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Original Article

Effects of chidamide and its combination with decitabine on proliferation and apoptosis of leukemia cell lines

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Abstract: Chidamide, a novel histone deacetylase inhibitor (HDACI), shows anticancer ability against leukemia and solid tumors. Decitabine (5-Aza-2'-deoxycytidine, DAC), an anti-leukemic drug, is effective in treating acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). In our study, we investigated the anti-leukemic ability of chidamide, as well as its combination with decitabine in leukemia cells (HL60 and NB4). The results showed that the inhibitive effect of chidamide was dose- and time-dependent at concentration of 0.25-8 μM. The proliferation of HL60 and NB4 cells were significantly inhibited by chidamide or its combination with decitabine. The combination had a remarkable synergistic anti-leukemic effect. Chidamide increased the levels of acetylated histone H3 in both HL60 and NB4 cells by effectively inhibiting histone deacetylases (HDAC) enzymatic activities. The cells were blocked in G_0/G_1 phase by chidamide, but when chidamide was combined with decitabine, the cell cycle was mainly blocked in G_2/M phase, accompanied by the induction of p21 expression. In both cases (chidamide or chidamide combined with decitabine), apoptosis of tumor cells was induced through up-regulation of Bax and Caspase-3, and down-regulation of Bcl-2, showing a synergistic cytotoxicity. In conclusion, our results suggested that chidamide in combination with decitabine might be an effective therapy for AML.

Keywords: Chidamide, acute myeloid leukemia, decitabine, apoptosis

Introduction

Recent studies have shown that cancer can be caused not only by the change of DNA sequence, but also by two typical epigenetic modifications: DNA promoter methylation and histone modification [1, 2]. These two epigenetic modifications can remodel chromatins, alter cell phenotypes, regulate gene expression, and promote cancer development [3, 4]. Both DNA methylation and histone modification have a reversible nature [2], which is most intriguing in designing therapeutic strategies. In addition, methylation and acetylation modifications of specific sites can regulate cellular pathways involved in cell cycle control and apoptosis [5]. Therefore, the epigenetic leukemic epigenome has become a new target of the treatment with histone deacetylase inhibitors (HDACI) and hypomethylating agents (HMAs) [6].

DNA methylation has been widely studied in epigenetic genetics. In acute myeloid leukemia (AML), abnormal DNA methylation can silence the expression of tumor suppressor genes (TSGs) [7, 8]. Histone deacetylases (HDAC) can modulate gene expression and chromatin modification by inducing acetylation of histones and non-histone proteins [9]. In addition, the conversion of HDAC from open state to the compact configuration is closely related to epigenetic gene silencing [10]. Besides HDAC-induced gene silencing, DNA methylation is also engaged in histone methylation [10, 11]. This mechanism provides that the combination of HDACI and HMAs may be effective to fight against leukemia.

As a synthetic HDACI of the benzamide class, chidamide (CS055/HBI-8000) has been researched in many clinical trials in the USA and

Table 1. Primers used for quantification measurements of mRNA expression

Gene	Forward	Reverse
Bcl-2	CTGGGAGAACAGGGTACGATAA	GGCTGGGAGGAGAAGATGC
Bax	TCATCCAGGATCGAGCAGG	TGTCCACGGCGGCAAT
Caspase-3	AGGCAGGCGACGAGTT	TTCCCATAGAGTTCCACAAA

China, and ratified to treat cutaneous T-cell lymphoma (CTCL) and peripheral T-cell lymphoma (PTCL) in China [12]. In our study, we detected the anti-leukemic effects of chidamide combined with decitabine on AML cells. The results showed that chidamide and its combination with decitabine had a strong anti-leukemic ability through inducing cell cycle arrest, inhibiting cell proliferation and accelerating apoptosis of AML cells. These results reveal that chidamide is a potential drug for leukemia, especially when it is combined with decitabine.

Materials and methods

Reagents

Chidamide supplied by Chipscreen Biosciences Ltd. (Shenzhen, China) was dissolved in dimethyl sulfoxide (DMSO) (Invitrogen, USA) at a 20 mM concentration for stock solutions. The stock solutions were stored at -80°C. Decitabine was purchased from Sigma (St. Louis, Mo, USA) and also dissolved in DMSO at a 20 mM concentration and kept at -80°C. The stock solutions were diluted to working concentrations in the subsequent experiments with growth media.

Cell lines and cell culture

AML cell lines HL60 and NB4 were obtained from the Cell Biology Research Institute (Chinese Academy of Science, Shanghai, China). The cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS, Gibco, Life Technologies, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (1 × P/S), in an incubator at 37°C in a humidified atmosphere consisting of 95% air and 5% CO_2.

Cell viability assay

The cytotoxic effect of chidamide or decitabine alone, or the combination of the two on the AML cell lines was determined by cell counting kit-8 (CCK-8, Dojindo, Japan) assay. Cells were seeded at a density of 1-3 × 10^4/mL in 96-well flat-bottomed microtiter plates, 100 μL/well, and exposed to chidamide (0-8 μM) or decitabine (0-100 μM) with varying concentrations, alone or in combination for 72 h, then subjected to a standard CCK-8 assay. The plate was read at a wavelength of 490 nm under a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The combination index (CI) value was calculated from the fraction-affected value of each combination according to the Chou-Talalay method using CompuSyn software (ComboSyn, Inc.). The CI value reflected the degree of drug interaction: CI < 1, CI = 1, or CI > 1 indicated synergistic, additive, or antagonistic effects, respectively. Statistical analysis and IC_{50} determination were calculated in Graph Pad 5 (Prism software). All experiments were performed in triplicate.

HDAC activity assay

HDAC activity was determined as described in the Colorimetric HDAC Activity Assay kit (Epigentek Group Inc.). Each reaction (100 μL) contained nuclear protein (50 μg) extracted from leukemia cells (isolated by Nuclear and Cytoplasmic Extraction reagents; Pierce) and HDAC substrate. To test the effect of HDACI, chidamide was added to the mixtures and incubated at 37°C for 30 min. Fluorescence intensity was measured at an excitation of 360 nm and an emission of 460 nm using a Synergy TM2 microplate reader from BioTek (Winooski, VT, USA). Each compound concentration was performed at least twice.

Cell cycle analysis

The cells were treated with chidamide alone or chidamide combined with decitabine for 72 h. Then, the cells were collected and washed with PBS and then fixed overnight in 75% ice-cold ethanol at 4°C. After that, the fixed cells were harvested, stained with propidium iodide/RNase (BD Pharmingen, San Diego, CA, USA) and incubated in the dark at room temperature for 15 min after being washed with PBS. The DNA content was analyzed by flow cytometry. ModFit LT software (Verity Software House, Inc., Topsham, ME) was used for data analysis.

