

Chidamide combined with paclitaxel effectively reverses the expression of histone deacetylase in lung cancer

Na Zhang^a, Pan Sun^a, Haizhen Jin^a, Yuqin Yang^b, Qingya Zhao^a, Lin Zhou^a, Lili Guo^a, Xiaohua Yang^a and Liming Lu^c

The role of histone deacetylases (HDACs) in lung cancer has been extensively studied. Inhibition of HDAC activities have been used as a new cancer treatment strategy. To date, many HDAC inhibitors have been shown to induce apoptosis and inhibit tumorigenesis. Chidamide (CS055) is a new member of HDAC inhibitors. In China, Chidamide has been approved for the treatment of relapsed or refractory peripheral T-cell lymphoma. However, the efficacy of Chidamide in non-small cell lung cancer remains unclear. In this study, we used lung cancer primary cells and investigated the effects of Chidamide combined with paclitaxel on lung cancer. We found that Chidamide combined with paclitaxel effectively inhibited the expression and activity of HDAC in primary lung cancer cells, induced their apoptosis and blocked cell

cycle. Chidamide combined with paclitaxel may therefore provide a new promising therapeutic treatment for lung cancer. *Anti-Cancer Drugs* 31: 702–708 Copyright © 2020 The Author(s). Published by Wolters Kluwer Health, Inc.

Anti-Cancer Drugs 2020, 31:702–708

Keywords: apoptosis, cell cycle, Chidamide, histone deacetylase, paclitaxel

^aCentral Laboratory, Shanghai Chest Hospital, Shanghai JiaoTong University, ^bLaboratory animal Center, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine and ^cDepartment of Immunology, School of Medicine, Shanghai Jiaotong University, Shanghai, China

Correspondence to Liming Lu, PhD, Department of Immunology, School of Medicine, Shanghai Jiaotong University, Shanghai 200025, China
Tel: +13916235624; fax: +021 63846383; e-mail: lulunew2003@163.com

Received 16 August 2019 Revised form accepted 10 March 2020

Background

Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-related death worldwide [1]. In recent years, the role of histone deacetylases (HDACs) in lung cancer has been extensively studied, and this family of enzymes plays an important role in lung cancer. To date, four classes of HDACs have been identified based on function, subcellular distribution, and DNA sequence similarity. Each HDACs contains several members. The first two classes are considered to be typical HDACs, whose activity can be inhibited by Trichostatin A; the third class is a class of NAD⁺-dependent proteins whose activity is related to NAD and is not affected by Trichostatin A; the four classes are considered to be atypical HDACs because their members are classified as HDACs based solely on their DNA sequence similarity to other HDACs [2]. HDACs remove the N-terminal lysine residues of histones, in contrast to histone acetyltransferases. Studies have demonstrated that HDACs are activated in lung cancer and their activation promotes proliferation and tumorigenesis of NSCLC cells *in vitro* and *in vivo*. Inhibition of HDAC activity may offer a new cancer treatment strategy [3].

To date, various HDAC inhibitors have been shown to induce apoptosis and to inhibit angiogenesis in tumor cells. Vorinostat (SAHA) has been approved by the US FDA for the treatment of cutaneous T-cell lymphoma [4].

Paclitaxel is extracted from plant shupi and induce apoptosis by blocking cell cycle, it is used as a single agent in first-line chemotherapy of advanced and metastatic NSCLC. However, the treatment of paclitaxel-resistant patients remains a key clinical challenge [5,6]. Finding and designing new and effective treatment including HDAC inhibitors would provide a new and promising therapeutic direction for NSCLC patients.

Chidamide (CS055) is a new HDAC inhibitor. Studies have demonstrated that Chidamide selectively inhibits HDAC1, HDAC2, HDAC3, and HDAC10 [7], and exerts antitumor effects by inducing growth arrest and apoptosis of blood and lymphoid tumor cells. Additionally, Chidamide can reverse the drug resistance of tumor cells. Chidamide also shows good tolerance and antitumor activity in advanced solid tumors such as colon cancer and liver cancer [8,9]. In China, Chidamide is currently used for the treatment of relapsed or refractory peripheral T-cell lymphoma. However, the efficacy of Chidamide in NSCLC is not known [10]. In this study, we first established cultures of primary NSCLC cells, and examined the consequences of combining the new HDAC inhibitor – Chidamide and paclitaxel. Our results demonstrated that the Chidamide and paclitaxel combination effectively inhibited the expression and activity of HDACs in the primary NSCLC cells, induced apoptosis and blocked cell cycle. Interestingly, Chidamide and paclitaxel treatment substantially decreased MYC expression. The Chidamide and paclitaxel combination may therefore offer a potentially new therapy for NSCLC.

This is an open-access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CC-BY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.

