Chidamide induces necroptosis via regulation of c-FLIP$_L$ expression in Jurkat and HUT-78 cells

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Abstract. T-cell acute lymphoblastic leukemia (T-ALL) is a hematopoietic malignancy, which is associated with a poor prognosis. It is difficult to achieve complete remission or long-term survival with conventional chemotherapy, partly due to decreased apoptosis. However, necroptosis can serve as an alternative pathway to induce cell death. The present study investigated whether the selective histone deacetylase (HDAC) inhibitor chidamide exerted a therapeutic effect on T-ALL and explored the underlying mechanism. The results revealed that HDAC expression was increased in Jurkat and HUT-78 cells treated compared with the control cell line (H9), and was accompanied by elevated cellular Fas-associated death domain-like interleukin-1β converting enzyme inhibitory protein long form (c-FLIP$_L$) levels. Chidamide treatment (2 μmol/l) also induced mitochondrial dysfunction, necroptosis and apoptosis in T-ALL cells *in vitro*. Furthermore, necroptosis was increased when apoptosis was blocked in T-ALL cells. Additionally, chidamide (2 μmol/l) downregulated c-FLIP$_L$, HDAC1 and HDAC3 expression, and increased receptor-interacting protein kinase 3 expression and the phosphorylation of mixed lineage kinase domain-like pseudokinase in Jurkat and HUT-78 cells. The results obtained in the present study revealed that chidamide may induce necroptosis via regulation of c-FLIP$_L$ expression when apoptosis is inhibited in Jurkat and HUT-78 cells.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is a type of malignant hematopoietic tumor caused by malignant transformation of T lymphocytes. T-ALL has a poor prognosis and it is difficult to achieve complete remission after recurrence through chemotherapy alone (1). Therefore, there is a requirement for novel therapeutic agents. Abnormal proliferation, disordered differentiation and inhibition of apoptosis are implicated in the pathogenesis and recurrence of leukemia. Cellular Fas-associated death domain-like interleukin-1β converting enzyme inhibitory protein long form (c-FLIP$_L$) is an important anti-apoptotic protein, which has been reported as an important factor affecting apoptosis sensitivity and has been demonstrated to exert a significant inhibitory effect on apoptosis (2). A previous study revealed that c-FLIP$_L$ is highly expressed in leukemia (3). It has been reported that c-FLIP$_L$ is highly expressed in bone marrow mononuclear cells obtained from patients with T-ALL and is associated with the malignancy and prognosis of T-ALL.

Histone deacetylase (HDAC) inhibitors (HDACis) comprise an important class of epigenetic drugs with high efficacy and low toxicity, which are becoming valuable antitumor drugs. It has been reported that HDACis successfully inhibit proliferation and increase apoptosis in various malignant tumor cells by decreasing c-FLIP$_L$ transcription and translation (4). Chidamide is a novel HDACi that has been independently researched and developed in China, which has been approved by the US Food and Drug Administration. Zheng *et al* (5) reported that patients with peripheral T-cell lymphoma exhibited increased c-FLIP$_L$ expression, and that HDACis inhibited the expression of c-FLIP$_L$ and increased apoptosis in T-cell lymphoma (5).

Necroptosis is a novel type of cell death that exhibits characteristics of apoptosis and necrosis, and is regulated by a series of signaling molecules independent of caspases (6). Furthermore, necroptosis is inhibited by the specific inhibitor necrostatin-1 (Nec-1) (7). Necroptosis has attracted increasing attention due to its close association with inflammation, ischemia-reperfusion injury, and tumor occurrence and development (6,8). In general, activation of death receptors induces apoptosis; however, cytokine-mediated apoptosis can also initiate necroptosis if apoptosis is inhibited or blocked (9). Certain leukemia cell lines (such as Jurkat cells) have congenital caspase function deficiency; hence, necroptosis is more likely to occur in these cell lines (10). The present study investigated the necroptosis-inducing effects of chidamide on Jurkat and HUT-78 T-cell leukemia cells and explored the underlying mechanism.

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Materials and methods

Cell culture. Jurkat, HUT-78 and H9 cells (Shanghai Cell Bank of Chinese Academy of Sciences) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc.), 100 units penicillin and 100 mg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2.

Reverse-transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from Jurkat, HUT-78 and H9 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using a Super MMLV RT kit (Takara Bio, Inc.), according to the manufacturer's instructions. The reaction was carried out at 37˚C for 15 min, 85˚C for 5 sec and then terminated at 4˚C. For qPCR, the reaction solution was prepared on ice in five replicate wells per cell type. The 20-μl reaction mixture consisted of 10 μl 2X SYBR Premix Ex Taq II (Takara Bio, Inc.), 0.8 μl forward primer (10 μmol/l), 0.8 μl reverse primer (10 μmol/l), 0.4 μl ROX Reference Dye or Dye II (50X), 6 μl cDNA solution and 6 μl dH_2O. The primer sequences were as follows: c-FLIP$_L$, forward, 5'-GCGAAGCCACCTGTTGAT G-3' and reverse, 5'-CTGCTCCGCAAAACCAGAA-3'; and β-actin, forward, 5'-TGGCACCCAGCACAATGAA-3' and reverse, 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'. The amplification conditions were as follows: Pre-denaturation at 95˚C for 30 sec, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 34 sec. The mRNA expression levels of c-FLIP$_L$ were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference gene β-actin (11).

