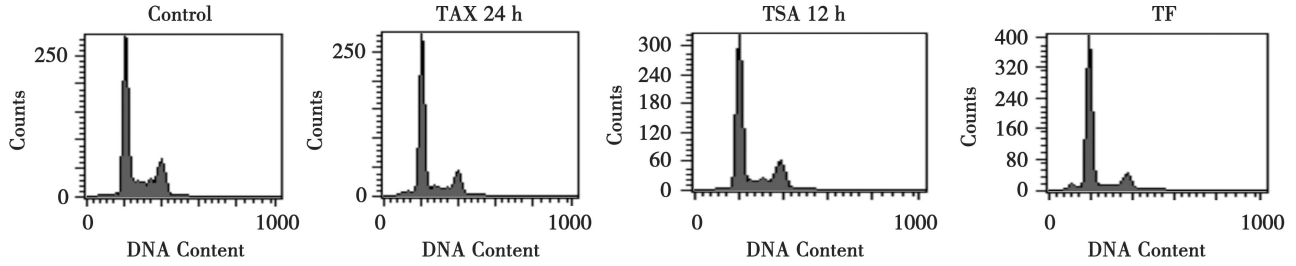

Figure 4 Flow cytometric analysis of each group of H1299 cells

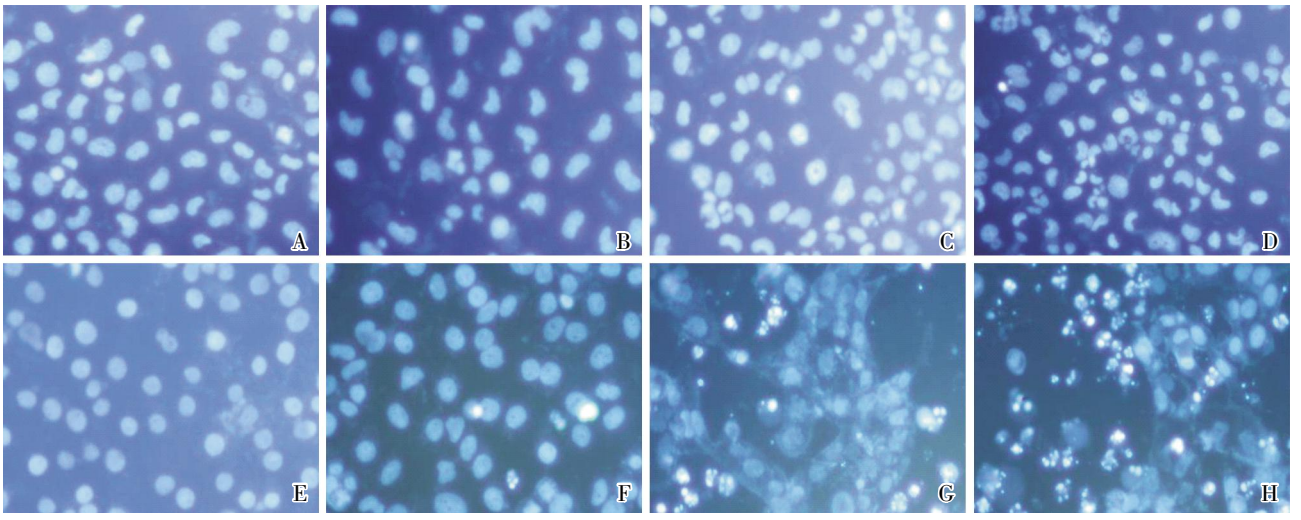

Figure 5 Nuclear morphology of each group of cells stained with Hoechst 33342 (×20)

A, H1299 cells control (H1299 cells nuclei are round and oval, and a small number of them half-moon);

B, H1299 cells treated with TSA for 12 h (H1299 cell nuclei show horseshoe-like changes accompanied by multi-core and death of the phenomenon, such as vacuolar degeneration);