Materials and methods

Materials and reagents

Chidamide and paclitaxel were donated by Shenzhen Microcore Biotech Co., Ltd., (Nanshan district, Shenzhen) RPMI1640 medium and fetal bovine serum were purchased from Hyclone (Logan, Utah, USA); DMSO and thiazolyl blue (MTT) were purchased from Sigma (USA); Annexin V-FITC /PI apoptosis detection kit was purchased from Roche; (Basel, Switzerland) bicinchoninic acid (BCA) protein quantification kit was purchased from Tiangen Biochemical Co., Ltd.; (Beijing, China) anti-HDAC3(85057) antibody and anti-HDAC6 antibody(7612s) were CST products, anti- β -actin and horseradish peroxidase (HRP)-labeled secondary antibody were all derived from Beijing Zhongshan Jinqiao Biological Co., Ltd (Beijing, China). patient-derived tumor xenograft (PDX) mice were purchased from Bikai Experimental Animal Co., Ltd. (Xuhui district, Shanghai), and the use of experimental mice complied with the ethical charter of experimental animals; the acquisition of patient tumor tissue complied with biomedical ethics.

Culture of primary lung cancer cells

The patient's tumor tissues were separated and washed with phosphate buffer saline (PBS) containing penicillin-streptomycin solution. The tumor tissues were cut into 1 mm³ millet, 0.2% type I collagenase digested for 2 h and filtered through a 200 mesh filter. The cells were

washed with PBS, cultured with RPMI1640 medium. And the fibroblasts were removed, the monoclonal cells were identified for subsequent experiments.

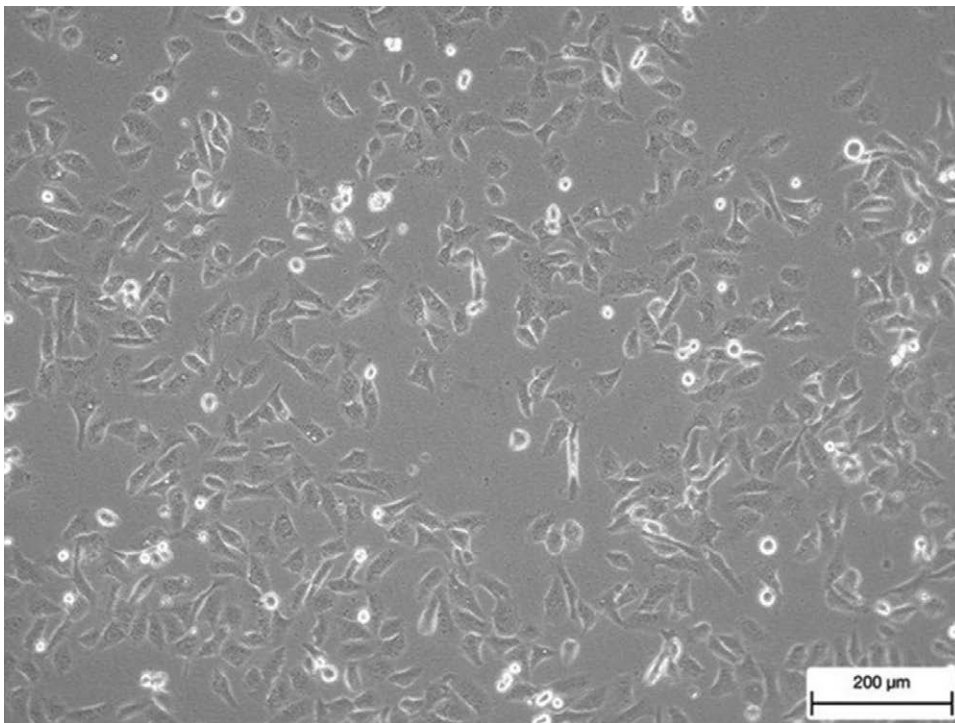
Western blot

About 1×10^7 CAFQ1 cells, PC-9 cells, and H1975 cells were gathered after pretreatment for the indicated time periods as described previously (addition of Chidamide and paclitaxel). Collect protein samples and detect the protein concentration using the BCA protein concentration assay kit. After protein quantification, protein samples were fractionated by 10–15% SDS-PAGE and then electrically transferred onto polyvinylidene difluoride (PVDF) membranes. Mouse or rabbit primary antibodies and appropriate HRP-conjugated secondary antibodies were used to detect the designated proteins. The bound secondary antibodies on the PVDF membrane were reacted with ECL detection reagents (Pierce, Rockford, Illinois, USA). Results normalized to the internal control β -actin.

