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ORIGINAL RESEARCH

# Selinexor Synergistically Promotes the Antileukemia Activity of Venetoclax in Acute Myeloid Leukemia by Inhibiting Glycolytic Function and Downregulating the Expression of DNA Replication Genes

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**Introduction:** The BCL-2 inhibitor venetoclax has been widely used in the treatment of acute myeloid leukemia (AML); however, AML patients treated with venetoclax gradually develop resistance. The exportin-1 (XPO1) inhibitor selinexor can synergistically promote the antileukemia activity of venetoclax, but the mechanism remains unclear.

**Methods and Results:** Annexin V/7-aminoactinomycin D assays were used to examine the effects of a combination of venetoclax and selinexor (VEN+SEL) on AML cell lines and primary AML cells. RNA sequencing and oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) determinations by a Seahorse XF analyzer were employed to investigate the molecular mechanism of the toxicity of the VEN+SEL combination to AML cells. The cytotoxicity of NK cell combined with VEN+SEL combination was assessed in vitro using flow cytometry. VEN+SEL enhanced the apoptosis of AML cells (KG-1A and THP-1) and primary AML samples in vitro. The ECAR and OCR results demonstrated that the VEN+SEL combination significantly inhibited glycolytic function. RNA sequencing of THP-1 cells demonstrated that DNA replication-related genes were downregulated after treatment with the VEN+SEL combination.

**Conclusion:** This study indicated that selinexor can synergistically enhance the antileukemia activity of venetoclax in AML cells in vitro by inhibiting glycolytic function and downregulating DNA replication-related genes. Based on our experimental data, combining selinexor with venetoclax is an appropriate advanced treatment option for AML patients.

**Keywords:** venetoclax, selinexor, acute myeloid leukemia, glycolytic function, DNA replication-related genes

## Introduction

Acute myeloid leukemia (AML) is a common hematological malignancy that increasingly appears with age, with a median age of 68 years at diagnosis. The standard treatment regimen for AML consists of intensive induction chemotherapy (anthracyclines plus cytarabine) to achieve complete remission (CR), followed by consolidation with high-dose cytarabine or hematopoietic stem cell transplantation (HSCT).[1] Despite the high response rates, most patients with AML eventually relapse and die from the disease. In addition, AML diagnosed in older patients is often accompanied by high-risk clinicopathological features, which confer resistance to conventional chemotherapy.[2]

B-cell lymphoma 2 (BCL-2), an overexpressed protein in AML blasts, acts as a key regulator of mitochondrial apoptosis pathways.[3] The BCL-2 family genes coding mediated related proteins that mediate either pro-apoptotic or anti-apoptotic activity, and the imbalance between these proteins promotes the survival of AML cells.[4] Venetoclax, a selective

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and effective inhibitor of BCL-2, was verified to bind directly to BCL-2, activating the caspase proteins that lead to cancer cell apoptosis.[5] In clinical practice, combining venetoclax and hypomethylating agents (HMAs) combination has been demonstrated to be effective in patients with newly diagnosed or relapsed/refractory AML (R/R AML).[6–9] Because of remarkable activity of Venetoclax-based therapies, the introduction of these combinations is re-shaping the landscape of frontline therapy in AML across all ages as well as in advanced AML. However, the development of venetoclax resistance has been reported in several studies of long-term AML treatment.[10,11] A retrospective study demonstrated that salvage therapies seem ineffective after patients become refractory/relapsed to Venetoclax-based therapies.[10] Therefore, it is necessary to continue to explore the possibilities of other venetoclax-based combinations for AML treatment to achieve more potent and curative effects.

Exportin-1 (XPO1) plays a significant role in the nuclear export of several cargoes, such as tumor suppressor proteins (TSPs). Dysregulation of XPO1-mediated nuclear export is evident in several haematologic malignancies and solid tumours, leading to enhanced transport of TSPs out of the nucleus.[12–14] Selective inhibitor of nuclear export (SINE) has demonstrated therapeutic potential in several diseases and cancers. In clinical practice, selective inhibition of nuclear export has been demonstrated to be effective in patients with several haematologic malignancies and advanced solid tumors.[15,16] Selinexor, an oral antagonist of XPO1 and classical SINE, can inactivate XPO1 by binding to regions located far from the basic residues of XPO1.[13] Previous studies have demonstrated that selinexor exhibits antitumor effects in several hematological malignancies.[17–19] These studies have shown that selinexor has the potential for treatment in AML.

The venetoclax-SINE combination is justified because it can significantly inhibit cell viability in hematologic malignancies;[20] however, the mechanisms involved in the effect of such combinations remain unknown. In this study, we showed that the venetoclax response is enhanced by the XPO1 inhibitor selinexor, resulting in synergistic antileukemic activity against AML cells, which can be explained by metabolic mechanisms and genomics analyses.

# Materials and Methods

## Ethics Statement

This study was approved by the Ethics Committee of The First Affiliated Hospital of University of Science and Technology of China and was performed in line with the principles of the Declaration of Helsinki. All informed consent was obtained from all the participants before enrollment in this cohort. Ethical Review Board approval number is 2022KY340.