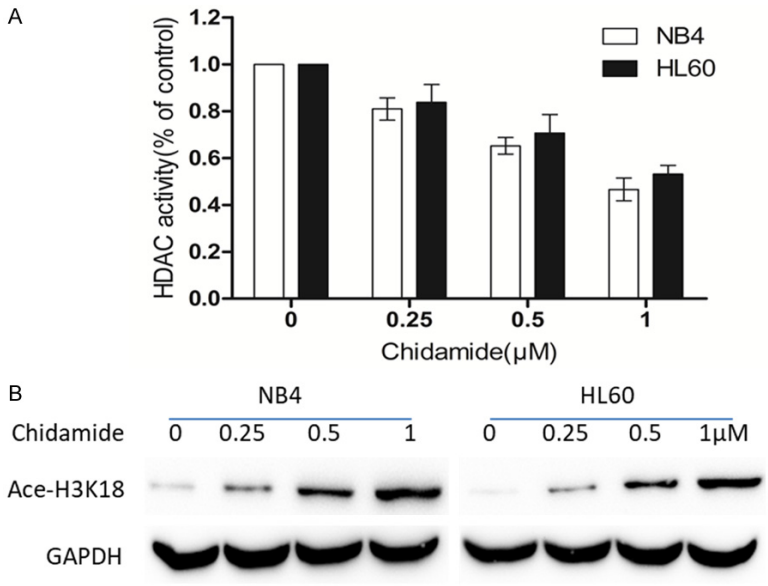


Figure 1. Induction of acetylated histone H3 and the inhibition on HDAC enzymes of chidamide. A. The inhibition of HDAC on HL60 and NB4 cells were tested by Elisa assay. Leukemia cells were incubated with chidamide for 72 h at the concentrations indicated. HDAC inhibitory activity of chidamide was shown in the graph. The plots were means ± SD for three independent experiments. B. Chidamide promoted histone H3 acetylation in HL60 and NB4 cell lines. HL60 and NB4 cell lines were treated with either DMSO or chidamide at indicated doses for 72 h and then subjected to Western blot analysis to examine histone H3 acetylation levels. GAPDH expression was determined as a loading control.

Cell apoptosis assay

The effects of chidamide alone or chidamide combined with decitabine on apoptosis in HL-60 or NB4 cell clines were analyzed by flow cytometry analysis. Briefly, cells were harvested, washed with PBS, and resuspended in 400 μL Annexin Binding Buffer. Then 1 μL FITC-conjugated Annexin-V and 5 μL of PI (BD Pharmingen, San Diego, CA, USA) were added to each sample. Stained samples were analyzed by flow cytometer (FACS Calibur; BD Biosciences, Franklin Lakes, NJ, USA). DNA fragmentation was detected as described in the DNA Ladder assay kit (Beyotime Biotech).

Total RNA isolation and qRT-PCR

Total cellular RNA was extracted from cells using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocols. RNA was eluted with RNase-free water, quantified at an absorbance at 260/280 nm, and used for reverse transcription reaction. Total mRNA was reverse transcribed into cDNA using RT reagent kit (TaKaRa, Dalian, China). Primers (Shenggong Bioengineering Co. Shanghai, China) used for quantification measurements are shown in **Table 1**. GAPDH was used as an internal standard.

Real-time quantitative reverse transcription-PCR (qRT-PCR) was performed with the Fast Start Universal SYBR Green Master (ROX) (Roche, Germany) following the instruction of the supplier. The RT-PCR conditions were as follows: 1 cycle at 94°C for 10 min, 40 cycles at 94°C for 10 sec, 60°C for 30 sec, and one cycle at 72°C for 3 min. The results were analyzed using 2-ΔΔCt, in which ΔCt = Ct (target gene)-Ct (internal reference), ΔΔCt = ΔCt (sample)-ΔCt (control). Each sample was measured in triplicate.

Western blotting analysis

Cultured cells were harvested, washed with PBS and then lysed in RIPA buffer. The protein lysates were clarified by centrifugation at 12000 g for 30 min at 4°C and the supernatant was collected. The protein of each sample was quantified by a BCA (bicinchoninic acid) assay (Pierce). Equal amounts of proteins were separated by SDS/PAGE and then electrotransferred onto a PVDF membrane (Millipore). The membranes were blocked with 5% skim milk, incubated with primary antibodies at 4°C overnight in TBS-T (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Tween 20). The primary antibodies were as follows: anti-acetylated histone H3, -Bax, -Bcl-2, -p21, -Caspase 3. GAPDH (Cell Signaling, Herts, UK) was used as a loading control. 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).

Statistical analysis

All data were presented as means ± standard deviation (SD). SPSS 17.0 Student's *t* test was

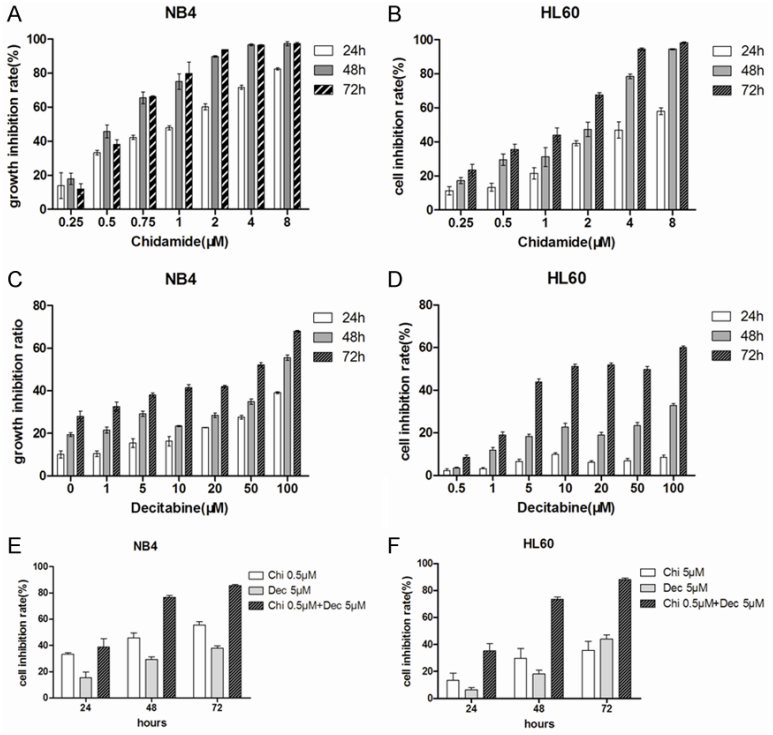


Figure 2. Chidamide, decitabine and their combination inhibit cell proliferation in leukemia cells. A-D. NB4 and HL60 cells were exposed to chidamide for 72 h and then the cell viability was determined by CCK-8 assay. The data represents the time- and dose-dependent effects of chidamide and decitabine on cell proliferation in NB4 and HL60 cells. Results are means ± SD. E, F. NB4 and HL60 cells were exposed to chidamide (0.5 μM) combined with decitabine (5 μM) for 72 h and then the cell viability was determined by CCK-8 assay. Results are means ± SD for three independent experiments.

used to compare the two independent groups and corresponding bar graph or line charts were drawn by GraphPad Prism 5 software. One way analysis of variance (ANOVA) was used to compare the multiple independent groups. Differences of measurement data and enumeration data were compared respectively with Student's *t* test. Statistical $P < 0.05$ was considered significant.

Results

HDAC inhibitory activity of chidamide

To assess the activity and efficacy of chidamide, we determined the HDAC inhibitory effect of chidamide. We treated HL60 and NB4 cell lines with 0, 0.25, 0.5 and 1 μM chidamide for 72 h. It was indicated *in vitro* that chidamide showed HDAC inhibition capability (**Figure 1A**). Then we examined its ability to change the lysine residues of histone H3 acetylation (**Figure 1B**). The results showed that chidamide suppressed HDAC and induced the accumulation of acetylated histone H3 in HL60 and NB4 cells.