CCK8 analysis

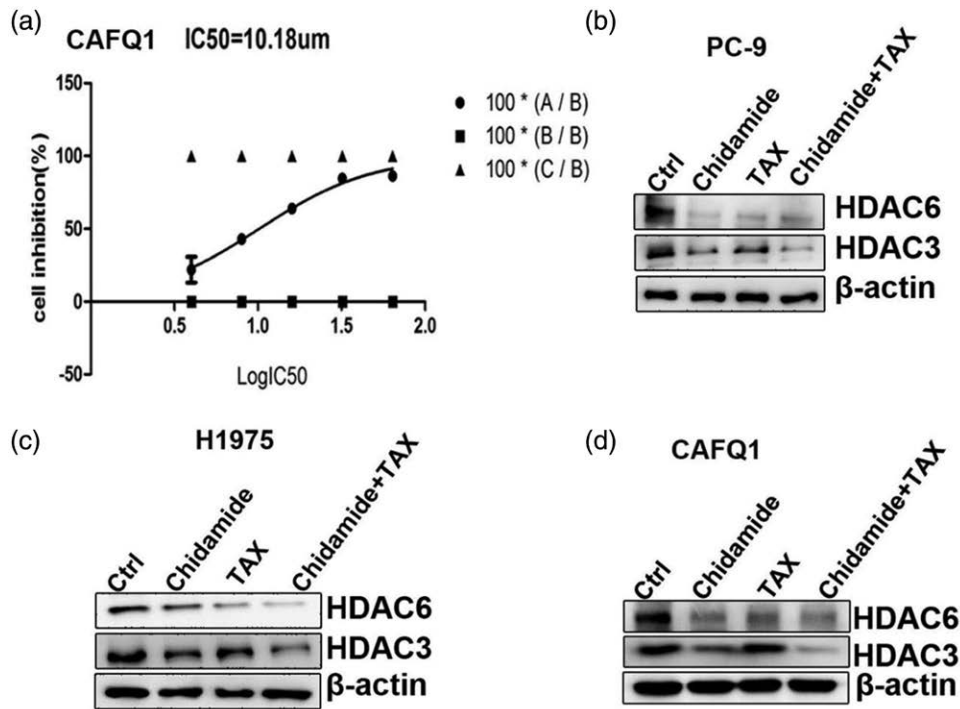
The CAFQ1 cells (50 cell/ μ l) were seeded to 96-well plates. After overnight incubation, the cells were treated with different concentrations (0, 4, 8, 16, 32 μ M) of Chidamide for 48 h. Then 10 μ l CCK8 MTT solution (2.5 mg/ml in PBS) was added to each well, and the plates were incubated for an additional 4 h at 37 C. Measure absorbance at 450 nm by a microplate reader.

Fig. 1



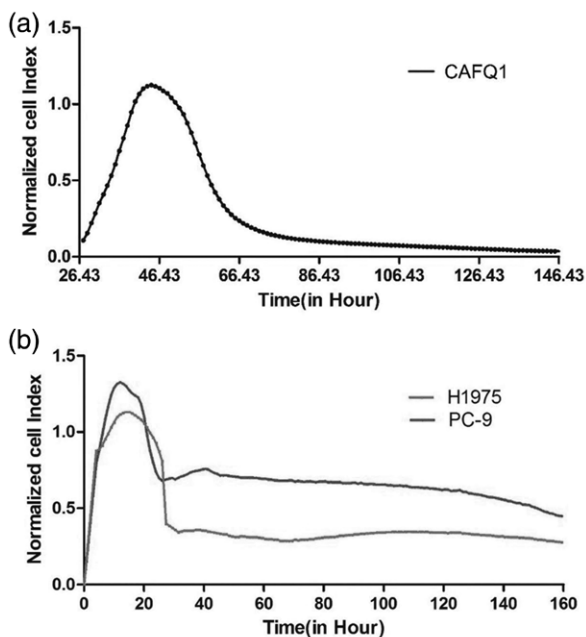
CAFQ1 cells in culture (200 \times).

Fig. 2



Chidamide and paclitaxel down-regulate the expression of HDAC3, HDAC6 in lung cancer cells. (a) Effect of Chidamide on the viability of CAFQ1 cells. CAFQ1 cells were treated with different concentrations (0, 4, 8, 16, 32 μM) of Chidamide for 48 h. (b) Effect of Chidamide and paclitaxel on HDAC expression. PC-9 cells were treated with 16 μM Chidamide and 10 μM paclitaxel for 48 h. (c) Effect of Chidamide and paclitaxel on HDAC expression. H1975 cells were treated with 16 μM Chidamide and 10 μM paclitaxel for 48 h. (d) Effect of Chidamide and paclitaxel on HDAC expression. CAFQ1 cell was treated with 16 μM Chidamide and 10 μM paclitaxel for 48 h.

Fig. 3



Effects of Chidamide and paclitaxel on lung cancer cells growth. (a) Effects of 16 μM Chidamide and 10 μM paclitaxel in CAFQ1 cell. (b) Effects of 16 μM Chidamide and 10 μM paclitaxel in PC-9 cells and H1975 cells.