## Cell Lines and Reagents

To confirm the effects of treatment with the venetoclax and selinexor combination, we first selected the KG-1A cell line, a venetoclax-sensitive AML cell line, to model venetoclax-sensitive AML cells.[21] Then, according to a previous study, THP-1 cells were selected to model moderate venetoclax-resistant AML cells, as they exhibit a higher single-agent venetoclax effective concentration (EC50) value.[22] The AML cell lines KG-1A and THP-1 were verified to be mycoplasma-negative. RPMI-1640 medium supplemented with 10% fetal calf serum (FBS) and 1% penicillin/streptomycin was applied to culture THP-1 cells. IMDM supplemented with 20% FBS and 1% penicillin/streptomycin was applied to culture KG-1A and primary AML blasts.

Bone marrow samples were gathered from 20 AML patients hospitalized at The First Affiliated Hospital of University of Science and Technology of China. The patient characteristics are summarized in Supplementary Table 1.

## Apoptosis Assays

After centrifugation at 4 °C at 168 g for 5 min, cell suspensions were washed twice with cold PBS. Next, the cells were incubated with flow cytometry antibodies directed against CD45, CD3 and CD33 at 4 °C for 30 minutes. Then, 1× PBS solution was employed to wash the cells. Alternatively, KG-1A cells, THP-1 cells and primary AML blasts were treated with venetoclax alone, selinexor alone or the venetoclax-selinexor combination (VEN+SEL) for 24 h. After incubation, the apoptosis of AML cells was evaluated by flow cytometry by staining with 7-aminoactinomycin D (7-AAD)/Annexin V. Data were acquired by a CytoFLEX flow cytometer and then analyzed by Flow Jo software. Annexin $V^+$/7-$AAD^-$ and

Annexin $V^+$/7-$AAD^+$cells represent early apoptotic and late apoptotic AML cells respectively. Results are shown as mean percentage of Annexin $V^-$ positive cells ± the standard error of the mean (SEM) of replicates from three independent experiments. The effects of the combinations were calculated by using the zero interaction potency (ZIP) model, which compares observed and expected combination effects.

## Oxygen Consumption Rate

A Seahorse bioscience extracellular flux (XFe96) analyzer was applied to measure the oxygen consumption rate (OCR) by determining basal respiration, maximum respiration, and coupled respiration. KG-1A and THP-1 cells were treated with venetoclax alone, selinexor alone or the VEN+SEL combination for 24 h. Then, AML cells were washed with PBS and plated at 50,000 cells per well in 5 replicates in polylysine (15 mg/mL)-precoated 96-well plates. The OCR was determined after successively injecting oligomycin (final concentration: 1.5 μM), carbonyl cyanide p-trifluoro-methoxyphenylhydrazone (FCCP; final concentration: 0.5 μM), and rotenone and antimycin A (Ro/AA; final concentration: 0.5 μM). XFe Wave software (Seahorse Bioscience Inc., MA) was used to analyze the results from the mitochondrial respiration assay, and the OCR was calculated (pmoles $min^{-1}$ per 50,000 cells).

## Glucose Consumption Rate

An XFe96 analyzer was applied to measure the extracellular acidification rate (ECAR) by determining basal, maximal and coupled respiration. AML cell line culture, harvested cell washing and plating were performed as described above. The ECAR was evaluated over time after injection of glucose (final concentration: 10 mM), oligomycin (final concentration: 2 μM), and 2-DG (final concentration: 5 mM). XFe Wave software was used to analyze the results from the Glyco Stress Test assay, and the results are expressed as the ECAR (mPH $min^{-1}$ per 50,000 cells).

## NK Cell-Mediated Cytotoxicity Assay

Cord blood mononuclear cells (CB-MNCs) were used to isolate NK cells, and NK cells were enriched with an EasySep™ Human NK Cell Isolation Kit (Stem Cell, USA). NK cells were cultured in 20% FBS IMDM medium and supplemented with IL-12 (10 ng/mL) and IL-15 (1 ng/mL) at 37 °C for 12 h. AML cells were incubated with CFSE at 37 °C for 30 minutes and washed to remove antibodies. KG-1A and THP-1 cells were cultured at a concentration of $10^5$ cells/mL and pretreated with different concentrations of venetoclax alone, selinexor alone or their combination for 24 h. Activated NK cells were cocultured at a 2.5:1 E:T ratio with KG-1A or THP-1 cells ($10^4$ cells/mL) for 6 h. Apoptosis of AML cells was evaluated by flow cytometry by staining with 7-aminoactinomycin D (7-AAD)/Annexin V. The toxicity to NK cells was calculated by Supplementary Formula 1. Annexin $V^+$/7-AAD- and Annexin $V^+$/7-AAD+cells represent apoptotic AML cells mediated by NK cells. Results are shown as mean percentage of Annexin $V^-$ positive cells ± SEM of replicates from three independent experiments.