Western blotting. Cells were harvested and lysed in lysis buffer (Beyotime Institute of Biotechnology). Lysates were centrifuged at 16,099.2 x g for 1 min at 4˚C and the supernatants were collected. Total cellular protein was extracted by using lysis buffer (Wanleibio) and the protein concentration was determined by the bicinchoninic acid assay. In total, 20 μl protein extracts were separated by 10 or 12% polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (EMD Millipore). The membranes were blocked in 5% skim milk solution and incubated with primary antibodies against HDAC1 (ABclonal, Inc.; cat. no. A0238; 1:500), HDAC3 (ABclonal, Inc.; cat. no. A2603; 1:500), c-FLIP$_L$ (ProteinTech Group, Inc.; cat. no. 10394-1-AP; 1:800), mixed lineage kinase domain-like pseudokinase (MLKL) (Cell Signaling Technology, Inc.; cat. no. 14993S; 1:1,000), phosphorylated (p)-MLKL (Cell Signaling Technology, Inc.; cat. no. 91689S; 1:1,000), receptor-interacting protein kinase (RIP) 3 (BD Bioscience; cat. no. 610458; 1:1,500), caspase-3 (ABclonal Inc.; cat. no. A11021; 1:500), caspase-8 (ProteinTech Group, Inc.; cat. no. 13423-1-AP; 1:1,500), RIP1 (ProteinTech Group, Inc.; cat. no. 17519-1-AP; 1:1,000), cyclin-dependent kinase inhibitor 1A (p21; Wanleibio; cat. no. WL0362; 1:400), proliferating cell nuclear antigen (PCNA; Wanleibio; cat. no. WL02208; 1:500), β-actin (Wanleibio; cat. no. WL01845; 1:5,000) and GAPDH (ProteinTech Group, Inc.; cat. no. 60004-1-Ig; 1:10,000) at 4˚C overnight. Following primary antibody incubation, the membrane was incubated with a secondary antibody (Wanleibio; cat. no. WLA023; 1:5,000) 37˚C for 45 min. Protein bands were visualized using enhanced chemiluminescence (Wanleibio; cat. no. WLA003).

Cell treatment. Jurkat and HUT-78 cells were divided into five groups: i) Control group, untreated cells cultured at 37˚C; ii) chidamide-treated group [cells were treated with 2 μmol/l chidamide (Sigma-Aldrich; Merck KGaA) at 37˚C for 24 h]; iii) Z-VAD-FMK-treated group [following pretreatment with 50 μmol/l Z-VAD-FMK (Abcam) for 30 min, cells were treated with 2 μmol/l chidamide at 37˚C for 24 h]; iv) Nec-1-treated group [following pretreatment with 20 μmol/l Nec-1 (Abcam) for 1 h, cells were treated with 2 μmol/l chidamide at 37˚C for 24 h]; and v) Z-VAD-FMK and Nec-1-treated group (following pretreatment with 50 μmol/l Z-VAD-FMK for 30 min and 20 μmol/l Nec-1 for 1 h sequentially, cells were treated with 2 μmol/l chidamide at 37˚C for 24 h).

Apoptosis analysis. Annexin V-FITC/propidium iodide (PI) staining kit (BD Biosciences) was used to detect apoptosis in Jurkat and HUT-78 cells. Cells were collected by centrifugation at 71.552 x g for 5 min at 4˚C. The supernatant was removed and the cells were resuspended in 500 μl PBS. The cells were then incubated with 5 μl Annexin V-FITC and PI staining reagent in the dark for 15 min at room temperature. Living cells are negative to Annexin V-FITC and PI (Annexin V$^-$PI$^-$), early apoptotic cells are positive to Annexin V-FITC and negative to PI (Annexin V$^+$PI$^-$), late apoptotic cells and necrotic cells are double positive to Annexin V-FITC and PI (Annexin V$^+$PI$^+$). Necroptosis and apoptosis were subsequently analyzed using a flow cytometer (NovoCyte; ACEA Biosciences, Inc.) and quantified by NovoExpress 1.2.5 (ACEA Biosciences, Inc.).

Transmission electron microscopy. Jurkat and HUT-78 cells were fixed using 2.5% glutaraldehyde (Servicebio) at 4˚C for 2-4 h, dehydrated in a graded series of ethanol, embedded in epoxy resin and sectioned into ultra-thin sections (60-80 nm). The sections were subsequently stained with 2% uranyl acetate and lead citrate at room temperature for 15 min. Finally, features of necroptosis were visualized using transmission electron microscopy (JEOL; cat. no. JEM-1400) and analyzed by JEOL TEM Center Ver.1.7.15 (JEOL, Ltd.).

Mitochondrial function assay. JC-1 staining solution (Beyotime Institute of Biotechnology) was used to determine the mitochondrial membrane potential of treated Jurkat and HUT-78 cells, according to the manufacturer's protocol. The change in mitochondrial membrane potential was analyzed using a flow cytometer (NovoCyte) and quantified by NovoExpress (Novoexpress 1.2.5). In order to assess the production of reactive oxygen species (ROS), Jurkat and HUT-78 cells were treated with DCFH-DA solution at 37˚C for 20 min according to the manufacturer's instructions (KeyGEN BioTECH) and were subsequently quantified using a flow cytometer (NovoCyte) and quantified by NovoExpress (Novoexpress 1.2.5).

Patient samples. A total of 75 patients with T-ALL (46 men and 29 women; mean age, 20 years) were recruited from Shengjing Hospital of China Medical University (Shenyang,

China) between January 2014 and December 2015. A total of 30 healthy individuals (19 men and 11 women; mean age, 29 years) diagnosed with anemia or lymphadenectasis served as the control group. Blood samples were collected from the patients and controls. Blood sample collection was conducted after the participants provided written informed consent. Blood samples were analyzed using an automatic blood analysis pipe-line (XN-9000; SYSMEX Corporation). If the participants were <18 years old, written informed consent was obtained from their parents/guardians. The present study was in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Shengjing Hospital of China Medical University.

