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Research Article

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HDAC inhibitor chidamide synergizes with venetoclax to inhibit the growth of diffuse large B-cell lymphoma via down-regulation of MYC, BCL2, and TP53 expression

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Abstract: Diffuse large B-cell lymphoma (DLBCL) is an aggressive type of non-Hodgkin's lymphoma. A total of 10%–15% of DLBCL cases are associated with myelocytomatosis viral oncogene homolog (*MYC*) and/or B-cell lymphoma-2 (*BCL2*) translocation or amplification. BCL2 inhibitors have potent anti-tumor effects in DLBCL; however, resistance can be acquired through up-regulation of alternative anti-apoptotic proteins. The histone deacetylase (HDAC) inhibitor chidamide can induce BIM expression, leading to apoptosis of lymphoma cells with good efficacy in refractory recurrent DLBCL. In this study, the synergistic mechanism of chidamide and venetoclax in DLBCL was determined through in vitro and in vivo models. We found that combination therapy significantly reduced the protein levels of MYC, TP53, and BCL2 in activated apoptotic-related pathways in DLBCL cells by increasing BIM levels and inducing cell apoptosis. Moreover, combination therapy regulated expression of multiple transcriptomes in DLBCL cells, involving apoptosis, cell cycle, phosphorylation, and other biological processes, and significantly inhibited tumor growth in DLBCL-bearing xenograft mice. Taken together, these findings verify the in vivo therapeutic potential of chidamide and venetoclax combination therapy in DLBCL, warranting pre-clinical trials for patients with DLBCL.

Key words: Diffuse large B-cell lymphoma (DLBCL); Histone deacetylase (HDAC) inhibitor; Venetoclax; MYC; BCL2; TP53

1 Introduction

Diffuse large B-cell lymphoma (DLBCL) is a subtype of heterogeneous and aggressive non-Hodgkin's lymphoma (Li et al., 2018). Myelocytomatosis viral oncogene homolog (MYC) and B-cell lymphoma-2 (BCL2) proteins are up-regulated in 20%–30% of DLBCL cases, irrespective of the *MYC* and *BCL2* chromosomal rearrangement. This is called double-expressed lymphoma (DEL) and has a poor prognosis (Swerdlow et al., 2016; Riedell and Smith, 2018). In addition, based on genetic and proteomics studies, 7%–10% of DLBCLs harbor *MYC*, *BCL2*, and/or *BCL6* rearrangements, and are known as double-hit lymphoma (DHL) or triple-hit lymphoma (Sarkozy et al., 2015; Burotto et al., 2016; Rosenthal and Younes, 2017). The characteristics of DEL and DHL are rapid clinical progression that is refractory to aggressive treatment and has poor outcome following standard R-CHOP (rituximab plus cyclophosphamide, doxorubicin vincristine, and prednisone) therapy (Nowakowski et al., 2016; Friedberg, 2017). Therefore, current therapeutic outcomes are not ideal, and development of new targeted therapies is imperative. In recent years, there have been several novel studies on the treatment of DLBCL, using approaches such as bispecific chimeric antigen receptor (CAR)-T cell therapy, or monoclonal antibodies like tafasitamab and loncastuximab tesirine to target specific cell-surface antigens for direct cytotoxic activity, or altering immune-mediated mechanisms (Huang et al., 2020; Patriarca and Gaidano, 2021). It is also worth noting that some

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new small-molecule inhibitors have shown promise as treatments for DLBCL. For example, a recent two-part phase-I/II trial of a Bruton's tyrosine kinase (BTK) inhibitor in patients with relapsed/refractory (R/R) B cell malignancies, including DLBCL, showed an overall response rate (ORR) of 50% and a disease control rate of 78%; and two patients achieved a complete response (CR).

Tumorigenesis factors of hematological tumor diseases include genetic aberrations. For instance, B- or T-cell lymphoma occurrence is reportedly driven by epigenetic disorders (Rodríguez-Paredes and Esteller, 2011; Sermer et al., 2019). DLBCL pathogenesis is also strongly related to epigenetic perturbations, and high epigenomic heterogeneity correlates with a higher relapse rate and poorer outcome (Pan et al., 2015; Morin et al., 2016). Nevertheless, histone deacetylase (HDAC) has been indicated as a key enzyme in epigenetic regulation. Previous studies have shown that HDAC inhibitors are effective in treating refractory relapsed DLBCL by reducing expression of *c-Myc* and increasing phosphorylation of TP53 (Heideman et al., 2013; Santoro et al., 2013; Adams et al., 2016). Specifically, chidamide, an oral HDAC inhibitor, selectively inhibited HDAC1–3 and HDAC10 (Ning et al., 2012; Xu et al., 2017), and has been approved for treatment of R/R peripheral T-cell lymphoma (Chan et al., 2017; Shi et al., 2017). Furthermore, both in vitro and in vivo studies have shown that chidamide has good antitumor effects on lymphoma, leukemia, and multiple myeloma (Li et al., 2017; Yuan et al., 2019).

Targeting anti-apoptotic pathways involving BCL2 family proteins represents a treatment strategy for hematologic malignancies (Hata et al., 2015). BCL2 is an anti-apoptotic protein and an important determinant of cell survival in various hematological malignancies (Hata et al., 2015; Croce and Reed, 2016; Perini et al., 2018). The oral treatment venetoclax (ABT-199), a highly selective small-molecule BH3 mimetic with an even greater affinity for BCL2 but a lower affinity for (BCL-XL, has shown clinical potential in hematologic malignancies (Souers et al., 2013; Vandenberg and Cory, 2013). However, monotherapy has rarely been sufficient to provide sustained therapeutic responses.

As *BCL2* overexpression is synergistic with *MYC* and other oncogenes in promoting both progression of lymphoma and resistance to chemotherapy, targeting these genes along with epigenetic regulation might have therapeutic potential (Berendsen et al., 2020). In this study, we sought to evaluate potential anti-tumor synergistic effects of a regimen combining chidamide with ABT-199 in DLBCL.

2 Materials and methods

2.1 Data acquisition and processing

Data of 47 lymph node samples from patients with DLBCL were obtained from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov), and the whole-blood-cell sample data of 337 healthy subjects were obtained from the Genotype-Tissue Expression (GTEx) database (https://commonfund.nih.gov/GTEx). The datasets were matched using the "SVA" package in R software (Version 3.61) and batch effects were removed. The "limma" package was used to analyze the differences between the two datasets, and $|\log_2(\text{fold-change})| \geqslant 1$ with $P<0.05$ was used to determine the significant expression changes in *BCL2* and *HDAC* genes. We used the "clusterProfiler" package for gene function enrichment analysis and pathway enrichment analysis of these genes, and conducted correlation analysis of *BCL2* and *HDAC* family genes. B-cell receptor (BCR) pathway genes were obtained from the gene set enrichment analysis (GSEA) website (http://www.gsea-msigdb.org/gsea/index.jsp). DLBCL-related data were selected in cBioPortal (http://cbioportal.org), and the gene mutation option was selected from the sub item column to view the mutation of the target gene.

2.2 Cell lines and culture

The DLBCL cell line SUDHL-4 (*MYC*/*BCL2* positive in DLBCL cells with wild type (WT)-*TP53*) (Figs. S1a–S1c) was kindly provided by the Stem Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The DLBCL cell line DB (*MYC/BCL2*-rearranged DHL cells with mutant (MUT)-*TP53*) (Figs. S1d–S1f) was purchased from Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China). Both cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 complete medium at 37 °C in a 5% CO_2 humidified incubator, authenticated by short tandem repeat (STR) profiling, and tested for mycoplasma contamination.

2.3 Cell viability assay

Cells were inoculated into 96-well plates and treated with the designated drug, and cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was added after 24, 48, or 72 h of action. The absorbance was read at 450 nm with a microplate analyzer (Thermo Fisher Scientific, USA).