C, H1299 cells treated with TAX for 24 h (H1299 cell nuclei show horseshoe-like changes accompanied by multi-core and death of the phenomenon, such as vacuolar degeneration, a small number of apoptotic bodies);

D, H1299 cells treated with TF (H1299 cell nuclei show horseshoe-like changes; accompanied by multi-core and death of the phenomenon, such as vacuolar degeneration, a small number of apoptotic bodies);

E, H322 cells control (H322 cells show round nuclei, including a small number of oval-shaped nuclei);

F, H322 cells treated with TSA for 12 h (a small amount of H322 cell nuclei fragmentation and a small number of apoptotic bodies are shown);

G, H322 cells treated with TAX for 24 h (more H322 cell nuclei fragmentation, dissolution, and more apoptotic bodies are shown);

H, H322 cells treated with TF (more H322 cell nuclei fragmentation, dissolution, and more apoptotic bodies are shown).

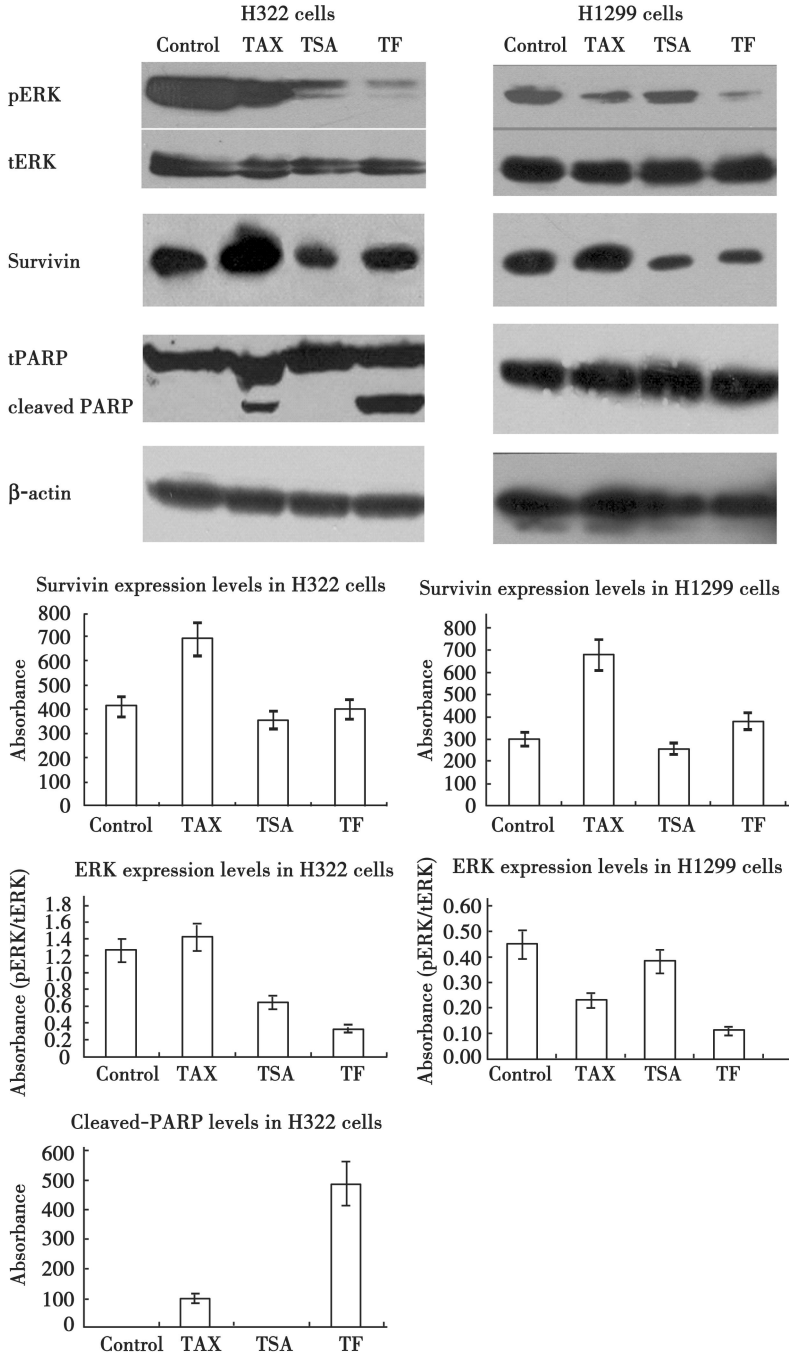

Figure 6 Expression levels of survivin, ERK, and PARP proteins in each group of H322 cells and H1299 cells

Abbreviations as in Figure 1.

respectively, of those in the normal control groups. TSA, when used in combination, sig nificantly downregulated the high expression level of survivin induced by TAX. The expression levels of the survivin protein was 51% and 33%, respectively, of those in the TAX groups (Fig. 6).

The PARP protein was detected in both the TAX and the TF groups. The TF group had significantly more PARP protein than the TAX group, while no cleaved PARP protein was detected in any of the groups of H1299 cells (Fig. 6).

Discussion

Chemotherapy is the major treatment approach for cancer. However, due to increasing rates of resistance, the response rate of chemotherapy has not been as good as expected. In recent years, studies have found that HDAC inhibitors could influence cell proliferation and sensitivity to chemotherapeutic agents by inhibiting the functions of the signal transduction network.[2] HDAC

inhibitors combined with chemotherapy can reduce the resistance to chemotherapy and increase the response rate to chemotherapy. Fuino *et al*.[3] used the HDAC inhibitor LAQ824 to treat breast cancer cells in combination with numerous kinds of chemotherapeutic agents, and found that LAQ824 could enhance the cell apoptosis induced by chemotherapeutic agents including TAX and gemcitabine, as well as increasing the sensitivity to chemotherapeutic agents in breast cancer cells. Imanishi *et al*.[4] demonstrated that all HDAC inhibitors could enhance cytocidal effects of TAX on tumor cells.

