

Original Article

Chidamide shows synergistic cytotoxicity with cytarabine via inducing G0/G1 arrest and apoptosis in myelodysplastic syndromes

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Abstract: Chidamide is a newly designed and synthesized histone deacetylase (HDAC) inhibitor that selectively inhibits HDAC 1, 2, 3, and 10. Our previous study demonstrated that chidamide induces G0/G1 arrest and apoptosis in myelodysplastic syndromes (MDS). Low-dose chemotherapy is effective in treating higher risk MDS patients, and here, we sought to determine whether the combination of chidamide with cytarabine at low doses would increase the cytotoxicity to MDS cells. In this study, the combination of chidamide (50 nM) with cytarabine (50 nM) showed synergistic inhibition on cell growth. The mean combination index values were 0.068, 0.158, and 0.226 in SKM-1, MUTZ-1, and KG-1 MDS cell lines, respectively. The combination increased the acetylation levels of histone H3 and decreased HDAC activity in MDS cells. A low concentration (25 and 50 nM) of chidamide combined with low-dose cytarabine (50 nM) inhibited cell proliferation and arrested the cell cycle in the G0/G1 phase via down-regulating CDK2 and up-regulating p21. Furthermore, the combined treatment induced cell apoptosis via down-regulating Bcl-2 and up-regulating cleaved caspase-3 protein. These results demonstrate the potential utility of combining chidamide and cytarabine in the treatment of MDS.

Keywords: Chidamide, cytarabine, HDAC inhibitor, myelodysplastic syndrome, cell cycle, apoptosis

Introduction

Chidamide is a novel benzamide chemical class of HDAC inhibitor (HDACi) that selectively inhibits HDAC 1, 2, 3, and 10. To date, it has been approved in China for the oral treatment of recurrent or refractory peripheral T-cell lymphoma (PTCL) [1-3]. It is well accepted that HDACs act as transcription repressors that control cell survival, proliferation, angiogenesis, and immunity. Importantly, multiple human cancers over express HDACs, especially class I HDACs [4, 5].

Myelodysplastic syndromes (MDS) are a group of clonal hematopoietic stem cell disorders. The most obvious characteristic of these syndromes is dysplasia of the hematopoietic cell lineages, which increases the risk of developing acute myeloid leukemia (AML) [6]. Low doses of cytarabine are considered one of the most promising strategies for treating high-risk MDS patients [7], because of its safety and efficacy. The combination of cytarabine with the DNA methyl transferase inhibitor decitabine

improves the outcomes in MDS patients [8]. Like decitabine, the HDACi, chidamide, acts as a type of epigenetic therapy for cancer. Our previous study has shown that chidamide induces apoptosis and growth inhibition in MDS cell lines [9], and the same results were obtained in other solid cancers such as lung cancer [10] and colon cancer [1]. Therefore, the combination of HDAC is with chemotherapy drugs at a low concentration is considered as one of the most promising strategies to increase the efficacy of cancer treatments. In this study, we sought to determine whether the combination of chidamide and cytarabine at low doses can synergistically enhance apoptosis in MDS cells, and to provide support for the clinical application of this regimen.

Materials and methods

Reagents

Chidamide was custom-synthesized by Shenzhen Chipscreen Biosciences Ltd. (Shenzhen,

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China) and dissolved in dimethyl sulfoxide (DMSO) at the concentration of 25, 50, 125, 250, and 500 nM. Cytarabine was a gift from Pfizer, Ltd. (China) and dissolved in DMSO at the concentration of 25, 50, 125, 250, and 500 nM. All the above stock solutions were stored at -20°C until use.

Cell culture

Two MDS cell lines (SKM-1 and MUTZ-1) and the AML cell line KG-1 were used in our study. SKM-1 was obtained from the Health Science Research Resources Bank in Japan [11]. MUTZ-1, a cell line established from Childhood Myelodysplastic Syndrome with del(5q), was obtained from the German Braunschweig cell center [12]. KG-1 was obtained from the American Type Culture Collection (Rockville, Maryland, USA). All cell lines were cultured in RPMI 1640 medium (Boehringer, Ingelheim, Germany) containing 10% heat-inactivated fetal calf serum (Boehringer), 100 µg/mL penicillin (Gibco), and 100 U/mL streptomycin (Gibco) in a humidified atmosphere (37.5°C and 5% CO₂).

Proliferation assay (CCK-8)

Cell viability was measured by using the tetrazolium salt-based CCK-8 assay (Dojindo Molecular Technologies Inc.). Cells were seeded into 96-well plates at 1.5×10⁵ cells/mL in 200 µL complete medium. Plates were put at 37°C in 5% CO₂, and then 20 µL of CCK-8 reagent was added to the wells and incubated for 1.5 h. The OD was then read at 450 nm within 15 min. This experiment was repeated three times and each sample had three replicates. Cell proliferation was calculated using the following equation:

$$\text{Proliferation (\%)} = \frac{\text{OD}_{450} \text{ of isogarcinol group}}{\text{OD}_{450} \text{ of control group}} \times 100\%$$