Statistical analysis. Data are expressed as the mean ± SD. SPSS software (version 17; SPSS, Inc.) was used to perform statistical tests. All experiments were repeated three times (n=3). Two sample groups were compared using an unpaired Student's t-test. Data from three or more groups were analyzed by one-way analysis of variance followed by Tukey's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

c-FLIP$_L$ is highly expressed in Jurkat and HUT-78 cells. The mRNA expression levels of c-FLIP$_L$ were significantly increased in Jurkat (4.94±0.14; n=5) and HUT-78 cells (4.15±0.17; n=5) compared with in human T lymphocyte H9 cells (0.39±0.03; n=5; Fig. 1; P<0.05).

Chidamide inhibits histone deacetylation in Jurkat and HUT-78 cell lines. As shown in Fig. 2A, the expression levels of HDAC1 and HDAC3 were increased in Jurkat and HUT-78 cells, indicating that HDAC levels were elevated in T-cell leukemia cell lines. Treatment with 2 μmol/l chidamide mark-edly reduced the expression levels of HDAC1 and HDAC3 in Jurkat and HUT-78 cells (Fig. 2B). Additionally, p21 and PCNA levels were increased and decreased, respectively, in 2 μmol/l chidamide-treated Jurkat and HUT-78 cells (Fig. 2C). These data indicated that chidamide inhibited histone deacetylation and regulated cell cycle arrest, as p21 and PCNA are involved in regulation of the cell cycle, in T-cell leukemia cell lines.

Chidamide induces necroptosis and apoptosis in Jurkat and HUT-78 cell lines. According to the fluorescence intensities of Annexin V and PI staining, Annexin V$^+$/PI$^+$ cells were defined as late apoptotic and necroptotic cells, whereas Annexin V$^+$/PI$^-$ cells were categorized as early apoptotic cells (12). As shown in Fig. 3A, cell death was markedly increased in Jurkat cells compared with in control cells following treatment with 2 μmol/l chidamide for 24 h (early apoptotic cells, 62.13%; necroptotic and late apoptotic cells, 26.87%); the proportion of early apoptotic cells was higher. Pretreatment of cells with 50 μmol/l Z-VAD-FMK (pan-caspase inhibitor) for 30 min followed by the addition of chidamide (2 μmol/l) significantly increased necroptosis and late apoptosis (93.39%). The proportion of necroptotic and late apoptotic cells induced by chidamide (2 μmol/l) was significantly reduced (1.71%) following Nec-1 treatment (20 μmol/l) for 1 h. Compared with the control group, Chidamide-induced cell death was not

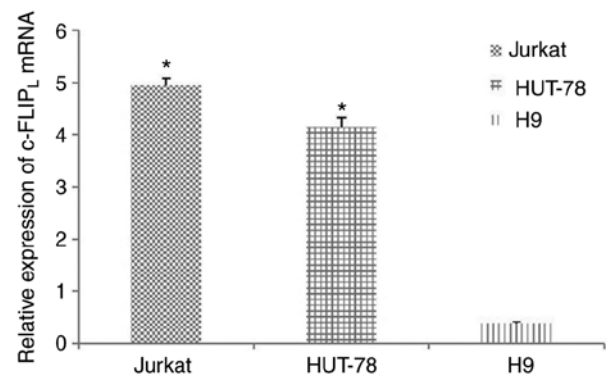Figure 1. c-FLIP$_L$ mRNA is highly expressed in Jurkat and HUT-78 cells. Data are presented as the mean ± SD. *P<0.05 vs. the control group. c-FLIP$_L$, cellular Fas-associated death domain-like interleukin-1β converting enzyme inhibitory protein long form.

significantly different following treatment with Z-VAD-FMK and Nec-1. HUT-78 cell death was also significantly increased following treatment with 2 μmol/l chidamide, and a high proportion of early apoptotic cells was observed compared with the control group (early apoptotic cells, 67.57%; necrop-totic and late apoptotic cells, 21.33%; Fig. 3B). Pretreatment with Z-VAD-FMK (50 μmol/l) significantly increased the proportion of necroptotic and late apoptotic cells (62.95%). However, Nec-1 treatment (20 μmol/l) significantly reduced the proportion of necroptotic and late apoptotic cells (0.14%). Following pretreatment with Z-VAD-FMK (50 μmol/l) and Nec-1 (20 μmol/l), chidamide-induced necroptosis/late apoptosis and early apoptosis was reduced to 0.13 and 0.11%, respectively. As shown in Fig. 3C and D, membranolysis, mito-chondrial swelling and organelle disappearance were observed in 2 μmol/l chidamide-treated cells. In addition, representa-tive apoptotic and necroptotic features could be reversed by Z-VAD-FMK (50 μmol/l) and Nec-1 (20 μmol/l) pretreatment. These results suggested that chidamide induced necroptosis in Jurkat and HUT-78 cell lines.

Chidamide aggravates mitochondrial dysfunction in Jurkat and HUT-78 cells. The effects of chidamide on mitochondrial function in Jurkat and HUT-78 cells were investigated. JC-1 fluorescent dye was used to detect alterations in mitochon-drial membrane potential following chidamide treatment (2 μmol/l). Chidamide induced mitochondrial dysfunction, which could be alleviated by single or combined pretreat-ment with Z-VAD-FMK (50 μmol/l) and Nec-1 (20 μmol/l) (Fig. 4A and B; P<0.05). ROS generation was also analyzed, and the results were consistent with the results of mitochon-drial function detection mentioned above (Fig. 4C and D; P<0.05). The present results suggested that chidamide induced mitochondrial dysfunction in Jurkat and HUT-78 cells.

