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Entinostat combined with Fludarabine synergistically enhances the induction of apoptosis in TP53 mutated CLL cells via the HDAC1/HO-1 pathway

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ABSTRACT

TP53 mutation is an indicator of poor prognostic in chronic lymphocytic leukemia (CLL). Worse still, CLL patients with TP53 mutation are associated with poor efficacy to current chemotherapeutic, such as Fludarabine. Here, we confirmed that high expression of HDAC1 in CLL patients with TP53 mutation, which is closely related to poor prognosis and drug-resistance. Subsequently, we demonstrated Entinostat (HDAC1 inhibitor) combination with Fludarabine significantly induced apoptosis in TP53 mutations CLL cells. Its mechanism was associated with up-regulation of the pro-apoptotic protein Bax and the down-regulation of HDAC1, HO-1 and BCL-2 proteins. More importantly, we also confirmed that upregulation of HDAC1 could resistant Entinostat-induced apoptosis in TP53 mutations CLL cells by activating the HDAC1/P38/HO-1 pathway. In vivo, we found that Entinostat combination with Fludarabine significantly induced tumor cells apoptosis and prolong survival time in xenograft mouse model. Finally, combining vitro and vivo experiments, we presented the first demonstration that Entinostat combination with Fludarabine had a synergistic effect on the induction of apoptosis in TP53 mutations CLL cells. In conclusion, we provide valuable pre-clinical experimental evidence for the treatment of CLL patients with poor prognosis, especially for TP53 mutations.

1. Introduction

Chronic lymphocytic leukemia (CLL) is a mature B lymph proliferative cancer that occurs predominantly in middle-aged and elderly populations. Its characteristics are monoclonal expansion of $CD5^+$ $CD19^+$ $CD23^+$ B lymphocytes in peripheral blood, bone marrow, spleen and lymph nodes [1–3]. CLL is a highly heterogeneous disease and varies widely in clinical course among different patients with survival ranging from months to decades [4]. Clinical staging system for CLL prognosis is the Rai [5] and Binet [6] systems. It has been reported that > 8% of CLL patients shown TP53 mutation, while TP53 mutation is an important indicator of poor prognostic [7–10]. We also searched the GEO database (GSE83566, PMID: 27909343) and confirmed TP53 mutations was associated with a shorter OS in CLL patients (Supplement Fig. 2).

Fludarabine has been approved for the treatment of CLL, which can inhibit DNA synthesis by interfering with ribonucleotide reductase and DNA polymerase, causing a change in apoptosis-related proteins (such as BCL-2 and Bax) and induce cell apoptosis [11,12]. Our team also confirmed that Fludarabine-mediated apoptosis of CLL cells is closely related to DNA fragmentation or cytochrome *c* release (Supplement Fig. 1). However, Fludarabine is ineffective in TP53 mutation CLL patients for clinical treatment [10,13].

In this study, we evaluated the feasibility of Entinostat combined with Fludarabine induced apoptosis in CLL cell line JVM-2 (TP53 wild type) and MEC-1 (TP53 mutation) cells apoptosis [14]. Finally, we presented the first demonstration that Entinostat combined Fludarabine significantly increased apoptosis of TP53-mutated CLL cells via

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HDAC1/P38/HO-1 pathway, in vitro and in vivo. In short, this study provides a valuable pre-clinical experimental evidence for the treatment of CLL patients with poor prognosis, especially those with TP53 mutations.

2. Materials and methods

2.1. Patient samples

According to the classification of the International Workshop on CLL and the National Cancer Institute sponsored working group on CLL criteria [15]. We collected peripheral blood samples from 10 normal donors and 12 newly diagnosed CLL patients from Guizhou Medical University, within the period of January 2016 to March 2018. According to the TP53 mutation classification, we divided into mutation and wild type groups of CLL patients. Note: We selected only with TP53 gene mutations alone as the subject of this study.

2.2. Cells and cell culture conditions

JVM-2 and MEC-1 were obtained from the China Academy of Shanghai Cell Bioresources and kept in a RPMI-1640 medium supplemented with 15% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin [16,17], which were purchased from Invitrogen (Carlsbad, CA, USA). The cells were maintained in a 37 °C incubator at 95% humidity and 5% CO_2.

2.3. Real-time PCR

Total RNA was extracted from the cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized using a Prime Script RT reagent kit (Takara, Dalian, China). The real-time PCR experiments were conducted in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA), using a SYBRGreen Real-time PCR Master Mix (Takara). Amplification was carried out as follows: denaturation at 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 35 s. The expression of the target gene was calculated using the 2-ΔΔCq method [18,19]. All experiments were conducted in triplicate.

2.4. The lentiviral vector and transduction

Sequences containing the human coding sequence of the HDAC1 gene (CDS-HDAC1) were selected using Invitrogen designer software. Retroviruses were generated by transfecting empty plasmid vectors containing HDAC1 and enhanced green fluoresence protein (EGFP) into 293 T packaging cells. Lentiviral stocks were concentrated using the Lenti-X concentrator, and titers were determined with the Lenti-X Q-PCR Titration kit (Shanghai Innovation Biotechnology Co., Ltd., China). Finally, two recombinant lentiviral vectors were constructed: lentivirus-HDAC1 (L-HDAC1) and lentivirus- EGFP (EGFP). The negative control was used as a scrambled non-targeting sequence. For transfection, MEC-1 cells were plated onto 12-well plates at 2.5×10^5 cells/well and infected with the lentiviral stocks at a multiplicity of infection in the presence of polybrene (10 μg/ml), and then analyzed by fluorescence microscopy (Olympus, Tokyo, Japan) and western blotting at 48 h post-transduction. MEC-1 cells were transduced with HDAC1 (HDAC1) and subsequently, EGFP (Vector) [20,21].

2.5. Chemicals

Fludarabine (99.72% purity, No. S1491), Entinostat (99.0% purity, No. S1053) and BIRB (99.17% purity, No. S1574) were purchased from Selleckchem (Houston, TX, USA), while DMSO (99.9% purity) was purchased from Solarbio (Beijing, China).

2.6. Cell viability assay

JVM-2 and MEC-1 cells were seeded at a density of 5000 cells per well in 96-well plates. The proliferations of JVM-2 and MEC-1 cells, as well as the response to Fludarabine and Entinostat were determined using Cell Counting Kit-8 (CCK-8) assay. The cells were exposed to different concentrations of fludarabine (1, 2, 4, 8, 12 and 20 μM) and Entinostat (1, 2, 4, 8, 12 and 20 μM) for 24, 48 and 72 h. After treatment, 10 μl of CCK-8 was added to each well. After 2 h of incubation at 37 °C, spectrometric absorbance at 450 nm was measured using a microplate reader. The experiments were performed 5 times for each group. The concentration that produced 50% cytotoxicity (IC50) was determined using GraphPad Prism v5.0 software (GraphPad Software Inc., San Diego, CA, USA) [20].