HDAC activity analysis

Chidamide (25 and 50 nM) and 50 nM cytarabine were added to the MDS cells and incubated at 37°C for 72 h. HDAC activity was detected as described in the Colorimetric HDAC Activity Assay kit (BioVision). Each reaction (100 µL) contained the nuclear protein (50 µg) extract from SKM-1, MUTZ-1, or KG-1 cells. The HDAC activities were measured with a microplate reader (SpectramaxM5) at 405 nm. The posi-

tive control (i.e., only the nuclear extract and vehicle) was set as 100% and double-distilled water containing 10 µM Trichostatin A, a strong HDACi, was used as a negative control and set as 0%.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of purified total RNA was used for the RT-PCR analysis with the SuperScript First-Strand Synthesis System (Invitrogen). The qRT-PCR was performed using SYBR Premix Ex Taq (Takara Biomedical, Siga, Japan) and the Thermal Cycler Dice Real Time system (Takara Biomedical) in a 96-well plate according to the manufacturer's instructions. Amplification proceeded through 40 cycles of 94°C for 10 s and 56.7°C for 30 s with an extension at 72°C for 30 s. The primers used for RT-PCR were as follows: human HDAC1 F: 5'-ACCGGGCAACGTTACGAAT-3', R: 5'-CTATCAAAGGACACGCCAAGTG-3'; HDAC2F: 5'-TCATTGGAAAATTGACAGCATAGT-3', R: 5'-CATGGTGATGGTGTGAAGAAG-3'; HDAC3 F: 5'-TTGAGTTCTGCTCGCTTACA-3' R: 5'-CCCAGTTAATGGCAATATCACAGAT-3'. Human β-actin was used as a housekeeping gene for quantity normalization, F: 5'-CTGGAACGGTGAAGGTGACA-3', R: 5'-AAGGGACTTCCTGTAACAATGCA-3'. The levels were calculated using the 2^{-ΔΔCt} method [(Ct, target gene Ct, β-actin) sample - (Ct, target gene Ct, β-actin) control] after normalizing the data according to the β-actin mRNA expression [13].

Cell cycle analysis by flow cytometry

Cell cycle analysis was performed with an Accuri C6 (BD Bioscience, Franklin Lakes, USA). Cells were fixed in 70% ethanol for at least 4 h at 4°C, and stained with 20 µg/mL propidium iodide (PI) containing 10 µg/mL RNase A for 30 min at room temperature. The resulting DNA distributions were analyzed by Modifit (Verify Software House Inc., Topsham, ME, USA) for the proportions of cells in the phases of the cell cycle.

Cell apoptosis analysis by flow cytometry

Cell apoptosis [14] was detected by flow cytometry and cells were stained with fluorescein-conjugated annexinV and PI. After washing with

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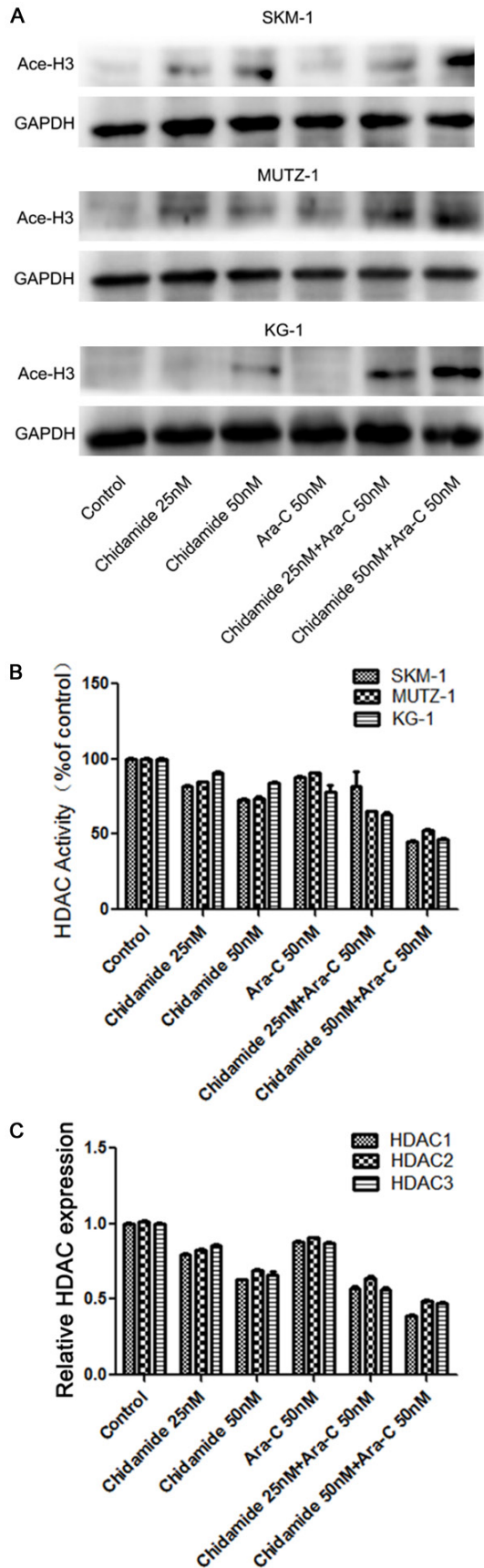