2.4 Flow cytometry

Cells were inoculated into six-well plates and cultured as mentioned above. Cells were then collected and stained for either Annexin V or prodium iodide (PI) (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Then, the cells were placed on a flow cytometer (BD FACSCanto, Franklin Lakes, NJ, USA) for detection of apoptosis.

2.5 Western blotting

Cells were inoculated into a culture flask and treated with the designated drug for 72 h. Cells were collected, total proteins were extracted using cell lysates (Sigma-Aldrich, St. Louis, MO, USA), and samples were quantified using a bicinchoninic acid (BCA) kit (ServiceBio, Wuhan, China). The same amount of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The PVDF membrane was sealed with 5% (0.05 g/mL) skim milk and incubated overnight with a primary antibody at 4 ℃. Following the wash steps, the PVDF membrane was incubated with a secondary antibody for 70 min and finally detected with a chemiluminescence substrate.

2.6 RNA sequencing analysis

The SUDHL-4 and DB cell lines were treated with drugs for 72 h, and then divided into the following groups: control, chidamide single-drug, venetoclax single-drug, and combination. We collected three biological replicates of RNA samples, and used total RNA for RNA sequencing (RNA-seq) analysis. Construction and sequencing of the complementary DNA (cDNA) library were conducted by the Wuhan Medical Laboratory of BGI using the DNBseq platform. The Bowtie 2 tool was used to compare the high-quality reads with the human reference genome (GRCh38). Using the expected maximization RNA-seq, the expression of each gene was normalized to the fragments per kilobase of transcript per million mapped reads (FPKM) exon model. The BGI bioinformatics service (https://biosys.bgi.com) used the combination of $\log_2$(ratio absolute value)≥1 and P≤0.05 to determine the significance of the differentially expressed genes (DEGs).

2.7 Xenograft mouse model of human DLBCL

This study used six-week-old female BALB/c nude mice (purchased from Hunan SJA Laboratory Animal Co., Ltd., China). Ethical approval for this study was obtained from the Medical Research Ethics Committee of the Second Affiliated Hospital of Nanchang University. Mice were subcutaneously inoculated with 1×10^7 SUDHL-4 cells on the right dorsal side and monitored daily. After two weeks, tumor-bearing mice (tumor size>100 mm^3) were randomly divided into four groups (five mice per group) receiving daily oral treatments: (1) control group (dimethylsulfoxide (DMSO), distilled water); (2) chidamide group (15 mg/kg); (3) venetoclax group (50 mg/kg); (4) combination group (15 mg/kg chidamide and 50 mg/kg venetoclax). Tumor size was measured daily to monitor treatment effectiveness. The mice were sacrificed on Day 21 of treatment and the tumors collected for further analysis.

2.8 Immunohistochemistry

The biopsy specimens of DLBCL xenograft mice were fixed with formalin, embedded with paraffin, and resected (thickness=4 μm). The slices were dewaxed in xylene and rehydrated into water by gradient ethanol. All sections were treated with 5 mmol/L citrate buffer (pH=6.0) for anti-genic repair and treated with 3% (volume fraction) H_2O_2 to inactivate endogenous peroxidase. After sealing for 30 min, sections and primary antibodies were incubated overnight at 4 ℃. After washing, sections were stained with secondary antibodies at 25 ℃ for 30 min. Diaminobenzidine and hematoxylin were used as either chromogenic substrates or for nuclear complex staining.

2.9 Statistical analysis

Data are presented as mean±standard deviation (SD) or median (quartile intervals). GraphPad Prism (Version 8.0) was used for statistical analysis, processing of image data, and calculating half maximal inhibitory concentration (IC_{50}) data. Student's t-test

was used for pair-wise comparison between groups, and one-way analysis of variance (ANOVA) was used for multivariate mean comparison. CompuSyn (Version 3.0.1) was used to calculate the dose–effect curves and generate the combination index (CI). $P<0.05$ was considered statistically significant.

3 Results

3.1 Correlation between *BCL2* and *HDAC* in DLBCL

By comparing 47 lymph-node sample data from patients with DLBCL with 337 normal whole-blood B-lymphocyte sample data, we obtained 2408 down-regulated and 2601 up-regulated genes (Fig. 1a). Expression of *HDAC4*, *HDAC5*, and *HDAC10* decreased in lymphoma, while expression of *HDAC1*, *HDAC7*, *HDAC9*, and *BCL2* increased in lymphoma tissue (Figs. 1a and 1b). The Gene Ontology (GO) enrichment analysis of the seven genes above had shown that these genes were closely related to the process of histone deacetylation (Fig. 1c). By using cBioPortal, we were able to find high mutation rates (including somatic mutations, DNA copy number alterations, and gene abundance changes) for *BCL2*, *HDAC4*, *HDAC7*, *HDAC9*, and *HDAC10* in the DLBCL samples (Fig. 1d). Finally, the correlation analysis of the seven genes showed that *HDAC1*, *HDAC7*, *HDAC9*, and *HDAC10* were strongly correlated with *BCL2* (Fig. 1e).

3.2 Effects of chidamide and venetoclax on proliferation of DLBCL cells

We examined the sensitivity of chidamide and venetoclax in DLBCL cell lines. First, we assessed the effects of different doses of chidamide or venetoclax monotherapy on SUDHL-4 and DB cells. The single-dose agents showed dose- and time-dependent proliferative inhibitory effects on both cell lines (Fig. 2a). Chidamide had the maximum inhibitory effect on both cell lines at 48 and 72 h, while venetoclax's maximum inhibitory effect occurred at 72 h. The IC_{50} values were calculated at 24 h (Fig. 2b). Next, we assessed the effect of drug combination on SUDHL-4 and DB cells over 24 h. Based on the Chou-Talalay method for drug combination (Chou, 2010), we set constant-ratio drug combinations using two-fold serial dilutions with several concentration points above or below the 24 h IC_{50} value. The results indicated that the inhibitory effect of the combination of chidamide with venetoclax was higher than those of the single agents (Fig. 2c). We graphically analyzed the CI value according to the Chou-Talalay method, as illustrated in Fig. 2c (right). At 50% cell proliferation (fraction affected (F_a)=0.5), the CI values were 0.7 and 0.3 in the SUDHL-4 and DB cell lines, respectively, suggesting strong synergism (CI<1) of the two drugs.

3.3 Effects of combination of chidamide and venetoclax on apoptosis in DLBCL cells and cell cycle at the G0/G1 phase

To further explore the synergistic effects of drug combinations, we first conducted apoptosis detection after combined drug treatments. As shown in Figs. 3a and 4a, SUDHL-4 cells were treated with chidamide (2 μmol/L) and venetoclax (0.5 μmol/L) for 24, 48, and 72 h; DB cells were treated with chidamide (5 μmol/L) and venetoclax (0.05 μmol/L) for 24, 48, and 72 h; and DMSO was used as the negative control. Either drug on its own could induce apoptosis of SUDHL-4 and DB cells, but the rate of apoptosis induced by either chidamide or venetoclax was significantly lower than that induced by a combination of the two drugs ($P<0.05$; Figs. 3b and 4b). Next, we exposed the corresponding cell lines to the same drug concentration as in the apoptosis assay for 72 h, followed by flow cytometry detection for cell-cycle distribution. The results showed that the combination of the two drugs could block progression of the DLBCL cell cycle in the G0/G1 phase (Figs. 3c, 3d, 4c, and 4d).

3.4 Effects of chidamide on transcriptomes in multiple genes and signaling pathways in DLBCL cells, including *MYC*, *BCL2*, and *HDAC* mRNA

The acetylated state of histones can affect gene silencing and expression; therefore, we measured transcriptome levels. As shown in Fig. 5a, for DB cells, chidamide regulated 6809 genes, venetoclax only 15 genes, and the combination group, a total of 6646 genes. For SUDHL-4 cells, chidamide regulated 4272 genes, venetoclax regulated 1041 genes, and the combination group, a total of 5200 genes. Therefore, it was evident that regulation of genes in the two DLBCL cell lines was primarily mediated by chidamide.

