Original Article

Trichostatin A sensitizes cisplatin-resistant A549 cells to apoptosis by up-regulating death-associated protein kinase

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Aim: To investigate the apoptosis-inducing effect of trichostatin A (TSA) in the human lung adenocarcinoma cisplatin-resistant cell line (A549/CDDP) and to examine whether TSA can enhance sensitivity to cisplatin treatment and the underlying molecular mechanisms of such an enhancement.

Methods: Cell viability was evaluated using the Neutral Red assay. Apoptosis was assessed using Hoechst 33258 staining and flow cytometry analysis. Protein expression was detected by Western blotting. To determine the role of Death-associated protein kinase (DAPK) in TSA-induced apoptosis in the A549/CDDP cell line, cells were transfected with pcDNA3.1(+)-DAPK, which has a higher expression level of DAPK compared to endogenous expression, and DAPK activity was inhibited by both over-expression C-terminal fragment of DAPK which may competitive binding DAPK substrates to inhibit the function of DAPK and RNA interference.

Results: TSA induced apoptosis in both A549 cells and A549/CDDP cells. TSA enhanced the sensitivity of A549/CDDP cells to cisplatin, along with concomitant DAPK up-regulation. When DAPK was over-expressed, A549/CDDP cells became sensitive to cisplatin and the cytotoxicity of TSA could be increased. Moreover, the cytotoxicity of TSA could be alleviated by inhibition of DAPK activity by the expression of a recombinant C-terminal fragment of DAPK or RNA interference.

Conclusion: TSA induced sensitivity to cisplatin treatment in cisplatin-resistant A549 cells. The up-regulation of DAPK is one of the mechanisms mediating sensitization to TSA-induced apoptosis in cisplatin-resistant cells.

Keywords: trichostatin A; cisplatin-resistance; lung cancer; death-associated protein kinase
ity could contribute to a general increase in gene expression including cell cycle inhibitor gene p21, p53, DAPK and the von Hippel-Lindau tumor suppressor genes as well as the pro-apoptotic genes Bax and Bad\(^{[18-21]}\), the molecular mechanisms of TSA-sensitized cytotoxicity to chemotherapeutic drugs remain largely unknown.

Death-associated protein kinase (DAPK), a modulator of apoptosis, is a cytoskeleton-localized Ca\(^{2+}\)/calmodulin (CaM)-regulated serine/threonine kinase that modulates cell death\(^{[22]}\). Recently, it was demonstrated that impaired translation of DAPK mRNA was involved in the acquisition of cisplatin resistance in human cancer cells\(^{[23]}\). However, how it is involved in the development of resistance to chemotherapy in cancer cells is unknown. Based on these observations, we hypothesized that DAPK might play an important role in TSA-induced apoptosis in the cisplatin (CDDP)-resistant A549 lung cancer cell line (A549/CDDP).

In this study, we report that TSA enhances the chemosensitivity of A549/CDDP cells, which correlated with the up-regulation of DAPK.

**Materials and methods**

**Cell culture**

Cells from the lung cancer cell line A549 and the CDDP-resistant derivative A549/CDDP were gifts from Cell Center of Cell Culture (Central South University, Changsha, China). They were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL, Grand Island, NY, USA) containing 100 µg/mL penicillin and 100 µg/mL streptomycin and supplemented with 10% calf blood serum (Sijiqing Laboratories, Hangzhou, China) at 37 °C in a humidified atmosphere with 5% CO\(_2\). Then 2 µmol/L cisplatin (Qilu Pharmaceutical Co, Ltd, Shandong, China) was added to the medium of the A549/CDDP cell line. A549/CDDP cells were cultured in complete DMEM medium without cisplatin for 3 d before being used in experiments.

**Plasmids and RNA interference**

The pcDNA3.1(+)–DAPK, pcDNA3.1(+)–DCTP, pcDNA3.1(+) and pDsRed1-N1-U6 shRNA vectors were gifts from Dr Haitao ZHANG (Guangdong Medical College, China).