Chidamide downregulates c-FLIP$_L$ expression in Jurkat and HUT-78 cells. The mRNA expression levels of c-FLIP$_L$ were significantly decreased in Jurkat and HUT-78 cells following chidamide treatment (2 μmol/l; Fig. 5A; P<0.05). In addi-tion, the protein expression levels of c-FLIP$_L$ were decreased in Jurkat and HUT-78 cell lines following treatment with chidamide (2 μmol/l; Fig. 5B), indicating that chidamide
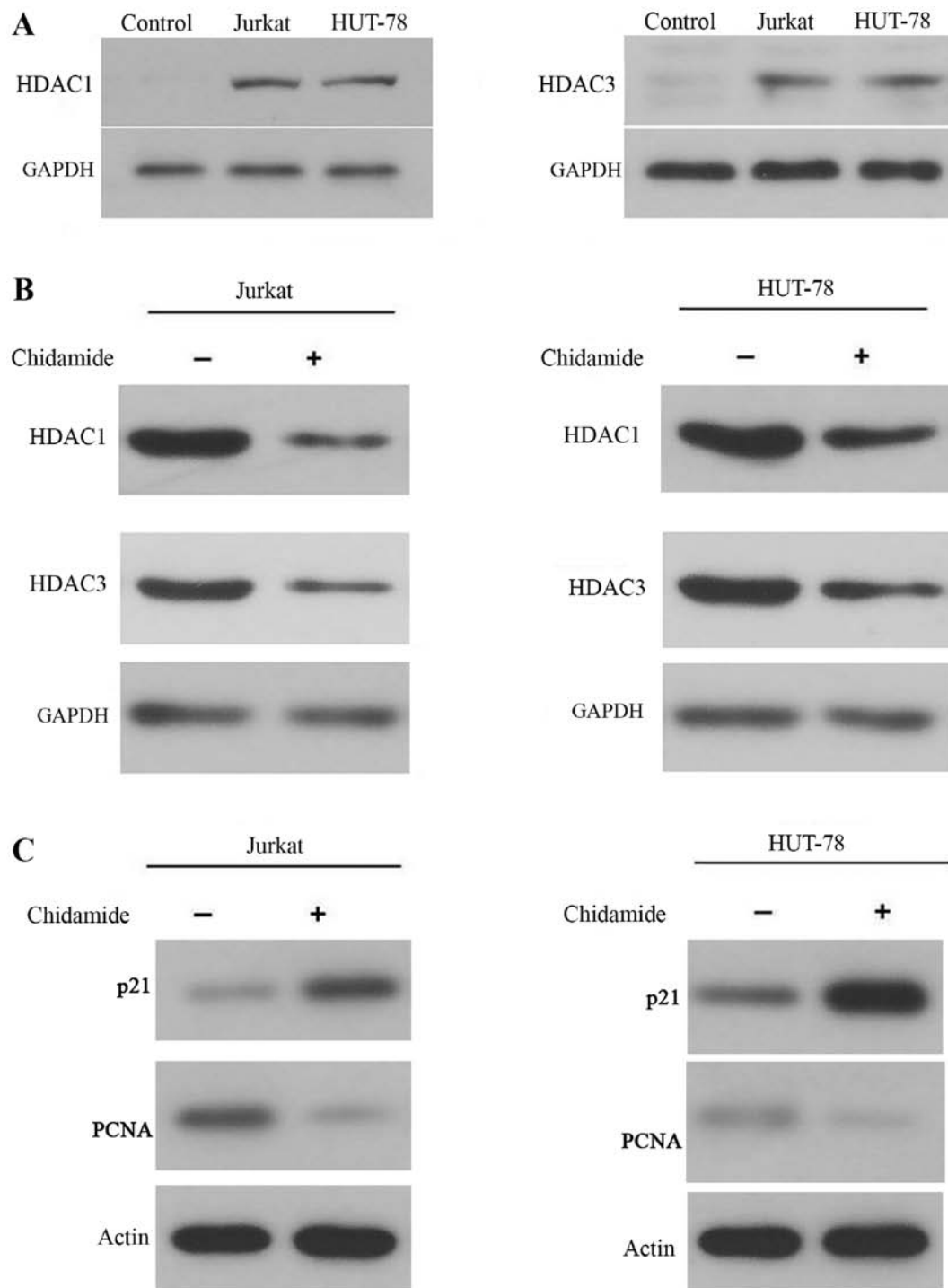
Figure 2. Chidamide inhibits histone deacetylation and regulates cell cycle-associated proteins in Jurkat and HUT-78 cell lines. (A) High protein expression of HDAC1 and HDAC3 in Jurkat and HUT-78 cell lines was observed. (B) Protein expression levels of HDAC1 and HDAC3 were markedly decreased following treatment with chidamide. (C) Chidamide upregulated p21 and downregulated PCNA expression in Jurkat and HUT-78 cell lines. HDAC, histone deacetylase; p21, cyclin-dependent kinase inhibitor 1A; PCNA, proliferating cell nuclear antigen.

downregulated c-FLIP$_L$ mRNA and protein expression in Jurkat and HUT-78 cells.