2.7. Apoptosis analysis

The MEC-1 cells were treated with Entinostat (10 μM), Fludarabine (6 μM) and DMSO (0.1%) for 24 h. Afterwards, the cells were harvested, washed with phosphate buffered saline (PBS), and stained with an Annexin V-FITC/PI apoptosis kit (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. The cells were measured with FCM and Cell Quest software (BD Biosciences) [22].

2.8. Western blot analysis

Western blot analysis was performed to analyze protein expression. Primary antibodies, such as HDAC1 and HO-1 for western blot analysis were obtained from Santa Cruz Biotechnology (Inc, CA, USA) [22,23]. We used P to represent phosphorylation and primary antibodies P-P38 were obtained from Cell Signaling Technology (Beverly, MA, USA). The secondary antibody for western blot analysis was obtained from Cell signaling Technology (Beverly, MA, USA). Equal amounts of protein lysate were used for the western blot analyses and β-actin expression was kept constant in all cases.

2.9. Xenograft mouse model of CLL

Nude mice, purchased from Beijing laboratory animal center, were exposed to 2.5 Gy X-ray at a dose rate of 1.2 Gy/min (RS2000Pro, Rad Source Technologies, USA) [24–26]. On withdrawal of X-ray after 2 days, the mice were randomly divided into four groups, MEC-1 cells (1×10^7 cells per animal were separately injected subcutaneously into the right abdomen side for all four groups). On the 12th day after inoculation, each group of mice consisting of four animals, were administered with Fludarabine (40 mg/kg), Entinostat (50 mg/kg) or normal saline (NS) intraperitoneally once a day from day 12 onwards. Tumor size was measured twice a day using a vernier calliper and calculated as $\pi/6$ length × width^2. The survival time of the mice were recorded and analyzed. All procedures were conducted in accordance with guidelines for the care and use of laboratory animals.

2.10. Statistical analysis

Each experiment or assay was performed in triplicate, and representative examples are shown. Data are reported as mean ± SEM. Statistically significant differences between the treatment groups were calculated using Student's *t*-test. Differences are considered statistically significant at p values < 0.05.

3. Results

3.1. Abnormal expression of Class I HDAC genes in CLL patients and CLL cell lines

We used flow cytometry to sort normal donor peripheral blood

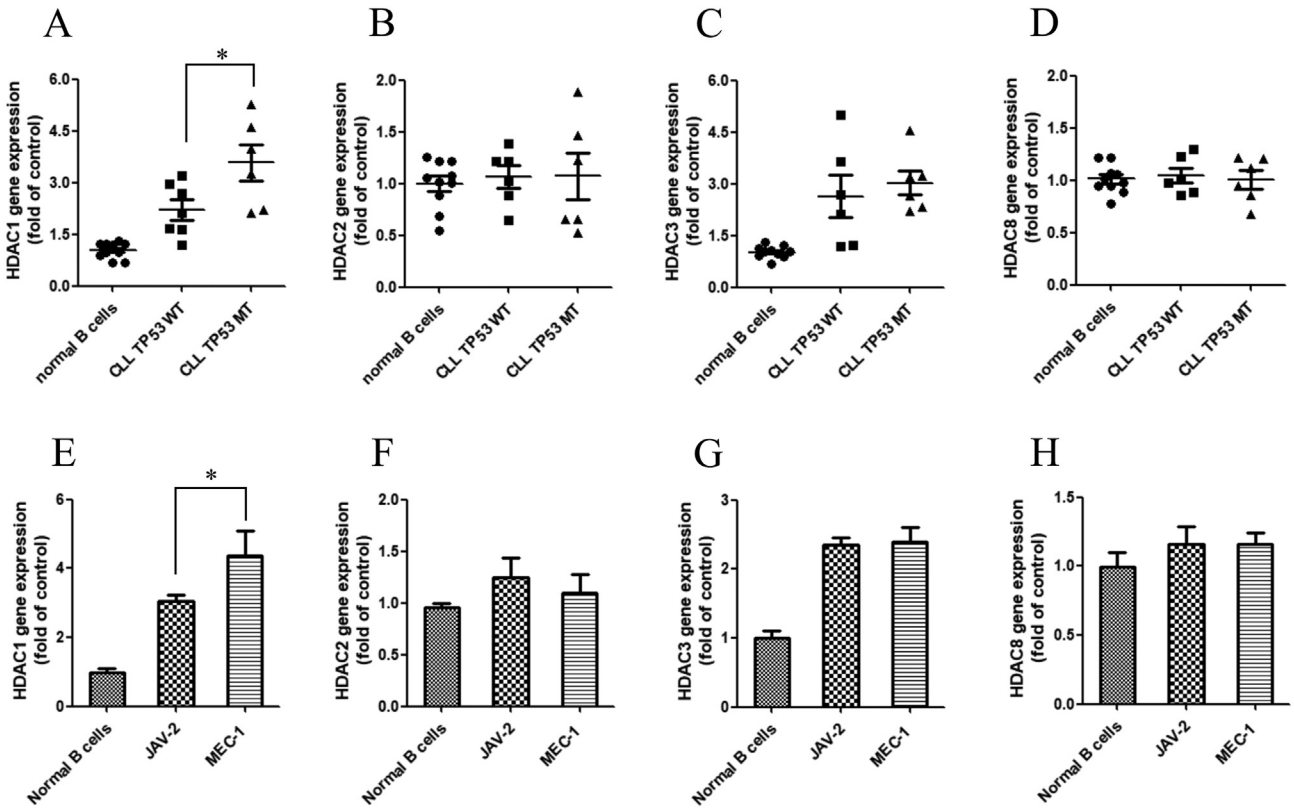

Fig. 1. Class I HDAC gene expression in CLL patients, in the peripheral blood of normal B cells and in CLL cell lines.
(A-D) HDAC1, HDAC2, HDAC3 and HDAC8 gene expressions was analyzed by real-time PCR on TP53 wild type and TP53 mutant CLL patients. A representative example (CLL = 12; normal B cells = 10). Normal B cell representation is of $CD19^+$ purified peripheral blood from normal B cells. (E–H) Expression of HDAC1, HDAC2, HDAC3 and HDAC8 was analyzed by real-time PCR in CLL cell lines (JVM-2 and MEC-1 cells). A representative example (normal B cells = 10). All experiments were performed in triplicate. *$p < 0.05$.

mononuclear cells $CD19^+$ B cells that were used as the normal control group [27]. Real-time PCR was used to detect Class I HDAC gene (HDAC1, HDAC2, HDAC3 and HDAC8) expressions in CLL patients and CLL cell lines. These results shown HDAC1 gene expression was higher in CLL patients with TP53 mutations and MEC-1 cells compared with normal B cells (Fig. 1). We also examined the expression of the HDAC1 gene in other cytogenetics of CLL patients and results shown that no significant difference (Supplement Fig. 3). Subsequently, we searched the TGCA (The Cancer Genome Atlas) database and found that HDAC1 was also elevated in CLL patients with TP53 mutations (Supplement Fig. 1). Similar reports also show that the HDAC1 significantly increases in CLL patients [28]. These results confirmed that HDAC1 is aberrantly expressed in CLL patients with TP53 mutations and CLL cell lines.