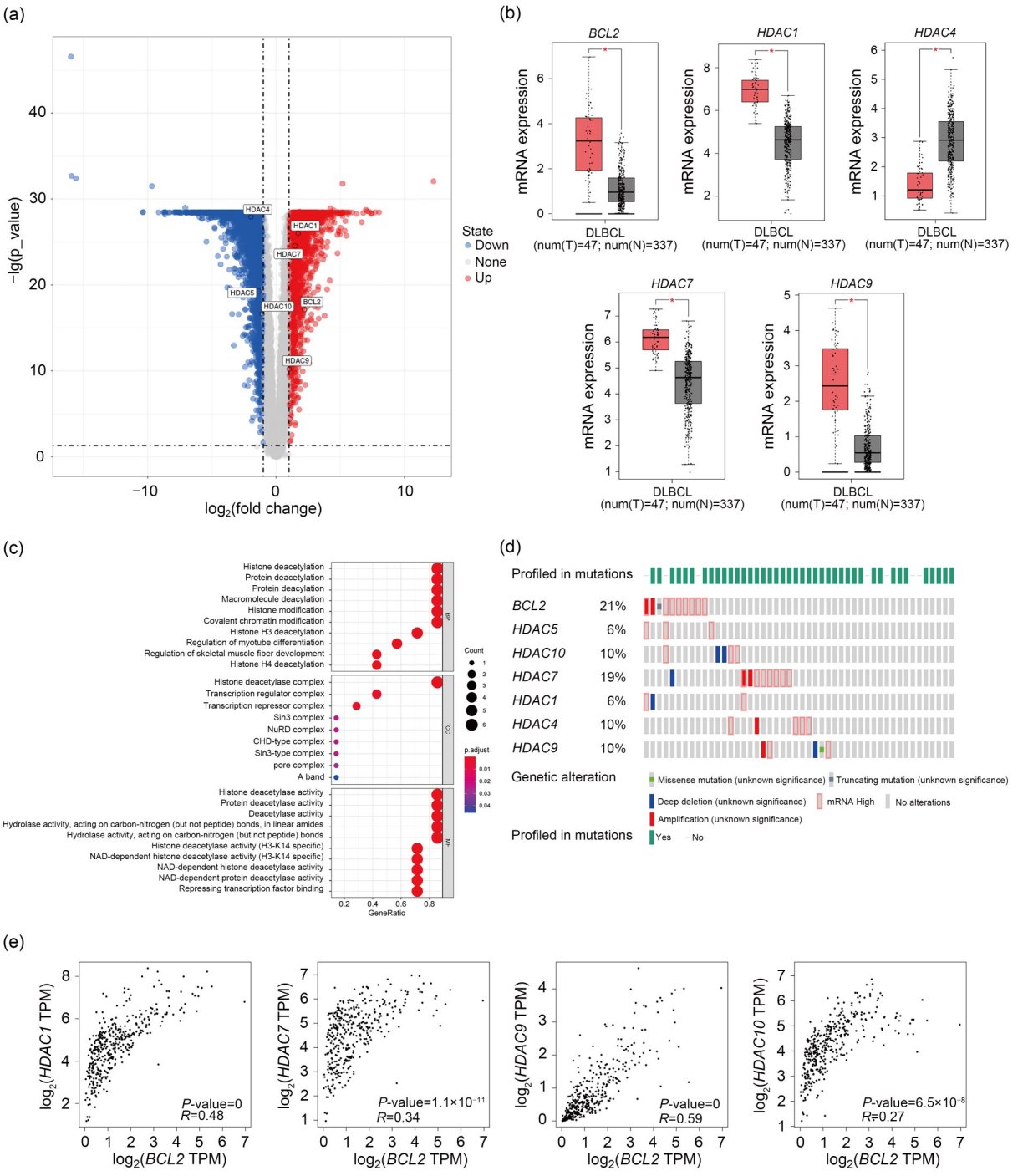
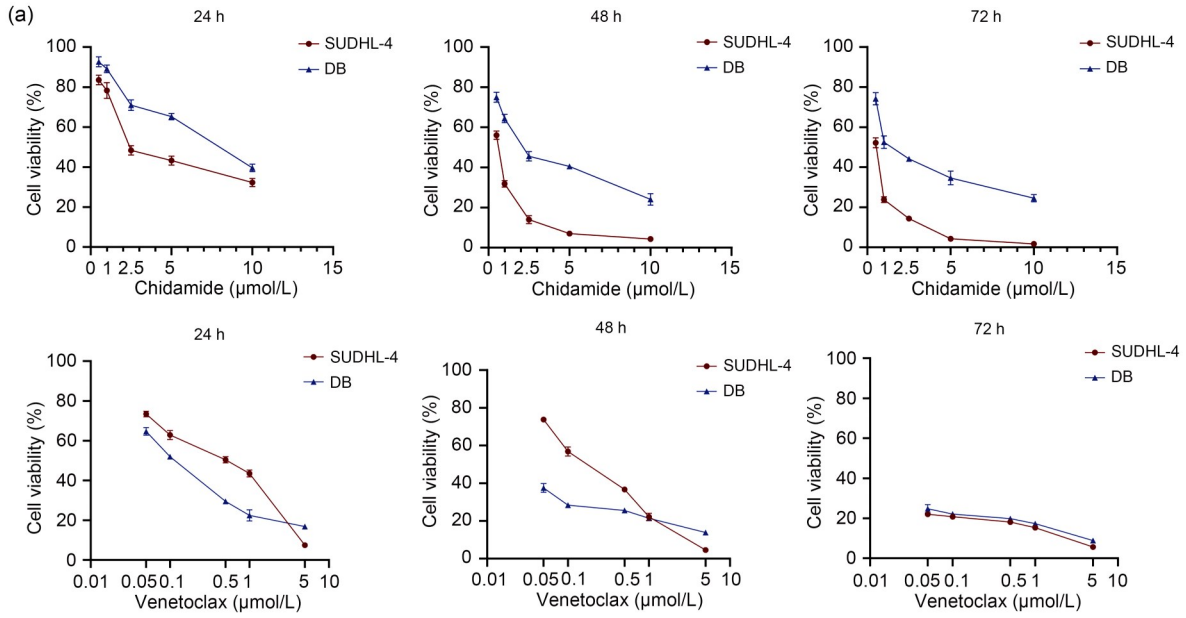


Fig. 1 Correlation between *BCL2* and *HDAC* genes in DLBCL. Comparison of 47 DLBCL patient lymph-node samples (T) with 337 normal B-lymphocyte samples (N). (a, b) Expression of *HDAC4*, *HDAC5*, and *HDAC10* decreased in DLBCL among 2408 down-regulated genes and 2601 up-regulated genes, while *HDAC1*, *HDAC7*, *HDAC9*, and *BCL2* were elevated. (c) The main biological functions of *HDAC1*, *HDAC4*, *HDAC5*, *HDAC7*, *HDAC9*, *HDAC10*, and *BCL2* genes (bubbles represent number of genes; color represents corrected *P* adjustment). (d) The mutation rates of *BCL2*, *HDAC4*, *HDAC7*, *HDAC9*, and *HDAC10* were higher in DLBCL tissue. (e) Correlation analyses of these seven genes showed that *BCL2* was strongly correlated with *HDAC1*, *HDAC7*, *HDAC9*, and *HDAC10*. Data are presented as median (quartile intervals). $^{*}P<0.05$. BCL2: B-cell lymphoma-2; *HDAC*: histone deacetylase; DLBCL: diffuse large B-cell lymphoma; TPM: transcripts per million.



