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Inhibitory effect of chidamide on the growth of human adenoid cystic carcinoma cells

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ABSTRACT

Adenoid cystic carcinoma (ACC) is a malignant epithelial neoplasm that limitedly responses to chemotherapy at the cost of significant toxicity. There is no single targeted drug approved by Food and Drug Administration (FDA) for ACC. Genomic landscape studies have revealed that frequently mutated pathways in ACC often involve in chromatin remodeling, which interfere multiple histone related proteins. Chidamide is a novel histone deacetylase inhibitor (HDACi) approved in clinical practice that was designed to increase the acetylation level of histone H3. It demonstrated anticancer effects in various cancers in preclinical study, but not in ACC. In this study, we aimed to investigate the anticancer effects of chidamide alone or in combination with cisplatin (cDDP) on ACC in vitro and in vivo. The results showed that chidamide alone or in combination with cDDP effectively inhibited the growth and proliferation of ACC cells in a dose- and time-dependent manner. Chidamide arrested cell cycle in G2/M phase by up-regulating the acetylation of histone H3 and interfering phosphorylation of AKT protein. Chidamide alone or in combination with cDDP did not induce distinct apoptosis in ACC cells. In vivo experiments showed that chidamide combining cDDP exerted significant inhibitory effects on ACC. These suggest that chidamide may be a promising candidate drug for the treatment of patients with ACC.

1. Introduction

Adenoid cystic carcinoma (ACC) is the most common malignant tumor originated from minor salivary glands and the second most prevalent cancer of parotid and sublingual salivary glands [1,2]. Despite its initially indolent course, it progresses relentlessly to a highly lethal malignancy. Compared with other malignancies, ACC has its unique characteristics, such as slow growth, diffuse invasion in the early stage, high recurrences rate and late distant metastases [3,4]. Radical surgical resection and postoperative radiotherapy can exert reasonable local control, but distant metastasis frequently develops independent of local or regional control [5]. Although the patient's early prognosis is favorable, estimated survival rates drop significantly after 10 and 15 years to 60% and 45%, respectively, and most patients die as a result of the disease progression in later decades [6]. Unfortunately, traditional chemotherapy has little activity in ACC [7]. Among them, cisplatin (cDDP) based regimens are commonly used. In addition, although extensive efforts had been made, there is no targeted drug approved by Food and Drug Administration (FDA) for the treatment of ACC [8]. As a result, systemic therapy had no significant impact on the natural course of advanced ACC, and the long term outcome of ACC patients remains dismal. Therefore, safe and effective treatments for ACC are desperately needed.

Histone deacetylases (HDACs) modify the structure of chromatin through removing acetyl groups from the acetylated lysine residues on histone and non-histone proteins [9,10]. The high level of HDAC silences the expression of many tumor suppressor genes and then promote the proliferation, migration and angiogenesis in various cancer cells [11,12]. It has been reported that 35–50% of ACC patients had gene mutations involved in histone modification and chromatin remodeling, such as ARID1A, KDM6A, EP300, BRD1, and CREBBP [13,14]. Furthermore, vorinostat (SAHA) has been proved effective in patients with ACC [12,15,16].

Chidamide (CS-055/HBI-8000), a novel benzamide chemical class

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of histone deacetylase inhibitor (HDACi) that is developed in China, has been approved for treatment of refractory peripheral T cell lymphomas (PTCLs) [17]. Comparing to SAHA, chidamide is more stable and it can selectively inhibit the activity of HDAC 1, 2, 3 and 10 [18]. Moreover, chidamide produces a longer median duration of response (DOR) (9.9 months) than SAHA (185 days) [19]. Our team reported one response out of three patients with advanced ACC after chidamide usage in a phase I trial [20]. Therefore, we carried out this preclinical study to investigate the anticancer effect and mechanism of chidamide alone or in combination with cDDP on ACC cells in vitro and in vivo.

2. Materials and methods

2.1. Chemicals and animals

Chidamide was kindly provided by Shenzhen Chipscreen Biosciences Ltd. (Shenzhen, China). cDDP was purchased from Hospira Australia Pty Ltd (LOT#434458, VIC 3170, Australia). BALB/c-Nu mice were purchased from Beijing Vital River Laboratory Animal Technology Co. (Beijing, China), housed with 12 h light/dark cycle and allowed free access to normal food and water. Experiments were approved by the animal control committee of National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China.

2.2. Cell lines and cell culture

The ACC-2 and ACC-3 human adenoid cystic carcinoma cell lines were obtained from our laboratory and cultured in Dulbecco's Modified Eagle's Medium (DMEM; BIOROC, Tianjin, China) and RPMI-1640 (BIOROC, Tianjin, China), respectively, supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Uruguay), at 37 °C in an atmosphere containing 5% CO_2.

2.3. Cell morphology observation and cell viability assay

The ACC-2 and ACC-3 cells were seeded in 6 cm-diameter culture dishes. When the cells grew to about 80% confluence, they were treated with 0 μM, 5 μM and 10 μM chidamide for 24 h, respectively. The morphology was observed under a 10-fold inverted microscope.