Figure 1. The combination of chidamide with cytarabine enhanced histone H3 acetylation and inhibited HDAC activity in MDS cell lines at 72 h. (A) The combination of chidamide with cytarabine promoted an increase in the level of histone H3 acetylation in human SKM-1, MUTZ-1, and KG-1 cells. (B) The combination of chidamide and cytarabine inhibited HDAC activity in SKM-1, MUTZ-1, and KG-1 cell lines. (C) The combination of chidamide and cytarabine decreased the expression of HDACs in SKM-1, MUTZ-1, and KG-1 cell lines.

phosphate-buffered saline (PBS) two times, cells were resuspended in binding buffer (10 mM N-2-hydroxyl piperazine-N0-2-ehane sulfonic acid/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). One hundred microliters of cell suspension were incubated with 10 μ L annex V-FITC and 10 μ L PI for 20 min at room temperature in the dark. Cells were then detected by flow cytometry within 30 min, and the percentage of apoptotic cells in the total number of cells in each group was compared.

Western blotting

Western blot was used to evaluate the content of acetyl-histone H3, p21, CDK2, cleaved caspase-3, Bcl-2, and Bax in cell extracts after using chidamide (0, 25, and 50 nM), cytarabine (50 nM), and the combination of chidamide with cytarabine (50 nM for both substances) for 72 h. Cells were lysed with a lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 1% NP-40, 1 μ g/mL leupeptin, 1 μ g/mL anti-pain, and 1 mM phenylmethylsulfonyl fluoride), and the protein concentrations were determined with the BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins (30 μ g) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis on an 8% SDS gel, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). PVDF membranes were blocked with a solution containing 5% skim milk and then incubated overnight at 4°C with the following antibodies: anti-acetyl-histone H3 (#9649), anti-p21 (#2947), anti-CDK2 (#2546), anti-cleaved caspase-3 (#9661), anti-Bcl-2 (#15071), anti-Bax (#5023), anti-GAPDH antibody (#5174) (Cell Signaling Technology, Beverly, MA, USA) antibody. After washing with Tris-buffered saline with Tween-20 (TBST), the membranes were incubated for 1 h at room temperature with anti-rabbit IgG

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Table 1. Measurement of IC₅₀ of chidamide and cytarabine in MDS cell lines

Cell lines	Chidamide (nM)			Ara-C (nM)		
	IC ₅₀	m	r	IC ₅₀	m	r
SKM-1	802.812	0.475±0.028	0.983	300.491	0.458±0.021	0.989
MUTZ-1	345.892	0.582±0.021	0.994	161.158	0.631±0.029	0.991
KG-1	301.291	0.731±0.025	0.994	71.803	0.698±0.045	0.979

m indicates the slope of the median-effect plot. *r* indicates the linear correlation coefficient of the median-effect plot. *r* = 1.0 indicates perfect conformity of the dose-effect data with respect to the median-effect principle of the mass-action law. *r* < 1.0 indicates less than a perfect correlation.

sheep antibody or anti-mouse IgG sheep antibody coupled to horseradish peroxidase (Amersham). Reactive proteins were visualized using a chemiluminescence kit (Millipore, Bedford, MA, USA).

Statistical analysis

All results are expressed as means and S.D. of several independent experiments. Pairwise comparison of CCK-8 results was done by the Student's *t*-test. Multiple comparisons of the PCR, HDAC activity, apoptosis, and cell cycle data were made using ANOVA test to determine statistical significance of the detected differences. A value of *P* < 0.05 was considered statistically significant.

Results

Chidamide synergistically enhanced the histone H3 acetylation level and inhibited HDAC activity with cytarabine

To explore whether the combination of chidamide and cytarabine might influence histone H3 acetylation in MDS cells, we investigated the histone H3 acetylation level when cells were exposed to just chidamide or cytarabine alone, and when they were given together. Chidamide and cytarabine together showed a higher acetylation level of histone H3 as compared to when chidamide was given alone (**Figure 1A**). The HDAC activity results indicate that the combination of chidamide with cytarabine caused greater HDAC inhibition as compared with chidamide alone (**Figure 1B**). Furthermore, we measured the expression of HDAC1, HDAC2, and HDAC3, since chidamide mainly suppresses the activity of class I HDACs. Our results showed a decrease in HDAC expression when chidamide with cytarabine were given together as compared to

when chidamide was given alone (**Figure 1C**). Thus, the combination of chidamide (50 nM) and cytarabine (50 nM) lead to higher levels of histone H3 acetylation and an inhibition of HDAC activity as compared to when MDS cells were exposed to chidamide alone.