(b)

Cell line	24 h IC_{50} value (μmol/L)	
	Chidamide	Venetoclax
SUDHL-4	3.278	0.405
DB	7.746	0.134

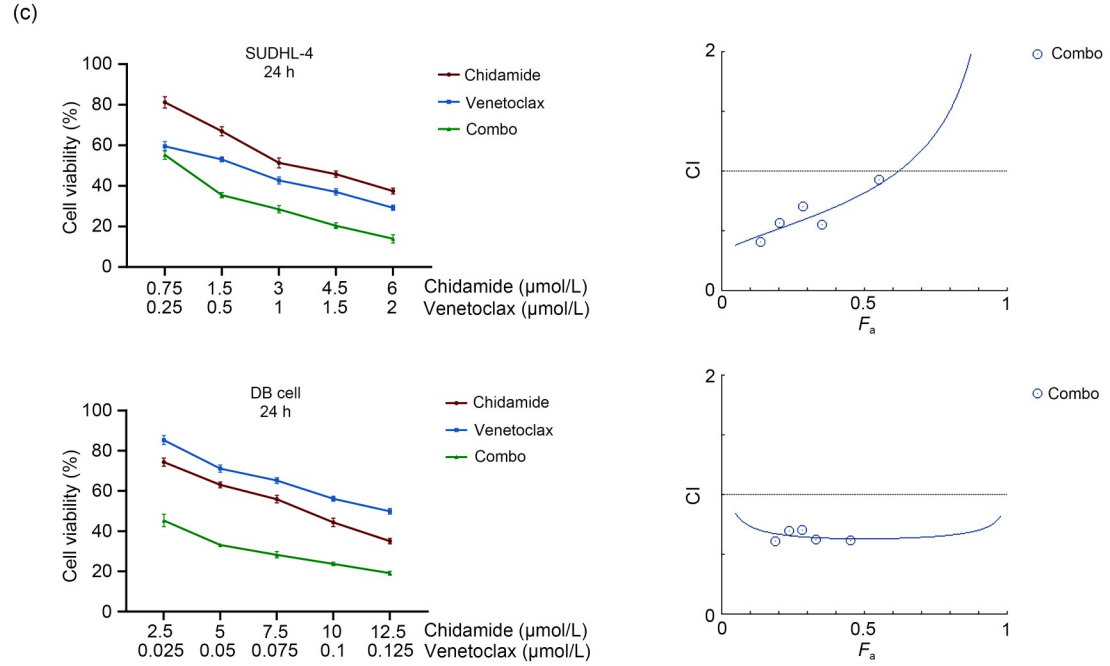


Fig. 2 Effects of chidamide or venetoclax on DLBCL cells. (a) SUDHL-4 and DB cell lines exposed to chidamide (0.5, 1, 2.5, 5, or 10 μmol/L) or venetoclax (0.05, 0.1, 0.5, 1, or 5 μmol/L) for 24, 48, or 72 h. Cell viability was detected by CCK-8 assay. (b) IC_{50} values calculated with linear regression based on CCK-8 data. (c) SUDHL-4 and DB cells treated with a constant-ratio drug combination for 24 h. The combo group was compared with the chidamide (2.5, 50, 7.5, 10, or 12.5 μmol/L) or venetoclax (0.025, 0.05, 0.075, 0.1, or 0.125 μmol/L) group. CI was calculated by CompuSyn (Version 3.0.1). Additive effect (CI=1), synergism (CI<1), and antagonism (CI>1). Data are presented as mean±standard deviation (SD). Data represent at least three independent experiments. DLBCL: diffuse large B-cell lymphoma; CCK-8: cell counting kit-8; IC_{50}: half maximal inhibitory concentration; CI: combination index; Combo: combination group.

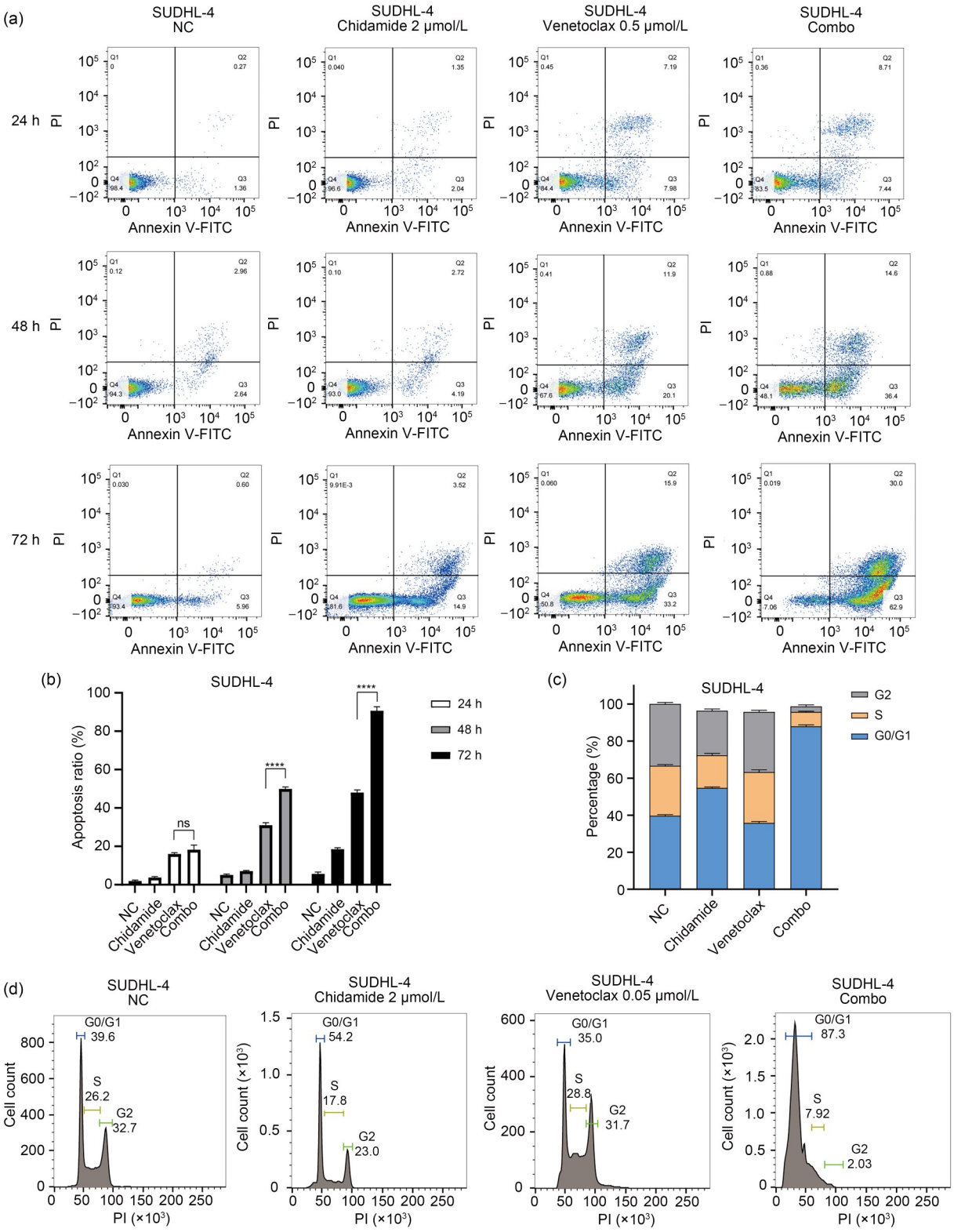


Fig. 3 Effects of combination of chidamide and venetoclax on apoptosis in SUDHL-4 cells. (a, b) The combination of chidamide (2 μmol/L) and venetoclax (0.5 μmol/L) induced cell apoptosis after 24, 48, and 72 h of treatment. (c, d) The cell cycle distribution of each group at 72 h was assessed through flow cytometry. (c, d) Data are presented as mean± standard deviation (SD). Data represent at least three independent experiments. **** P<0.0001; ^{ns} not significant. NC: negative control; PI: prodium iodide; Combo: combination group; FITC: fluorescein isothiocyanate.

