Biochemical and Biophysical Research Communications 392 (2010) 190–195

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel histone deacetylase inhibitor Chidamide induces apoptosis of human colon cancer cells

Lin Liu [a], Baoan Chen [a,*], Shukui Qin [b], Suyi Li [a], Xiangming He [a], Shaomin Qiu [c], Wei Zhao [c], Hong Zhao [c]

[a] Department of Oncology, Zhong-Da Hospital of Southeast University, Nanjing 210009, Jiangsu, PR China
[b] Chinese PLA Cancer Center, The 81st PLA Hospital, Nanjing 210002, Jiangsu, PR China
[c] Department of Internal Medicine, Nanjing Municipal Cancer Hospital, Nanjing 210003, Jiangsu, PR China

ARTICLE INFO

Article history:
Received 31 December 2009
Available online 7 January 2010

Keywords:
HDAC inhibitor
Apoptosis
Colon cancer cells
Chidamide

ABSTRACT

Many studies have demonstrated that histone deacetylase (HDAC) inhibitors induce various tumor cells to undergo apoptosis, and such inhibitors have been used in different clinical trials against different human cancers. In this study, we designed and synthesized a novel HDAC inhibitor, Chidamide. We showed that Chidamide was able to increase the acetylation levels of histone H3 and to inhibit the PI3K/Akt and MAPK/Ras signaling pathways, which resulted in arresting colon cancer cells at the G1 phase of the cell cycle and promoting apoptosis. As a result, the proliferation of colon cancer cells was suppressed in vitro. Our data support the potential application of Chidamide as an anticancer agent in treating colon cancer. Future studies are needed to demonstrate its in vivo efficacy.

Histone deacetylases (HDACs) are a family of enzymes that remove the lysine residues in the N-terminal tails of core histone proteins in the nucleosome. The action of HDACs is opposite to that of histone acetyltransferase. The acetylation and deacetylation of histones result in a remodeling of the chromatin structures and affect the access of transcription factors to the chromatin for gene transcription, which is an ongoing process that is required for normal cell growth and differentiation [1,2]. To date, four classes of HDACs have been identified, and each contains several members based on function, subcellular distribution, and DNA sequence similarity [3]. The first two classes are considered to be typical HDACs, and their activities can be inhibited by trichostatin A (TSA); the third class is a family of NAD^+-dependent proteins whose activities are not affected by TSA; and the fourth class is believed to be atypical because its members are classified as part of the family solely based on their DNA sequence similarity to the others [3].

Previous studies have demonstrated that HDAC activity is increased in many human cancers, and inhibition of HDAC activity has been used as a novel strategy in cancer therapeutics [4–7]. To date, a number of different HDAC inhibitors have been studied, and many of them have been shown to induce apoptosis and suppress angiogenesis and autophagy in different tumor cells [8–10]. One of them, suberoylanilide hydroxamic acid (SAHA), a hydroxamate, has been approved by the US FDA for treatment of cutaneous T-cell lymphoma (CTCL) and development of other HDAC inhibitors for effective control of solid tumors is in progress [11,12]. As a result, herein we present a novel HDAC inhibitor, *N*-(2-amino-5-fluorine benzyl)-4-[*N*-(pyridine-3-acrylyl) ammonia methyl] benzamide (Chidamide) (patent No. PCT/IB04/000401), and we describe its anti-tumor functions in colon cancer cells. We found that Chidamide treatment can induce accumulation of histone acetylation. Furthermore, treatment of colon cancer cells with Chidamide induced cell cycle arrest and apoptosis and subsequently inhibited proliferation of colon cancer cells in vitro.