Fludarabine interacts with Entinostat to induce apoptosis of JVM-2 and MEC-1 cells in a time-dependent and concentration-dependent manner.

Many studies have been reported Entinostat can induce apoptosis in CLL cells [29,30]. However, the mechanism of Entinostat combined with fludarabine on apoptosis of CLL cells with TP53 gene mutation was not elucidated.

In order to assess Entinostat combined with fludarabine on proliferation of CLL. Firstly, we performed CCK-8 assays and determined both Fludarabine (1–20 μM) and Entinostat (1-20 μM) can significantly inhibition proliferation of CLL cells (Fig. 2A–Fig. 2C). Subsequently, we performed FCM assays and determined both Fludarabine and Entinostat also cause apoptosis of CLL cells (Fig. 2F and Supplement Fig. 1). In addition, Fludarabine caused an increase of γ-H2AX protein expression with a time- and concentration-dependent manner in MEC-1 cells (Supplement Fig. 4). MEC-1 cells show stronger drug-resistance to Fludarabine than JVM-2 cells. Yet, there is no difference inhibition proliferation of JVM-2 and MEC-1 cells by Entinostat (Fig. 2D and Fig. 2E). Importantly, Fludarabine combined with Entinostat is able to significantly promote apoptosis of MEC-1 cells (Fig. 2F). Therefore, in subsequent experiments, we used MEC-1 cells for further study.

3.2. The combination of Fludarabine and Entinostat potentiates apoptosis-related protein expression in MEC-1 cells

Based on the results in Fig. 2, we used Fludarabine (1, 3 and 6 μM) and Entinostat (2, 4 and 10 μM) on MEC-1 cells. HDAC1, Ace-H3K9, Total histone3, HO-1, BCL-2 and Bax protein expressions was analyzed by western blot in MEC-1 cells. The results show that Bax is increased as Fludarabine concentration increased in MEC-1 cells (Fig. 3A). However, Ace-H3K9, Total histone3, HDAC1 and HO-1 protein expressions showed no had significant increased (Fig. 3A). Yet, Ace-H3K9 protein expression was increased with HDAC1 inhibition by Entinostat concentration increased (Fig. 3B). And BCL-2 protein expression was not significantly increased with Entinostat concentration increase (Fig. 3B). In brief, Fludarabine (6 μM) and Entinostat (10 μM) significantly increase the apoptosis of MEC-1 cells by decreasing HDAC1, HO-1, BCL-2 protein expression and increasing Bax protein expression (Fig. 3).

Lentivirus-mediated HDAC1 gene expression was upregulated in MEC-1 cells.

Consistent with our previous study, we used lentiviral-mediated HDAC1 upregulation in MEC-1 cells. Enhanced green fluorescent protein (EGFP) was detected with the use of fluorescence microscopy (Fig. 4A). HDAC1 gene and protein expression were detected by western blot and real-time PCR in lentiviral-transfected MEC-1 cells. The results showed that HDAC1 gene and protein expression in lentiviral-transfected MEC-1 cells significantly increased compared with Vector