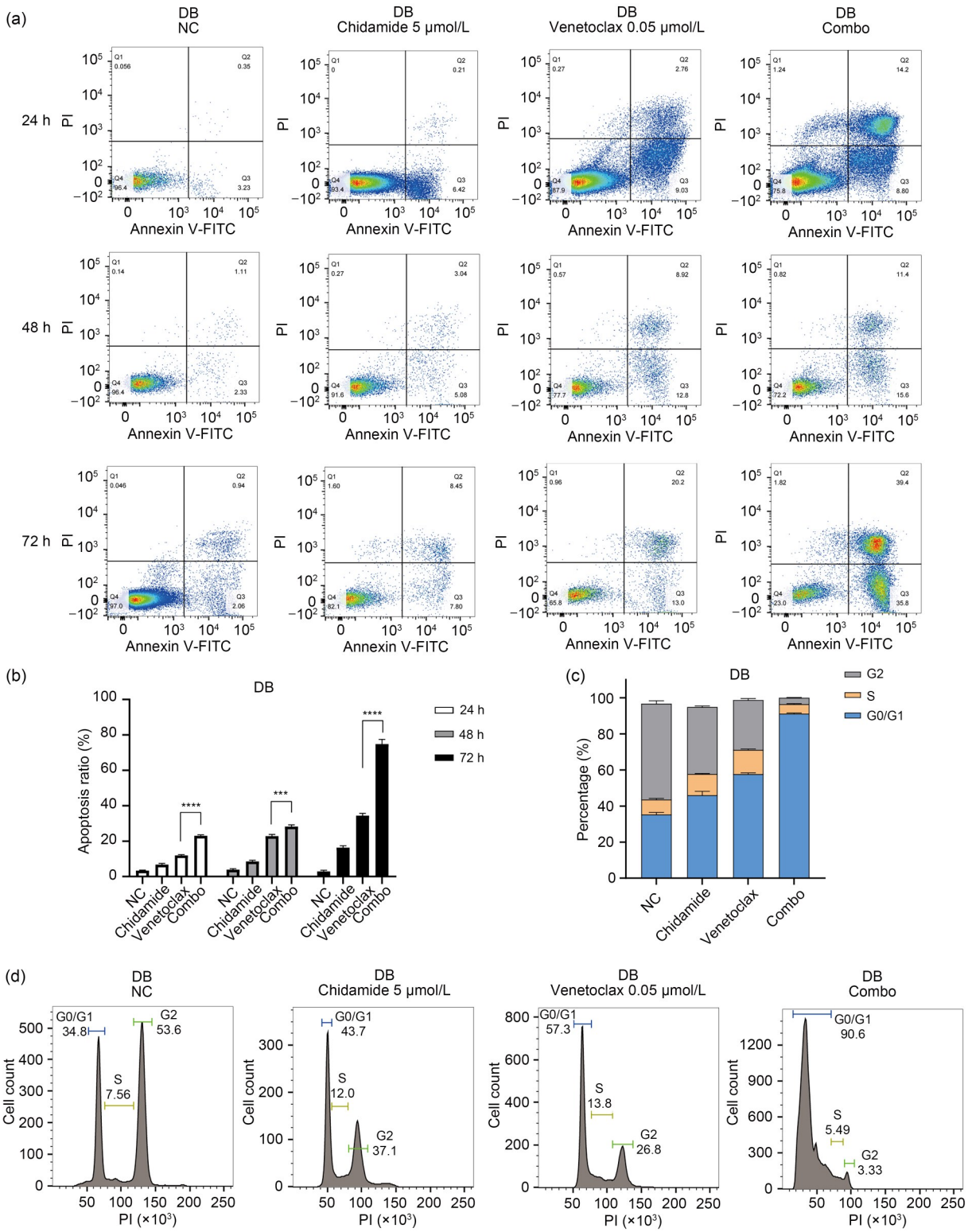


Fig. 4 Effects of combination of chidamide and venetoclax on apoptosis in DB cells. The DHL cell line DB was treated with chidamide (5 μmol/L), venetoclax (0.05 μmol/L), or a combination of the two for 24, 48, and 72 h. (a, b) The apoptosis rates induced by the combination and single-drug groups. (c, d) The cell cycle distribution at 72 h was detected by flow cytometry. *** P<0.001, **** P<0.0001. Data are expressed as mean±standard deviation (SD) at least three independent experiments. DHL: double-hit lymphoma; NC: negative control; PI: prodium iodide; combo: combination group; FITC: fluorescein isothiocyanate.

