Synergistic Effect of Chidamide and Venetoclax on Apoptosis in Acute Myeloid Leukemia Cells and Its Mechanism

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Research

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Abstract

**Background:** Acute myeloid leukemia (AML) is a hematological malignancy with a low remission rate and a high recurrence rate. The 5-year overall survival rate is only approximately 25%. The overexpression of the antiapoptotic protein Bcl-2 is associated with a lower overall survival rate in AML patients. Venetoclax (ABT199) is a selective inhibitor of Bcl-2 that has a significant effect in AML, but single-drug resistance often occurs due to the high expression of Mcl-1 protein. Chidamide is a histone deacetylase inhibitor that was independently developed in China. Studies have confirmed that chidamide can downregulate the expression levels of Bcl-2 and Mcl-1 and induce apoptosis. We examined whether the combination of venetoclax and chidamide could synergistically inhibit AML.

**Methods:** This study aimed to use AML cell lines and primary cells to study the effects of venetoclax and chidamide combination therapy on AML cell apoptosis, the cell cycle and changes in related signaling pathways in vitro; establish an AML mouse model to observe the efficacy and survival time of combination therapy in vivo; and analyze the drug effects with multi-omics sequencing technology. The changes in gene and protein expression before and after treatment were examined to clarify the molecular mechanism driving the synergistic effect of the two drugs.

**Results:** (1) Both venetoclax and chidamide promoted apoptosis in AML cell lines and primary cells in a time- and concentration-dependent manner. The effect was further enhanced when the two drugs were combined, and they had a synergistic effect ( Combination Index < 1).
(2) The cell cycle of the AML cell line was arrested in the G1 phase by chidamide monotherapy. The primary cells were arrested in S phase by monotherapy with chidamide or venetoclax. The cell-cycle-blocking effect was further strengthened by the combination of the two drugs. (3) At both the mRNA and protein levels, the expression of Mcl-1 was upregulated by venetoclax and downregulated by chidamide. The expression of Mcl-1 decreased further after combination treatment. (4) Transcriptome sequencing showed that the differentially expressed genes in the combination group compared with the venetoclax monotherapy group were mainly enriched in the PI3K-AKT pathway and JAK2/STAT3 pathway. Moreover, qRT-PCR and Western blotting confirmed the following results. 1) Chidamide downregulated the expression of the AKT gene and P-AKT protein, upregulated the expression of p21, and downregulated the expression of CDK2 and c-myc. 2) The expression of the SOCS3 gene and protein was upregulated, whereas the expression of the JAK2 gene and p-Jak2 protein was downregulated, by chidamide. 3) The expression of the HDAC1 gene and protein was upregulated in the chidamide monotherapy and combination therapy groups. (5) Compared with the monotherapy groups and control group, the combination therapy group exhibited significantly inhibited disease progression and a prolonged survival time among AML mice.

**Conclusion:** First, venetoclax combined with chidamide synergistically promoted apoptosis in AML cell lines and primary cells. Second, transcriptome sequencing, qRT-PCR and Western blotting showed that chidamide synergistically promoted the apoptosis of AML cells by inhibiting the activation of the PI3K/AKT pathway and Jak2/STAT3 pathway. Third, compared with monotherapy, combination therapy with chidamide and venetoclax significantly inhibited tumor progression and prolonged the survival time of mice.

**Background**

Acute myeloid leukemia (AML) is a genetically heterogeneous clonal malignancy originating from clonal hematopoietic stem cells and is characterized by chromosomal abnormalities, recurrent gene mutations, epigenetic modifications affecting chromatin structure, and microRNA dysregulation [1]. Although the complete remission (CR) rate of AML has greatly improved with the emergence of new drugs, targeted drugs and immunotherapy, 20-40% of patients still have difficulty achieving CR, and approximately 60% of patients eventually relapse after CR [2-4]. The 5-year overall survival rate of AML is only approximately 25% [4]. Pre-clinical and clinical studies have demonstrated that the selective and highly potent Bcl-2 inhibitor venetoclax has anti-leukemia activities against various hematological malignancies, including MDS, CLL, and AML [5-9]. Venetoclax is FDA approved for a subset of patients with CLL and AML [10,11]. The FDA-approved combination of venetoclax with decitabine or azacitidine is resulting in CR/CRi rates of 70-95% and good tolerability in elderly AML patients, but patients invariably relapse [9,12,13]. Resistance to apoptosis induced by venetoclax in AML is mediated by pre-existing and venetoclax-induced overexpression of Mcl-1 [9]. Overexpression of Mcl-1 has been reported in AML at relapse [14]. The mechanism by which Mcl-1 blocks the progression of apoptosis is through binding and sequestering the pro-apoptotic BH3-only proteins Bim, PUMA, Noxa, Bak, and Bax [15], preventing pore formation on mitochondrial membrane and the release of cytochrome c into the cytoplasm [16]. Therefore, it is important to identify clinically available agents that interfere with Mcl-1 to augment the therapeutic efficacy of venetoclax in AML [17]. Numerous studies combining venetoclax with other therapies are being trialed [12,18,19].