The synthetic sequences (sense, 5’-CGTAACCTATATCCCTCTAAGTCCAAGAGACTTAGGAGGATATGTTATTTTGGAG-3’, antisense, 5’-GATCCTTCCCAAAAAAAATAACCTATATCCTCTAAGTCTCTGAACTTAGGAGGATATAGGTTATTGGAAG-3’, antisense, 5’-GATCCTTCCAAAAAAATAACCTATATCCTCTAAGTCTCTGAACTTAGGAGGATATAGGTTATTGGAAG-3’, antisense, 5’-GATCCTTCCAAAAAAATAACCTATATCCTCTAAGTCTCTGAACTTAGGAGGATATAGGTTATTGGAAG-3’, antisense, 5’-GATCCTTCCAAAAAAATAACCTATATCCTCTAAGTCTCTGAACTTAGGAGGATATAGGTTATTGGAAG-3’, antisense) were annealed and cloned into pDsRed1-N1-U6 shRNA vectors.

**Cellular transfection**

The cells were transfected with different vectors using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s guidelines. Stable cell lines were cultured in medium containing 800 µg/mL G418 (Invitrogen). G418 concentration was reduced to 400 µg/mL after three weeks. Cells were treated with TSA or cisplatin and cell viability was determined by cytotoxicity assay using the Neutral Red assay.

**Treatment with cisplatin or TSA**

Both A549/DDP and A549 cells were cultured in 96-well plates (1.0×10\(^3\) cells/well) and treated with different concentrations of cisplatin or TSA (Sigma, St Louis, MO, USA) at 37 °C in a humidified atmosphere with 5% CO\(_2\) for 24 h.

**Combined treatment with cisplatin and TSA**

A549/CDDP cells were cultured in 24-well plates (3.0×10\(^5\) cells/well) and treated with different concentrations of cisplatin and TSA at 37 °C in a humidified atmosphere with 5% CO\(_2\) for 24 h. The cells were divided into three groups: the first group was cultured with different concentrations of cisplatin; the second group was cultured with 31.25 nmol/L TSA and cisplatin; and the third group was cultured with 62.5 nmol/L TSA and cisplatin.

**Cell viability assays**

Following the drug treatments described above, cytotoxicity assays were performed as described previously\(^{[24]}\). Briefly, the drug-containing medium from each well was replaced with 100 µL medium containing 50 mg/L Neutral Red for an additional 5-h incubation. Then, the cells were rinsed with PBS three times. A volume of 200 µL of 1% acetic acid/50% ethanol was added to each well to extract the dye from lysosomes of viable cells. The amount of dye was quantified at 540 nm using a Varioskan Flash Reader spectrophotometer (THERMO, USA). The inhibitory rate of cell growth was calculated as \[1 - \frac{A_{\text{test group}}}{A_{\text{control group}}} \times 100\%\].

**Flow cytometry analysis**

The cells were collected by centrifugating at 1000 r/min for 5 min after treatment, washed twice in PBS and fixed with ice-cold 70% ethanol overnight. Prior to flow cytometry analysis, the fixed cells were washed once with PBS and incubated with 100 µg/mL propidium iodide (Sigma, USA) plus 200 µg/mL Rnase (Promega,USA) for 10 min. The DNA profile was analyzed by flow cytometry (EPICS XL, Coulter, Fullerton, CA, USA). The percent of apoptosis is calculated as the percentage of the sub-G\(_1\) peak as determined by FACS analysis cell-cycle profile following propidium iodide staining\(^{[25]}\).

**Hoechst 33258 staining**

Apoptosis was evaluated by in situ uptake of propidium iodide and Hoechst 33258 as described by McKeague et al\(^{[26]}\). In brief, after being treated with TSA, the cells were washed with PBS, and incubated in PBS containing 40 µg/mL propidium iodide and 2.5 µg/mL Hoechst 33258 (Sigma, USA) for 10 min. A volume of 500 µL of methanol:acetic acid (3:1) fixative was then added directly to each well. Cells were viewed under fluorescence microscopy (Nikon Eclipse ET2000-E, Japan). Four replicate wells were analyzed for each treatment by quantitative and qualitative examination of four random fields in each well. The apoptotic index was calculated from the number of apoptotic nuclei vs total number of nuclei in each
visual field.