Our results suggest that HDAC inhibitor TSA in combination with TAX could improve the cytotoxicity of TAX on lung adenocarcinoma cells H1299 and H322. The combination significantly increased the inhibition rate on both cell lines with TAX, and also remarkably reduced the IC_{50} of TAX in both cell lines. That is, TSA significantly increased the inhibition sensitivity in both cell lines. However, when treated with the combination, the IC_{50} of TAX decreased to different extents in the two cell lines. The IC_{50} decreased to 13.2% in H322 cells and to 57.6% in H1299 cells. This finding suggests that the combination might induce different effects depending on different cell features.

HDAC inhibitors hinder tumor growth by keeping cells arrested in a certain phase of the cell cycle and inducing cell apoptosis. Different HDAC inhibitors may induce different cell-cycle-related changes in different cells. The study by Blagosklonny *et al*[5] showed that TSA and butyric acid kept A549 cells arrested in the G_1 phase, whereas the docking protein (DP) rendered A549 cells arrested in the G_2/M phase. Our study results revealed that TSA kept H1299 and H322 cells arrested in the G_1 phase, but the cell-cycle-related changes induced by the combination was far more complicated than by that of either drug alone. When TSA was used in combination with TAX, as many or more H1299 cells were arrested in the G_1 phase, while H322 cells were arrested in the G_2 and S phases. TAX mainly affected G_2-phase cells. More cells arrested in the G_1 phase resulted in fewer target cells for TAX. Therefore cells arrested in the G_1 phase might impact sensitivity to TAX.

In our study, we detected the early cell-cycle-related changes within the first 36 h. These changes, together with the results from cell growth inhibition curves, suggested that early G_1-phase arrest did not decrease the cell growth inhibition of TAX. Besides inducing G_1-phase arrest, HDAC inhibitors also triggered cell apoptosis or programmed cell death, which also resulted in a cytocidal effect on the tumor cells.[6] The inconsistent changes related to the cell cycle induced by the combination of TSA and TAX might be one of the reasons for the different synergic effects with the combination. Continued observation on the cell-cycle-related changes induced by the combination at various time points might better explain the influence of these inconsistent changes on the synergic effects of the combination.