Inhibitory effects of chidamide and decitabine on cell proliferation in leukemia cells

To determine the effect of chidamide and decitabine on proliferation, we first examined the proliferation of HL-60 and NB4 cells in response to chidamide and decitabine of various concentrations using CCK-8 assay. Results showed that the half-maximal inhibitory concentrations (IC_{50}) of chidamide on HL60 and NB4 cell lines were (1.544±0.050) μM and (0.460±0.039) μM, respectively. IC_{50} values of decitabine were (4.724±0.067) μM and (2.788±1.725) μM, respectively, in three independent experiments after a 72 h exposure. Chidamide and decitabine caused growth arrest of the HL60 and NB4 cell lines in a dose and time dependent manner (**Figure 2A-D**). Chidamide markedly inhibited cell proliferation at low concentrations (**Figure 2A**, **2B**). Next, we evaluated the effect of chidamide and decitabine combination on cell viability, we treated HL60 and NB4 cells with different drug concentrations in accordance with the IC_{50} of each compounds. The live or viable cells were determined using CCK-8 proliferation assay and the data was analyzed by Graph-Pad Prism software. The effects of the combination were evaluated by the CompuSyn software. As shown in **Figure 2E**, **2F**, chidamide combined with decitabine significantly enhanced growth arrest, as determined by CCK-8 assays. Calculating CI showed that chidamide combined with decitabine had a clear synergistic effect. CI < 1, indicative of synergism, was obtained in each of the drug combinations (**Figure 3A**, **3B**).

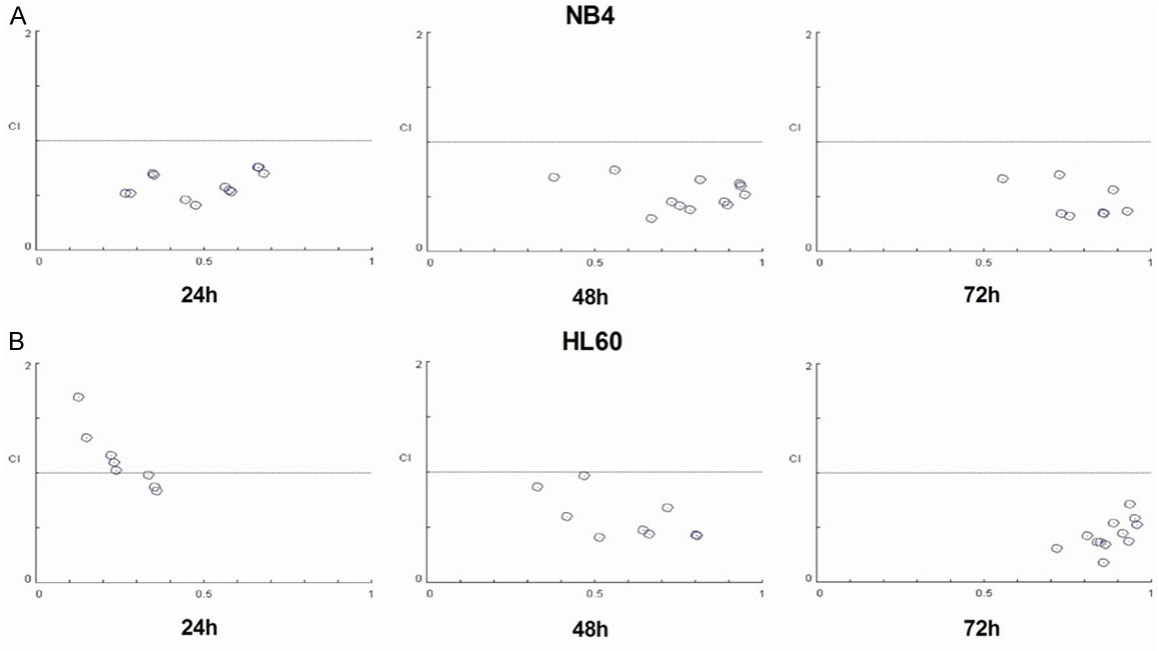


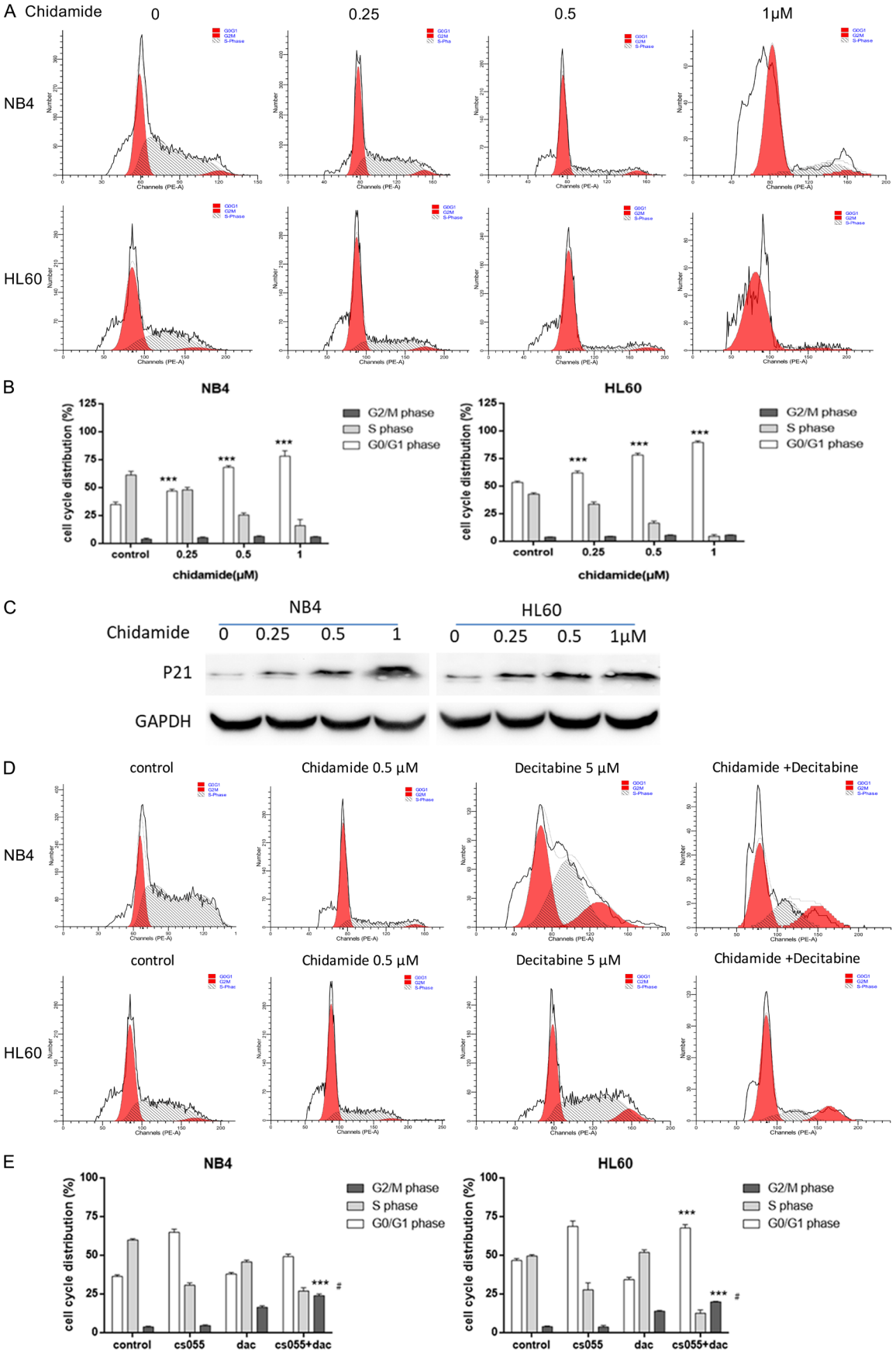
Figure 3. Chidamide synergies with decitabine in inhibiting the proliferation of leukemia cell lines. A, B. NB4 or HL60 cells were treated with chidamide combined with decitabine. Combination index values were calculated with the CompuSyn software, which was developed based on the median-effect method. CI < 1 indicates synergy; CI = 1 is additive; and CI > 1 means antagonism. The result showed that chidamide combined with decitabine had the most notable synergistic effect.