Effects of chidamide on caspase activation and RIP3/MLKL signaling. Chidamide induced apoptosis of Jurkat and HUT-78 cells. The expression levels of caspase-3 and caspase-8 were markedly reduced following Z-VAD-FMK pretreatment (50 μmol/l), indicating that chidamide-induced apoptosis was inhibited by caspase inhibition. Z-VAD-FMK pretreatment

(50 μmol/l) also increased the expression of the key necroptosis proteins RIP1 and RIP3, and enhanced phosphorylation of MLKL (Fig. 6). These results indicated that chidamide induced necroptosis via the RIP3/MLKL signaling pathway when apoptosis was inhibited.

High levels of c-FLIP$_L$ predict poor prognosis in patients with T-ALL. As shown in Table I, the mRNA expression levels of c-FLIP$_L$ were not associated with a specific age group or sex.
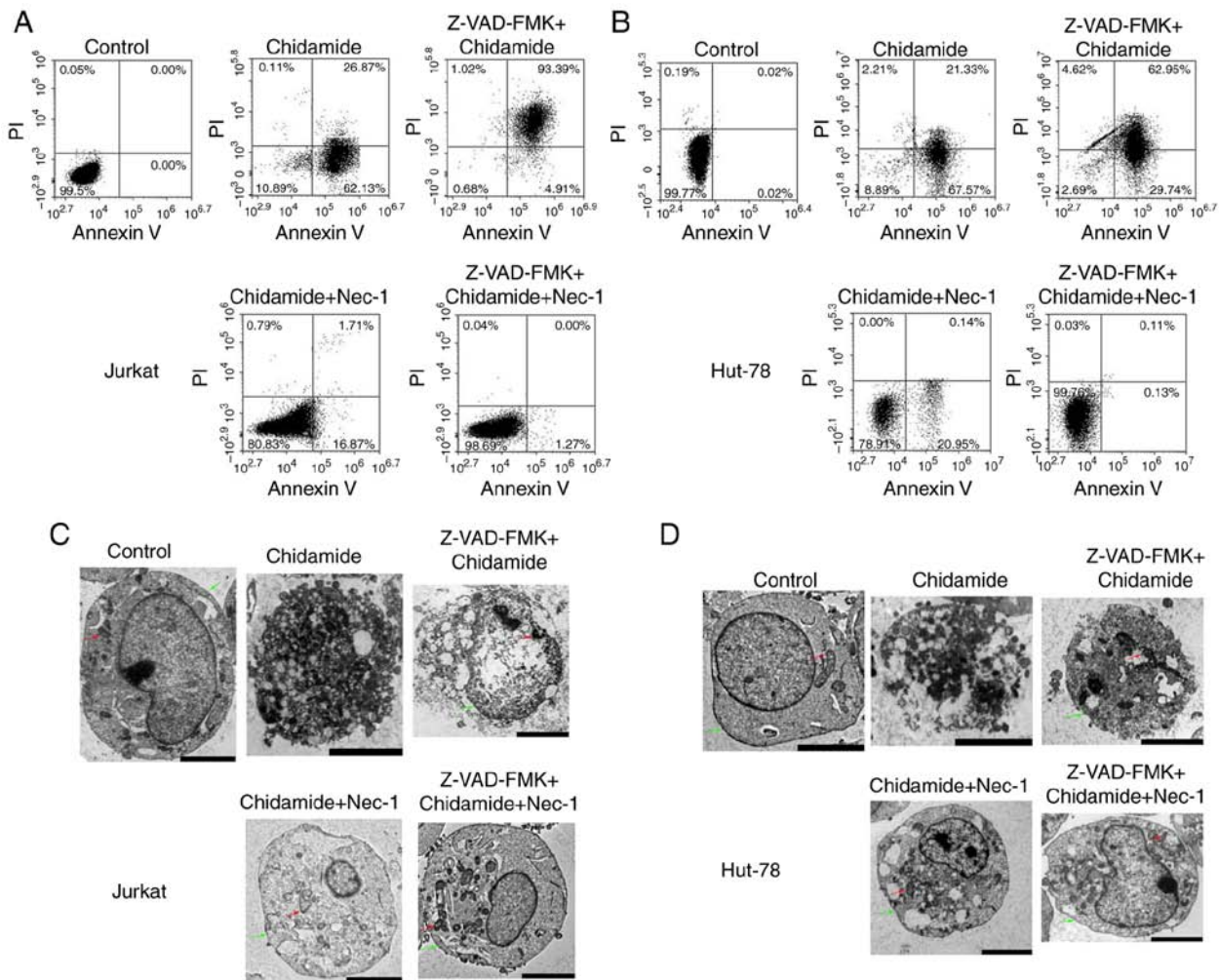
Figure 3. Chidamide induces necroptosis and apoptosis of Jurkat and HUT-78 cell lines. Detection of necroptosis and apoptosis by flow cytometry in (A) Jurkat and (B) HUT-78 cells. Transmission electron microscopy of (C) Jurkat and (D) HUT-78 cells at x1,000 magnification. Green arrows indicate the cell membrane; red arrows indicate the mitochondria. Nec-1, necrostatin 1.

However, according to the National Comprehensive Cancer Network Guidelines for diagnosing and treating pediatric acute lymphoblastic leukemia (13), high c-FLIP$_L$ mRNA expression was closely associated with dangerous degree, complete remission rate, levels of hydroxybutyrate and lactate dehydrogenase, leukocyte counts, CD45 expression and silver (mouse homolog) like-TAL bHLH transcription factor 1 erythroid differentiation factor gene fusion.