Cancer can be caused not only by changes in DNA sequences but also by typical epigenetic modifications, such as histone modifications. This type of epigenetic modification can remodel chromatin, change the cell phenotype, regulate gene expression, and promote cancer development [20,21]. Acetylation modifications at specific sites can regulate the pathways involved in the cell cycle and apoptosis [22].
Therefore, the leukemia epigenome has become a new target for histone deacetylase inhibitors (HDACis) \[23\]. Chidamide (CS055) is a histone deacetylase inhibitor (HDACi) that was independently developed in China and has been approved for the treatment of peripheral T cell lymphoma (PTCL) \[24-27\]. It has been suggested that chidamide can inhibit cell proliferation, increase apoptosis, induce cell cycle arrest in a time- and dose-dependent manner, and promote the apoptosis and differentiation of leukemia cells \[28\]. To further examine the synergistic killing effect and mechanism of combination therapy on AML, this study aimed to use AML cell lines and primary cells to study the effects of Venetoclax and chidamide combination therapy on AML cell apoptosis, cell cycle and changes in related signaling pathways in vitro; establish an AML mouse model to examine the efficacy and survival time of combination therapy in vivo; and analyze the drug effects with multi-omics sequencing technology. The changes in gene and protein expression before and after treatment were examined to clarify the molecular mechanism driving the synergistic effect of the two drugs to provide a new treatment strategy for clinical refractory/relapsed AML.

Materials And Methods

1. Cell lines, primary tumor cells, and reagents

The AML cell lines OCI-AML3, THP-1, MV4;11, and MOLM13 were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 with 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (100 µg/ml). All cell lines were cultured in an incubator with 95% humidity at 37.5°C with an atmosphere of 5% CO\(_2\). Primary AML cells were isolated from peripheral blood containing >50% blasts. These specimens were collected before chemotherapy. Venetoclax powder was purchased from Selleck Company (USA) and dissolved in DMSO at a concentration of 10 mM. Chidamide was received as a gift from Shenzhen Microchip Biotechnology Co. Ltd. and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mM.

2. Cell viability assay

The cells were plated in 96-well plates (5000 cells/well) and treated with different doses of venetoclax and chidamide for 24, 48 and 72 hours. At different time points, the cell number was measured using a Cell-Counting Kit-8 (CCK8) proliferation assay kit (Tongren Institute of Chemistry, Japan). Ten microliters of CCK-8 solution was added to each well of the plate. After incubation for 2 hours at 37°C, the plates were measured at 450 nm using a microplate reader (Biotech, NY, USA).

3. Flow cytometry analysis

3.1 Apoptosis

The AML cell lines were incubated with venetoclax and chidamide alone or in combination for 8 h and 24 h. Then, apoptosis was measured using Alexa Fluor 647-conjugated annexin V and propidium iodide (PI) (China Nanjing Kaiji Company) according to the manufacturer’s instructions. Briefly, the cells were harvested, washed twice with phosphate-buffered saline, and resuspended in 200 µL of binding buffer. Then, the cells were incubated with 2 µL Alexa Fluor 647-conjugated annexin V and 1 µL PI for 10 min. Apoptosis was analyzed using a Navios flow cytometer (Beckman Coulter, Brea CA, USA). The results are expressed as the percentage of Annexin V+ cells. AML cell line experiments were performed 3 independent times in triplicate, and the data presented are from one representative experiment; the patient sample experiments were performed once in triplicate due to the limited sample availability. The combination index (CI) values were determined using CompuSyn software. CI<1, CI=1, and CI>1 indicate synergistic, additive, and antagonistic effects, respectively.

3.2 Cell cycle analysis

The cells were treated with venetoclax alone or combined with chidamide for 72 h. Then, the cells were collected and washed with PBS and fixed overnight in 75% ice-cold ethanol at 4°C. 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 software (Verity Software House, Inc., Topsham, ME) was used for data analysis.

3.3 Analysis of the mitochondrial membrane potential

First, the optimal concentrations of venetoclax and chidamide in the OCI-AML3, THP-1, MV4;11, and MOLM13 cell lines were determined according to the results of the above apoptosis assays. Second, AML cells were treated with venetoclax and chidamide alone or in combination for 24 h, and then JC-1, a cationic lipid fluorescent dye, was used to stain the cells. During the process of apoptosis, the mitochondrial transmembrane potential decreases, JC-1 exists in the cytoplasm in the form of monomers, and the number
of polymers decreases. Flow cytometry was used to determine whether JC-1 existed in the form of monomers or polymers to detect changes in the mitochondrial transmembrane potential.

4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays

Total cellular RNA was extracted from cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol. RNA was eluted with RNase-free water, quantified at an absorbance of 260/280 nm, and used for reverse transcription reactions. Total mRNA was reverse transcribed into cDNA using an RT reagent kit (TaKaRa, Dalian, China). Primer sequences of qRT-PCR are shown in Table 2. β-actin was used as an internal standard. qRT-PCR was performed with Fast Start Universal SYBR Green Master Mix (ROX) (Roche, Germany) following the instructions of the supplier. The qRT-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 the 2-ΔΔCt method, in which ΔCt = Ct (target gene)-Ct (internal reference), and ΔΔCt = ΔCt (sample)ΔCt (control). Each sample was measured in triplicate.