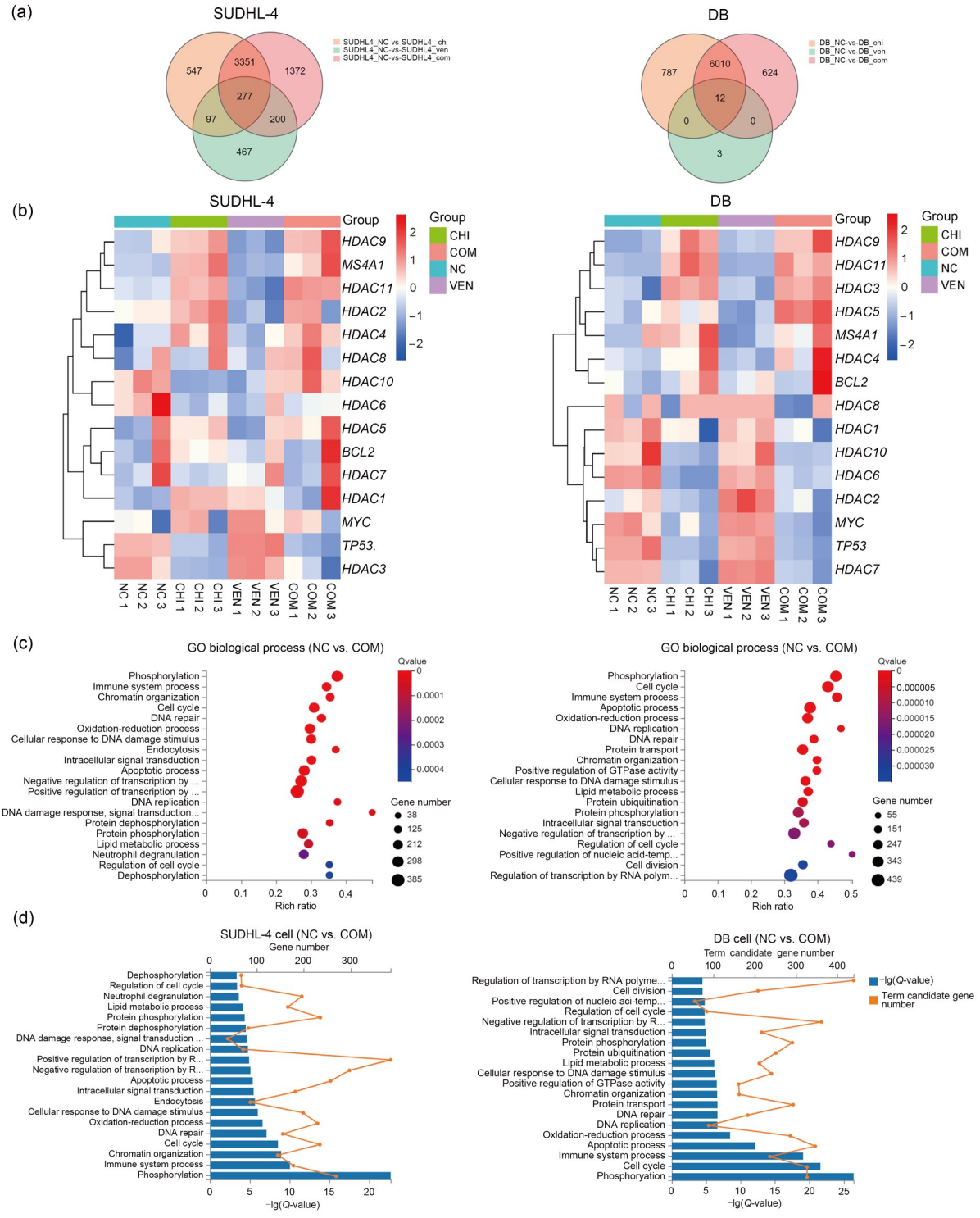


Fig. 5 Effects of CHI and VEN treatment on the transcriptome of DLBCL cells. (a) The number of DEGs after treatment with CHI, VEN, or their combination for 72 h. (b) Heatmap of the DEGs involved in multiple signaling pathways in DLBCL cells after CHI and/or VEN treatment for 72 h. (c, d) GO enrichment analyses of the DEGs analyzed by GSEA, and the pathways affected by CHI, VEN, or their combination in the SUDHL-4 and DB cells. DLBCL: diffuse large B-cell lymphoma; DEGs: differentially expressed genes; CHI: chidamide; VEN: venetoclax; COM: combination of CHI and VEN; GSEA: gene set enrichment analysis; *HDAC*: histone deacetylase; *MS4A1*: membrane spanning 4-domains A1; *MYC*: myelocytomatosis viral oncogene homolog; GO: Gene Ontology; NC: negative control.

We analyzed the transcription levels of genes in the related signaling pathways, and the results showed that the mRNA levels of *MYC*, *TP53*, *HDAC1*, *HDAC2*, *HDAC6*, *HDAC7*, and *HDAC10* in DB cells were down-regulated in the combination group, while the mRNA levels of *HDAC3*, *HDAC4*, *HDAC5*, *HDAC9*, and membrane spanning 4-domains A1 (*MS4A1*, *CD20*) were up-regulated (Fig. 5b). For SUDHL-4 cells, the mRNA levels of *TP53*, *HDAC3*, and *HDAC6* were down-regulated, while the mRNA levels of *HDAC2*, *HDAC4*, *HDAC9*, *HDAC11*, and *MS4A1* were up-regulated in the combination group. The drug combination thus had different effects on HDAC subtypes and showed inconsistency in its regulation of *MYC* and *TP53* between the two cell types.

GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of the DEGs between the combined and control groups showed that most DEGs were concentrated in phosphorylation modification, cell cycle, immune regulation, apoptosis, DNA damage repair, and chromatin remodeling (Figs. 5c and 5d). These results suggested that chidamide regulates a variety of biological processes in DLBCL cells together with venetoclax, and thus produces a series of anti-tumor effects.

We obtained 75 BCR pathway genes from the GSEA website and constructed a heatmap to describe the mRNA levels of these genes after different treatments in DB and SUDHL-4 cells (Fig. S2). The results showed that expression of key genes (*BTK*, phosphatidylinositol-3-kinase (*PI3K*), spleen tyrosine kinase (*SYK*), and protein kinase B (*AKT*)) of the BCR pathway was down-regulated in the combination group. This indicated that the combined use of chidamide and venetoclax may play a therapeutic role in DLBCL by inhibiting the BCR pathway.

3.5 Effects of chidamide on c-Myc and TP53 proteins in DLBCL

To determine the changes in MYC, BCL2, and TP53 protein levels in response to chidamide, SUDHL-4 and DB cell lines were treated with chidamide and venetoclax for 72 h. We observed a consistent decrease in c-Myc protein levels across the two cell lines evaluated, suggesting that chidamide suppressed *MYC* regardless of *MYC* rearrangement status (Fig. 6a). Simultaneously, the combination of chidamide and venetoclax significantly reduced the level of TP53 protein and significantly increased levels of acetyl-histone-H3 and -H4 proteins (Fig. 6b). The BCL2 protein level in the combination group also decreased, while that of the pro-apoptotic protein BIM increased. Furthermore, the increased ratio of BCL2/BIM protein levels is one of the mechanisms of drug resistance when using BCL2 inhibitors (Niu et al., 2016). Therefore, we speculated that the combination of chidamide and venetoclax could reduce the occurrence of BCL2 inhibitor resistance by increasing levels of pro-apoptotic proteins.

3.6 Effects of chidamide and venetoclax on tumor growth in the DLBCL xenograft mouse model

To determine whether chidamide enhances venetoclax-mediated DLBCL tumor growth inhibition in vivo, we inoculated six-week-old female BALB/c nude mice with human DLBCL SUDHL-4 cells. Two weeks later, tumor-bearing xenograft mice were treated daily with 15 mg/kg chidamide and/or 50 mg/kg venetoclax for another three weeks. The tumors were collected, and the tumor load was measured (Fig. 7a). Treatment with chidamide or venetoclax alone did not significantly reduce DLBCL tumor size, but the combination of the two drugs significantly inhibited tumor growth (Fig. 7b). Immunohistochemical analysis confirmed that co-treatment with venetoclax and chidamide significantly increased levels of acetyl-histone-H3 in mouse DLBCL tissues, decreased BCL2 protein levels, and increased CD20 levels (Fig. 7c). Taken together, these results indicated that chidamide enhanced venetoclax activity in the DLBCL xenograft mouse model in vivo.

4 Discussion

In this study, chidamide, a novel HDAC inhibitor, increased the level of BIM, decreased expression of *c-Myc* and *TP53*, and significantly synergized venetoclax-induced tumor-growth inhibition. Evidently, we identified a synergistic effect of chidamide and venetoclax in the treatment of DLBCL in vivo and in vitro, which effectively controls the growth of DLBCL.

Our biological information analysis showed that expression of the epigenetic *HDAC* genes was increased in lymphoma tissues, and was strongly

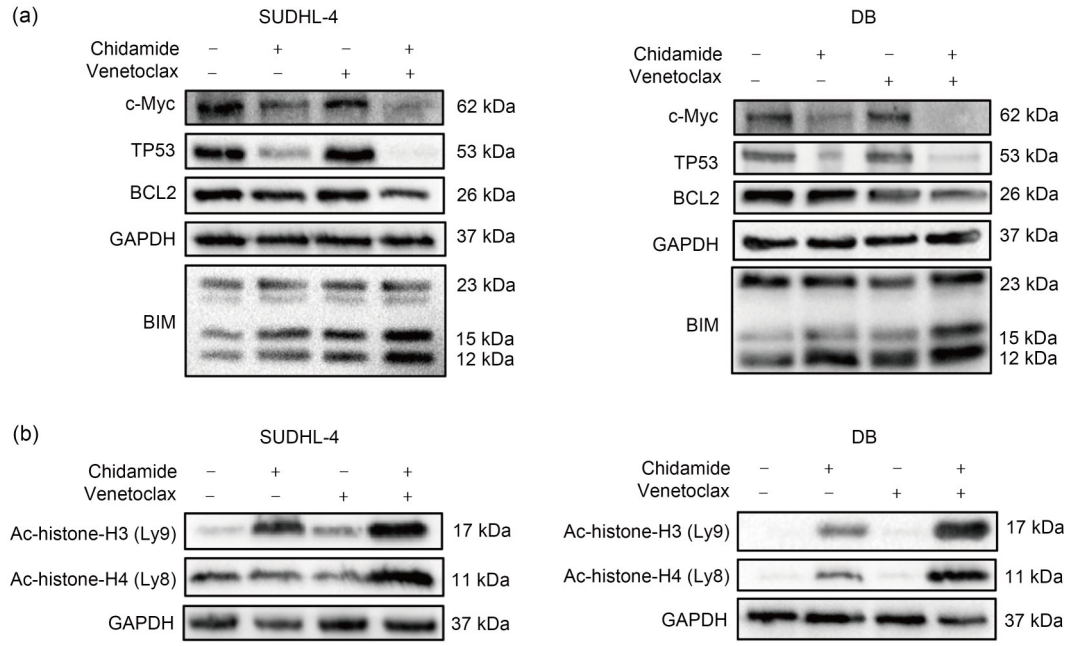


Fig. 6 Effects of chidamide and venetoclax on c-Myc, TP53, BCL2, and acetyl-histone. (a) SUDHL-4 or DB cells treated with chidamide and/or venetoclax for 72 h. Western blotting results showed that c-Myc, TP53, and BCL2 protein levels were significantly decreased in both drug combination groups. (b) Levels of acetyl-histone-H3 and -H4 proteins increased. GAPDH was used as a loading control. Myc: myelocytomatosis viral oncogene homolog; BCL2: B-cell lymphoma-2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Ac-histone: acetyl-histone.