Cell cycle arrest induced by chidamide and its combination with decitabine in leukemia cells

Since chidamide, decitabine and their combination inhibited cell proliferation, we intended to testify whether cell cycle arrest could be contributed to their synergistic antitumor effects. First, we examined the effect of chidamide on cell cycle arrest in leukemia cells. After treating the HL60 and NB4 cells with chidamide for 72 h, cells arrested at the G_0/G_1 phase in a dose dependent manner (**Figure 4A**, **4B**). Then, we explored the distribution of cell cycle phases treated with chidamide-decitabine combination for 48 h. Chidamide caused an arrest in G_0/G_1 phase, however, decitabine induced G_2/M phase cell arrest in HL60 and NB4 cell lines. In the combined group, the proportions of G_0/G_1 phase and G_2/M phase were higher than the control group, but mainly in G_2/M phase, especially in NB4 cells (**Figure 4D**, **4E**). Further, in order to monitor the molecular mechanism of chidamide, we detected the proteins involved in cell cycle regulation by Western blot. As shown in **Figure 4C**, p21 protein expression was up-regulated when treated with chidamide alone, which was in accordance with the result of cell cycle analysis. The up-regulation of p21 expression induced by chidamide could inhibit cell growth and lead to cell cycle arrest, while chidamide combined with decitabine could also up-regulate p21 protein expression in a similar way (**Figure 4F**).

Apoptosis induced by chidamide and its combination with decitabine in leukemia cells

Since cytotoxicity could lead to cell apoptosis, we investigated whether chidamide could induce apoptosis in leukemia cells. To examine the effects of chidamide on cell viability, we carried out a DNA ladder assay and a flow cytometry analysis with Annexin V/PI double staining. We observed Annexin V positive cells and DNA fragmentation in HL60 and NB4 cells 72 h after chidamide treatment (**Figure 5A**, **5B**). The results indicated that apoptosis occurred in these cells with a significantly higher early apoptotic rate than the late apoptotic rate. Similarly, DNA fragmentation was observed in HL60 and NB4 cells after being exposed to chidamide for 72 h (**Figure 5C**). These results showed that chidamide induced cells apoptosis in a dose dependent manner (**Figure 5A**, **5B**).


A
Chidamide
0
0.25
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1μM
NB4
HL60
Channels (PE-A)
Number
B
NB4
HL60
G2/M phase
S phase
G0/G1 phase
cell cycle distribution (%)
control
0.25
0.5
1
chidamide(μM)
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C
NB4
HL60
Chidamide
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0.5
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1μM
P21
GAPDH
D
control
Chidamide 0.5 μM
Decitabine 5 μM
Chidamide +Decitabine
NB4
HL60
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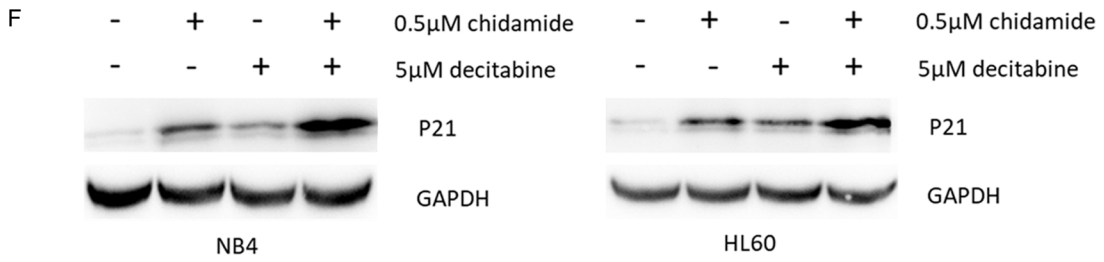


Figure 4. Chidamide combined with decitabine induced cell cycle arrest in leukemia cells, cooperatively enhanced cell cycle arrest. A. NB4 and HL60 cells were treated with chidamide at indicated concentrations (0, 0.25, 0.5, 1 μM) for 72 h, and cell cycle analysis was performed by flow cytometry. B. The data were presented as means ± SD of three independent experiments (Statistical differences were calculated by one-way ANOVA. ***$P < 0.001$ *vs* control group). C. NB4 and HL60 cells were treated with indicated doses of chidamide for 72 h and then p21 was assessed using Western blot analysis. GAPDH was used as a loading control. D. NB4 or HL60 cells were treated with the combination of chidamide and decitabine simultaneously for 48 h, and then harvested. Cell cycle analysis was determined by PI staining and flow cytometry. E. Distribution of cell cycle phases were presented as means ± SD of three independent experiments. Statistical differences were calculated by one-way ANOVA. ***indicated $P < 0.001$, while # indicated $P < 0.001$, relative to control or individual drug treatments. F. HL60 and NB4 cells were exposed to chidamide and decitabine simultaneously at indicated doses for 48 h, and then subjected to Western blotting. Western blot analyses were conducted with antibodies against p21. GAPDH was measured as a loading control.

Then the synergic antitumor function of chidamide combined with decitabine was determined by observing apoptosis in cells. We found that apoptosis in HL60 and NB4 cells was greatly enhanced when the cells were disposed with 0.5 μM chidamide combined with5 μM decitabine simultaneously for 48 h (**Figure 5E**, **5F**). The results also showed that chidamide alone mainly induced early apoptosis, while the combination of chidamide and decitabine caused late apoptosis.

The B-cell lymphoma 2 (Bcl-2) family proteins and caspases are critical regulators of the apoptotic pathways. In order to understand the molecular mechanism underlying chidamide-induced apoptosis, we explored the expression levels of Bcl-2, Bax, and Proaspase-3 in HL60 and NB4 cells during the 72 h chidamide treatment. The results of Western blot showed that chidamide significantly down-regulated Bcl-2 and Procaspase-3, while Bax protein levels were up-regulated in a dose-dependent manner (**Figure 5D**). Meanwhile, chidamide combined with decitabine also caused similar regulation in the Bcl-2 family proteins as those treated with single chidamide (**Figure 5F**). Therefore, we speculated that chidamide combined with decitabine can also induce apoptosis through the Bcl-2 pathway.