5. Western blotting analysis

After treating cells with venetoclax and chidamide for 24 hours, cultured cells were harvested, washed with PBS and then lysed with ice-cold lysis buffer. The protein lysates were clarified by centrifugation at 14000 g for 15 min at 4°C, and the supernatant was collected. The protein level in 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 and incubated with primary antibodies at 4°C overnight in TBS-T (10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.1% Tween 20). The primary antibodies against the following proteins were used: γ-H2AX, PARP, Caspase3, Bcl-2, Mcl-1, Bim, Bax, Bak, AKT, P-AKT, SOCS3, P-JAK2, and P-STAT3. β-actin (Cell Signaling, Herts, UK) was used as a loading control. The blots were washed, exposed for 1 hour to the corresponding HRP-conjugated secondary antibodies, and finally detected by chemiluminescence reagents (Millipore, Billerica, MA, USA). The target protein bands were visualized using ECL and exposed to X-ray film. Immunoreactive proteins were visualized using an Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE), as described by the manufacturer. Densitometry measurements were made using an Odyssey V3.0 (Li-Cor) and normalized to β-actin.

6. HDAC inhibitory activity of chidamide

The HDAC1 gene level was detected by qRT-PCR. Meanwhile, HDAC1 protein, acetylated histone H3 and histone H4 levels were detected by Western blotting to determine the ability of chidamide to inhibit histone deacetylation.

7. In vivo experiment

MOLM13 cells transfected with luciferase were injected into mice (5×10⁶ cells) via the tail vein in mice with severe immunodeficiency (NOD/SCID). Four days after the injection, in vivo imaging was performed to confirm the successful establishment of an AML xenotransplantation mouse model. The mice were randomly divided into groups with 9 mice in each group. The mice were treated with venetoclax (100 mg/kg, intragastric administration, QD) and chidamide (25 mg/kg, intragastric administration, QD) alone or in combination. The untreated group was considered the control. The tumor growth was observed twice a week. Three mice in each group were sacrificed on the 10th day of treatment. The liver and spleen were collected for immunohistochemical staining with a human CD45 antibody (hCD45), and the expression of CD45+CD33+ cells in the bone marrow was analyzed by flow cytometry. The survival time of the remaining 5 mice in each group was observed, and the survival curve of tumor-bearing mice was generated. The weight of the mice in the four groups was measured every other day, and a weight curve was generated after 14 days of continuous monitoring.

8. Statistical analysis

Each experiment was performed at least three times, and the data are presented as the mean ± standard deviation for the indicated number of separate experiments. A t-test was used to compare the mean of each group with that of the control group in experiments. All analysis was performed with SPSS 22.0 System. The results were considered significant if the P-value was less than 0.05.

Results
1. Venetoclax and chidamide synergistically inhibit cell proliferation

To determine the effect of venetoclax and chidamide on proliferation, we first examined the proliferation of OCI-AML3, THP-1, MV4;11, and MOLM13 cells in response to venetoclax and chidamide at various concentrations using a CCK-8 assay. The half-maximal inhibitory concentration (IC50) of venetoclax and chidamide for the OCI-AML3, THP-1, MV4;11, and MOLM13 cell lines is shown in Fig. 1a-c; measurements were performed in three independent experiments after 24 h, 48 h, and 72 h of exposure. Venetoclax and chidamide caused growth arrest in the OCI-AML3, THP-1, MV4;11, and MOLM13 cells in a concentration- and time-dependent manner (Fig. 1d-e). We believe that an IC50>1 µM is indicative of a relatively resistant cell line. Therefore, OCI-AML3 and THP-1 cells are relatively resistant to venetoclax and chidamide, and MV4;11 and MOLM13 cells are relatively sensitive to venetoclax and chidamide.

2. Venetoclax and chidamide synergistically promote apoptosis in AML cells

According to previous reports, both venetoclax and chidamide can induce apoptosis. Therefore, we measured the apoptosis rate in response to monotherapy and combination treatment with flow cytometry. Fig. 2a, b shows that venetoclax and chidamide induced apoptosis in four AML cell lines and primary AML cells in a concentration- and time-dependent manner, and the combination of venetoclax and chidamide had a synergistic effect on apoptosis (CI<1). With patient consent, the bone marrow (primitive or immature cells >50%) of 5 newly diagnosed AML patients (AML#01-AML#05) was extracted to isolate mononuclear cells. Clinical characteristics of AML patients is described in Table 1. Additionally, we detected changes in apoptosis-related genes at the mRNA level with qRT-PCR. As shown in Fig. 2d, the expression of the antiapoptotic gene Bcl-2 was decreased in the venetoclax and chidamide monotherapy groups, and the expression level of Bcl-2 was further decreased in the combination therapy group; the difference was statistically significant (P<0.05). The expression of the antiapoptotic gene Mcl-1 was increased in the venetoclax monotherapy group, decreased in the chidamide monotherapy group, and further decreased in the combination therapy group; the difference was statistically significant (P<0.05). The expression of the antiapoptotic gene Bcl-x1 was significantly decreased in the monotherapy groups and the combination therapy group (P<0.05).