correlated with *BCL2* expression. Meanwhile, the mutation rates of *BCL2*, *HDAC7*, and *HDAC10* in DLBCL tissues reached 21%, 19%, and 10%, respectively. HDACs as epigenetic regulators can regulate the histone tail, chromatin conformation, transcription, and protein–DNA interaction. It is known that a direct effect on chromatin involves pro-apoptotic *BCL2* family activation (Matthews et al., 2012; Stazi et al., 2019). Therefore, these data suggest that dual inhibition of BCL2 and HDAC might provide an effective strategy for cancer treatment.

Rearrangement of *MYC* and *BCL2* is a hallmark of DHL and leads to an increase in c-Myc and BCL2 protein levels. c-Myc is a transcription factor involved in regulation of the cell cycle, DNA damage repair, metabolism, protein synthesis, and other processes (Baluapuri et al., 2020; Duffy et al., 2021). In this study, SUDHL-4 was used as a model of DEL DLBCL cells with *WT-TP53*, without *MYC*/*BCL2* chromosome rearrangement, whereas DB was used as a model that represented *MYC*/*BCL2*-rearranged DHL cells with *MUT-TP53*. Both cell lines were of the germinal center B-cell-like lymphoma (GCB) subtype. Li et al. (2019) showed that targeting epigenetic regions is effective in reducing *MYC* expression, i.e., bromodomain and extra-terminal domain (BET) inhibitors can mitigate the effects of *MYC* overexpression by blocking cell–signal transduction. Furthermore, HDAC inhibitors can reduce c-Myc expression by affecting the chromatin structure of *MYC* (Nebbioso A et al., 2017; Ecker et al., 2021). *MYC* rearrangement status may lead to stronger cell tolerance and drug sensitivity. Our experiments also confirmed that DB cells with *MYC*/*BCL2* rearrangement were less sensitive to chidamide than SUDHL-4 cells. However, when SUDHL-4 and DB cells were treated with a combination of chidamide and venetoclax, the inhibition rate of cell proliferation was higher than those in the single-drug groups (P<0.05). Although different *MYC* states can lead to different drug sensitivities in cells, our synergistic indices (all CI<1) indicated that the combination of chidamide and venetoclax had synergistic effects on proliferation inhibition of both DLBCL cell types.

HDAC inhibitors have been employed to reverse post-translational deacetylation of cancer cells. Studies have also shown that HDAC inhibitors reduce expression of *c-Myc* and *TP53*, and subsequently arrest the cell cycle in the G0/G1 phase by influencing the cyclin pathway, while promoting levels of the

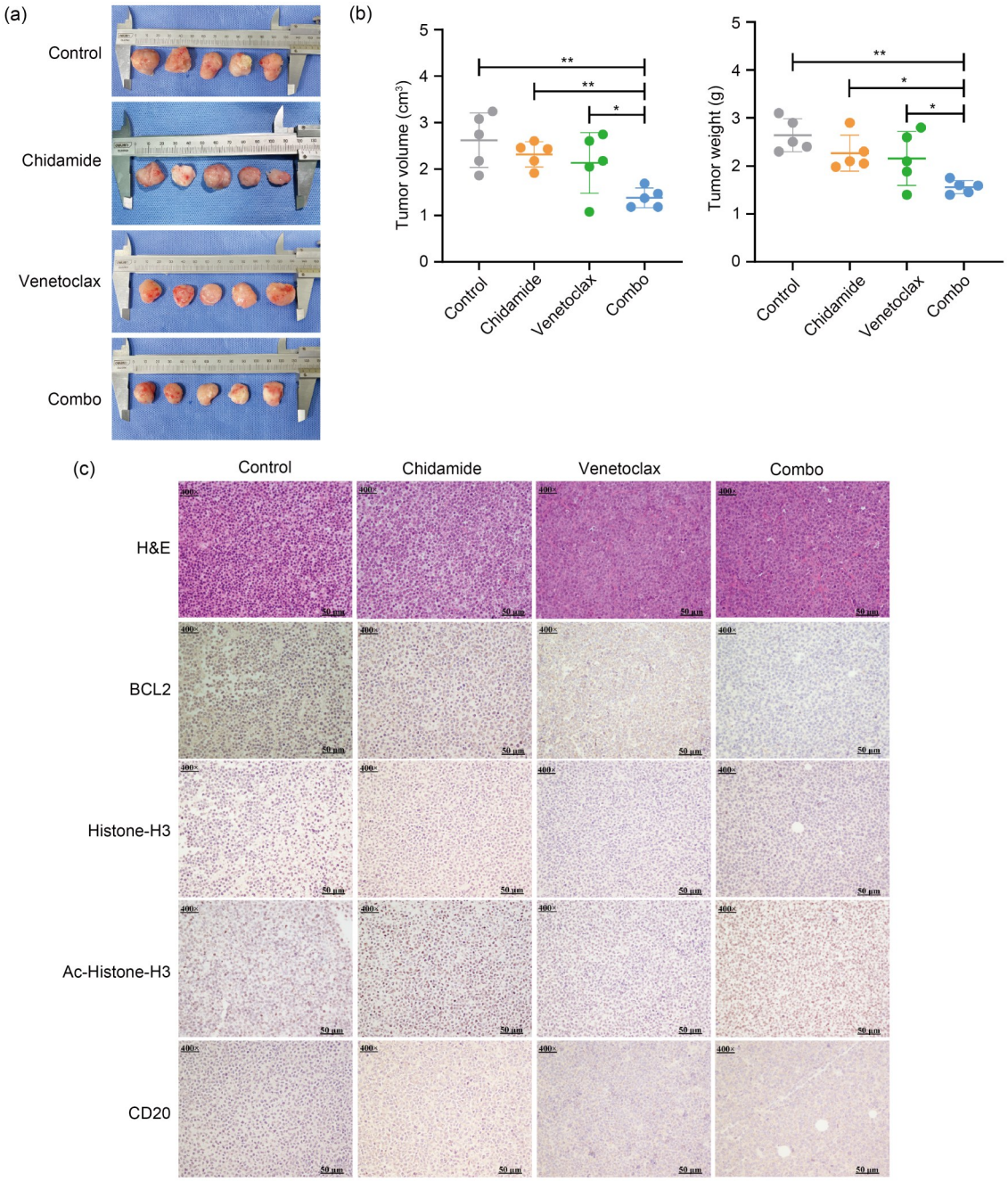


Fig. 7 Effects of chidamide and venetoclax in a DLBCL-cell xenograft mouse model. (a) Subcutaneous tumor size in human DLBCL tumor-bearing xenograft mice after treatment with chidamide (15 mg/kg), venetoclax (50 mg/kg), or a combination of the two drugs for three weeks. (b) Volume and weight of tumors in different treatment groups. Compared with the single-drug group, the combined group displayed the most significant inhibition of tumor growth. (c) H&E and IHC staining of tumors in different treatment groups. The combination group decreased the level of BCL2 and increased the levels of acetyl-histones-H3 and CD20. Data are presented as median (quartile intervals). * P<0.05, ** P<0.01. Data represent at least three independent experiments. DLBCL: diffuse large B-cell lymphoma; H&E: hematoxylin & eosin; IHC: immunohistochemistry; BCL2: B-cell lymphoma-2; Combo: combination group.