**Western blot analysis of DAPK expression**

The cells were collected, washed twice with PBS and incubated with cell decomposition buffer (pH 8.0) that contained 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, 0.05% PMSF, 2 µg/mL aprotinin (Sigma, USA), and 2 µg/mL leupeptin (Sigma, USA). The level of proteins was determined by Western blot with a DAPK monoclonal antibody (clone-55, Sigma, USA), anti-DAPK-phosphoserine308 monoclonal antibody (Sigma, USA), and anti-β-actin monoclonal antibody (clone AC-74, Sigma, USA). Primary antibodies were detected with goat anti-mouse or goat anti-rabbit antibodies conjugated to horseradish peroxidase using enhanced chemiluminescence (Beyotime Institute of Biotechnology, Jiangsu, China). Photographs were taken with the FluerChem SP8900 automatic photomicrograph system (ALPHA, USA) and analyzed.

**Statistical analysis**

Data are presented as mean±SD and were analyzed using the Student’s t-test and Student-Newman-Keuls (SNK) test. P<0.05 was considered to be statistically significant.

**Results**

**Effect of TSA on A549 cells and A549/CDDP cells**

Consistent with our previous reports[27], here we showed that the IC50 of cisplatin to A549 cells and A549/CDDP cells was 2.36±0.10 µmol/L and 30.27±1.50 µmol/L, respectively (Figure 1A). A549/CDDP cells were 12.8 times more resistant than the parental A549 cells. Western blot analysis showed that A549/CDDP cells had a lower expression of DAPK than the A549 cell line (Figure 1B).

As shown in Figure 2A, morphological evaluation using Hoechst 33258/PI staining with fluorescence microscopy revealed a significant increase in the number of cells showing nuclear condensation and fragmentation after incubation with TSA for 24 h in both A549 and A549/CDDP cells.

A significant decrease in cell viability was detected by the Neutral Red assay after cells had been treated with different concentrations of TSA for 24 h. The assay showed that the IC50 of TSA in A549 cells and A549/CDDP cells was 418.66±25.12 nmol/L and 446.59±27.32 nmol/L, respectively (P>0.05, Figure 2B). Apoptosis was measured as the percentage of sub-G1 DNA content, as well as DNA condensation with Hoechst 33258 staining. The results showed that TSA induced apoptosis in a dose-dependent manner (Figure 3).

**TSA enhances the sensitivity of A549/CDDP cells to cisplatin**

Low concentrations of TSA (31.25 nmol/L and 62.5 nmol/L) had little effect on cell viability and apoptosis of A549/CDDP cells. The inhibitory rate of cell growth was 1.08% and 2.33%, respectively (Figure 4). Thus, these two concentrations of TSA were selected to determine whether TSA could sensitize A549/CDDP cells to cisplatin-induced apoptosis.

The results showed that the IC50 of cisplatin to A549/CDDP cells was at 31.34±1.23 µmol/L in the absence of TSA, whereas the IC50 was 20.61±1.80 µmol/L and 18.48±0.70 µmol/L in the presence of 31.25 nmol/L and 62.5 nmol/L TSA, respectively (P<0.05 vs control) (Figure 4). The reverse fold was calculated according to the formula (IC50 of resisted-drug cell line)/(IC 50 of combining reversal drug to resistanted-drug cell line). The results showed that the reverse fold was 1.52 and 1.70 in the presence of 31.25 nmol/L and 62.5 nmol/L TSA, respectively. This indicates that TSA treatment increases the sensitivity of A549/CDDP cells to cisplatin.

**TSA up-regulates DAPK expression in A549/CDDP cells**

The results showed that the expression of DAPK was elevated when A549/CDDP cells were treated with different concentrations of TSA for 24 h (Figure 5A). In addition, DAPK expression was up-regulated in a time-dependent manner (Figure 5C). The phosphorylation of Serine-308 deactivates DAPK and dephosphorylation relieves auto-inhibition and stimulates the pro-apoptotic activities of DAPK[28, 29]. Our results showed that the phosphorylation level of DAPK was unchanged or only slightly increased with TSA treatment (Figure 5A, 5C). However, the relative phosphorylation level actually decreased (Figure 5B, 5D). Furthermore, we confirmed that cisplatin had no effect on DAPK expression in A549/CDDP cells (Figure 6).
DAPK expression and its activity affect the sensitivity of A549/CDDP cells to cisplatin