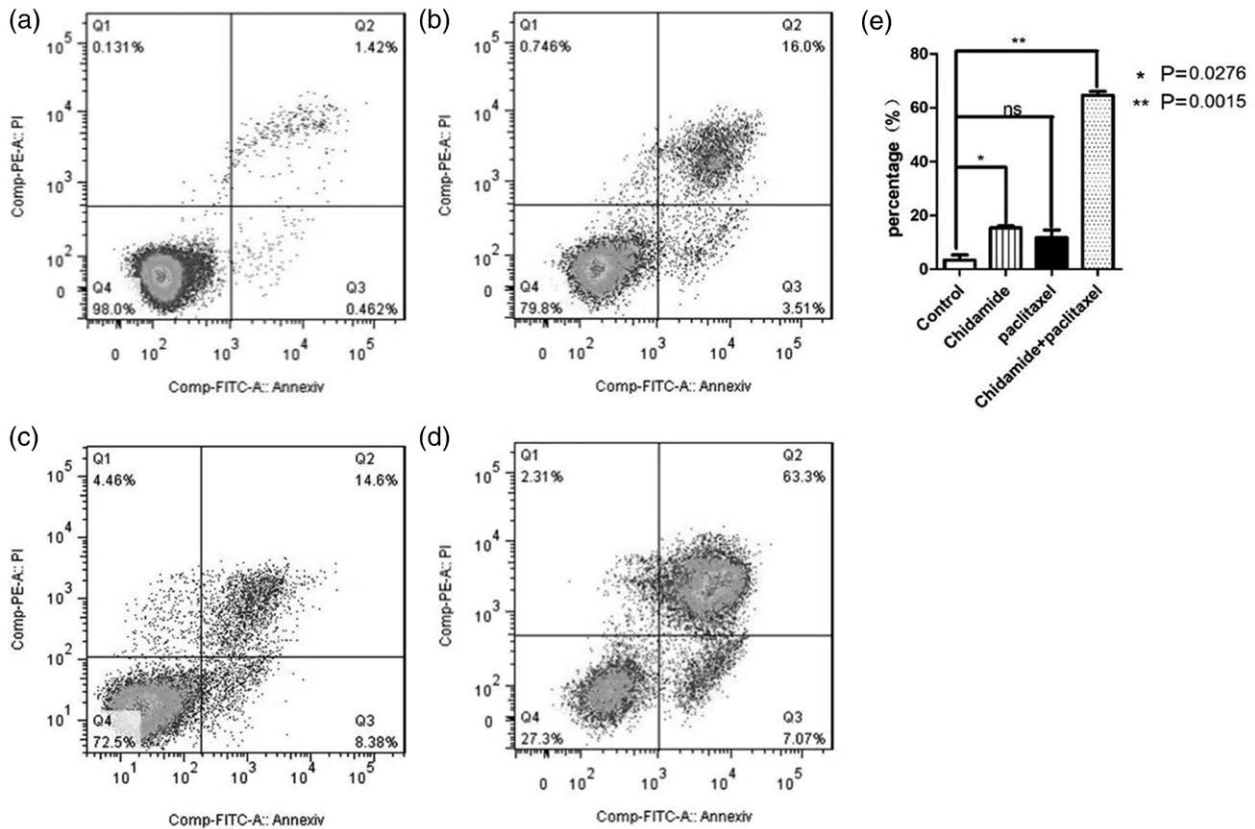
RTCA analysis

The cell proliferation was measured by ACEA Biosciences (San Diego, California): xCELLigence real-time unlabeled cell analysis technique (RTCA) DPlus. First, 50 μl of medium were added to the wells of E-Plate 96. And then, the E-Plate 96 was placed on the RTCA Station to automatically check the contact and the background: Make sure the selected wells are in normal contact, and the Cell Index of all wells is lower than 0.063. Next, took out E-Plate 96 and added 100 μl of the well-mixed CAFQ1 cells, PC-9 cells and H1975 cells (50 cell/ul) to the wells. Placed E-Plate 96 in a clean bench for 30 minutes at room temperature, and then, placed the E-Plate 96 in the incubator. After overnight incubation, the cells were treated with Chidamide (16 μM) and paclitaxel (10 μM) to detect the cell proliferation curve for 1 week at 37 C. The RTCA system would automatically generate curves and values that can be used directly or added values to GraphPad for graphing.