Approximately 5000 cells were plated into each well of 96-well plates and grew overnight. The next day, chidamide alone or in combination with cDDP was added at indicated final concentration in triplicate, and the cells were cultivated for additional 3 days or indicated time points. After cell medium was removed, 100 μl of MTS solution diluted with culture medium free of serum was added into each well of the plates, and the cells were then incubated for 1 h. The plate was read with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 490 nm. The inhibitory rates of cell proliferation were determined by the equation $[1-(OD_{treated}-OD_{blank})/(OD_{control}-OD_{blank})] \times 100\%$. OD was the optical value from the reader. The experiments were independently repeated four times.

2.4. Cell apoptosis and cell cycle analysis by flow cytometry

ACC-2 and ACC-3 cells in logarithmic growth were cultured in 6 cm-diameter culture dish (5×10^5 cells/well) overnight. Chidamide was added in the final concentration of 0 μM, 5 μM and 10 μM, respectively, and cDDP was added at indicated final concentration. 24 h later, the cells were collected and the cell suspension was adjusted to 1×10^6 cell/ml. The apoptosis of ACC-2 and ACC-3 cells was detected by Annexin V-FITC/propidium iodide (PI) double staining (#556547, BD Pharmingen™, USA). Stained samples were analyzed by flow cytometry.

For cell cycle assay, the collected cells were washed with phosphate buffered saline (PBS) and fixed with 70% ethanol at −20 °C overnight. Finally, the cells were centrifuged, washed with PBS, and then incubated with 50 μg/ml PI, 100 μg/ml RNase A and 0.2% Triton X-100 working solution for 15 min. Flow cytometry assay was performed.

2.5. Western blots

ACC-2 and ACC-3 cells in logarithmic growth were treated with chidamide at concentrations of 0 μM, 5 μM and 10 μM for 24 h, respectively. The total protein lysates were extracted and protein concentrations were measured by Coomassie brilliant blue method. Lysates containing 40 μg proteins were separated on SDS–PAGE gel and transferred onto PVDF membrane (IPVH00010, Millipore, USA). The membrane was blocked with PBS containing 5% non-fat dry milk for 1 h, and then incubated with antibodies specific for Akt (#4691, Cell Signaling Technology, USA; 1:1000 dilution), p-Akt (#4060, Cell Signaling Technology, USA; 1:2000 dilution), Caspase-3 (#9665, Cell Signaling Technology, USA; 1:1000 dilution), acetyl-Histone H3 (JM8009, Jiamay Biotech, Beijing, China; 1:1000 dilution), β-actin (A5316, Sigma-Aldrich, USA; 1:5000 dilution) at 4 °C overnight. Next day, the membrane was incubated for 2 h with the HRP-conjugated secondary antibodies, anti-Rabbit (ZB-2301, ZSGB-BIO, Beijing, China; 1:5000 dilution) and anti-Mouse (ZB-2305, ZSGB-BIO, Beijing, China; 1:5000 dilution) at room temperature. Proteins were visualized by super ECL plus (P1010, PPLYGEN, Beijing, China) under a chemiluminescence and fluorescence imaging system (LAS-4000, FUJIFILM, Japan). Image J software was used to quantify the western blots results.

2.6. In vivo treatment experiments

To evaluate the anti-cancer effect of chidamide in vivo, 20 nude mice were used for in vivo assay. ACC-2 cells (4×10^6/200 μl) were subcutaneously injected in the right armpit of each nude mouse. When exnografts grew to 100 mm^3, the mice were randomized into 4 groups (vehicle, cDDP 3 mg/kg, chidamide 10 mg/kg/d and cDDP 3 mg/kg + chidamide 10 mg/kg/d). cDDP was injected intraperitoneally every three days and chidamide was fed with a chidamide solution (10 mg/kg) by gastric gavage every day. Body weight of the mice and tumor volume were recorded every three days. Measurements of tumor were taken manually by collecting the longest dimension (length) and the longest perpendicular dimension (width). Tumor volume was estimated with the formula: $(L \times W^2)/2$. The mice were sacrificed 21 days after treatment. Tumors were isolated, weighed and taken photos.

2.7. Statistical analysis

All data were expressed as the means ± SD and analyzed using two tailed Student's *t*-test. $P < .05$ was considered to be statistically significant.

The IC_{50} was defined as the drug concentration required of reducing viability to 50% of the control, and was calculated by IBM SPSS Statistics 19 software. Drug interaction was assessed by using the combination index (CI) which was calculated using the following equation:

$$CI = (D)_A/(D_{50})_A + (D)_B/(D_{50})_B$$

Where $(D_{50})_A$ and $(D_{50})_B$ were the concentrations of the single drugs required to reduce cell viability by 50% compared to control, $(D)_A$ and $(D)_B$ were the concentration of in combination treatments which also reduce cell viability by 50% compared to control. When $CI < 1$ was considered synergistic, $CI = 1$ was considered additive and $CI > 1$ was considered an antagonistic interaction [21,22].