Various expression and interference DAPK vectors were transfected into A549/CDDP cells and the effect of DAPK on the sensitivity of A549/CDDP cells to cisplatin was evaluated. DAPK expression increased in cells transfected by pcDNA3.1(+)−DAPK, whereas there was no significant difference in the DAPK level in cells transfected by pcDNA3.1(+)−DCTP or pcDNA3.1(+) as compared to un-transfected cells (Figure 7A). With TSA treatment, DAPK expression increased in cells transfected by pcDNA3.1(+)−DCTP, pcDNA3.1(+) or pcDNA3.1(+)−DAPK (Figure 7B). DCTP, a C-terminal fragment of DAPK, has been reported to mediate inhibition of DAPK activity[30, 31].

The results showed that the IC50 of TSA to A549/CDDP cells, transfected by different vectors, was 439.00±21.16 nmol/L (pcDNA3.1(+)−PC) and 220.45±9.92 nmol/L (pcDNA3.1(+)−DAPK), whereas the IC50 of TSA to A549/CDDP cells was 19.05±0.57 μmol/L (pcDNA3.1(+)−DAPK) (P<0.05 vs control) (Figure 7D). These data suggest that DAPK could mediate A549/CDDP cell death induced by cisplatin.

In addition, endogenous DAPK expression was decreased by RNA interference to evaluate the role of DAPK in TSA- or cisplatin-induced A549/CDDP cell death (Figure 8A, 8B). These results show that knock-down of DAPK increases the IC50 of TSA on A549/CDDP cells to 869.08±34.73 nmol/L, whereas the IC50 of TSA on control A549/CDDP cells was 465.99±19.57 nmol/L (non-transfected group) and 485.87±23.16 nmol/L (control vector group) (P<0.05 vs control) (Figure 8C). Also, the same results were obtained with cisplatin treatment. The results showed that the IC50 of cisplatin on the three groups of A549/CDDP cells was 30.90±1.03 μmol/L (non-transfected group), 30.14±0.87 μmol/L (control vector-transfected group) and 40.63±1.00 μmol/L (RNA interference vector-transfected group, P<0.05 vs control vector-transfected group) (Figure 8D). These results suggest that inhibition of DAPK expression could cause A549/CDDP cell resistance to cisplatin-induced cell death.

Discussion

Apoptosis is usually deregulated in cancer because of increased expression of anti-apoptotic proteins or decreased expression of pro-apoptotic proteins. In human lung cancers, resistance to drug therapy has been often found to be associ-
ated with the silencing of pro-apoptotic proteins including p16INa, Bax, and DAPK[32, 33]. Therefore, the up-regulation of apoptosis-inducing factors might be a promising approach to enhance the efficacy of chemotherapy treatment of multidrug-resistant cancer.

HDAC inhibitors have emerged as a new class of anti-cancer therapeutic agents for both hematologic and solid cancers. More importantly, these inhibitors also display promising anti-tumor activity in clinical trials [34, 35]. A recent report showed that HDAC inhibitors induced apoptosis in cells of cisplatin-resistant ovarian cancer associated with overexpression of Bad[36]. Moreover, Muscolini et al also reported that an HDAC inhibitor overcome apoptosis resistance to cisplatin by restoring both p73 and Bax[15]. These results demonstrate that HDAC inhibitors overcome resistance to cisplatin by up-regulating the expression of apoptosis-inducing factors. However, less is known about the role of DAPK in the development of chemotherapy resistance in cancers cells, although DAPK has been reported to play an important role in IFN-γ, tumor necrosis factor (TNF)-α, or Fas-ligand induced apoptosis[37]. Previous studies have shown that DAPK was expressed at a basal level in A549 cells[33], but we found that DAPK was downregulated in A549/CDDP cells, suggesting its involvement in the cisplatin resistance of A549/CDDP cells.