Discussion

Aberrant apoptosis and proliferation are important mechanisms underlying the pathogenesis and recurrence of T-ALL. The majority of chemotherapeutic drugs used in leukemia induce apoptosis of cancer cells, which are initially sensitive to apoptosis inducers but may develop resistance following prolonged treatment. Additionally, hyper-malignant T-ALL cells demonstrate primary drug resistance. A previous study has revealed that multidrug resistance in leukemia cells caused by P-glycoprotein, multidrug resistance associated protein 1, ATP binding cassette subfamily G member 2 (Junior blood group), BCL2 apoptosis regulator and BCL2 like 1 may be overcome by inducing necroptosis (14).

c-FLIP$_L$ is an apoptosis-inhibiting protein widely found in viruses, eukaryotes and mammals, which was first identified in malignant melanoma by Irmler et al (15) in 1997. Similar to the function of pro-caspase-8, c-FLIP binds to Fas-associated protein with death domain and competes with caspase-8 for a binding site on the death-inducing signaling complex. This leads to incomplete activation of caspase-8, thereby inhibiting apoptosis mediated by death receptors, such as Fas cell surface death receptor, tumor necrosis factor-related apoptosis-inducing ligand receptor and tumor necrosis factor receptor 1. A recent study has demonstrated that c-FLIP$_L$ expression is significantly increased in certain solid tumors and is associated with advanced disease progression (16). c-FLIP$_L$ expression decreases during disease remission, but remains high in drug-resistant tumor cells not undergoing apoptosis. However, c-FLIP$_L$ expression is downregulated by c-FLIP$_L$ inhibitors, which in turn induce apoptosis (17). High c-FLIP$_L$ expression is associated with a poor prognosis in patients with leukemia (18), which is consistent with a previous clinical investigation (4). Therefore, it is important to investigate gene-targeting drugs that can specifically modulate the expression levels of c-FLIP$_L$. The results of the current study revealed that Jurkat and HUT-78 cells expressed increased levels of c-FLIP$_L$ compared with control cells. Therefore, the
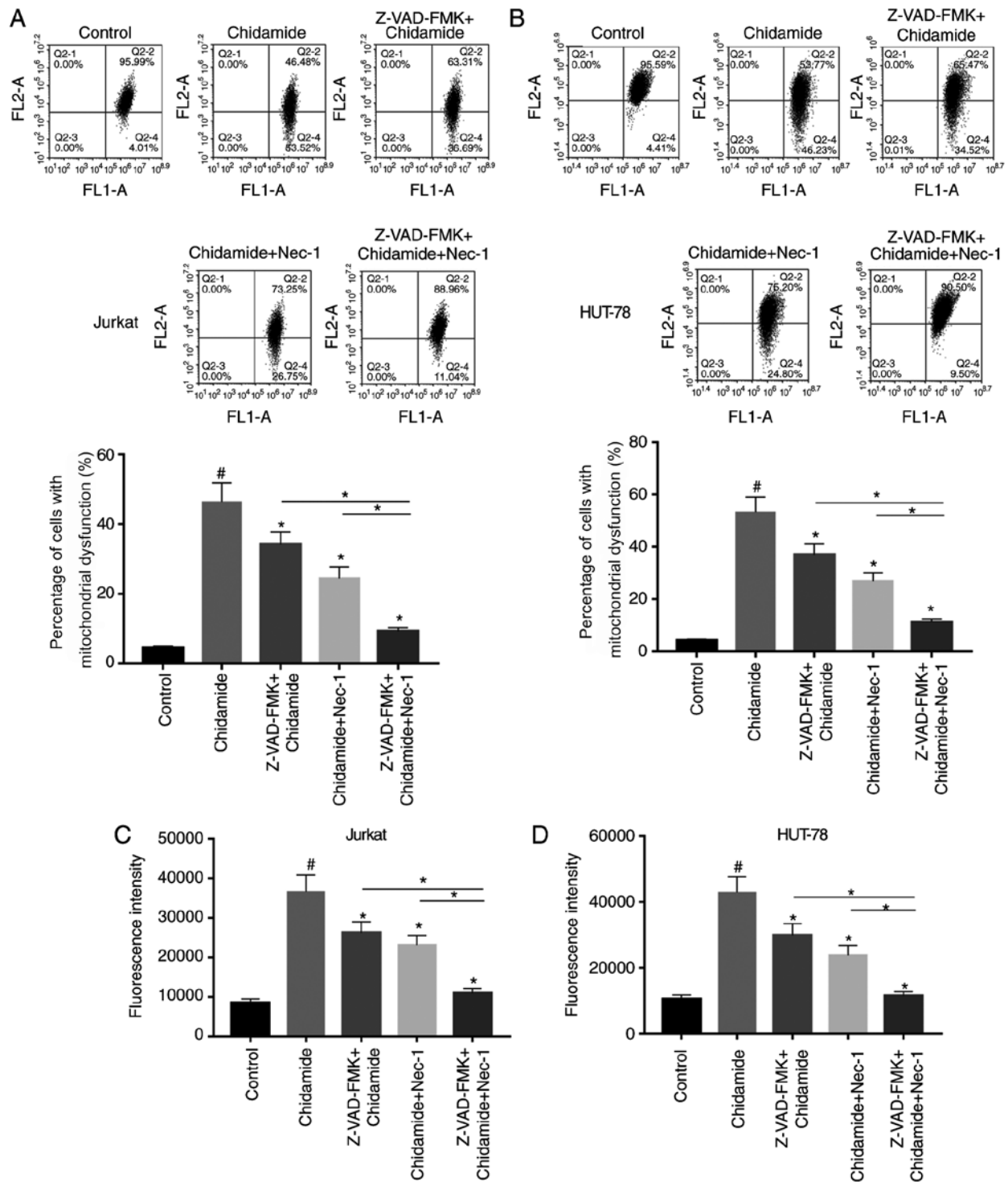
Figure 4. Chidamide aggravates mitochondrial dysfunction in Jurkat and HUT-78 cells. Mitochondrial membrane potential in (A) Jurkat and (B) HUT-78 cells was detected by JC-1 staining and the proportion of cells with mitochondrial dysfunction quantified using a flow cytometer. Reactive oxygen species production in (C) Jurkat and (D) HUT-78 cells. Data are reported as the mean ± SD. #$P<0.05$ vs. control and *$P<0.05$ vs. the chidamide group or the indicated group. Nec-1, necrostatin 1.