In addition, we also examined apoptosis-related proteins in the mitochondrial pathway and Bcl-2 family apoptosis regulatory proteins by WB. Fig. 2e shows that the expression of the apoptosis-related protein cf-Caspase-3 in the monotherapy groups was higher than that in the control group, and this effect was further enhanced in the combination therapy group. The protein expression of cf-PARP1, whose cleavage is considered to be an important indicator of apoptosis and caspase 3 activation, in the combination group was significantly higher than that in the control group. The expression levels of γ-H2AX protein in the two monotherapy groups were higher than those in the control group, and the effect was further enhanced in the combination therapy group. Moreover, Fig. 2f shows that the antiapoptotic protein Bcl-2 was decreased in the chidamide monotherapy group and the combination therapy group. The antiapoptotic protein Mcl-1 was increased by venetoclax monotherapy, decreased by chidamide monotherapy, and further decreased by combination therapy. The expression of the 'BH3-only' proapoptotic protein Bim in the venetoclax and chidamide monotherapy groups was decreased, and this effect was further enhanced in the combination therapy group. The expression of the proapoptotic protein Bax/Bak in the monotherapy groups and the combination therapy group was higher than that in the control group.

3. Venetoclax and chidamide synergistically decrease the mitochondrial membrane potential

As shown in Fig. 3, venetoclax and chidamide monotherapy reduced the mitochondrial membrane potential of AML cells in the four AML cell lines. In the combination therapy group, the effect was significantly enhanced, and the mitochondrial membrane potential was significantly different from that in the monotherapy groups and the control group.

4. Venetoclax and chidamide synergistically induce cell cycle arrest

As shown in Fig. 4a, venetoclax and chidamide blocked the cell cycle of the AML cell lines in the G1 phase and of primary cells in S phase, thus inhibiting proliferation. The blocking effect was further enhanced by the combination of the two drugs. Fig. 4b shows that the apoptosis inhibited gene p21 was upregulated and that the downstream factors CDK2 and myc were downregulated, as detected by qRT-PCR, resulting in cell cycle arrest. Fig. 4c shows that apoptosis inhibitory protein p21 was upregulated, and the downstream factors CDK2 and c-myc were downregulated, as detected by WB, resulting in cell cycle arrest.
5. Transcriptome sequencing and cluster analysis showed that the differentially expressed genes were enriched in the PI3K-AKT and JAK2/STAT3 pathways in the venetoclax monotherapy group vs the combination therapy group.

As shown in Fig. 5a-c, the number of differentially expressed genes in the venetoclax monotherapy and combination groups was examined after 24 hours of treatment. B showed the venetoclax monotherapy group, and E shows the venetoclax+chidamide group. After 24 hours of drug treatment, the differentially expressed genes in each group were clustered. By counting the number of differentially expressed genes in different layers and the levels of KEGG pathways, we can determine in which metabolic pathways and signaling pathways the differentially expressed genes are mainly involved. Cluster analysis of transcriptome sequencing showed that after venetoclax and chidamide were added to OCI-AML3 cells, the gene clustering of the venetoclax monotherapy group and negative control group was basically the same, and the expression levels of most genes changed after chidamide was added to the cells. We found that in OCI-AML3 cells, genes related to the PI3K-AKT pathway and JAK2/STAT3 were differentially expressed in the venetoclax monotherapy group and venetoclax+chidamide group.

6. qRT-PCR confirmed that the PI3K-AKT and JAK2/STAT3 pathways were inhibited and that the expression of HDAC1 was increased.

We used qRT-PCR to analyze the mRNA expression levels of the above two pathways and HDAC1. The results are shown in Fig. 5d. 1) In the PI3K-AKT pathway, the expression of AKT was downregulated, thus upregulating the expression of the downstream factor p21 and downregulating the expression of CDK2 and c-myc (P<0.05). 2) For the JAK2/STAT3 pathway, the expression of SOCS3 was upregulated, thus downregulating the expression of the downstream factors JAK2 and STAT3, and the difference was statistically significant (P<0.05). 3) For HDAC1, the expression of HDAC1 was upregulated in the chidamide group and the combination group, and the difference was statistically significant (P<0.05).

7. Western blotting showed that the PI3K-AKT and JAK2/STAT3 pathways were inhibited and that the expression of HDAC1 also increased.

According to Western blot analysis, the PI3K-AKT and JAK2/STAT3 pathways were both inhibited, and the expression of HDAC1 was upregulated. The results are shown in Fig. 5e. (1) Chidamide inhibited the activation of the PI3K-AKT pathway by downregulating the expression of P-AKT protein, upregulating the expression of the downstream factor apoptosis-inhibited protein p21, downregulating the expression of CDK2 and c-myc, and resulting in cell cycle arrest. (2) Chidamide inhibited the activation of the JAK2/STAT3 signaling pathway by upregulating SOCS3 protein expression and downregulating P-JAK2 protein expression. Thereby, it upregulated downstream p21 protein expression, inhibited downstream CDK2 and c-myc protein expression of STAT3, caused cell cycle arrest and inhibited proliferation. At the same time, it inhibited the expression of Bcl-2, Bcl-xl and Mcl-1 and promoted apoptosis. (3) In regard to the HDAC1 protein level, the expression of HDAC1, acetyl-H3 and H4 was upregulated, resulting in the prevention of histone deacetylation and an increased degree of histone acetylation, which caused cell proliferation to be inhibited by upregulating p21 protein levels.