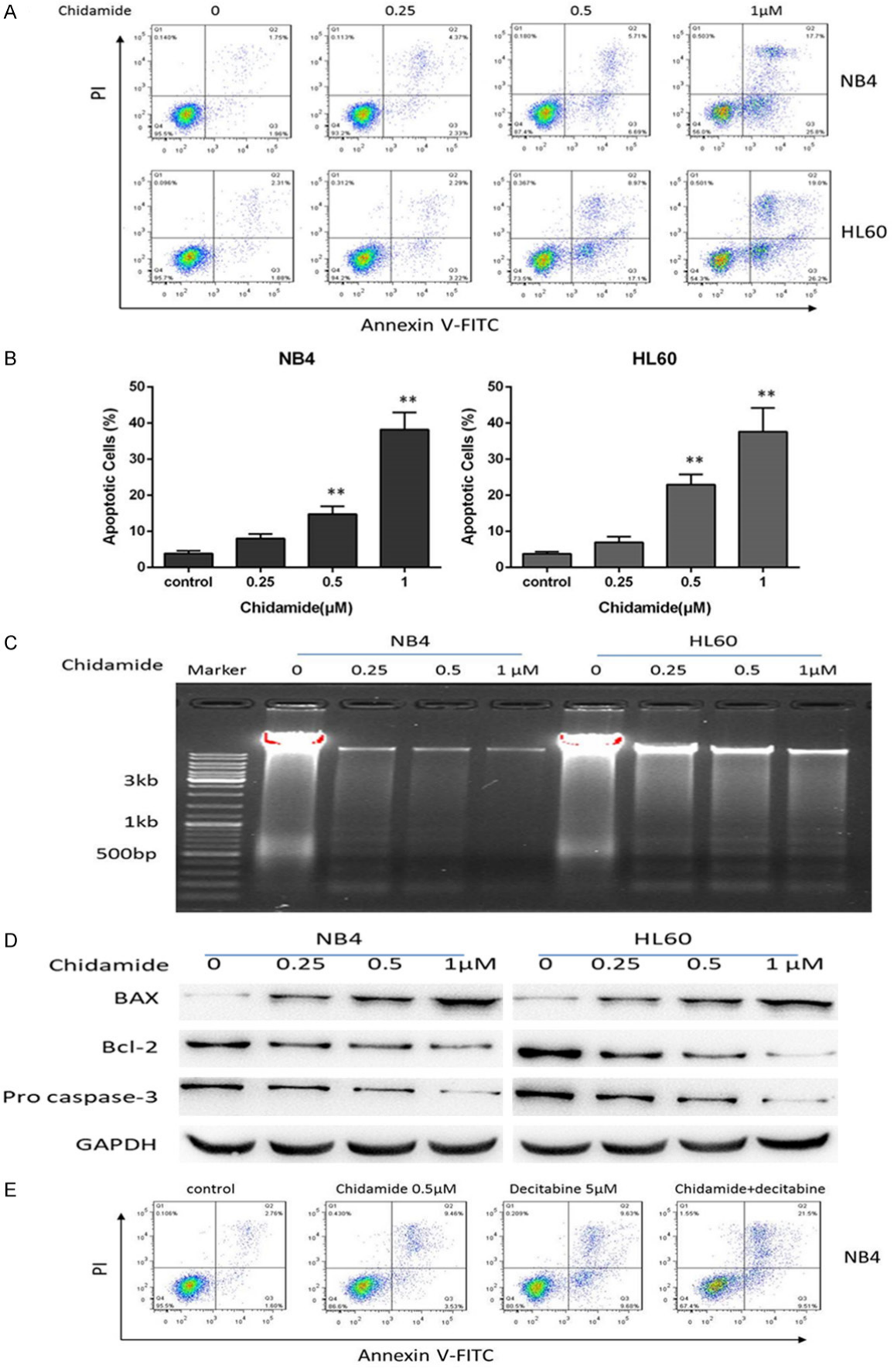
The effects of chidamide alone or combined with decitabine on mRNA levels of Bcl-2, Bax and Caspase-3 were explored by qRT-PCR. Bcl-2 mRNA levels were significantly down-regulated in HL60 and NB4 cells when they were exposed to chidamide alone for 48 h, meanwhile, the levels of Bax and Caspase-3 were significantly up-regulated. The cells exposed to chidamide combined with decitabine also demonstrated the similar pattern regarding to the mRNA levels of Bcl-2, Bax, and Caspase-3.

Discussion

Epigenetic aberrations occur during tumor genesis [13, 14]. Genes that can be silenced by abnormal methylation or acetylation are promising targets for cancer therapies [15]. In clinical practice, using HDACI or HMAs alone is not an effective strategy, but their combination or with other anti-tumor drugs may be different.

To investigate this strategy, hypomethylating combination trials for AML have been carried out [16-19], including the application of HDACI [20, 21]. HMAs have already shown promising effects in clinical treatment of AML [22]; besides, the significant anti-tumor effects of HMAs combined with HDACI have been observed in several *in vitro* and *in vivo* studies [6, 10, 23, 24]. In our study, we investigated the potential of chidamide combined with decitabine in leukemia treatment by inducing cell cycle arrest and apoptosis.

Chidamide is anovel HDACI with a similar chemical structure to entinostat (MS-275). It can


A
Chidamide
0
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Annexin V-FITC
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NB4
HL60
Apoptotic Cells (%)
control
0.25
0.5
1
Chidamide(μM)
C
Chidamide
Marker
NB4
HL60
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0.25
0.5
1 μM
3kb
1kb
500bp
D
Chidamide
BAX
Bcl-2
Pro caspase-3
GAPDH
E
control
Chidamide 0.5μM
Decitabine 5μM
Chidamide+decitabine
PI
NB4
Annexin V-FITC