Our study detected the PARP protein an early marker of cell apoptosis.[7] Cleaved PARP protein was detected when H322 cells were treated with TAX or the combination, while the expression of the cleaved PARP protein with the combination increased significantly compared to that with TAX alone, indicating that, compared to TAX alone, TSA combined with TAX increased apoptosis in H322 cells. The results of flow cytometry also showed that TSA combined with TAX increased the apoptosis in H322 cells compared to TAX alone. Results from the Hoechst 33342 staining further confirmed that TSA-enhanced cell apoptosis induced by TAX.

Unlike H322 cells, H1299 cells hardly showed apoptosis. In the combination group, apoptosis was detected in only 1.47% of the cells by flow cytometry. This was higher than the cell apoptotic rate induced by TAX alone, but such a low level of apoptosis was not enough to explain the changes in the inhibition curve. On the other hand, no cleaved PARP was seen in the PARP protein measurement. Hoechst 33342 staining revealed morphological changes in the cell nucleus and minimal apoptotic bodies. All these results suggested that the combination of TSA and TAX did not produce the synergism on H1299 cells by inducing cell apoptosis; they might induce nonapoptotic cell death. Chobanian *et al*.[8] also suggested that apoptosis in ovarian cancer cells, as induced by HDAC inhibitors TSA and SAHA combined with TAX, was only partially responsible for the ultimate cell death. Therefore, we believed that, in early stages of the treatment with TAX alone or TAX in combination with TSA, cell apoptosis was not the main mechanism underlying the inhibition of H1299 cells; there might be nonapoptotic death involved. Whether different death mechanisms in the two cell lines were related to the different extents of IC_{50} decrease in the two cell lines when treated by the combination has to be further investigated.

Antitumor agents activate a series of death-related genes in tumor cells and thus trigger cell necrosis and apoptosis. At the same time, they also trigger self-protective mechanisms in tumor cells by releasing antiapoptotic genes and activating cell signaling pathways to keep tumor cells proliferating and avoiding cell death. Among these protective responses, increased expression of the survivin gene and the activation of the ERK pathway have important roles. In addition, these protective responses are closely related to the staging, prognosis, and the sensitivity to drugs in patients with lung cancer, and chemotherapy efficacy can be improved by blocking these protective responses.[9,10]

Our study results also showed that, when H322 and H1299 cells were treated by TAX at the concentration of 10 nmol/L for 24 h, the expression level of survivin was 1.71 times and 1.6 times, respectively, of that in the normal control group. The expression of pERK increased slightly in the H322 cells and decreased in the H1299 cells. The changes in pERK were related to treatment time and the concentration of TAX. Studies revealed that the ERK pathway could be activated and the expression of pERK increased by TAX only with prolonged treatment at lower concentrations or treatment at higher concentrations.[11] In our study, we treated cells with TAX at 10 nmol/L for 24 h. Such treatment concentration or time might not be enough to activate the ERK pathway. When used alone or in combination with TAX, TSA significantly inhibited the expression of survivin and pERK in both cell lines. Importantly, the expression of both survivin and pERK with the com bination was remarkably decreased when

compared to TAX alone, indicating that TSA inhibited the self-protective responses in both cell lines and might be the mechanism of synergistic cytotoxicity of TAX and TSA.

In conclusion, the HDAC inhibitor TSA in combination with the chemotherapeutic agent TAX could improve the growth inhibition of TAX on the lung cancer cell lines H1299 and H322. The synergism of the combination was seen as increased cell apoptosis in H322 cells, whereas in H1299 cells, there might be nonapoptotic factors involved. When used in combination, TSA inhibited the expression of survivin and pERK, which might be the mechanism underlying the synergistic cytotoxicity of TAX and TSA.

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