8. The signaling pathways of venetoclax combined with chidamide in AML cells

Finally, to summarize the above conclusions, the signaling pathways of chidamide and venetoclax in AML cells are summarized as follows:

9. In vivo experiment

Compared with the monotherapy groups and control group, the combination therapy group exhibited significantly inhibited disease progression and prolonged the survival time of AML mice. We can see that: (1) Venetoclax alone prolonged the survival time of mice (P<0.05), but chidamide had no significant effect on the survival time of mice. The survival time of mice was further prolonged after venetoclax was combined with chidamide (P<0.01) (Fig. 7a). (2) Compared with that in the control group, the fluorescence intensity decreased in the chidamide group, but there was no significant difference. The fluorescence intensity decreased in the venetoclax group and further decreased in the combination group, and the difference was statistically significant (P<0.001) (Fig. 7b, c); thus, venetoclax alone significantly inhibited the growth of tumors in AML xenograft mice, and this effect was further enhanced after combination with chidamide. (3) On the 10th day of treatment, 3 mice in each group were sacrificed and dissected. In vivo imaging showed that AML
leukemia cells infiltrated the lungs, liver, spleen, kidneys and femurs of the mice. The liver, spleen and lung of the mice in each group were stained with HE, and immunohistochemical staining was performed with an hCD45 antibody. The expression intensity of hCD45 was as follows: the control group > chidamide group > venetoclax group > combination group (Fig. 7g-h). Flow cytometry showed that compared with that in the control group, the number of CD45+CD33+ cells in the bone marrow of the monotherapy groups and the combination treatment group decreased, and the combination group had the lowest number; the difference was statistically significant (P<0.05) (Fig. 7d, e). (4) On the 14th day, the body weight of the mice in the chidamide group and the control group was significantly lower than that in the venetoclax group and the combination group, and the difference was statistically significant (P<0.01) (Fig. 7f).

Discussion

We found that both venetoclax and chidamide inhibited the proliferation of AML cells in a time-dependent and concentration-dependent manner, which was consistent with previous reports [29,30]. At the same time, we measured the apoptosis rate by flow cytometry and confirmed that both venetoclax and chidamide promoted the apoptosis of AML cell lines and primary cells in a time-dependent and concentration-dependent manner. The combination of the two drugs further enhanced the effect apoptosis promotion and had a synergistic effect (CI>1). Venetoclax promotes apoptosis in AML cells mainly through the mitochondrial pathway. Kroemer et al. [31] reported that the mitochondrial transmembrane potential is necessary to maintain normal mitochondrial function. In the early stage of apoptosis, the mitochondrial permeability pore (PT pore) opens, which leads to a decrease in the mitochondrial transmembrane potential and the release of JC-1 from the mitochondria. Early apoptosis can be detected by measuring the fluorescence intensity of JC-1 before morphological changes occur in apoptotic cells. Therefore, we detected changes in the mitochondrial membrane potential before and after treatment. Both venetoclax and chidamide decreased the mitochondrial membrane potential of AML cells, and the effect was further enhanced after the two drugs were combined, which indirectly confirmed the synergistic effect of venetoclax and chidamide on apoptosis in AML cells.

The qRT-PCR results showed that Bcl-2, Mcl-1 and Bcl-xl were decreased after the two drugs were combined. These three genes are related to inhibiting apoptosis. Treatment with venetoclax resulted in an increase in Mcl-1 protein levels in intrinsic drug-resistant AML cell lines. The possible reason is that the binding of Mcl-1 and Bim increases the stability of Mcl-1 protein. The sequestration of Bim by Mcl-1 is a mechanism that drives the drug resistance to venetoclax in AML cells. At the same time, Mcl-1 may reduce the level of free Bim and prevent it from inducing apoptosis [32]. Choudhary et al. [33] confirmed that the increased mRNA level and increased Mcl-1 protein stability led to the increased protein level in an acquired drug-resistant AML cell line model. In our study, venetoclax monotherapy upregulated Mcl-1, while the expression of Mcl-1 decreased after combination therapy, which may be one of the mechanisms by which chidamide reduces the resistance to venetoclax. Venetoclax reduced the association between Bcl-2 and Bim; however, once released, Bim/Mcl-1 binding exhibits a compensatory increase, especially in venetoclax-resistant cell lines, preventing apoptosis [32]. By reducing Bim/Mcl-1 and releasing Bim, Mcl-1 inhibition can terminate this association and eliminate venetoclax resistance [34-36]. Therefore, the combined inhibition of Mcl-1 and Bcl-2 seems to be necessary to effectively induce apoptosis through this mechanism. Venetoclax decreased the binding of Bcl-2 to Bim but increased the binding of Mcl-1 to Bim.