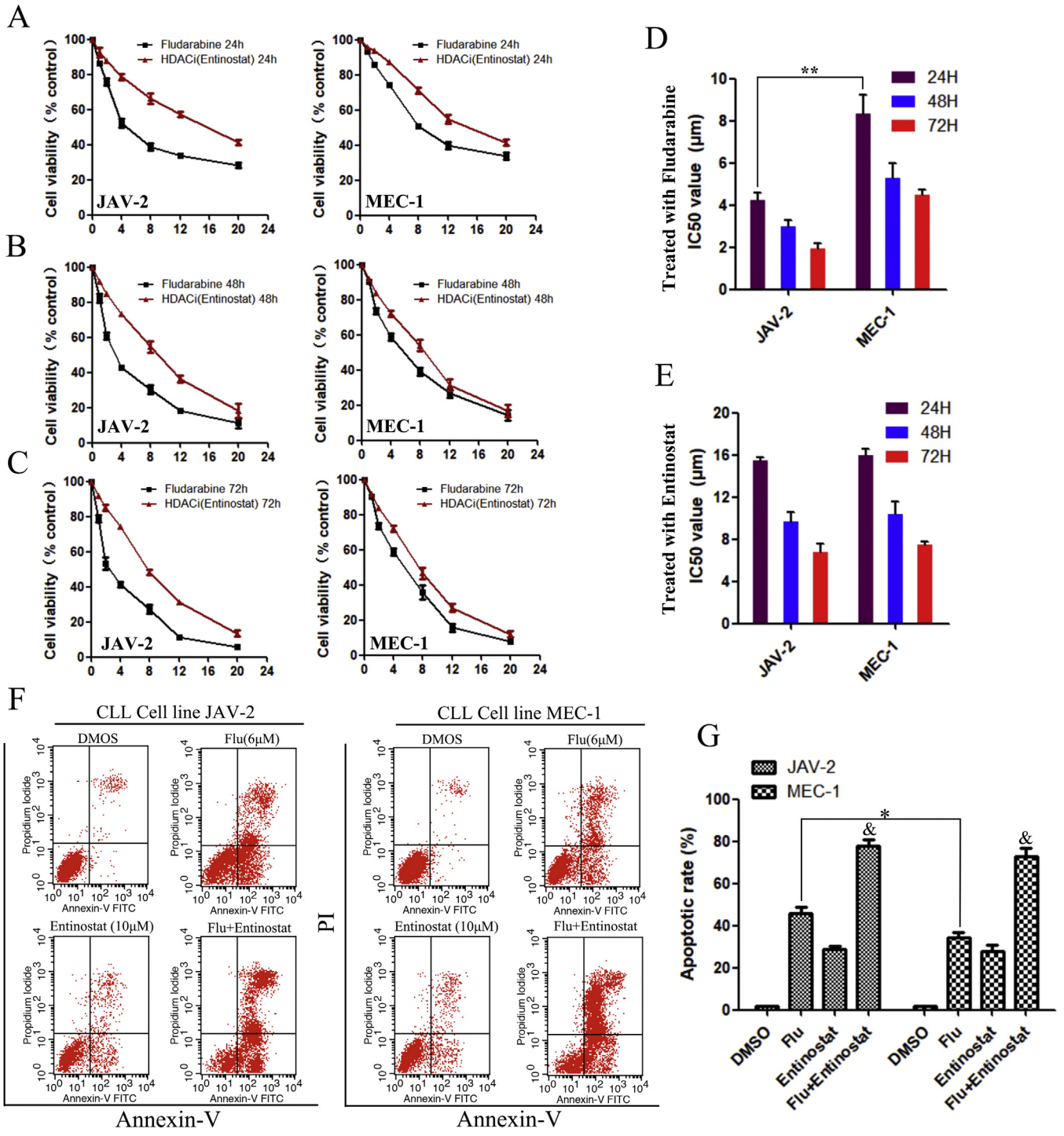

Fig. 2. Fludarabine interacts synergistically with Entinostat to induce apoptosis in JVM-2 and MEC-1 cells in a time-dependent and concentration-dependent manner. (A-C) JVM-2 and MEC-1 cells treated with Fludarabine (1-20 μM) and Entinostat (1-20 μM) for 24, 48 and 72 h. CCK-8 assay was used to detect cell viability. (D, E) IC-50 values of JVM-2 and MEC-1 cells treated for 24, 48 and 72 h with Fludarabine and Entinostat. (F) Flow cytometry was used to detect the apoptosis rate after JVM-2 and MEC-1 cells were treated with Entinostat (10 μM), Fludarabine (6 μM) and DMSO (0.1%) for 24 h. Graphs show the number of apoptotic cells in each group of cells. Data were analyzed using Prism v5.0 (GraphPad Software, San Diego, CA, USA). All experiments were performed in triplicate. * for Fludarabine (JVM-2) compared with Fludarabine (MEC-1) group - $p < 0.05$. & for Fludarabine + Entinostat compared with Fludarabine groups - $p < 0.01$.

group (Fig. 4B and Fig. 4C).

3.3. Overexpression of HDAC1 protects MEC-1 cells for Fludarabine and Entinostat induced apoptosis

MEC-1 (control), MEC-1 (vector), and MEC-1 (HDAC1) cells were treated with Fludarabine (6 μM), Entinostat (10 μM) and Fludarabine (6 μM) combined with Entinostat (10 μM) for 24 h. The apoptosis rate was detected by flow cytometry. As shown in Fig. 4D, we confirmed apoptosis rate of the HDAC1 group significantly decreased ($p < 0.05$). These results demonstrated HDAC1 protein is able to protect MEC-1 cells from Fludarabine and Entinostat induced apoptosis.

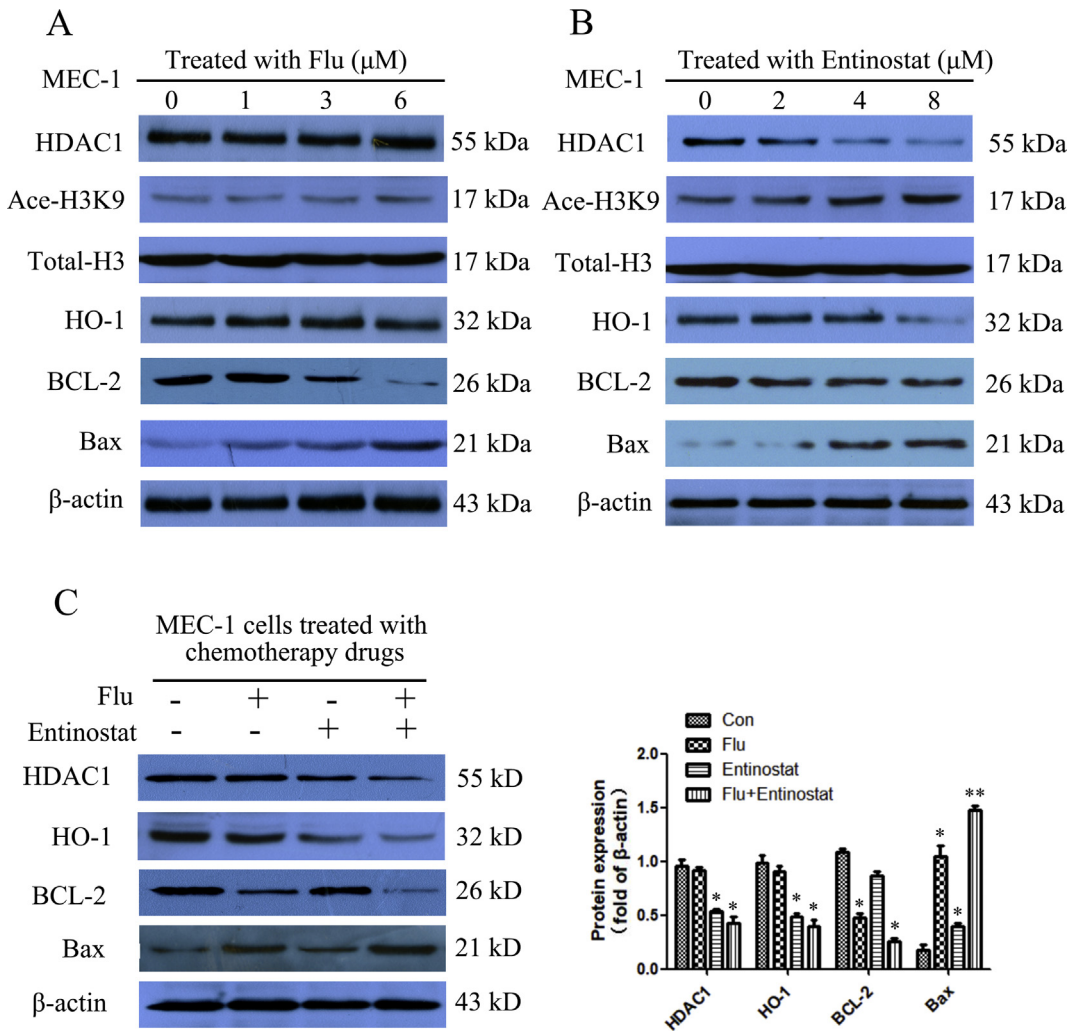

Fig. 3. Treatment of MEC-1 cells with Fludarabine, Entinostat and Fludarabine (Flu) + Entinostat significantly decreases HDAC1, HO-1 and BCL-2 protein expressions.