Chidamide synergistically enhanced the inhibitory effects in MDS cells with cytarabine

The concentration of chidamide in human plasma is 25-250 nM [15], and a low dose of cytarabine (50-500 nM) is widely accepted as a therapy for clinical MDS patients [16]. Considering that the combination of chidamide (50 nM) and cytarabine (50 nM) increased histone H3 acetylation, we next tested their effects in MDS cells. To this end, the IC₅₀ of chidamide and cytarabine in SKM-1, MUTZ-1, and KG-1 cell lines was measured. The IC₅₀ for chidamide varied from 301.29-802.81 nM and the IC₅₀ for cytarabine varied from 71.80-300.49 nM (**Table 1**). To evaluate the effects of chidamide and cytarabine on MDS cell proliferation, we used a fixed ratio (1:1) of chidamide (25, 125, 250, and 500 nM) and cytarabine (25, 125, 250, and 500 nM). The combination of chidamide and cytarabine showed significant anti-proliferative/anti-survival effects on MDS cells as compared with cytarabine or chidamide alone at all four doses (**Figure 2B**). The combination index (CI) values were calculated according to the Chou-Talalay equation [17]. The mean CIs of the combined chidamide and cytarabine group were 0.068, 0.158, and 0.226 in the SKM-1, MUTZ-1, and KG-1 cell lines, respectively (**Table 2**); all of the CIs were lower than 1, which suggests good synergy with both drugs (**Figure 2A**). Thus, chidamide synergistically enhanced cell toxicity with cytarabine in MDS cells.

Combination of chidamide with cytarabine resulted in cell cycle arrest in the G0/G1 phase

Flow cytometry analysis was used to investigate the cell cycle. The results showed the percentage of cells in the G0/G1 phase increased significantly in the combination of chidamide (25 and 50 nM) and cytarabine (50 nM) group as compared to the chidamide alone (25 and

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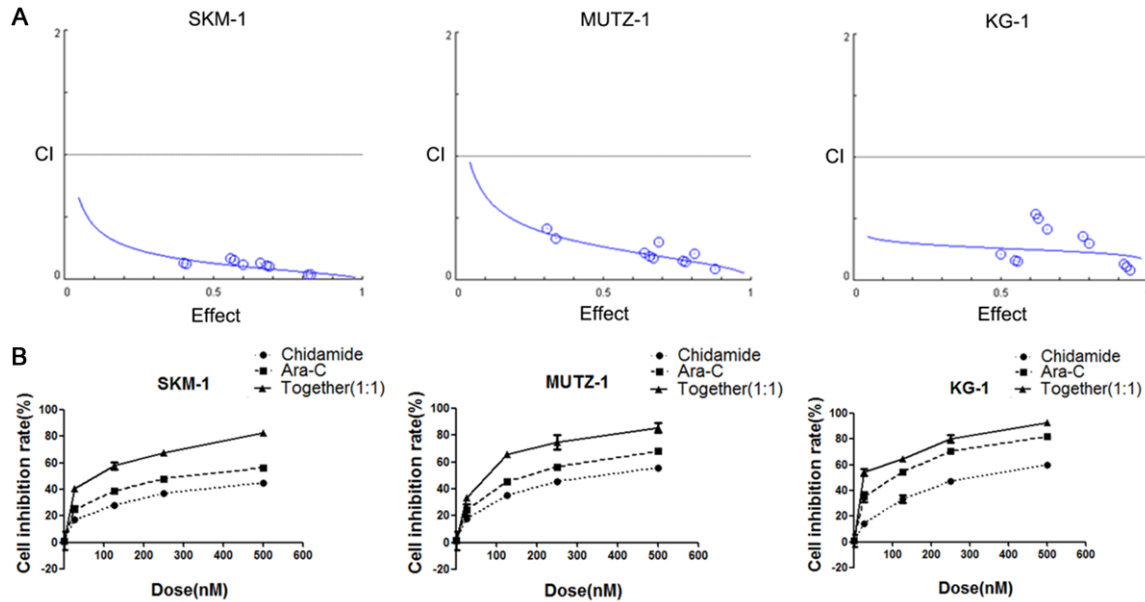


Figure 2. Chidamide and cytarabine synergistically inhibited cell proliferation in MDS cells at 72 h. (A) Combination index (CI) values for chidamide combined with cytarabine in MDS cells were lower than 1. The effects of these combinations were estimated using the CompuSyn software, which was developed based on the Clequation of Chou-Talalay. $CI < 1$ indicates synergism; $CI = 1$ indicates additive effect in the absence of synergism or antagonism; $CI > 1$ indicates antagonism. (B) The combination of chidamide and cytarabine inhibited cell proliferation. Cell inhibition rate (%) = $(1 - OD_{450}$ of isogarcinol group / OD_{450} of control group) $\times 100\%$.

Table 2. Combination index value of the combination of chidamide and cytarabine in MDS cell lines

Cell lines	Combination index				
	ED50	ED75	ED90	ED95	Mean
SKM-1	0.131	0.072	0.041	0.027	0.068
MUTZ-1	0.271	0.171	0.109	0.081	0.158
KG-1	0.262	0.234	0.211	0.195	0.226

Combination index values < 1.0 are consistent with synergism, and the lower the value, the greater the synergism.

50 nM) group at 72 h ($P < 0.05$; **Figure 3A** and **3B**). Western blot analysis indicated that CDK2 expression was down-regulated and p21 was up-regulated after treatment with both drugs, which was consistent with the cell-cycle analysis (**Figure 3C**). The decreased level of CDK2 and increased level of p21 suggests a possible mechanism by which cell-cycle arrest is occurring in the G₀/G₁ phase after treatment with both chidamide and cytarabine.