Bim interacts with Bax/Bak, leading to Bax/Bak activation. Activated Bax/Bak forms pores in the outer membrane of mitochondria, leading to the release of cytochrome c and subsequent apoptosis. Bcl-2 was used to sequester Bim and thus prevent Bim from inducing apoptosis. The release of Bim from Bcl-2 by venetoclax reduced the association between Bim and Bcl-2, which was offset by the increased interaction between Bim and Mcl-1, stabilizing Mcl-1 and leading to resistance to venetoclax. In venetoclax-resistant cells, treatment with venetoclax does not change the permeability of the mitochondrial outer membrane, which is a necessary event for intrinsic apoptosis. Mcl-1 has previously been shown to reduce DNA damage and to be necessary to inhibit Bak and Bax activation [37-40]. Therefore, the change in the balance of Bcl-2 family members is the main cause of drug resistance. In our study, the downregulation of Bim was induced in both the chidamide monotherapy and combination therapy groups. Therefore, the binding of Bim to Mcl1 decreased, which reduced the resistance to venetoclax. Jonathan Schwartz et al. [30] confirmed that panobinostat upregulated Bim (a kind of HDACI). In the presence of ABT-199, Bim still increased. We speculated that there might be other mechanisms that drive Bim upregulation, which eventually led to an increase in Bax/Bak and apoptosis.

To determine how the expression of Mcl-1 protein was reduced by chidamide, we first studied it with qRT-PCR. At 24 hours after treatment, the transcription level of Mcl-1 was significantly decreased by chidamide, and the decreased level was maintained in cells treated with the combination therapy (Fig. 2d), indicating that the transcription level of Mcl-1 was downregulated by chidamide. To determine whether chidamide affects the stability of Mcl-1 protein, we used chidamide and venetoclax alone or in combination to treat AML cell lines and primary cells for 24 hours. The results showed that the protein level of Mcl-1 was decreased in the chidamide monotherapy group and the combination therapy group, and the decrease was more obvious in the combination therapy group (Fig. 2f). These results indicate that
chidamide decreases the expression of Mcl-1 at both the transcriptional and protein levels. Venetoclax releases Bim from Bcl-2, but Bim can be sequestered by Mcl-1 later. Therefore, the combined effect of Bcl-2 inhibition and Mcl-1 downregulation/inhibition is necessary to effectively induce apoptosis. Mcl-1 is downregulated by chidamide, although the exact molecular mechanism remains undetermined. We speculate that in venetoclax-sensitive cells, there may not be enough Mcl-1 to sequester all of the released Bim, leading to the accumulation of free Bim and allowing Bim to activate Bax/Bak, thus leading to apoptosis. In contrast, in venetoclax-resistant cells, Bim released from Bcl-2 was sequestered by Mcl-1, which stabilized Mcl-1 and eventually led to the survival of venetoclax-resistant cells. Chidamide reduced the interaction between Mcl-1 and Bim, reduced the sequestration of Bim, allowed the activation of Bax/Bak, and eventually led to apoptosis.

It has been proven that JAK2/STAT3 signaling inactivation can inhibit growth and induce apoptosis in many cancer cells and tumors. Therefore, the JAK2/STAT3 pathway is considered to be a target for anticancer therapy for many human cancers. The JAK-STAT signaling pathway is widely activated in leukemia cells. For example, STAT1, STAT3 and STAT5 are the most common signaling proteins in leukemia. MPN is characterized by the somatic acquisition of a mutation in either JAK2 (JAK2 V617F) or calreticulin (CALR) in a hematopoietic stem cell. SOCS3 protein, a negative regulator of JAK2/STAT3 signal transduction, plays a role as a tumor suppressor. We found that JAK2-STAT3 signaling pathway-related genes were differentially expressed based on transcriptome sequencing. Then, we verified that SOCS3 expression was upregulated and JAK2 and STAT3 gene expression was downregulated with qRT-PCR. At the same time, we further verified the correlation at the protein level with Western blot analysis. The results also showed that the expression of SOCS3 was upregulated, and the expression of P-Jak2 and P-STAT3 was downregulated. Therefore, we concluded that the JAK2/STAT3 signaling pathway was inhibited. The downstream related factors in the STAT3 pathway can regulate the cell cycle and apoptosis process and inhibit the expression of the CDK2 and c-myc genes, which are downstream of STAT3, by upregulating the expression of p21. At the same time, it inhibited the expression of Bcl-2 and Mcl-1 and promoted apoptosis.