(A, B) MEC-1 cells treated with Fludarabine (1, 3 and 6 μM) or Entinostat (Entinostat) (2, 4 and 10 μM) for 24 h. Expression of HDAC1, phosphorylated JAK2, JAK2, phosphorylated STAT3, STAT3, phosphorylated P38, P38 and HO-1 was analyzed by western blot. (C) Combined treatment of Fludarabine (6 μM) and Entinostat (10 μM) for 24 h. Expression of HDAC1, HO-1, BCL-2 and Bax was analyzed by Western blot. Western blot bands were quantified using Quantity One software. Each sample was normalized using related β-actin expression. All experiments were performed in triplicate. *$p < 0.05$, **$p < 0.01$.

3.4. Upregulation of HDAC1 gene expression resistance to Entinostat-induced MEC-1 cell apoptosis through enhancement of the HDAC1/P38/HO-1 axis

To explore the mechanism by which HDAC1 protects MEC-1 cells for apoptosis, we examined several important pathways related to apoptosis. Firstly, MEC-1 (control), MEC-1 (Vector), and MEC-1 (HDAC1) cells were treated with Entinostat (10 μM) and DMSO (0.1%) for 24 h. HDAC1, P-JAK2, JAK2, P-STAT3, STAT3, P-P38 and P38 proteins expression were detected by western blot. The results show that HDAC1 expression was no correlation with JAK2/STAT3, but was positively correlated with the P38 pathway (Fig. 4E). Interesting, we found upregulation of HDAC1 paralleled with the level of P-P38 protein expression (Fig. 4E). In order to verify whether increased P-P38 protein expression is able to protect the MEC-1 cells from apoptosis, we treated MEC-1 (control), MEC-1 (Vector), and MEC-1 (HDAC1) cells with Entinostat (10 μM) or Entinostat (10 μM) and the P38 pathway inhibitor, BIRB (200 nM). The results show that BIRB can inhibit the P-P38 protein expression in MEC-1 cells. Moreover, our results confirmed that blocking HO-1 gene expression with small interfering RNA can not only directly lead to apoptosis of CLL cells, but also increase the chemotherapy effect of fludarabine on CLL cells. In Fig. 4F, BIRB can inhibit expression of P-P38 in MEC-1 cells and overexpression of the HDAC1 paralleled with the increases in P-P38 expression, and the activation of HO-1 protein. These results demonstrated HDAC1 confers Entinostat resistance in CLL cells via activating the P38/MAPK signaling pathway.

3.5. The combination of Fludarabine and Entinostat inhibited tumor growth in a CLL xenograft mouse model

Hypodermic MEC-1 cells (1×10^7 cells) in nude mice flank were used to establish a xenograft mouse model of CLL. The mice were treated every day with 0.9% saline, 40 mg/kg Fludarabine, 50 mg/kg Entinostat and Fludarabine combined with Entinostat (40 mg/kg Flu and 50 mg/kg, respectively) when the tumors were palpable (day 12). The xenograft mouse models of CLL were then euthanized on the 14th day after treatment with Fludarabine and Entinostat. Measurements were taken and tumor volumes were calculated (Fig. 5A). Compared with the Fludarabine and Entinostat treated groups, the control groups of the xenograft CLL mouse models treated with normal saline developed tumors more rapidly (Fig. 5B and Fig. 5C). Meanwhile, Fludarabine group was slower growth rate of mouse tumors than Entinostat group (Fig. 5B and Fig. 5C). Furthermore, combination of Fludarabine