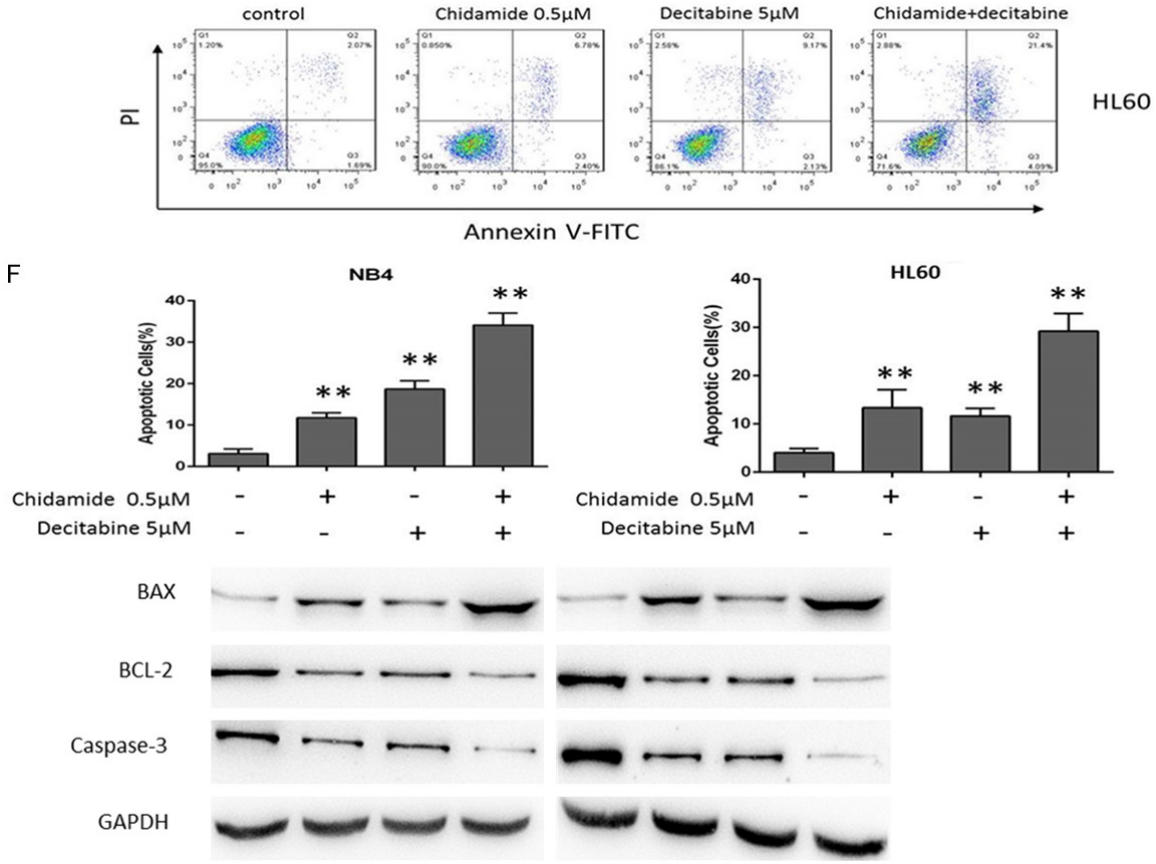

Figure 5. Chidamide induced apoptosis in leukemia cells, and cooperatively induced apoptosis with decitabine. A. NB4 and HL60 cells were exposed to chidamide at indicated concentrations (0, 0.25, 0.5, 1 μM) for 72 h and apoptosis was determined by Annexin-V/PI staining. B. The quantitative data for the percentage of apoptotic cells were pooled from three independent experiments. Results represented the means ± SD (Statistical differences were calculated by one-way ANOVA. $^{**}P < 0.01$ compared with untreated cells). C. DNA ladder assay. HL60 and NB4 cells were treated with chidamide for 72 h and then harvested. The genomic DNA was then extracted and analyzed by agarose gel electrophoresis. D. HL60 and NB4 cells were exposed to chidamide at indicated doses for 72 h and then subjected to Western blotting. Western blot analyses were done with antibodies against pro-caspase-3, Bcl-2 and Bax. GAPDH was measured as a loading control. E. Apoptosis was determined by Annexin-V/PI staining after HL60 and NB4 cells were treated with chidamide combined with decitabine at indicated concentrations for 48 h. F. The percentage of apoptotic cells (Annexin-V and PI positive) was indicated in the histogram. Results represented the means ± SD (Statistical differences were calculated by one-way ANOVA. $^{**}P < 0.01$ compared with untreated cells). F. Expression of Bcl-2, Bax and procaspase-3 were assayed by a standard Western blot method after HL60 and NB4 cells were treated with chidamide combined with decitabine at indicated concentrations for 48 h.

selectively inhibit HDAC1, HDAC2, HDAC3 and HDAC10, especially for HDAC2 and HDAC3 [12]. Chidamide is highly toxic to leukemia cells in a concentration- and time-dependent manner (**Figure 2A**). Compared with MS-275, chidamide is less toxic, more tolerant and more stable [13].

In this study, it is indicated that chidamide could inhibit growth and induce apoptosis of leukemia cells in a dose-dependent manner. Many experiments have confirmed that HDACI induces cell cycle arrest in the G_0/G_1 phase *in vitro* [12, 25-28].

Decitabine increased the cell number in G_2 phase, while chidamide caused an arrest in G_0/G_1 phase in both NB4 and HL60 cell lines. The combination of the two drugs induced a marked increase of cells in G_2/M, showing that the effects were overlaid. According to Western blot assay, the expression level of p21 protein in the combination group was up-regulated, which could induce cell cycle arrest and inhibit

cell proliferation. In addition, the combination of chidamide and decitabine could activate the p53 signaling pathway and induce apoptosis [5, 27, 29].

HDAC activity and the high acetylation level of histone are closely related to low p21 expression levels in tumor cells [30]. The p21 protein is a cyclin dependent kinase (CDK) inhibitor with an extensive kinase activity. Our study has shown that chidamide could inhibit HDAC activity and up-regulate p21 expression levels. HDACI could also inhibit the expression of cyclin D, resulting in cell cycle arrest in G_1 phase [12, 31, 32]. However, the interaction between p21 and CDK is not included in this study. So it is necessary to further confirm this mechanism in the future research.

Epigenetic drugs could overcome drug resistance by inducing cell cycle arrest in G_2/M phase and reactivating the previously silenced genes. The combination of two epigenetic drugs could be more effective in lowering drug resistance than the use of one drug [33], and the synergistic effect could be more obvious in inducing apoptosis.

Bcl-2 family proteins are pivotal to cell fate because they can regulate cells in two ways: anti- and pro-apoptosis [34, 35]. The results of our study demonstrated that chidamide-induced apoptosis in NB4 and HL60 cells via the anti-apoptotic Bcl-2 family proteins, the up-regulation of pro-apoptotic Bcl-2 family protein Bax and the activation of Caspase-3. In addition, various HDACI in combination with decitabine shortened the tumor cell survival at mRNA and protein levels [36, 37]. The effects of HDACI are multifactorial. Mitochondria membrane potential and ROS generation can induce apoptosis [26]. Many studies have reported HDACI alone or its combination with decitabine can induce ROS production and change mitochondria membrane potential. For this reason, we excluded the detection of mitochondria membrane potential and ROS generation in our experiment.

When chidamide was combined with decitabine, chidamide enhanced the cytotoxicity in both HL60 and NB4 cell lines potently and synergistically. This synergistic cytotoxicity was obvious because the two agents could cooperatively induce cell cycle arrest and apoptosis. This study showed that the decitabine-induced inhibition was incomplete (**Figure 2C**, **2D**), but when combined with chidamide, the inhibition was significantly improved, showing a marked synergistic effect. The possible reason is that the two drugs can block different cell cycles.

In the study of U937 and SKM-1 cell lines, it was found that decitabine combined with other drugs could exert an anti-leukemia effect by regulating MAPK and Wnt signaling pathway [38]. Chidamide can also induce apoptosis through inhibiting PI3K/Akt and MAPK/Ras signal pathway [39] and activating JNK signal pathway [26] to fulfill the anti-leukemia function. Therefore, many antitumor drugs have multi-functions and multi-targets. HDACI combined with decitabine at different concentrations might have multiple pathways to induce cell cycle arrest and differentiation. In a word, these results suggested that chidamide induced apoptosis through intrinsic apoptotic pathways [40].

Chidamide in combination with decitabine has a synergistic anti-leukemia effect *in vitro*, and can be taken as a therapeutic strategy for acute myeloid leukemia.

Additional files: All original western images were showed in Supplementary Figure 1.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

[1] Bhatla T, Wang J, Morrison DJ, Raetz EA, Burke MJ, Brown P and Carroll WL. Epigenetic reprogramming reverses the relapse-specific gene expression signature and restores chemosensitivity in childhood B-lymphoblastic leukemia. Blood 2012; 119: 5201-5210.

[2] Schonheit J, Leutz A and Rosenbauer F. Chromatin dynamics during differentiation of myeloid cells. J Mol Biol 2015; 427: 670-687.

[3] Falkenberg KJ and Johnstone RW. Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. Nat Rev Drug Discov 2014; 13: 673-691.

[4] Roy DM, Walsh LA and Chan TA. Driver mutations of cancer epigenomes. Protein Cell 2014; 5: 265-296.

[5] Brodska B, Holoubek A, Otevrelova P and Kuzelova K. Combined treatment with low concentrations of decitabine and SAHA causes cell death in leukemic cell lines but not in normal peripheral blood lymphocytes. Biomed Res Int 2013; 2013: 659254.