Materials and methods

Reagents. Chidamide was custom-synthesized by Shenzhen Chipscreen Biosciences Ltd. (Shenzhen, China) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 32 mM and then kept at −80 °C until use. Methyl thiazolyl tetrazolium (MTT) was purchased from Sigma (St. Louis, MO, USA). Antibodies recognizing acetylated histone H3, cleaved Caspase-3, poly (ADP-ribose) polymerase (PARP), CDK4, p-Akt, Akt, p-mTOR, mTOR, p-p70S6k, p70S6k, p-Erk1/2, Erk1/2, and β-actin were obtained from Cell Signaling Technology Corporation (Beverly, MA, USA), and antibodies recognizing p2l, Raf, and p-Raf were obtained from Santa Cruz Biotechnology Corporation (Santa Cruz, CA, USA). The electrochemiluminescence (ECL) and BCA reagents were obtained from PIERCE Biotechnology, Inc. (Rockford, IL, USA). Laemmli sample buffer was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Cell lines and culture. Human colon cancer cell lines LoVo and HT-29 were obtained from the Cell Biology Research Institute of Shanghai, Chinese Academy of Science (Shanghai, China). The cells were grown in RPMI-1640 medium supplemented with 10% fetal

* Corresponding author. Fax: +86 25 83272011.
E-mail address: wenyu811@126.com (B. Chen).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.01.011

calf serum, penicillin/streptomycin (100 U/ml each), $NaHCO_3$ (2 g/l), and Hepes (2.4 g/l) (all from Invitrogen, Carlsbad, CA, USA) in a incubator at 37 °C with 95% air and 5% CO_2.

MTT assay. Approximately 6000 cells were plated into each well of 96-well plates and grown overnight. The next day, Chidamide was added at a final concentration of 2, 4, 8, 16, or 32 μM in hexaplicate, and the cells were cultivated for additional 3 days. After that, 20 μl of MTT solution (5 mg/ml) was added into the cell culture medium, and the cells were then incubated for 4 h. The cell medium was then removed and 150 μl DMSO was added to the 96-well plates to dissolve the MTT crystals. The plate was read with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 490 nm. The suppression rate of cell proliferation was determined by the equation $(1 - OD_{treated}/OD_{control}) \times 100$. OD is the optical number from the reader. The experiments were repeated four times.

Cell cycle analysis. The colon cancer cells were grown and treated with Chidamide in cell culture dishes for 48 h. At the end of the treatment, the cells were washed with phosphate buffered saline (PBS) and fixed with 70% ethanol containing 1% fetal calf serum (FCS) overnight at −20 °C. The next day, the cells were washed again with PBS and treated with RNaseA (at a final concentration of 200 μg/ml) for 30 min. The cells then were washed and resuspended in 0.5 ml PBS for propidium iodide (PI) incubation at a final concentration of 100 μg/ml, and the cells were incubated for 1 h. Finally, the cells were analyzed with the FACS Vantage SE (BD Corporation, Franklin Lakes, NJ, USA), and Cell Quest software version 2.0 (BD) was used for data analysis.

Annexin-V staining. The cells were grown and treated with or without Chidamide for 48 h. The cells then were washed with PBS and subjected to apoptosis assay (Annexin-V staining kit from Keygen Biotechnology Co. Ltd., Nanjing, China) following the kit protocols. Briefly, the cells were resuspended in 500 μl of the binding buffer followed by the addition of 1 μl of FITC-conjugated Annexin-V and 5 μl of PI to each sample. The cells were incubated for 5 min and then analyzed using Becton-Dickenson FACScan flow cytometry and Cell Quest software version 2.0 (BD).

Transmission electron microscopy (TEM). The cells were grown and treated with or without Chidamide for 48 h. The cells were then washed with PBS and fixed in 2% glutaraldehyde and 1% osmium tetroxide solution for 2 h. The fixed cells (approximately 1×10^7) were embedded in Epon812 (E Micron Technologies Limited, Shanghai, China), and TEM sections were prepared at a thickness of 50–100 nm with a microtome (Ultrotome-V, LKB, Bromma, Sweden). The sections were viewed under the TEM (JEM1011, JEOL Limited, Akishima-shi, Tokyo, Japan). More than 50 cells per sample were observed from three independent experiments, and photographs were taken.