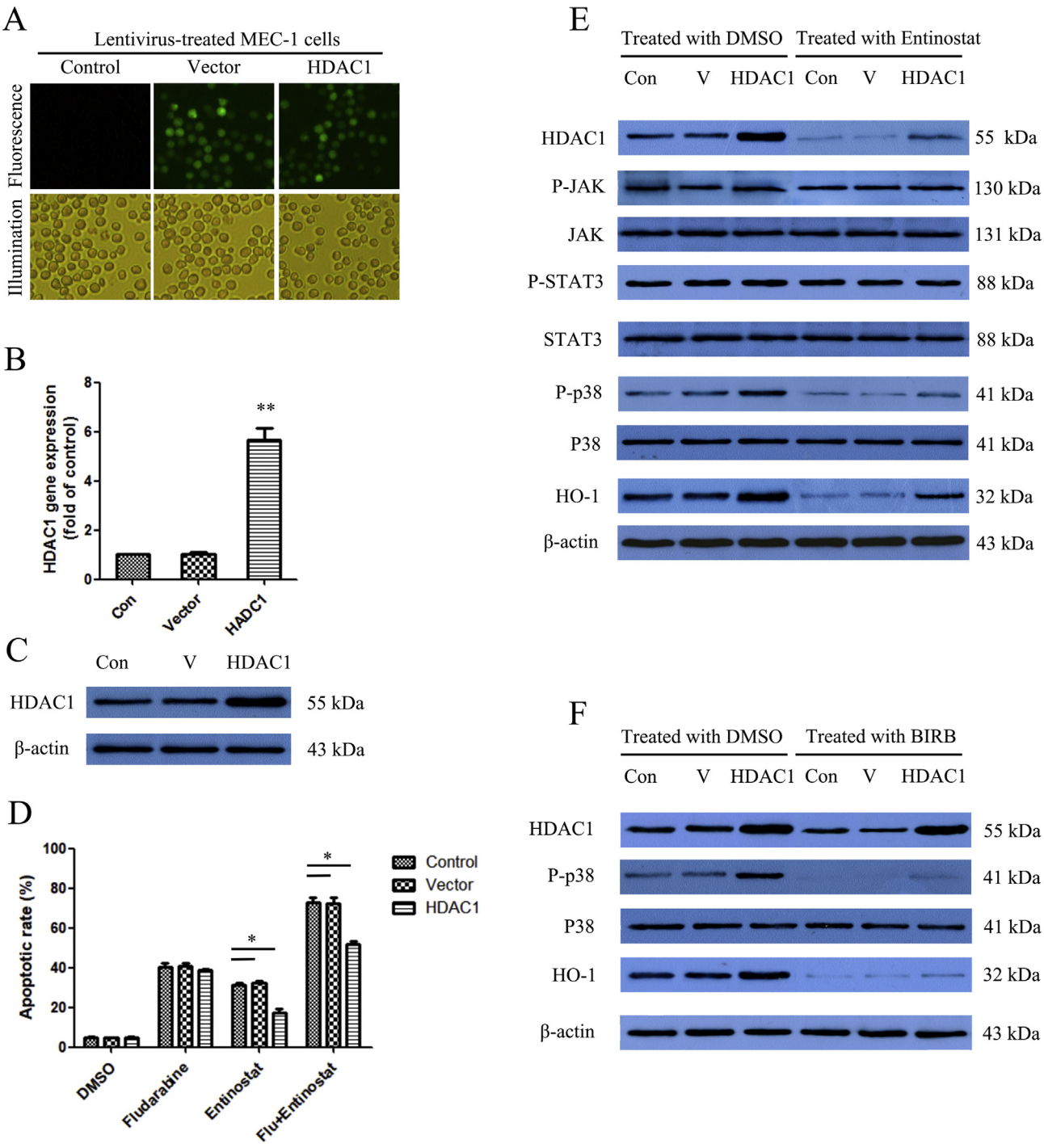

Fig. 4. Upregulation of HDAC1 gene expression induces resistance of MEC-1 cells to apoptosis induced through Entinostat treatment.
(A) MEC-1 cells were grouped as MEC-1 (control), MEC-1 (vector1), and MEC-1 (HDAC1). The positivity of lentivirus-mediated HDAC1 transduction (> 95%) was observed by fluorescence microscopy. (B) HDAC1 gene expression was detected using real-time PCR. (C) HDAC1 protein expression was analyzed by western blot. (D) MEC-1 (control), MEC-1 (Vector), and MEC-1 (HDAC1) cells were treated with Fludarabine (6 μM), Entinostat (10 μM) and DMSO (0.1%) for 24 h. The apoptosis rate was detected by flow cytometry. Graphs show the number of apoptotic cells in each group of MEC-1 cells. (E) MEC-1 (control), MEC-1 (Vector), and MEC-1 (HDAC1) cells were treated with Entinostat (10 μM) and DMSO (0.1%) for 24 h. Expression of HDAC1, phosphorylated JAK2, JAK2, phosphorylated STAT3, STAT3, phosphorylated P38, P38 and HO-1 proteins was analyzed by Western blot. (F) MEC-1 (control), MEC-1 (Vector), and MEC-1 (HDAC1) cells were treated with a P38 inhibitor (BIRB, 200 nM) and DMSO (0.1%) for 24 h. Expression of HDAC1, phosphorylated P38, P38 and HO-1 proteins was analyzed by western blot. Each sample was normalized using related β-actin expression. All experiments were performed in triplicate. *$p < 0.05$, **$p < 0.01$.

and Entinostat was able to significantly reduce the tumor volume in the xenograft mouse model of CLL (Fig. 5D). At the same time, we observed that overall survival was prolonged in the CLL mouse model treated with Fludarabine or Entinostat, especially in the group in which Fludarabine was combined with Entinostat ($p < 0.05$) (Fig. 5E).

3.6. HDAC1, HO-1, BCL-2 and Bax gene and protein in the CLL xenograft mouse model

Consistent with vitro experiment, the expression of the HDAC1 gene and protein in tumors of the Fludarabine treatment group were not

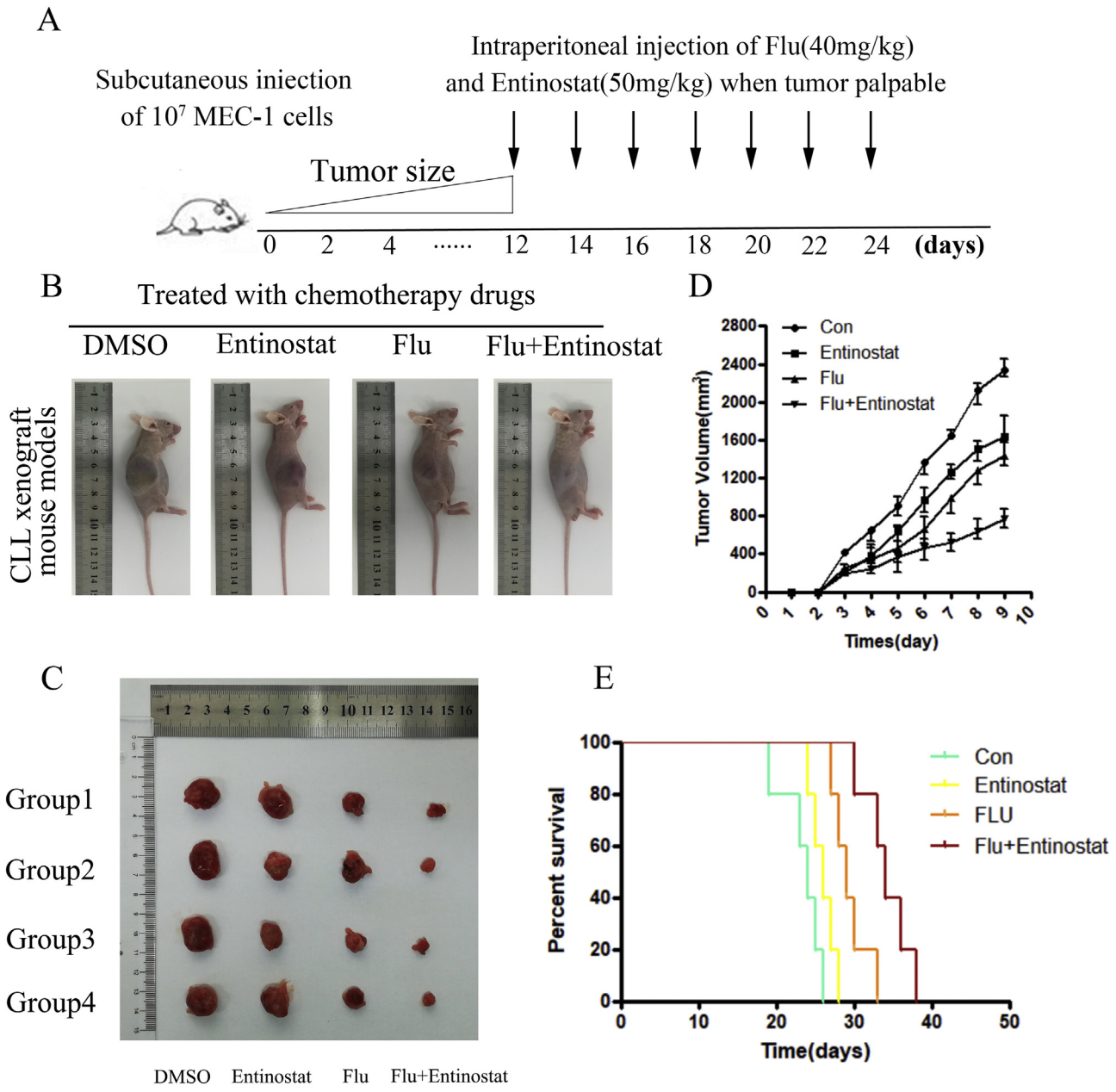

Fig. 5. Combination of Fludarabine and Entinostat can significantly inhibit tumor growth in a xenograft mouse model.
(A-C) Nude mice were subcutaneously inoculated with MEC-1 cells (1×10^7 cells) in flank to establish xenograft mouse models of CLL. The mice were treated with 0.9% saline, 20 mg/kg Fludarabine, 50 mg/kg Entinostat and Fludarabine in combination with Entinostat (20 mg/kg Fludarabine and 50 mg/kg Entinostat) once a day when tumors were palpable (day 12). The xenograft mouse models of CLL were euthanised on the 14th day after treatment with Fludarabine or/and Entinostat. (D) Measurements were taken using a ruler and tumor volumes were calculated. (E) Survival curves of each individual group were evaluated from the first day of treatment until death using Kaplan-Meier curves. The results are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$.