Combination of chidamide and cytarabine induces apoptosis in MDS cells

To further investigate the mechanism by which cell proliferation is arrested in MDS cells treat-

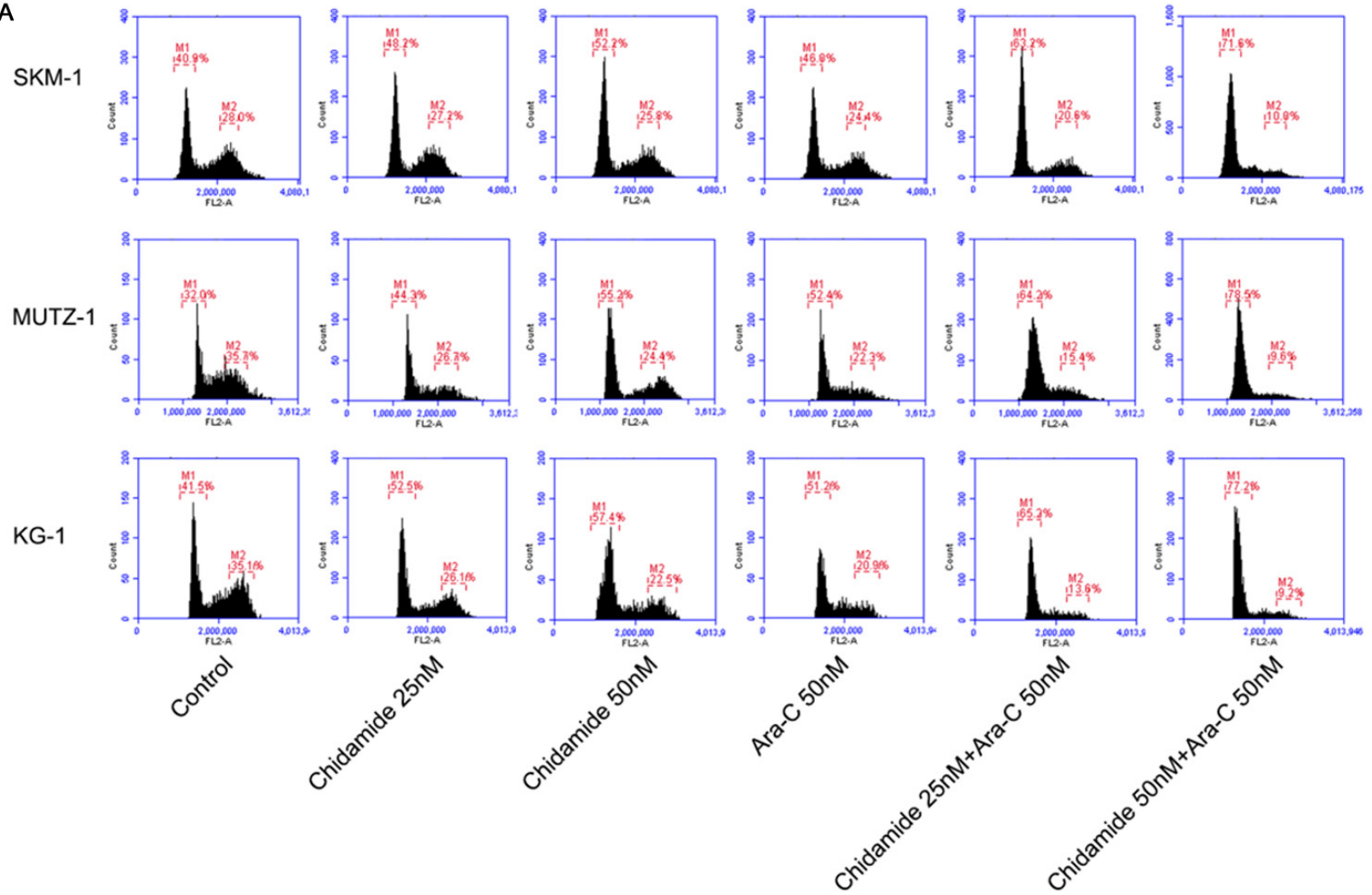
ed with chidamide with cytarabine, cell apoptosis was tested by flow cytometry. Our results indicated that the number of apoptotic cells significantly increased when cells were treated with a combination of chidamide (25 and 50 nM) with cytarabine (50 nM) as compared to chidamide (25 and 50 nM) or cytarabine (50 nM) alone for 72 h ($P < 0.05$; **Figure 4A** and **4B**). Furthermore, the expression of Bcl-2 was down-regulated, and cleaved caspase-3 and Bax were up-regulated when cells were exposed to both compounds (**Figure 3C**).