Flow-cytometry analysis

Analyses for apoptosis were also conducted with an Annexin V-FITC Apoptosis Detection Kit (BioVision, San Diego, California). CAFQ1 cells (1×10^6) were exposed to Chidamide (16 μM) and/or paclitaxel (10 μM) for 48 h. They were collected by centrifugation and

Fig. 4



Effects of Chidamide and paclitaxel on CAFQ1 cells apoptosis. (a) Ctrl. (b) 16 μ M Chidamide treatment. (c) 10 μ M paclitaxel. (d) 16 μ M Chidamide and 10 μ M paclitaxel. (e) Bar chart shows statistical analysis of the flow cytometry results.

resuspended in 500 μ l of $1 \times$ binding buffer. Annexin V–fluorescein isothiocyanate (FITC; 5 μ l) and PI (5 μ l) were added to the cells. After incubation at room temperature for 5 minutes in the dark, cells were analyzed by FACS using a flow cytometer (Becton Dickinson, Bergen, New Jersey, USA). Cells that were stained for Annexin V–FITC were analyzed.

PDX establishment

The patient's tumor tissues were separated and washed with PBS containing penicillin-streptomycin solution and were cut into four pieces of 3–5 mm^3 . And then, implanted into mice. Intra-gastric administration with 30 mg/kg Chidamide combined with 10 mg/kg paclitaxel for 18 days and examined tumor volume and HDAC expression.

Statistical analysis

The study was analyzed by GraphPad Prism 5.0 software, and the mean \pm SEM was used. The mean difference between the groups was analyzed by unpaired *t*-test. *P* < 0.05 was considered statistically significant.

Results

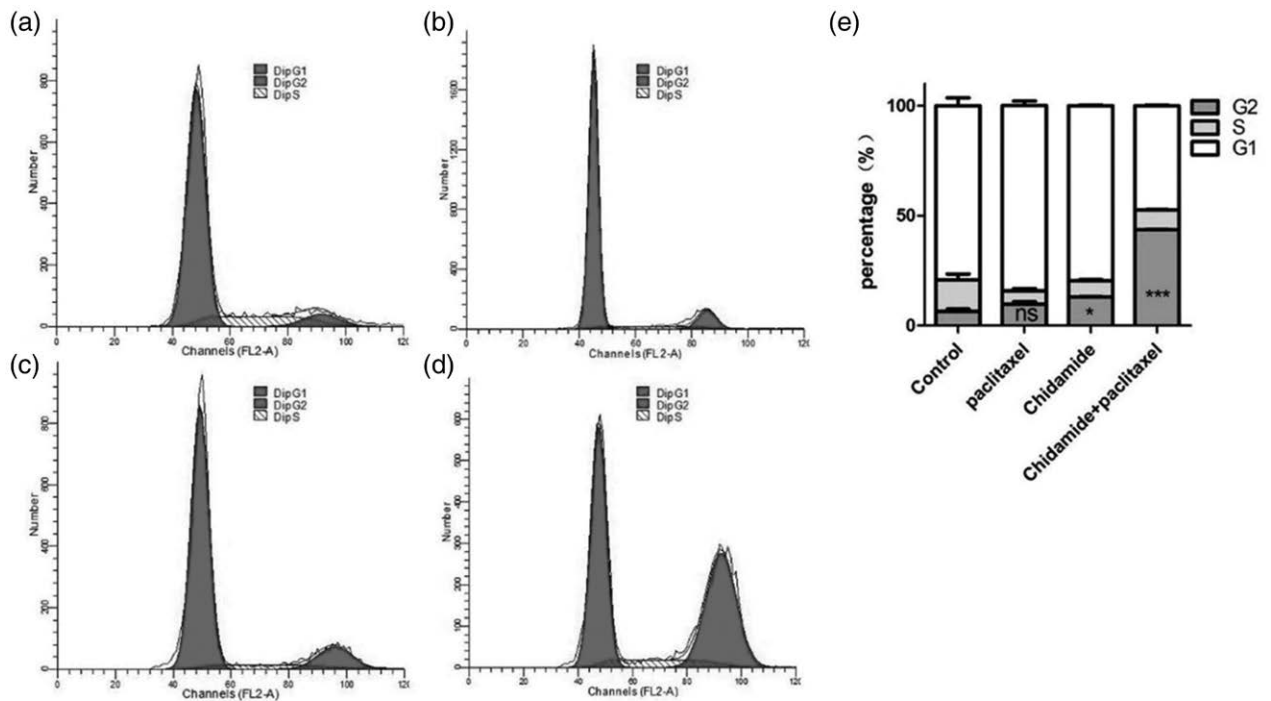
Derivation of new non-small cell lung cancer primary cell lines

We have recently derived a number of new NSCLC primary cell lines from Chinese patients' tumor tissues. One of the new cell lines, CAFQ1 (patent pending), has been subcloned and extensively characterized including short tandem repeat (STR) and karyotype analysis. Flow-cytometry analysis found that they expressed high Epcam and Vimentin. In this study, we used CAFQ1 primary cell (Fig. 1) and investigated the effect of Chidamide and paclitaxel focusing on to HDACs.