effect of inducing necroptosis in Jurkat and HUT-78 cells by downregulating c-FLIP$_L$ expression was investigated.

Epigenetic alterations affect gene transcription through the chemical modification of DNA without altering the DNA sequence (19). Histone modifications, including phosphorylation, methylation, acetylation and ribosylation, represent important epigenetic mechanisms that serve an important role in the regulation of gene expression (20). Acetylation by

histone acetyltransferase promotes gene expression and HDAC gene expression can lead to silencing of tumor suppressor genes (21). As a key enzyme regulating gene expression, HDAC has an important role in the occurrence and development of hematological malignancies via the promotion of cell proliferation and inhibition of cell apoptosis (22). There are 18 types of HDACs in mammals, which are divided into four groups: Class I, IIa, IIb and IV, according to their structure and
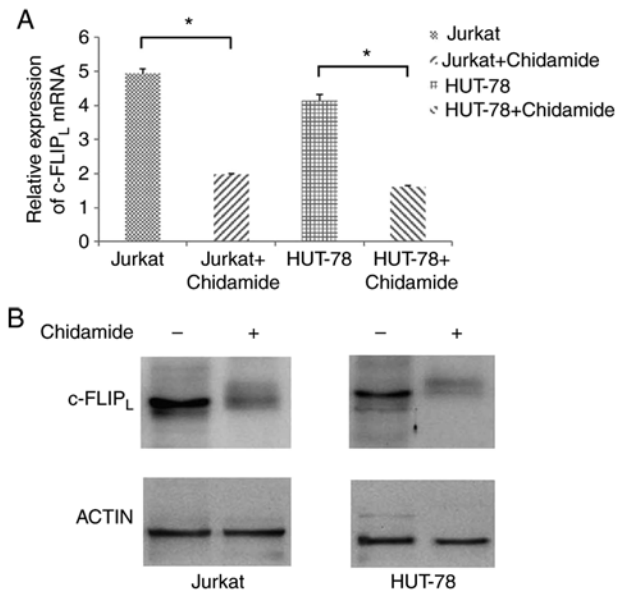
Figure 5. Chidamide downregulates c-FLIP$_L$ expression in Jurkat and HUT-78 cells. (A) c-FLIP$_L$ mRNA and (B) c-FLIP$_L$ protein expression levels were markedly decreased in Jurkat and HUT-78 cells following treatment with chidamide. Data are presented as the mean ± SD. *P<0.05 vs. the indicated group. c-FLIP$_L$, cellular Fas-associated death domain-like interleukin-1β converting enzyme inhibitory protein long form.
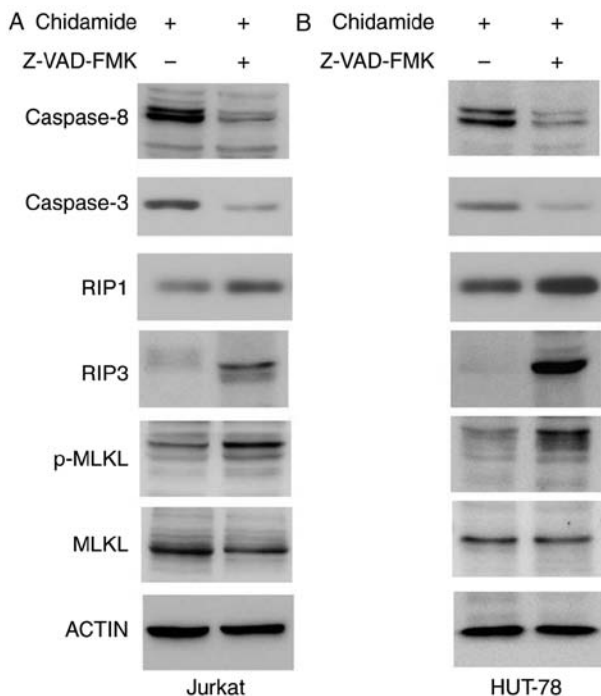
Figure 6. Effects of chidamide on caspase activation and RIP3/MLKL signaling. Western blot analysis of caspase-8, caspase-3, RIP1, RIP3 and p-MLKL in chidamide-treated (A) Jurkat and (B) HUT-78 cells. MLKL, mixed lineage kinase domain like pseudokinase; p, phosphorylated; RIP, receptor-interacting protein kinase.

function. Class I HDACs, including HDAC1, HDAC2, HDAC3 and HDAC8, are mainly distributed in the nucleus and regulate histone acetylation levels. Recent studies have demonstrated that class I HDACs serve an important role in regulating differentiation, proliferation and apoptosis (23-25). HDACis

target and inhibit HDACs to increase histone acetylation levels and trigger chromatin remodeling, thereby regulating the expression levels of key proto-oncogenes or tumor suppressor genes. Furthermore, HDACis may potentially inhibit proliferation, and induce differentiation and apoptosis. HDACis have exhibited efficacy in the treatment of hematopoietic malignancies and four HDACis, including vorinostat, romidepsin, panobinostat and belinostat, have been approved by the US Food and Drug Administration for clinical use (26).