[6] Tan P, Wei A, Mithraprabhu S, Cummings N, Liu HB, Perugini M, Reed K, Avery S, Patil S, Walker P, Mollee P, Grigg A, D'Andrea R, Dear A and Spencer A. Dual epigenetic targeting with panobinostat and azacitidine in acute myeloid leukemia and high-risk myelodysplastic syndrome. Blood Cancer J 2014; 4: e170.

[7] Figueroa ME, Lugthart S, Li Y, Erpelinck-Verschueren C, Deng X, Christos PJ, Schifano E, Booth J, van Putten W, Skrabanek L, Campagne F, Mazumdar M, Greally JM, Valk PJ, Lowenberg B, Delwel R and Melnick A. DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. Cancer Cell 2010; 17: 13-27.

[8] Deneberg S, Grovdal M, Karimi M, Jansson M, Nahi H, Corbacioglu A, Gaidzik V, Dohner K, Paul C, Ekstrom TJ, Hellstrom-Lindberg E and Lehmann S. Gene-specific and global methylation patterns predict outcome in patients with acute myeloid leukemia. Leukemia 2010; 24: 932-941.

[9] Bolden JE, Peart MJ and Johnstone RW. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov 2006; 5: 769-784.

[10] Momparler RL, Cote S, Momparler LF and Idaghdour Y. Epigenetic therapy of acute myeloid leukemia using 5-aza-2'-deoxycytidine (decitabine) in combination with inhibitors of histone methylation and deacetylation. Clin Epigenetics 2014; 6: 19.

[11] Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, Morey L, Van Eynde A, Bernard D, Vanderwinden JM, Bollen M, Esteller M, Di Croce L, de Launoit Y and Fuks F. The polycomb group protein EZH2 directly controls DNA methylation. Nature 2006; 439: 871-874.

[12] Gong K, Xie J, Yi H and Li W. CS055 (Chidamide/HBI-8000), a novel histone deacetylase inhibitor, induces G1 arrest, ROS-dependent apoptosis and differentiation in human leukaemia cells. Biochem J 2012; 443: 735-746.

[13] Chan TA and Baylin SB. Epigenetic biomarkers. Curr Top Microbiol Immunol 2012; 355: 189-216.

[14] Hatziapostolou M and Iliopoulos D. Epigenetic aberrations during oncogenesis. Cell Mol Life Sci 2011; 68: 1681-1702.

[15] Ganesan A, Nolan L, Crabb SJ and Packham G. Epigenetic therapy: histone acetylation, DNA methylation and anti-cancer drug discovery. Curr Cancer Drug Targets 2009; 9: 963-981.

[16] Qin T, Youssef EM, Jelinek J, Chen R, Yang AS, Garcia-Manero G and Issa JP. Effect of cytarabine and decitabine in combination in human leukemic cell lines. Clin Cancer Res 2007; 13: 4225-4232.

[17] Esteller M, Guo M, Moreno V, Peinado MA, Capella G, Galm O, Baylin SB and Herman JG. Hypermethylation-associated inactivation of the cellular retinol-binding-protein 1 gene in human cancer. Cancer Res 2002; 62: 5902-5905.

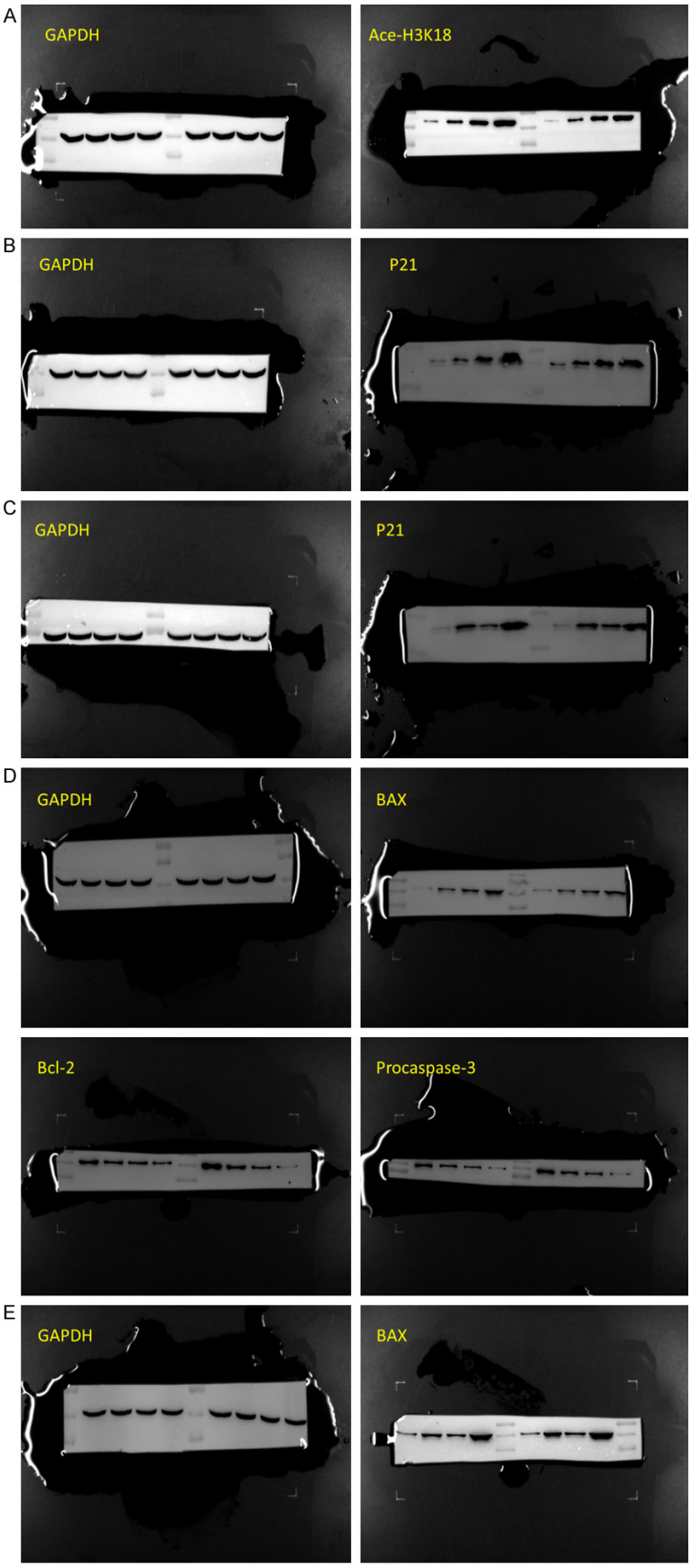
[18] Peng CY, Jiang J, Zheng HT and Liu XS. Growth-inhibiting effects of arsenic trioxide plus epigenetic therapeutic agents on leukemia cell lines. Leuk Lymphoma 2010; 51: 297-303.

[19] Anzai H, Frost P and Abbruzzese JL. Synergistic cytotoxicity with 2'-deoxy-5-azacytidine and topotecan in vitro and in vivo. Cancer Res 1992; 52: 2180-2185.

[20] Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, Yang H, Rosner G, Verstovsek S, Rytting M, Wierda WG, Ravandi F, Koller C, Xiao L, Faderl S, Estrov Z, Cortes J, O'Brien S, Estey E, Bueso-Ramos C, Fiorentino J, Jabbour E and Issa JP. Phase 1/2 study of the combination of 5-aza-2'-deoxycytidine with valproic acid in patients with leukemia. Blood 2006; 108: 3271-3279.