Discussion

Our previous study found that chidamide induces cell cycle arrest and apoptosis in MDS and another study demonstrated that chidamide inhibits the viability of MDS and AML cells [9, 18]. Indeed, chidamide is a potential therapeutic agent in MDS patients. The deoxycytidine analogue 1-h-D-arabinofuranosylcytosine (cytarabine, ara-C) is the most commonly used drug in the treatment of AML [19]. Moreover, use of low dose of cytarabine is beneficial for elderly patients with high-risk MDS [16]. Ara-C exerts its cytotoxicity by inhibiting DNA polymerase or by terminating chain elongation upon being incorporated into newly synthesized DNA [20,

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A



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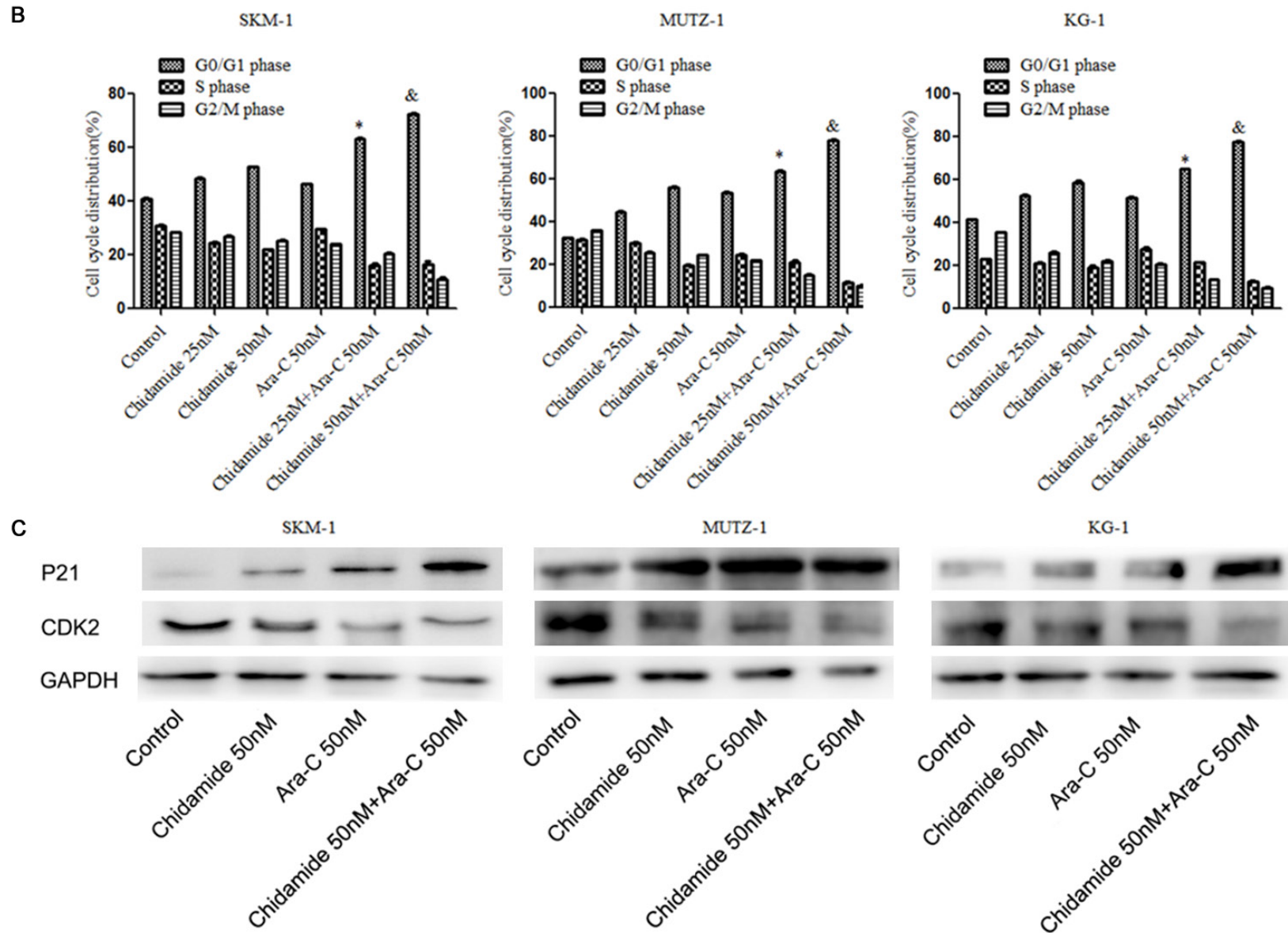
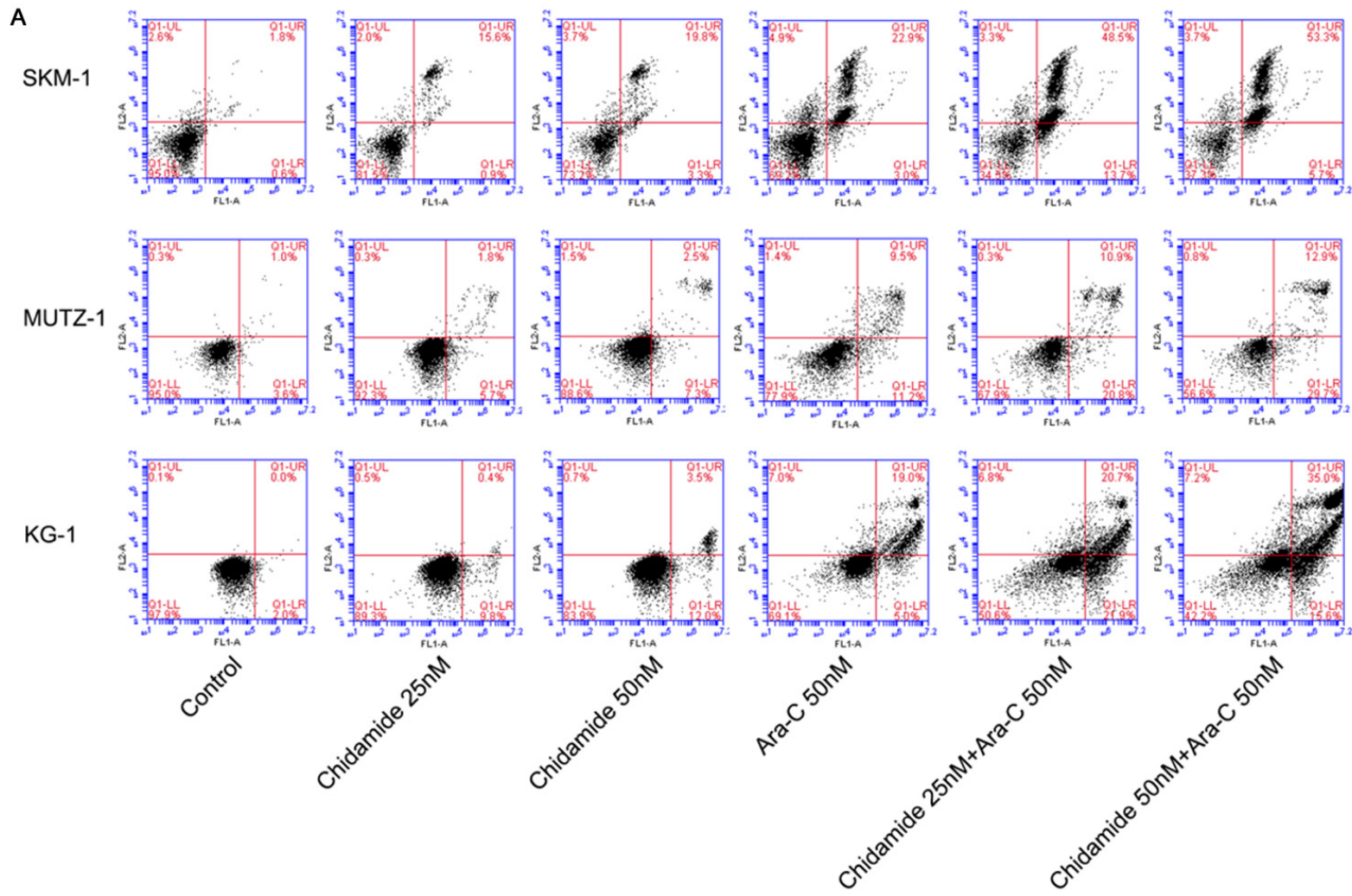