Chidamide combined with paclitaxel decreased HDAC3 and HDAC6 in lung cancer cells

We selected CAFQ1 cells, PC-9 cells, and H1975 cells to study the inhibitory effect of Chidamide and paclitaxel on HDACs in lung cancer cells. To test the inhibiting kinetics of Chidamide in CAFQ1 cells, we first tested different concentrations of Chidamide for 48^h and identified $\text{IC}_{50} = 10.18 \mu\text{M}$ in CAFQ1 cells (Fig. 2a). Subsequently, CAFQ1 cells, PC-9 cells and H1975 cells were treated

Fig. 5



Effects of Chidamide and paclitaxel on cell cycle in CAFQ1 cells. (a) Ctrl. (b) 10 μ M paclitaxel. (c) 16 μ M Chidamide. (d) 16 μ M Chidamide and 10 μ M paclitaxel.

with Chidamide and paclitaxel for 48 h, and were harvested for detecting HDAC3 and HDAC6 in Western blot. Chidamide decreased HDAC3 and HDAC6 protein levels. Significantly, Chidamide combined with paclitaxel demonstrated synergetic effects of decreasing HDAC3 and HDAC6 (Fig. 2b–d).

Chidamide combined with paclitaxel inhibited lung cancer cells growth

To investigate the combined effect of two drugs in inhibiting lung cancer cells growth, we treated CAFQ1 cells, PC-9 cells and H1975 cells with Chidamide and paclitaxel for 7 days to detect the cell growth. In CAFQ1 cells, Chidamide and paclitaxel combination dramatically decreased the cell growth in 46.43 h (Fig. 3a). In PC-9 cells and H1975 cells, Chidamide and paclitaxel combination dramatically decreased the cell growth in 20 h (Fig. 3b). Chidamide and paclitaxel together substantially inhibited lung cancer cells growth.

Chidamide combined with paclitaxel-induced CAFQ1 cells apoptosis

Activation of HDAC in lung cancer promotes proliferation of NSCLC cells *in vitro* and *in vivo*. To investigate whether Chidamide combined with paclitaxel inhibited the proliferation of CAFQ1 cells, we treated CAFQ1 cells with Chidamide and paclitaxel for 48 h to detect

apoptosis. In the control group, 1.42% were undergoing apoptosis (Fig. 4a), whereas, Chidamide group significantly increased the apoptosis cell to 16% ($P = 0.0276$, Fig. 4b), and paclitaxel treatment to 14.6% (Fig. 4c). Chidamide and paclitaxel combination dramatically increased apoptotic rate to as high as 63.3% ($P = 0.0015$, Fig. 4d).

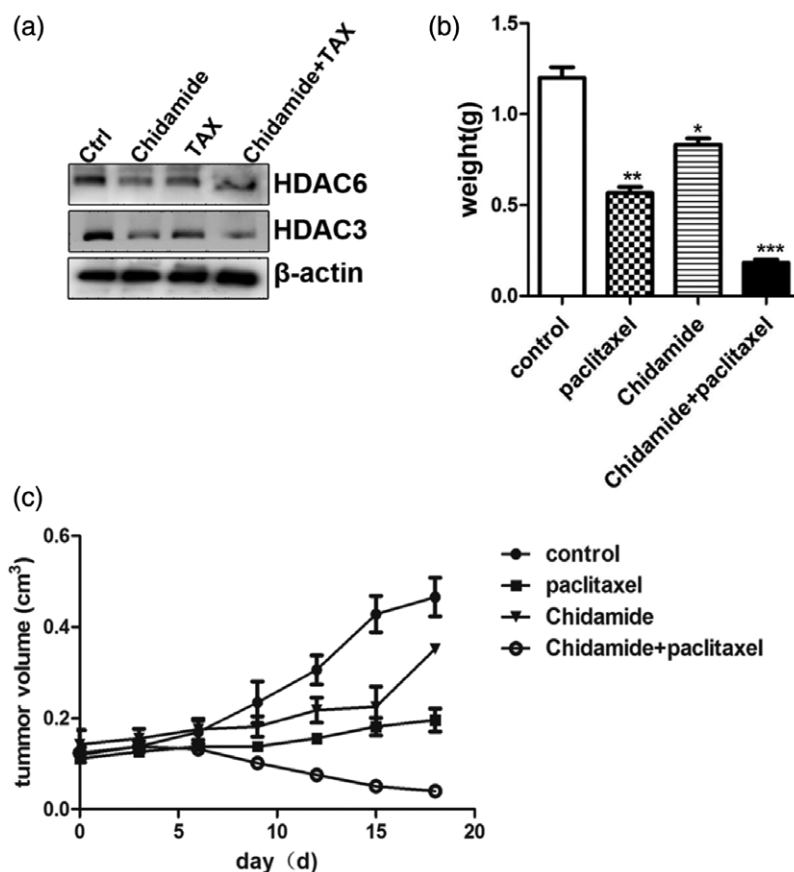
Chidamide combined with paclitaxel blocks CAFQ1 cell in cell cycle G2 phase