[21] Sekeres MA, List AF, Cuthbertson D, Paquette R, Ganetzky R, Latham D, Paulic K, Afable M, Saba HI, Loughran TP Jr and Maciejewski JP. Phase I combination trial of lenalidomide and azacitidine in patients with higher-risk myelodysplastic syndromes. J Clin Oncol 2010; 28: 2253-2258.

[22] Griffiths EA and Gore SD. Epigenetic therapies in MDS and AML. Adv Exp Med Biol 2013; 754: 253-283.
[23] Salmon JM, Bots M, Vidacs E, Stanley KL, Atadja P, Zuber J and Johnstone RW. Combining the differentiating effect of panobinostat with the apoptotic effect of arsenic trioxide leads to significant survival benefit in a model of t(8;21) acute myeloid leukemia. Clin Epigenetics 2015; 7: 2.
[24] Kirschbaum M, Gojo I, Goldberg SL, Bredeson C, Kujawski LA, Yang A, Marks P, Frankel P, Sun X, Tosolini A, Eid JE, Lubiniecki GM and Issa JP. A phase 1 clinical trial of vorinostat in combination with decitabine in patients with acute myeloid leukaemia or myelodysplastic syndrome. Br J Haematol 2014; 167: 185-193.
[25] Li X, Yan X, Guo W, Huang X, Huang J, Yu M, Ma Z, Xu Y, Huang S, Li C, Zhou Y and Jin J. Chidamide in FLT3-ITD positive acute myeloid leukemia and the synergistic effect in combination with cytarabine. Biomed Pharmacother 2017; 90: 699-704.
[26] Li Y, Chen K, Zhou Y, Xiao Y, Deng M, Jiang Z, Ye W, Wang X, Wei X, Li J, Liang J, Zheng Z, Yao Y, Wang W, Li P and Xu B. A new strategy to target acute myeloid leukemia stem and progenitor cells using chidamide, a histone deacetylase inhibitor. Curr Cancer Drug Targets 2015; 15: 493-503.
[27] Liu Z, Ding K, Li L, Liu H, Wang Y, Liu C and Fu R. A novel histone deacetylase inhibitor Chidamide induces G0/G1 arrest and apoptosis in myelodysplastic syndromes. Biomed Pharmacother 2016; 83: 1032-1037.
[28] Zhao S, Guo J, Zhao Y, Fei C, Zheng Q, Li X and Chang C. Chidamide, a novel histone deacetylase inhibitor, inhibits the viability of MDS and AML cells by suppressing JAK2/STAT3 signaling. Am J Transl Res 2016; 8: 3169-3178.
[29] Yang H, Hoshino K, Sanchez-Gonzalez B, Kantarjian H and Garcia-Manero G. Antileukemia activity of the combination of 5-aza-2'-deoxycytidine with valproic acid. Leuk Res 2005; 29: 739-748.
[30] Fang JY, Chen YX, Lu J, Lu R, Yang L, Zhu HY, Gu WQ and Lu LG. Epigenetic modification regulates both expression of tumor-associated genes and cell cycle progressing in human colon cancer cell lines: Colo-320 and SW1116. Cell Res 2004; 14: 217-226.
[31] Sandor V, Senderowicz A, Mertins S, Sackett D, Sausville E, Blagosklonny MV and Bates SE. P21-dependent g(1)arrest with downregulation of cyclin D1 and upregulation of cyclin E by the histone deacetylase inhibitor FR901228. Br J Cancer 2000; 83: 817-825.
[32] Wong JC, Guo L, Peng Z, Zhang W, Zhang N, Lai W, Zhang Z, Zhang C, Zhang X, Song S, Pan D, Xie C, Li J, Ning Z, Lu X, He Y and Chen L. Application of p21 and klf2 reporter gene assays to identify selective histone deacetylase inhibitors for cancer therapy. Bioorg Med Chem Lett 2011; 21: 110-116.
[33] Vijayaraghavalu S, Dermawan JK, Cheriyath V and Labhasetwar V. Highly synergistic effect of sequential treatment with epigenetic and anticancer drugs to overcome drug resistance in breast cancer cells is mediated via activation of p21 gene expression leading to G2/M cycle arrest. Mol Pharm 2013; 10: 337-352.
[34] Krishna S, Low IC and Pervaiz S. Regulation of mitochondrial metabolism: yet another facet in the biology of the oncoprotein Bcl-2. Biochem J 2011; 435: 545-551.
[35] Gillies LA and Kuwana T. Apoptosis regulation at the mitochondrial outer membrane. J Cell Biochem 2014; 115: 632-640.
[36] Kaminskyy VO, Surova OV, Vaculova A and Zhivotovsky B. Combined inhibition of DNA methyltransferase and histone deacetylase restores caspase-8 expression and sensitizes SCLC cells to TRAIL. Carcinogenesis 2011; 32: 1450-1458.
[37] Yang L, Xu RR, Song GQ, Hang HM, Liu H, Jiang SH, Wang XF and Ding XS. Effect of decitabine combined with trichostatin A on MDS cell line SKM-1 in vitro. Zhongguo Shi Yan Xue Ye Xue Za Zhi 2008; 16: 819-823.
[38] Li K, Hu C, Mei C, Ren Z, Vera JC, Zhuang Z, Jin J and Tong H. Sequential combination of decitabine and idarubicin synergistically enhances anti-leukemia effect followed by demethylating Wnt pathway inhibitor promoters and downregulating Wnt pathway nuclear target. J Transl Med 2014; 12: 167.
[39] Liu L, Chen B, Qin S, Li S, He X, Qiu S, Zhao W and Zhao H. A novel histone deacetylase inhibitor Chidamide induces apoptosis of human colon cancer cells. Biochem Biophys Res Commun 2010; 392: 190-195.
[40] Ning ZQ, Li ZB, Newman MJ, Shan S, Wang XH, Pan DS, Zhang J, Dong M, Du X and Lu XP. Chidamide (CS055/HBI-8000): a new histone deacetylase inhibitor of the benzamide class with antitumor activity and the ability to enhance immune cell-mediated tumor cell cytotoxicity. Cancer Chemother Pharmacol 2012; 69: 901-909.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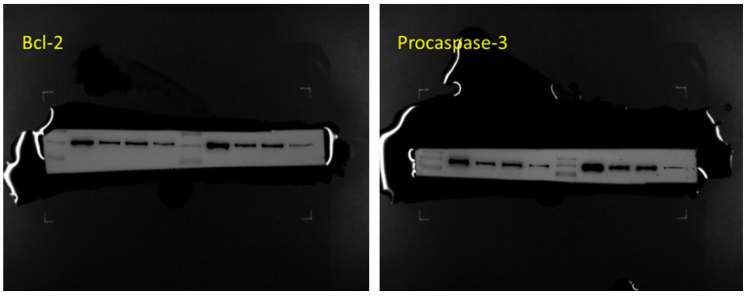

Supplementary Figure 1. A. Chidamide promoted histone H3 acetylation in HL60 and NB4 cell lines. B. NB4 and HL60 cells were treated with indicated doses of chidamide for 72 h and then p21 was assessed using Western blot analysis. C. HL60 and NB4 cells were exposed to chidamide and decitabine simultaneously at indicated doses for 48 h, and then subjected to Western blotting. D. HL60 and NB4 cells were exposed to chidamide at indicated doses then subjected to Western blotting. E. Expression of Bcl-2, Bax and procaspase-3 were assayed by a standard Western blot method after HL60 and NB4 cells were treated with chidamide combined with decitabine.