Figure 3. The combination of chidamide and cytarabine arrested the cell cycle in the G0/G1 phase. (A, B) The combination of chidamide and cytarabine induced cell cycle arrest at the G0/G1 phase. (C) Western blot analysis showed the expression of cell-cycle protein p21 was increased and CDK2 was down-regulated. * indicates a significant difference as compared with 25 nM chidamide. & indicates a significant difference as compared with 50 nM chidamide.

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Anti-MDS cell activity of chidamide and cytarabine

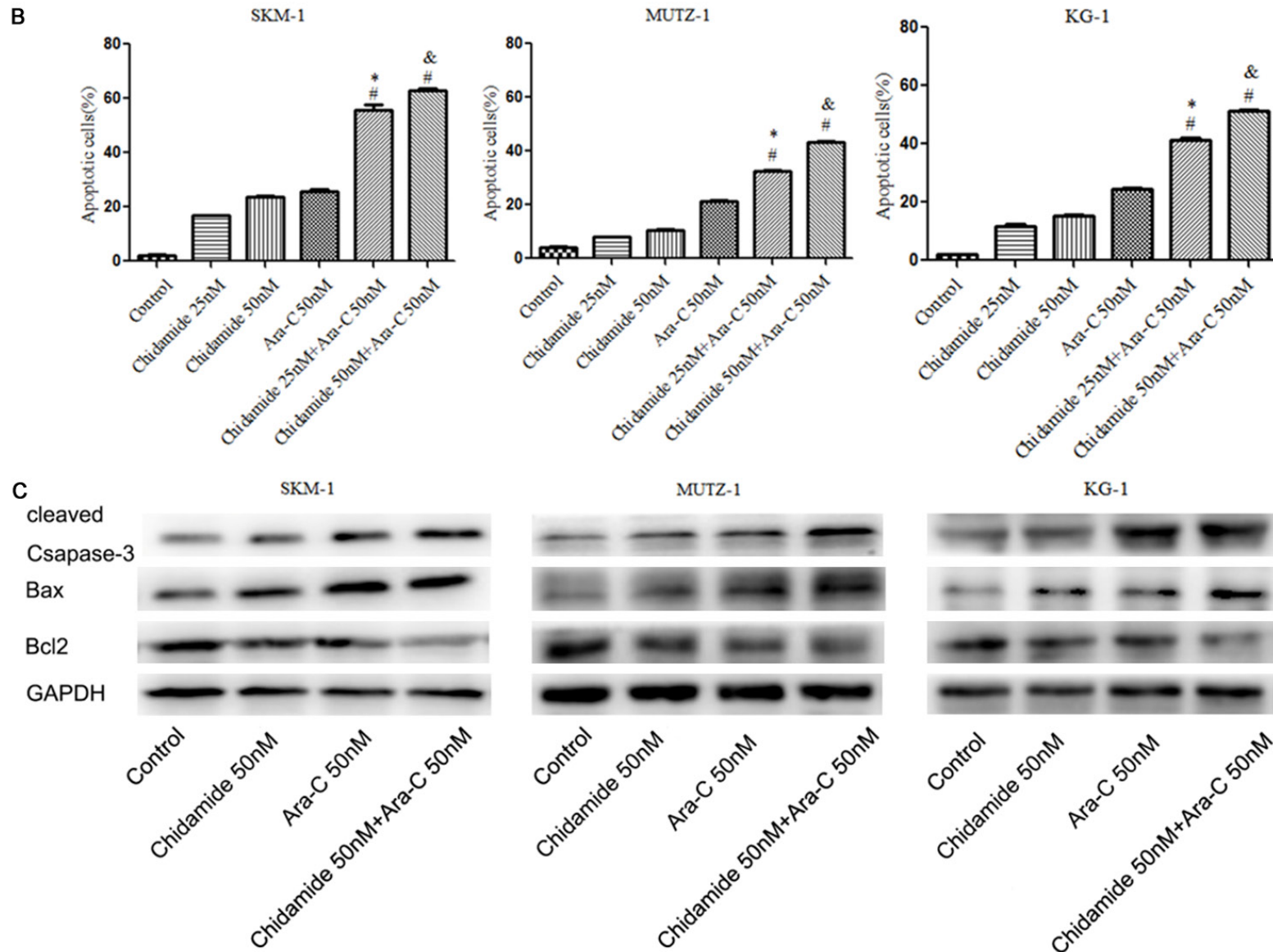


Figure 4. The combination of chidamide and cytarabine induced cell apoptosis in MDS cells at 72 h. (A, B) The combination of chidamide and cytarabine induced cell apoptosis. (C) Western blot analysis showed the expression of the cell apoptosis protein cleaved caspase-3 was up-regulated, but Bcl-2 and Bax were down-regulated following treatment with both agents. * indicates a significant difference as compared with 25 nM chidamide. & indicates a significant difference as compared with 50 nM chidamide. # indicates a significant difference as compared with 50 nM cytarabine.

21]. During a demethylation therapy, chidamide increases the acetylation level of histone H3 and inhibits HDAC activity. Both chidamide and ara-C are effective in treating myeloid leukemia through different mechanisms, thus triggering interest in combining them as a therapeutic treatment for MDS. However, there is little known regarding the effects of their combination on MDS cells.

Our current study showed that chidamide and cytarabine synergistically enhanced histone H3 acetylation and inhibited HDAC activity in MDS cells. The effect was significant in the group that received 50 nM of both chidamide and cytarabine. Therefore, our results indicate that the fixed ratio (1:1) of chidamide to cytarabine is more efficient in killing MDS cells. Moreover, our CCK-8 results further support our hypothesis regarding the synergistic effect of both compounds on MDS cell proliferation. These results provide some clues as to the potential application of the combination of chidamide and cytarabine in MDS patients. However, the mechanism by which these chemicals inhibit MDS cell proliferation and survival needs to be clarified.

HDACs induce cell cycle arrest in both normal cells and cancer cells at low concentration [22, 23]. Chidamide induces G1 arrest and ROS-dependent apoptosis in human leukemia cells [24].

Our results showed that the expression of CDK2, which is a positive regulator of the cell cycle, [25] was down-regulated and the expression of p21, which is a negative regulator of the cell cycle, was up-regulated in the chidamide/cytarabine combination group. All of these modulations resulted in cell growth arresting at the G0/G1 phase. Furthermore, several critical apoptotic regulators were modulated by the administration of chidamide and cytarabine. Specifically, we observed a down-regulation of Bcl-2 (a negatively regulator of cell apoptosis) [26, 27], and an up-regulation of cleaved caspase-3 and Bax (a positively regulator of cell apoptosis) [28, 29] expression with the combination of chidamide and cytarabine treatment in MDS cell lines. All of these modulations resulted in cell apoptosis.

In summary, we provide compelling evidence that a non-toxic dose of chidamide and cytarabine synergistically induced cell cycle arrest

and apoptosis in MDS cells. This is an interesting clinical therapy strategy for MDS, which combines epigenetic and traditional chemical drugs at safe concentrations. Our data suggest that chidamide in combination with cytarabine at low concentration may be a promising treatment for the patients with MDS. Future studies will likely need to focus on the *in vivo* efficacy of such a treatment regimen.

Acknowledgements

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Disclosure of conflict of interest

None.

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