significantly different from that of the control, yet BCL-2 significantly decreased, while Bax significantly increased. Meanwhile, the expression of the BCL-2 in tumors of the Entinostat treatment group did not show significant increased, but the expression of the HDAC1 and HO-1 significantly reduced. Interestingly, compared with the other three groups, the HDAC1, HO-1 and BCL-2 in tumor tissues of the Fludarabine combined with Entinostat treatment group of the CLL model significantly decreased, while the expression of the Bax gene and protein significantly increased (Fig. 6).

4. Discussion/Conclusion

Histone deacetylases (HDACs) is a potential therapeutic target in CLL [27–29,31,32]. In recent years, we found more and more novel histone deacetylase inhibitors (HDACi) have been developed [33]. Vorinostat is the first FDA-approved HDACi, which has anti-tumor effects in various malignancies, especially in lymphomas [34–36]. However, Vorinostat compared with other HDAC inhibitors has poor HDACs selectivity and more side effects. Recent research has shown that HDAC1, -2, and -3 are frequently overexpressed in human tumors, and the knockdown of HDAC1 is sufficient to reduce tumor growth in vivo [37]. Entinostat is an orally available potent HDAC1 targeted inhibitor that has been shown to display anti-tumor activity in hematological malignancies (acute myeloid leukemia, chronic myelomonocytic leukemia and multiple myeloma) [38,39]. Importantly, Entinostat is a highly suitable HDAC1 inhibitor for incorporation into currently

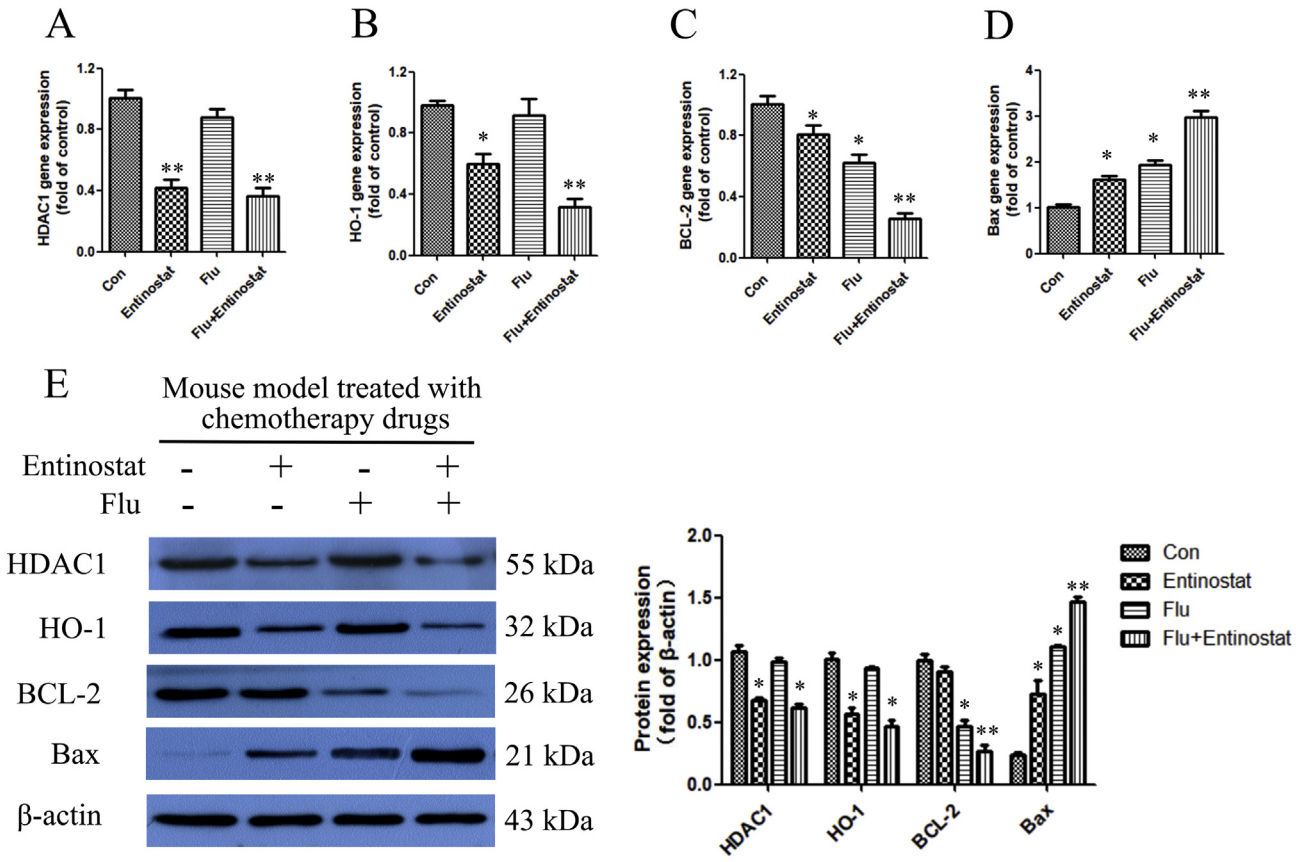

Fig. 6. Combination of Fludarabine and Entinostat can significantly inhibit HDAC1, HO-1 and BCL-2 protein expression in a xenograft mouse model.
(A-E) HDAC1, HO-1, BCL-2 and Bax expression in the xenograft mouse model tumor tissue were analyzed by real-time PCR or western blot. Each sample was normalized by related β-actin expression. Western blot bands were quantified using Quantity One software. All experiments were performed in triplicate. *$p < 0.05$, **$p < 0.01$.

available drug-resistance reversing regimens [40]. Moreover, many studies also shown Entinostat combined with other drugs have synergistic antitumor effects [40–43]. Therefore, Entinostat is a potentially potent therapeutic drug for CLL patients, which may lead to better clinical efficacy and fewer clinical side effects.