Chidamide is a benzamide HDACi developed in China, which selectively inhibits HDAC class I subtypes 1, 2 and 3, and class IIb subtype 10. Numerous *in vitro* experiments have demonstrated the effects of chidamide on various tumors (27-29). The results of the present study revealed that Jurkat and HUT-78 cells expressed high levels of HDAC1 and HDAC3, which were significantly decreased following treatment with chidamide. Additionally, chidamide regulated cell cycle arrest, as p21 and PCNA are involved in regulation of the cell cycle, and induced mitochondrial dysfunction and necroptosis by downregulating c-FLIP$_L$ transcription and translation in Jurkat and HUT-78 cells.

Degterev *et al* (30) proposed the term necroptosis in 2005 and revealed that the small molecule Nec-1 acted specifically on RIP1 without affecting apoptosis. Necroptosis is defined as cell death mediated by death receptors, which is specifically inhibited by Nec-1, but not the apoptosis inhibitor Z-VAD-FMK. The current study revealed that the proportions of Jurkat and HUT-78 cells undergoing necroptosis and late apoptosis were significantly increased following pretreatment with the apoptosis inhibitor Z-VAD-FMK. Furthermore, treatment with the necroptosis-specific inhibitor Nec-1 significantly reduced the proportion of cells undergoing necroptosis induced by chidamide. Chidamide-induced cell death was also significantly inhibited in Jurkat and HUT-78 cells following pretreatment with both Z-VAD-FMK and Nec-1. Similarly, membranolysis, mitochondrial swelling and organelle disappearance were observed following chidamide treatment. However, representative apoptotic and necroptotic features, and mitochondrial dysfunction were alleviated by Z-VAD-FMK and Nec-1 pretreatment.

The expression levels of caspase-3 and caspase-8 were markedly decreased after Z-VAD-FMK treatment in chidamide-treated cells. Chidamide increased the expression of the key necroptosis proteins RIP1 and RIP3 and phosphorylation of MLKL in Jurkat and HUT-78 cells, when apoptosis was inhibited. The RIP3 gene is located on human chromosome 2q33, which is subject to multiple gene mutations and chromosomal damage, particularly in nasopharyngeal carcinoma and leukemia (31). Additionally, RIP3 is a key molecule that regulates the transformation between apoptosis and necrosis, and RIP3-deficient cells are only able to undergo apoptosis. Downregulation of RIP3 prevents necroptosis without affecting apoptosis (32). Tumor necrosis factor-α, which is considered to be a specific factor controlling necroptosis, does not induce necroptosis in mouse embryonic fibroblasts lacking the RIP3 gene but with wild type RIP1 expression (33). In addition, RIP3 expression is positively correlated with necroptosis and its expression level is a key factor determining the ability of cells to undergo necroptosis (34,35). MLKL is an important downstream effector of RIP3, and serves as an

Table I. Clinical features of patients with T-ALL in the present study.

Clinical feature	n	c-FLIP$_L$ mRNA expression	P-value
Age (years)			0.536
<1	1	3.16±0.00	
1-14	25	2.94±0.25	
≥14	20	3.80±0.34	
Sex			0.066
Male	28	3.65±0.22	
Female	18	2.80±0.03	
Dangerous degree			<0.0001
High	27	4.37±0.33	
Low	19	1.82±0.23	
Leukocyte count (10^9/l)			<0.0001
≥100	11	4.90±0.37	
<100	35	2.82±0.04	
HBDH (U/l)			<0.0001
≥1,000	17	4.37±0.41	
<1,000	29	2.70±0.40	
LDH (U/l)			0.002
≥1,000	14	4.37±0.45	
<1,000	32	2.86±0.44	
CD45			<0.0001
Positive	33	3.99±0.29	
Negative	13	1.62±0.46	
Fusion gene			0.003
Negative	40	3.09±0.46	
SIL-TAL1	5	5.22±0.47	
CR in initial chemotherapy			0.011
Yes	41	3.15±0.54	
No	5	4.65±0.64	

c-FLIP$_L$, cellular Fas-associated death domain-like interleukin-1β converting enzyme inhibitory protein long form; CR, complete remission; HBDH, hydroxybutyrate dehydrogenase; LDH, lactate dehydrogenase.

important switch for RIP3-mediated necroptosis (36,37). In the present study, Z-VAD-FMK pretreatment followed by chidamide treatment increased RIP3 expression and MLKL phosphorylation in Jurkat and HUT-78 cells, indicating that chidamide induced necroptosis when apoptosis was inhibited in T-ALL cells.

In conclusion, the present study revealed that Jurkat and HUT-78 cells expressed high levels of c-FLIP$_L$. The HDACi chidamide induced necroptosis in T-ALL cells by suppressing the transcription and translation of c-FLIP$_L$, which may serve as a novel target for the treatment of T-ALL.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WY designed and directed the study. ZC was the major contributor in writing the manuscript and analyzed the data. HG, HL, BZ and MG participated in performing the experiments. BW participated in writing and was involved in the design of the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was in accordance with the Declaration of Helsinki and was approved by The Ethics Committee of

Shengjing Hospital of China Medical University. All patients and controls, or their parents/guardians, provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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