To further investigate the effect of Chidamide combined with paclitaxel, we treated CAFQ1 cells with Chidamide and paclitaxel for 48 h and analyzed cell cycle defects. In the control group cells in G2 phase were 7.45% (Fig. 5a), and up to 10.83% ($P = 0.1515$, Fig. 5b) in cells treated with paclitaxel, and was up to 13.27% in the Chidamide group ($P = 0.0212$, Fig. 5c). The combination of Chidamide and paclitaxel increased G2 phase cells, up to 43.53% ($P = 0.0007$, Fig. 5d). We concluded that Chidamide and paclitaxel together substantially blocked CAFQ1 cell cycle.

Chidamide combined with paclitaxel Inhibit the expression of HDAC3 and HDAC6 in the PDX model

We transplanted the NSCLC patient's tumor tissue into the mouse to establish PDX models, and investigated the effect of Chidamide and paclitaxel on the

Fig. 6



Effects of Chidamide and paclitaxel in the PDX model. (a) Effect of Chidamide and paclitaxel on HDAC expression in the PDX model. 30 mg/kg Chidamide combined with 10 mg/kg paclitaxel for 18 days. (b) Effect of Chidamide and paclitaxel on tumor weight in the PDX model. 30 mg/kg Chidamide combined with 10 mg/kg paclitaxel for 18 days. (c) Effect of Chidamide and paclitaxel on tumor volume in the PDX model. 30 mg/kg Chidamide combined with 10 mg/kg paclitaxel for 18 days. PDX, patient-derived tumor xenograft.

PDX model by intragastric administration. The results showed that in the PDX model, Chidamide decreased expression of HDAC3 and HDAC6. Again, the downregulation of HDAC3 and HDAC6 by Chidamide and paclitaxel was more significant (Fig. 6a). Chidamide and paclitaxel individual treatment inhibited the growth of tumor measured by its volume and weight ($P = 0.0118$, Fig. 6b). In the Chidamide and paclitaxel combination group, tumor volume is significantly reduced (Fig. 6c), and tumor weight could be reduced to 0.2 g ($P = 0.0035$, Fig. 6b). Therefore, *in-vivo* Chidamide combined with paclitaxel effectively inhibited tumor growth.

Chidamide combined with paclitaxel significantly reduced the expression of c-myc and Bcl-2 in CAFQ1 cells

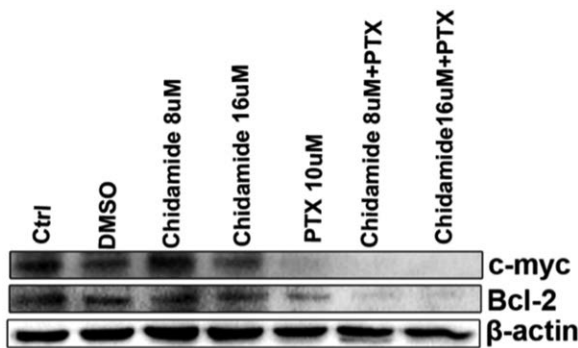
Oncogenes and tumor suppressor genes can regulate both tumor cell proliferation and differentiation, and apoptosis. The proto-oncogene c-myc can cause cell division and proliferation in the presence of growth factors. The Bcl-2

gene can inhibit apoptosis. To explore the regulatory possible mechanism of Chidamide and paclitaxel in CAFQ1 cells, they were stimulated with different concentrations of Chidamide and paclitaxel for 48 h and harvested for detecting the expression of c-myc and Bcl-2. Chidamide decreased both c-myc and Bcl-2 in CAFQ1 cells, and this decrease was more prevalent when combined with paclitaxel (Fig. 7). Downregulation of c-myc and Bcl-2 may represent an important mechanism in CAFQ1 cells treated with both Chidamide and paclitaxel.

Discussion

Previous studies have shown that HDAC inhibitors can increase the levels of histone acetylation, cause transcriptional changes, induce cell cycle arrest and apoptosis, and alter carcinogenic signals, blocking proliferation of various tumor cells *in vitro* and *in vivo* [11]. However, the mechanism of HDAC inhibitors in the treatment of tumors is not fully understood. Studies have shown that HDAC inhibitors can affect gene expression in tumor cells, and, may induce tumor cell apoptosis and inhibit

Fig. 7



Chidamide combined with paclitaxel down-regulates c-myc and Bcl-2 expression in CAFQ1 cells.

tumor cell growth [12]. HDAC inhibitors have been widely used clinically, but still have some clinical limitations. Patients with HDAC inhibitor resistance remain a key clinical challenge, finding and designing more potent and effective HDAC inhibitors is needed.