Our previous study found that inhibition of HO-1 could increase apoptosis in CLL cell (Supplement Fig. 5). Interestingly, in this study we found that Entinostat can also induced apoptosis and significantly inhibit HO-1 expression in CLL cells. And the mechanism by which Entinostat induces apoptosis in CLL cells is not yet fully elucidated. So, this study focuses on the mechanism of the Entinostat combined with Fludarabine significantly induced apoptosis of TP53-mutant CLL cells. Likewise, many studies have confirmed HDACi can inhibit HDACs expression, restore the expression of the HO-1 protein and lead to apoptosis of haematolological tumor cells [22,44,45]. In order to understand the underlying mechanism of Entinostat combined with Fludarabine significantly induced apoptosis of TP53-mutant CLL cells. We used lentivirus to upregulation HDAC1 gene expression in MEC-1 cells, and confirmed HDAC1 overexpression causes resistance to the effect of Entinostat on MEC-1 cell apoptosis in vitro. Similarly, some studies demonstrated that HDAC1 plays a key role in transcriptional regulation and is involved in the pathogenesis of malignancies [46,47]. HDAC1 has a unique role in modulating the transcriptional activities of nuclear receptors, leading to chromatin condensation and gene expression regulation, and confirmed HDAC1 integrates the expression of TP53 mutants in pancreatic cancer [48]. We also found that HDAC1 is highly expressed of TP53-mutated CLL patients in TGCA database. So, we speculate that HDAC1 is a potential target for reducing the chemotherapeutical resistance of TP53-mutant CLL patients.

In this study, we found that enhancing HDAC1 protein expression increased the phosphorylation of P38 protein expression, while a P38 inhibitor (BIRB, 200 nm) can reverse the above phenomenon. Yet, enhancing HDAC1 protein expression had no significant effect on JAK2 and STAT3 pathway. These results demonstrated that HDAC1 could resistant Entinostat-induced apoptosis in TP53 mutations CLL cells by activating the P38 pathway. P38 mitogen-activated protein kinase (P38/MAPK) pathway is closely related to CLL B-cell biology and apoptosis, such as in 2003, Pepper reported that EB1089 (a novel vitamin D3 analog), which is potential drugs as a treatment for CLL, importantly, EB1089-induced apoptosis was associated with activation of P38/MAPK and lead to activation of downstream caspase-3 pathway in CLL cells [49]. Similarly, in 2004 year, Ringshausen and colleague confirmed P38/MAPK pathway was constitutively activated in B-CLL. In addition, researchers demonstrated that MMP-9 protein is elevated in the serum of CLL patients and correlate with an unfavorable prognosis. Constitutive expression of MMP-9 was dependent on P38-activity and inhibition of P38 strongly down regulated MMP-9 expression, and demonstrated that P38/MAPK pathway play a crucial role in CLL biology [50]. In 2013, Bai found OSU-DY7, a novel D-tyrosinol derivative targeting P38/MAPK, which can mediates cytotoxicity in lymphocytic cell lines representing CLL (MEC-1), Burkitt lymphoma (Raji and Ramos) and primary B cells from CLL patients in a dose- and time-dependent manner. OSU-DY7-induced cytotoxicity is dependent on caspase activation, as evidenced by induction of caspase-3 activation and poly (ADP-ribose) polymerase (PARP) cleavage and rescue of cytotoxicity by *Z*-VAD-FMK. Interestingly, OSU-DY7-induced cytotoxicity is mediated through activation of P38/MAPK pathway, as evidenced by increased phosphorylation of P38 protein expression [51]. In recent years, Paiva

Table 1
Characteristics of CLL patients.

Sample	Age	Sex	ALC (/L)	HGB (g/L)	PLT (/L)	Binet stage	Rai stage	Cytogenetic
1	52	F	6.5×10^9	149.5	292×10^9	A	0	TP53 wild type
2	67	M	8.1×10^9	136.2	206×10^9	A	0	TP53 wild type
3	56	M	17.2×10^9	175.1	332×10^9	A	I	TP53 wild type
4	69	F	6.2×10^9	136.5	192×10^9	A	II	TP53 wild type
5	60	M	9.7×10^9	152.8	252×10^9	A	I	TP53 wild type
6	53	F	11.2×10^9	123.3	122×10^9	B	II	TP53 wild type
7	72	M	37.6×10^9	52.9	32×10^9	C	IV	TP53 mutation
8	67	F	17.5×10^9	81.6	81×10^9	C	III	TP53 mutation
9	72	M	47.7×10^9	103.1	85×10^9	B	III	TP53 mutation
10	63	M	217.2×10^9	85.9	61×10^9	C	IV	TP53 mutation
11	83	M	37.1×10^9	96.7	75×10^9	C	III	TP53 mutation
12	48	F	136.3×10^9	75.5	63×10^9	C	IV	TP53 mutation

Note: Absolute Lymphocyte Count - ALC; Hemoglobin - HGB; Platelets - PLT.

reported that P1446A (cyclin-dependent kinase inhibitor) induces apoptosis in P38/MAPK-dependent manner in CLL cells [52]. We also confirmed that upregulation of phosphorylation-P38 protein can protects MEC-1 cells from fludarabine-induced apoptosis. In summary, P38/MAPK pathway plays an important role in the biological processes of CLL cells. Therefore, we speculate that HDAC1 may affect the P38/MAPK pathway, leading to the upregulation HO-1 protein expression and resistance of MEC-1 cells to apoptosis (Fig. 4). However, it is necessary to further confirm the effect of HDAC1 gene knockout in a mouse model.

In vivo, Entinostat combined with Fludarabine efficiently enhanced the effects of chemotherapy, resulting in significantly prolonged survival time and decreased HDAC1, HO-1 and BCL-2 protein expressions (Fig. 5). These results further demonstrated that Entinostat is able to enhance the ability of Fludarabine induced apoptosis of MEC-1 cells, and there is synergetic action between them. In conclusion, Entinostat is a potential epigenetic therapeutic drug and this study provides valuable preclinical experimental evidence for the treatment of poor prognosis of CLL patients, especially for TP53-mutated patients (Table 1).

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Author contributions

Jishi Wang, Zhen Zhou and Dan Ma conceived and designed the study. Peifan Li, Dan Ma, Zhengchang He, Nana Zhe, Mei Ren, Bingqing Chen, Danna Wei, Qin Zhong, Jun Wang and Qin Fang performed research. Peifan Li and Qin Fang made substantial contributions to interpretation of data. Jun Wang and Zhen Zhou drafted the original manuscript. All authors were involved in critically revising the manuscript for important intellectual content and gave final approval of the published version.

Statement of ethics

In the animal experiments section, all procedures were conducted in accordance with Guidelines for the Care and Use of Laboratory Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of Guiyang Medical University (NO: 1801121), and this study was approved by the Ethics of Human Investigation Committee of Guizhou Medical University (NO: 20160002).

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Appendix A. Supplementary data

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