BH3-only proteins BIM and BCL2-binding component 3 (PUMA) (Muthalagu et al., 2014; Mrakovcic et al., 2019). In this study, both the transcription and translation processes of the oncoproteins c-Myc and TP53 were reduced by the combined chidamide and venetoclax treatment. For BCL2, it appears that

the inhibition occurred on the post-translational level. Studies have shown that anti-apoptotic protein overexpression represents a key compensatory factor for adaptive resistance to ABT-199; for example, Mcl-1 can act to re-sequester BIM released from BCL2 (Kapoor et al., 2020; Hafezi and Rahmani, 2021). HDAC inhibitors may reverse the ratio of anti-apoptotic to pro-apoptotic proteins by increasing the level of BIM and down-regulating BCL2 or Mcl-1 (Gong et al., 2019; Chen et al., 2020; Laszig et al., 2020). This information inspired us to speculate that chidamide could affect post-transcriptional modifications to affect the expression of BCL2 protein. Venetoclax has a high affinity for BCL2, and can displace BCL2-bound pro-apoptotic proteins such as BIM, BCL2-associated X (BAX), and BCL2 antagonist/killer (BAK), leading to permeabilization of the mitochondrial outer membrane, pro-apoptotic cytochrome *c* release, and caspase activation, and eventually resulting in apoptosis (Bhola and Letai, 2016). Mechanistically, the synergy between HDAC inhibitors and venetoclax induced cellular apoptosis through down-regulation of gene expression (*MYC*/*TP53*) and the anti-apoptotic signaling pathway, as well as up-regulation of the pro-apoptotic protein BIM in DLBCL cells (Fig. 8).

HDAC mainly induces cell differentiation, growth arrest, apoptosis, senescence, suppression of the immune system, and angiogenesis (Wang P et al., 2020; Wang XG et al., 2020). We found down-regulated mRNA levels of *TP53*, *HDAC6*, and *HDAC7* in the combination group and the chidamide group. The biological processes were analyzed by GO enrichment, and the results showed that the combination of chidamide and venetoclax affected cell phosphorylation, apoptosis, cell cycle, immune system, and DNA damage repair. In addition, compared with the untreated controls, the combination group showed better anti-tumor effects in the tumor-bearing nude mice. The IHC staining results showed that levels of BCL2 were decreased while those of acetylated histones H3 and CD20 were increased in the combination group. Guan et al. (2020) previously demonstrated that HDAC can

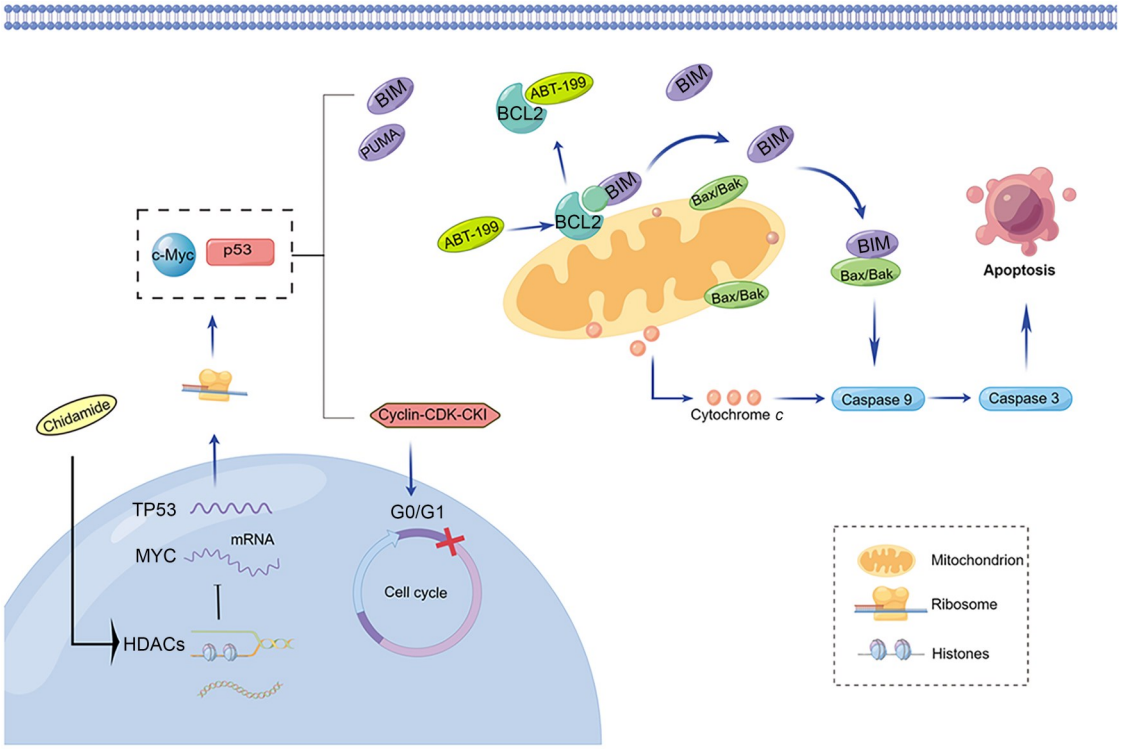


Fig. 8 Mechanism of chidamide and venetoclax treatment. Chidamide suppresses MYC/TP53 transcription and translation and alters the balance of pro-apoptotic BIM vs. anti-apoptotic BCL2 proteins, by which chidamide interacts with venetoclax to amplify the anti-tumor effect in DLBCL. This figure was drawn with Figdraw. Myc: myelocytomatosis viral oncogene homolog; DLBCL: diffuse large B-cell lymphoma; BCL2: B-cell lymphoma-2; HDAC: histone deacetylase; CDK: cyclin dependent kinase; CKI: CDK inhibitor; Bax: BCL2-associated X; Bak: BCL2 antagonist/killer.

mediate gene silencing to decrease *CD20* expression, and use of the HDAC inhibitor chidamide can up-regulate *CD20* gene expression. Moreover, several in vivo and in vitro trials of DLBCL treated with combinations of HDAC inhibitors and rituximab (targeting CD20) have shown good anti-tumor effects (Shimizu et al., 2010; Bobrowicz et al., 2017). Notably, studies have revealed that the combination of HDACs and small-molecule inhibitors, such as PI3K inhibitors, can achieve a better ORR and median response duration in patients with DLBCL than single small-molecule inhibitors (Patriarca and Gaidano, 2021). Therefore, combination therapy with small-molecule inhibitors, especially epigenetic inhibitors, could be a more effective way to treat DLBCL in the future.

5 Conclusions

In summary, our study determined the synergistic anti-tumor effect of a combination regimen of the epigenetic modulator chidamide and the BCL2 inhibitor venetoclax in DLBCL. This synergistic effect was also observed in DHL cells with *MYC*/*BCL2* rearrangement. The combination of chidamide and venetoclax simultaneously regulates epigenetic changes and affects the expression of genes such as *MYC*, *TP53*, and *BCL2* to achieve highly effective and precise therapeutic effects. Our data strongly supports the potential therapeutic effect of epigenetic modulators with secondary agents in DLBCL, and possibly in patients with DHL. Therefore, a preclinical trial using a combination of HDAC and BCL2 inhibitors for treating patients with DLBCL could be beneficial.

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Author contributions

Cancan LUO performed research design, the experimental research, and data analysis, wrote and edited the manuscript. Tiantian YU performed the experimental research and data analysis. Ken H. YOUNG performed the research design and data analysist. Li YU performed research design and data analysis, wrote and edited the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and securityof the data.

Compliance with ethics guidelines

Cancan LUO, Tiantian YU, Ken H. YOUNG, and Li YU declare that they have no conflict of interest.

This study was carried out in accordance with the recommendations of the Ethics Committee of the Second Affiliated Hospital of Nanchang University. The protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

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