Our results reported here found that Chidamide combined with paclitaxel effectively decreased HDAC3 and HDAC6 in primary lung cancer cells. In the mouse PDX model, Chidamide combined with paclitaxel also effectively inhibited HDAC3 and HDAC6, and substantially reduce the volume of the tumors.

Chidamide regulates the expression of p21 protein in tumor cells, and consequently blocks cell cycle and induces apoptosis [13]. Our data demonstrated that Chidamide combined with paclitaxel also significantly reduced the expression of c-myc and Bcl-2 in primary lung cancer cells, arrested cells in G2 phase, and induced apoptosis. One possible mechanism is that Chidamide caused low expression of both c-myc and p21, which are well-characterized factors in cell proliferation and cell survival.

In summary, this study presents data that Chidamide combined with paclitaxel effectively reduced HDAC protein levels, blocked cell cycle, and induced apoptosis in primary lung cancer cells. In addition, Chidamide combined with paclitaxel potently inhibited tumor growth in a PDX model. Our study indicates that the combination of Chidamide and paclitaxel may offer a potentially new second-line lung cancer treatment.

Acknowledgements

We thank the patients for their participation and support. We gratefully acknowledge financial support from the National Key R&D Program Key Special Project (2017YFA0104600), National Natural Science Foundation Project (81671579), Shanghai Science and Technology Commission Experimental Animal Research Project (18140903800), Shanghai Jiaotong University Affiliated Chest Hospital Major Project (YJXT20190106), Shanghai Transportation Collaborative Self-help Project of Collaborative Innovation Center of University Translational Medicine (TM201522, TM201721).

Conflicts of interest

There are no conflicts of interest.

References

- Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**:87–108.
- Zhang J, Kan S, Huang B, Hao Z, Mak TW, Zhong Q. Mule determines the apoptotic response to HDAC inhibitors by targeted ubiquitination and destruction of HDAC2. *Genes Dev* 2011; **25**:2610–2618.
- Ropero S, Esteller M. The role of histone deacetylases (hdacs) in human cancer. *Mol Oncol* 2007; **1**:19–25.
- Liu L, Chen B, Qin S, Li S, He X, Qiu S, et al. A novel histone deacetylase inhibitor chidamide induces apoptosis of human colon cancer cells. *Biochem Biophys Res Commun* 2010; **392**:190–195.
- Wang L, Li H, Ren Y, Zou S, Fang W, Jiang X, et al. Targeting HDAC with a novel inhibitor effectively reverses paclitaxel resistance in non-small cell lung cancer via multiple mechanisms. *Cell Death Dis* 2016; **7**:e2063.
- Zhou J, Sun M, Jin S, Fan L, Zhu W, Sui X, et al. Combined using of paclitaxel and salinomycin active targeting nanostructured lipid carriers against non-small cell lung cancer and cancer stem cells. *Drug Deliv* 2019; **26**:281–289.
- Zhou Y, Pan DS, Shan S, Zhu JZ, Zhang K, Yue XP, et al. Non-toxic dose chidamide synergistically enhances platinum-induced DNA damage responses and apoptosis in non-small-cell lung cancer cells. *Biomed Pharmacother* 2014; **68**:483–491.
- Chan TS, Tse E, Kwong YL. Chidamide in the treatment of peripheral T-cell lymphoma. *Onco Targets Ther* 2017; **10**:347–352.
- Shi Y, Jia B, Xu W, Li W, Liu T, Liu P, et al. Chidamide in relapsed or refractory peripheral T cell lymphoma: a multicenter real-world study in china. *J Hematol Oncol* 2017; **10**:69.
- Hu X, Wang L, Lin L, Han X, Dou G, Meng Z, Shi Y. A phase I trial of an oral subtype-selective histone deacetylase inhibitor, chidamide, in combination with paclitaxel and carboplatin in patients with advanced non-small cell lung cancer. *Chin J Cancer Res* 2016; **28**:444–451.
- Xu WS, Perez G, Ngo L, Gui CY, Marks PA. Induction of polyploidy by histone deacetylase inhibitor: a pathway for antitumor effects. *Cancer Res* 2005; **65**:7832–7839.
- Raha P, Thomas S, Thurn KT, Park J, Munster PN. Combined histone deacetylase inhibition and tamoxifen induces apoptosis in tamoxifen-resistant breast cancer models, by reversing bcl-2 overexpression. *Breast Cancer Res* 2015; **17**:26.
- Sachweh MC, Drummond CJ, Higgins M, Campbell J, Lain S. Incompatible effects of p53 and HDAC inhibition on p21 expression and cell cycle progression. *Cell Death Dis* 2013; **4**:e533.