Protein isolation and Western blot analysis. The cells were grown and treated with or without Chidamide for 48 h. The cells then were washed with PBS and lysed in laemmli sample buffer (100 μl/10^6 cells), and the protein lysates were centrifuged at 10,000*g* for 10 min at 4 °C to remove cell debris. Next, the total protein lysates were heated at 99 °C for 8 min and then put into ice for cooling. The protein concentration was determined using BCA reagent. After this, 50 g of the total protein lysate were separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The PVDF membranes then were subjected to Western blot analysis. Briefly, the membrane was first blocked in Tris-buffered solution (TBS) containing 5% non-fat milk for 30 min, and different primary antibodies (see *Reagents*) were added and incubated overnight at 4 °C. The next day, the membranes were washed with TBS containing 0.2% Tween-20 three times for 5 min each, and then the secondary antibody conjugated with HRP in 3% non-fat milk was added to the membranes and incubated for 1 h at room temperature. The membranes then were washed again in TBS containing 0.2% Tween-20. The target protein bands were visualized using ECL and exposed to X-ray film. The abundance of proteins was quantified using imaging processing software (Peiqing Biotech Ltd., Shanghai, China) with β-actin as the loading control.

Statistical analysis. Statistical analysis was conducted according to the type of data. Most data are shown as means ± SD, and a Student *t*-test usually was performed to compare data. $p < 0.05$ was considered to be significant.

Results

Chidamide increased histone H3 acetylation levels and inhibited growth of colon cancer cell lines

We designed a novel HDAC inhibitor based on a benzamide structure backbone (Fig. 1A) [13–15]. To test the activity and efficacy of this novel HDAC inhibitor, Chidamide, we first examined its ability to change histone H3 acetylation levels, the target of HDAC. We treated two colon cancer cell lines with 4, 8, or 16 μM Chidamide for 48 h. Trichostatin A, a well-known HDAC inhibitor, was used as the positive control. As shown in Fig. 1B, Chidamide was able to increase the acetylation level of histone H3. These results imply that Chidamide inhibited the histone deacetylases and

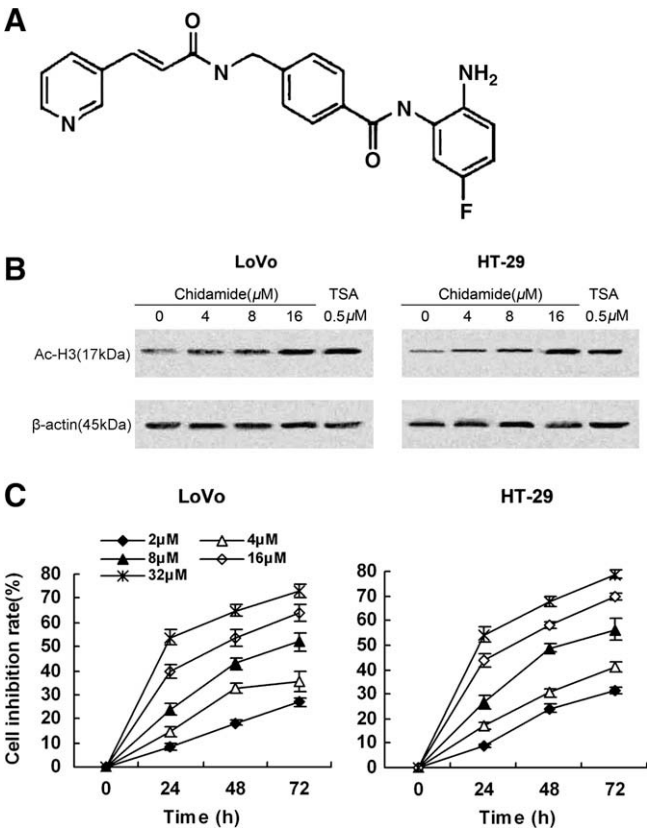

Fig. 1. Accumulation of acetylated histone H3 and inhibition of colon cancer cell proliferation by Chidamide, a novel HDAC inhibitor. (A) The chemical structure of Chidamide. (B) Chidamide promotion of histone H3 acetylation in human colon cancer cell lines. Colon cancer LoVo and HT-29 cell lines were treated with either DMSO or Chidamide at indicated doses for 48 h and then subjected to Western blot analysis to examine histone H3 acetylation levels. The corresponding β-actin levels are shown as loading controls. (C) The colon cancer cell lines LoVo and HT-29 were treated with Chidamide at the indicated doses and time points. The cells were then subjected to MTT assay. The inhibition rate (%) was determined using the equation $(1 - OD_{treated}/OD_{control}) \times 100$. The plot shows the average inhibition rate ± SD obtained from four independent experiments.