Mcl-1 are also downstream of PI3K/AKT pathway, which are frequently constitutively activated in AML. The PI3K/AKT signaling pathway plays a key role in tumor occurrence, development and radiation resistance, is involved in various cellular activities and metabolic regulation; and is closely related to the proliferation and apoptosis, cell cycle regulation, angiogenesis and invasion and metastasis of cancer cells. The PI3K/AKT pathway inhibits apoptosis through a variety of mechanisms. AKT upregulates the expression of c-myc by upregulating the transcription of c-myc; AKT can also inhibit GSK3β kinase activity via phosphorylation to prevent cyclin D1 degradation, change the cell cycle distribution and promote cell proliferation in S phase. We found that there were differences in the expression of PI3K-AKT pathway-related genes after chidamide was combined with venetoclax. By qRT-PCR, we verified that AKT gene expression was downregulated in this pathway, while Western blot analysis was used to verify that chidamide reduced the protein expression of P-AKT. After combination treatment with venetoclax, the P-AKT level further decreased, and the activation of the PI3K-AKT pathway was inhibited. Through the upregulation of p21 protein expression, the expression of CDK2 and c-myc was downregulated, which caused cell cycle arrest. Chidamide can inhibit the activation of the PI3K-AKT signaling pathway to have anti-acute myeloid leukemia effects by cooperating with venetoclax.

Regarding cell cycle regulation, the p21 gene controls the synthesis of p21 protein in human cells, which can inhibit DNA replication. Venetoclax and chidamide can increase the p21 gene and protein levels, and chidamide can also inhibit the PI3K/AKT pathway. Western blot results showed that P-AKT protein was downregulated after the addition of chidamide, and CDK2 was an important regulator of the G1/S phase transition. On the other hand, the downregulation of P-AKT also led to the downregulation of c-myc, which is a gene that promotes cell division, and c-myc is also a downstream factor of STAT3 in the JAK2/STAT3 pathway. After the JAK2/STAT3 pathway is inhibited, c-myc is downregulated. CDK2 and c-myc are downregulated, which together cause cell cycle arrest and inhibit the proliferation of AML cells.

Moreover, the expression of HDAC1 was upregulated, resulting in the prevention of histone deacetylation. Thus, the degree of histone acetylation increased, and cell proliferation was inhibited by upregulating p21 protein levels.

**Conclusion**

Our study generated the following three conclusions. First, venetoclax combined with chidamide can synergistically promote apoptosis in AML cell lines and primary cells. Second, transcriptome sequencing, qRT-PCR and Western blotting showed that chidamide synergistically promotes the apoptosis of AML cells by inhibiting the activation of the PI3K/AKT pathway and Jak2/STAT3 pathway. Third, compared with the monotherapy group, the combination of chidamide and venetoclax significantly inhibited tumor progression and prolonged the survival time of mice. The purpose of this study was to explore the antileukemia effect and mechanism of action of venetoclax combined with
chidamide. The research results of this study provide a new strategy and theoretical basis for solving the problem of drug resistance to venetoclax in the clinic.

**Declarations**

**Ethics approval and consent to participate**

This study was approved by the Medical Ethics Committee of the Affiliated Cancer Hospital of Zhengzhou University (approval no. 2018111). Written informed consent was obtained from all participants or their appropriate surrogates.

**Consent for publication**

Not applicable.

**Availability of data and materials**

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

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

GPL collected the patient information, analyzed the data, designed the tables, and wrote the manuscript. DBL, CC, FFY and LC collected clinical information and reviewed the literature. XDW designed the study, analyzed the data, and wrote and revised the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1  Clinical characteristics of AML patients

| case number | gender | age (years) | disease status | FAB type | prime /immature cells | molecular mutation | fusion gene | chromosome |
|-------------|--------|-------------|----------------|----------|-----------------------|-------------------|------------|-------------|
| #01         | male   | 22          | newly diagnosed | M2b      | 86.8%                 | c-kit /ABCB1      | AML1-ETO   | 45,XY,t(8;21)(q22;q22) |
| #02         | female | 56          | newly diagnosed | M2a      | 51.6%                 | ABCB1             | WT1        | 46,XX,t(2;22;21) (q21;q37;q21)/46,xx[19] |
| #03         | male   | 61          | newly diagnosed | M2b      | 65%                   | C-kit/ABCB1       | AML1-ETO   | 45,XY,t(8;21)(q22;q22) |
| #04         | male   | 23          | newly diagnosed | M2b      | 54.8%                 | negative          | AML1-ETO   | 45,XY,t(8;21)(q22;q22) |
| #05         | female | 45          | newly diagnosed | M1       | 96%                   | FLT3-ITD,NPM1,IDH2,TET2,ABCB1 | negative | 46,XX[16] |