In this study, we found that TSA induced dose-dependent apoptosis in both A549 cells and cisplatin-resistant A549/CDDP cells. We also provide new evidence that A549 cells and cisplatin-resistant A549/CDDP cells undergo similar level of apoptosis with TSA treatment. However, a low concentration of TSA (31.25 nmol/mL) can sensitize A549/CDDP cells to cisplatin-induced cell death. We found that DAPK was up-regulated with TSA treatment in A549/CDDP cells in a dose- and time-dependent manner. DAPK is known to be negatively regulated by auto-phosphorylation of serine-308. The Serine308 phosphorylated DAPK has lower activity than its non-phosphorylated counterpart. The dephosphorylation of serine-308 on DAPK can activate DAPK and induce apoptosis[28, 29]. Our study demonstrated that the serine-308-

Figure 3. Apoptosis (sub-G1) analyzed by flow cytometry. DNA profile was analyzed by PI staining with flow cytometry. The percent apoptosis is calculated as the percentage sub-G1 peak as determined by cell-cycle analysis (arrow indicated sub-G1 peak).

![Graphs](image-url)
phosphorylation level of DAPK remained about the same after TSA treatment (Figure 5B, 5D). This observation suggests that the relative ratio of serine308 phosphorylated DAPK to the total DAPK decreased with TSA treatment. Therefore, TSA induces elevation of the active form of DAPK, since the level of Serine308 phosphorylated DAPK remained unchanged as compared to the untreated control. However, DAPK expression did not significantly change in A549/CDDP cells treated with cisplatin.

Cell-transfection experiments reconfirmed that increased DAPK expression increased A549/CDDP cells sensitivity to TSA or cisplatin. We also observed that transient DAPK treatment could promote cell apoptosis. To determine whether DAPK kinase contributes to cisplatin resistance, we generated a stable-transfection cell line, A549/CDDP/DAPK, which had a higher expression level of DAPK than A549/CDDP cells. Our data showed that stable expression of DAPK in A549/CDDP could sensitize cells to TSA or cisplatin treatment, which is consistent with previous reports that DAPK is a pro-apoptotic factor and may be involved in the mechanisms increasing the chemosensitivity of anticancer drugs of 5-fluorouracil, paclitaxel and TNF-related apoptosis-inducing ligand\[16, 33\]. Additionally, auto-phosphorylation on Serine-308, the activity of DAPK is also regulated by its C-terminal fragment (DCTP). It has been reported that the expression of DCTP can suppress DAPK activity without affecting the protein level of DAPK. Moreover, DCTP was shown to inhibit PC12 apoptosis induced by DAPK\[33\].

Figure 4. The effect of TSA on the sensitivity of A549/CDDP cells to cisplatin. The IC50 of cisplatin to A549/CDDP cells is at 31.34±1.23 μmol/L in absence of TSA, while the IC50 values are at 20.61±1.80 μmol/L and 18.49±0.70 μmol/L in presence of 31.25 nmol/L and 62.5 nmol/L TSA, respectively. n=4. Mean±SD.

In summary, our present results reveal that treatment with TSA induces apoptosis of A549/CDDP cells and enhances the sensitivity of the cells to cisplatin, possibly mediated by the up-regulation of DAPK.
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Author contribution
Cheng-ping HU and Jun WU designed research; Jun WU and Min SONG performed research; Qi-hua GU contributed new analytical tools and reagents; Ye-peng LI analyzed data; Cheng-ping HU and Jun WU wrote the paper.

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Figure 8. TSA changes the sensitivity of A549/CDDP cells to the cisplatin treatment by affecting DAPK expression. (A) Western blot analysis of DAPK expression in A549/CDDP cells transfected by interference vectors. 1: Non-transfected group 2: control vector group; 3: Endogenous DAPK expression silence group. (B) Western blot analysis of DAPK expression in TSA-treated A549/CDDP cells transfected by interference vectors. 1: non-transfected group in absence of TSA; 2: non-transfected group induced by 500 nmol/L TSA; 3: Control vector group in absence of TSA; 4: Control vector group induced by 500 nmol/L TSA; 5: Endogenous DAPK expression silence group in absence of TSA; 6: Endogenous DAPK expression silence group induced by 500 nmol/L TSA. (C) Effect of endogenous DAPK expression silence on TSA-induced A549/CDDP cells death. n=4. Mean±SD. (D) Effect of endogenous DAPK expression silence on cisplatin-induced A549/CDDP cells death. n=4. Mean±SD.
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