Table 2  Primer sequences of qRT-PCR
| Gene       | Forward Sequence | Reverse Sequence |
|------------|------------------|------------------|
| Bcl-2      | 5'-CGACTTCGCGAGATGTCAG-3' |  |
| Bcl-2 reverse | 5'-CGGGTTCAAGCTACTCAATGAC-3' |  |
| Mcl-1      | 5'-GCCGCTGACGCCATCATGTC-3' |  |
| Mcl-1 reverse | 5'-CAACTCGTCCTCCTCCTCCTVTC-3' |  |
| Bcl-xl     | 5'-GTCGCGTGGAGAGCGTAGACAG-3' |  |
| Bcl-xl reverse | 5'-AGAGCGACCCAGCGAGAC-3' |  |
| Jak2       | 5'-CGGTCGGTAAATGTGTCAGAGAGG-3' |  |
| Jak2 reverse | 5'-CGGCTGGGAGTGTGACTTTTC-3' |  |
| STAT3      | 5'-AGGGAGCAGAGATGGGAATGG-3' |  |
| STAT3 reverse | 5'-TCGTTGTTGAGGAGAGAAGAC-3' |  |
| SOCS3      | 5'-GGTCACCCACAGCAAGTGTCCC-3' |  |
| SOCS3 reverse | 5'-CACCAGCTTGAGCAGTGC-3' |  |
| AKT        | 5'-GTGGCTATTGTGAAGGAGG-3' |  |
| AKT reverse | 5'-GCAGGCGAGCCGATGAGAAG-3' |  |
| HDAC1      | 5'-TTACGCCCCTCAAGCTAAGGC-3' |  |
| HDAC1 reverse | 5'-CTTGCCCAAGAACAGAGTAC-3' |  |
| P21        | 5'-GCGTTTGAGTGGTAGAAC-3' |  |
| P21 reverse | 5'-GCCCATCAAAGCTACTCTGAC-3' |  |
| CDK2       | 5'-GGCCATCAAGCTAGCAGACT-3' |  |
| CDK2 reverse | 5'-GAATCCAGGGAATAGGGC-3' |  |
| C-myc      | 5'-CCACAGCAACCTCCCTCAGC-3' |  |
| C-myc reverse | 5'-GCAGGATAGCTCCGTCGAATG-3' |  |
| β-actin forward | 5'-CGCTTCGGTGCTGTCGACA-3' |  |
| β-actin reverse | 5'-GTCAGCGACGATTTCCGCT-3' |  |

**Figures**
Figure 1

a IC50 values of four AML cell lines treated with venetoclax for 24 h, 48 h and 72 h. b IC50 values of four AML cell lines treated with chidamide for 24 h, 48 h and 72 h. c IC50 values of the two drugs in four AML cell lines at 24 h, 48 h and 72 h. d The proliferation inhibition curve of venetoclax for OCI-AML3 and MOLM13 at 24 h, 48 h and 72 h. e The proliferation inhibition curve of chidamide for OCI-AML3 and MOLM13 at 24 h, 48 h and 72 h.
Figure 2

a Apoptosis histograms of the OCI-AML3 treated with venetoclax or chidamide alone or in combination for 8h and 24h. b Apoptosis histograms of the OCI-AML3 treated with venetoclax or chidamide alone or in combination for 8h and 24h. c. Venetoclax and chidamide monotherapy and combination treatment of primary AML cells. d Changes in apoptosis-related proteins detected by qRT-PCR. e Changes in apoptosis-related proteins detected by WB. e Changes in Bcl-2 family apoptotic regulatory proteins detected by WB.

Figure 3

Histogram of changes in the mitochondrial membrane potential in the OCI-AML3, THP1, MV4;11, and MOLM13 cell lines treated with venetoclax and chidamide.
Figure 4

a. Cell cycle analysis after venetoclax and chidamide monotherapy and combination therapy in different AML cell lines and primary AML cells with flow cytometry. b. Changes in cell cycle-related genes before and after treatment, as detected by qRT-PCR. The data were statistically analyzed with a t-test (*, #P<0.05; **, ##P<0.01; ***, ###P<0.001). c The changes in cell cycle-related proteins before and after treatment, as detected by WB.
Figure 5

a Volcano map of differentially expressed genes. b The genes with the most obvious differences in expression. c Enrichment analysis of KEGG pathways. d The mRNA levels of AKT, HDAC1, SOCS3, JAK2 and STAT3 expressed in OCI-AML, MOLM13 and primary cells treated with venetoclax and chidamide for 24 hours, as analyzed by qRT-PCR. The data were statistically analyzed by a t-test (*, #P<0.05; **, ##P <0.01; ###,###P <0.001). e PI3K-AKT and JAK2/STAT3 signaling pathway-related proteins and HDAC1 protein expression levels in the OCI-AML3 and MOLM13 cell lines and AML primary cells after 24 hours of treatment with venetoclax and chidamide.

Figure 6
The signaling pathway of venetoclax combined with chidamide in AML cells.

**Figure 7**

a Survival curve of AML xenograft mice treated with venetoclax (ABT199) and chidamide alone or in combination. b The fluorescence intensity of AML xenograft mice in the monotherapy groups and the combination therapy group. c The tumorigenesis of AML xenograft mice treated with venetoclax and chidamide alone or in combination. d On the 10th day of administration, 3 mice in each group were sacrificed for in vivo imaging. e The number of CD45+CD33+ cells in the bone marrow of AML xenotransplantation mice treated with venetoclax and chidamide alone or in combination for 10 days. f The body weight curve of mice with AML xenotransplantation treated with venetoclax and chidamide for 14 days. g Ten days later, the mice were sacrificed, and the liver, spleen and lung were stained with HE. h Ten days later, the mice were sacrificed, and the liver, spleen and lung were stained for hCD45+ with immunohistochemistry. A t-test was used to analyze the data (*P< 0.05, ** P <0.01, and *** P<0.001, n.s., not significant).