## RNA Sequencing

THP-1 cells were treated with venetoclax alone, selinexor alone or their combination at the IC50 concentration of the VEN+SEL combination for 24 h. Total RNA was isolated by TRIzol (TIANGEN, China), and RNA sequencing (RNA-seq) was performed by Azenta Life Sciences (Jiangsu, China). Differentially expressed genes (DEGs) were defined as genes whose varied expression levels were ≥2.0 between the two groups and whose signal values were higher than those of the background signal. An adjusted p value was used to determine the DEGs. The p values displayed for each gene set were significant.

## Western Blot Analysis

THP-1 cells were treated with venetoclax alone, selinexor alone or their combination at the IC50 concentration of the VEN+SEL combination for 24 h. Whole-cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, USA) and immunoblotted with anti-POLA2 (Absin, China), anti-POLD2 (Absin, China), anti-POLE2 (Absin, China) or anti-GADPH (Cell signaling, USA) antibody. Western blots were repeated at least 2 times, and one representative blot is shown.

## RT–qPCR Assays

THP-1 cells ($1.0\times10^7$) were centrifuged at 300 *g* for 7 min. Total RNA was isolated by TRIzol (Beyotime, China). Then, 3 μg of total RNA was applied to reverse-transcribe into cDNA with a reverse transcription kit (Biosharp, China). qPCR using 2 μL of cDNA reaction with SYBR Green Premix Pro Tap HS qPCR Kit reagent (Precision Biology) plus designated Prime time qPCR probes manufactured by Sangon Biotech was performed and detected in a 20 μL system. LightCycler 96 (Roche) was used to perform qPCRs in a 96-well PCR plate. All gene expression levels were quantified by the $2^{-\Delta\Delta Ct}$ method, and the target POLA2, POLD2, and POLE2 probes were normalized to the expression of the control gene (GAPDH). Finally, their relative gene expression was calculated by comparison with the control. Fold changes were calculated using relative percentages.

## Statistical Analysis

Statistical analyses were performed with GraphPad Prism software v9. Flow cytometry data were analyzed in FlowJo software, v10. Results are presented as the mean± SD. A value of $p < 0.05$ was considered to indicate statistical significance.

# Results

## Selinexor Synergizes with Venetoclax to Enhance the Apoptosis of AML Cells in vitro

To verify the synergistic potential between selinexor and venetoclax in vitro, we treated KG-1A and THP-1 cells with venetoclax and selinexor (VEN+SEL) at different concentrations for 24 hours and stained the cells with annexin V and 7-AAD. To verify the specific effect, we determined the IC50 values of the two agents and their combination in KG-1A and THP-1 cells. We found that apoptosis of the AML cells treated with the VEN+SEL combination was significantly different from that of each single agent in vitro (KG-1A: 250 nM and THP-1: 950 nM) (Figure 1A-C). In addition, as determined by the IC50 constructed from different concentrations of compounds, VEN+SEL had a strong synergistic effect on the apoptosis of AML cells, while treatment with the single-agent venetoclax showed a moderate effect and selinexor alone had little effect (Figure 1D and E). In addition, the drug ZIP synergy scores were calculated with the online SynergyFinder software (Supplementary Figure 1), and the results indicated that the average (and maximum) proportions of the anti-leukemia response attributable to the VEN +SEL combination were 10.54 (19.22) in THP-1 cells and 13.75 (21.13) in KG-1A cells. Indeed, treatment with Selinexor and Venetoclax suggested synergistic effects in promoting cell apoptosis of AML cells (ZIP synergy scores >10). As shown in Supplementary Figure 1, the white rectangle indicates the region of the maximum synergistic area.

## Selinexor and Venetoclax Synergize in Primary AML Cells to Increase Apoptosis

To further verify that selinexor synergizes with venetoclax to increase the apoptosis of AML cells, 20 primary AML blasts from 14 newly diagnosed and 6 relapsed/refractory AML patients were treated with selinexor, venetoclax and their combination (Supplementary Table 1). There were 11 primary AML patients with WT1 mutation, 2 with FLT3-ITD mutation and 3 with NPM1 mutation mutations. The flow gating strategy of AML primary cell subsets is shown in Figure 2A. From the results, we found that VEN+SEL treatment tended to increase the apoptosis of primary AML cells compared with single treatment with selinexor or venetoclax in newly diagnosed AML (Figure 2B) or R/R AML patients (Figure 2C). The VEN+SEL combination at a concentration of 500 nM significantly enhanced the apoptosis of primary AML cells compared with single-agent treatment in newly diagnosed (Figure 2D) and R/R AML samples (Figure 2E).

## The Selinexor and Venetoclax Combination Inhibits the Glycolytic Function of AML Cells

To further explore the synergistic mechanism of the VEN+SEL combination, KG-1A and THP-1 cells were treated with selinexor, venetoclax and their combination at different concentrations (KG-1A: 250 nM and THP-1: 950 nM) for 24

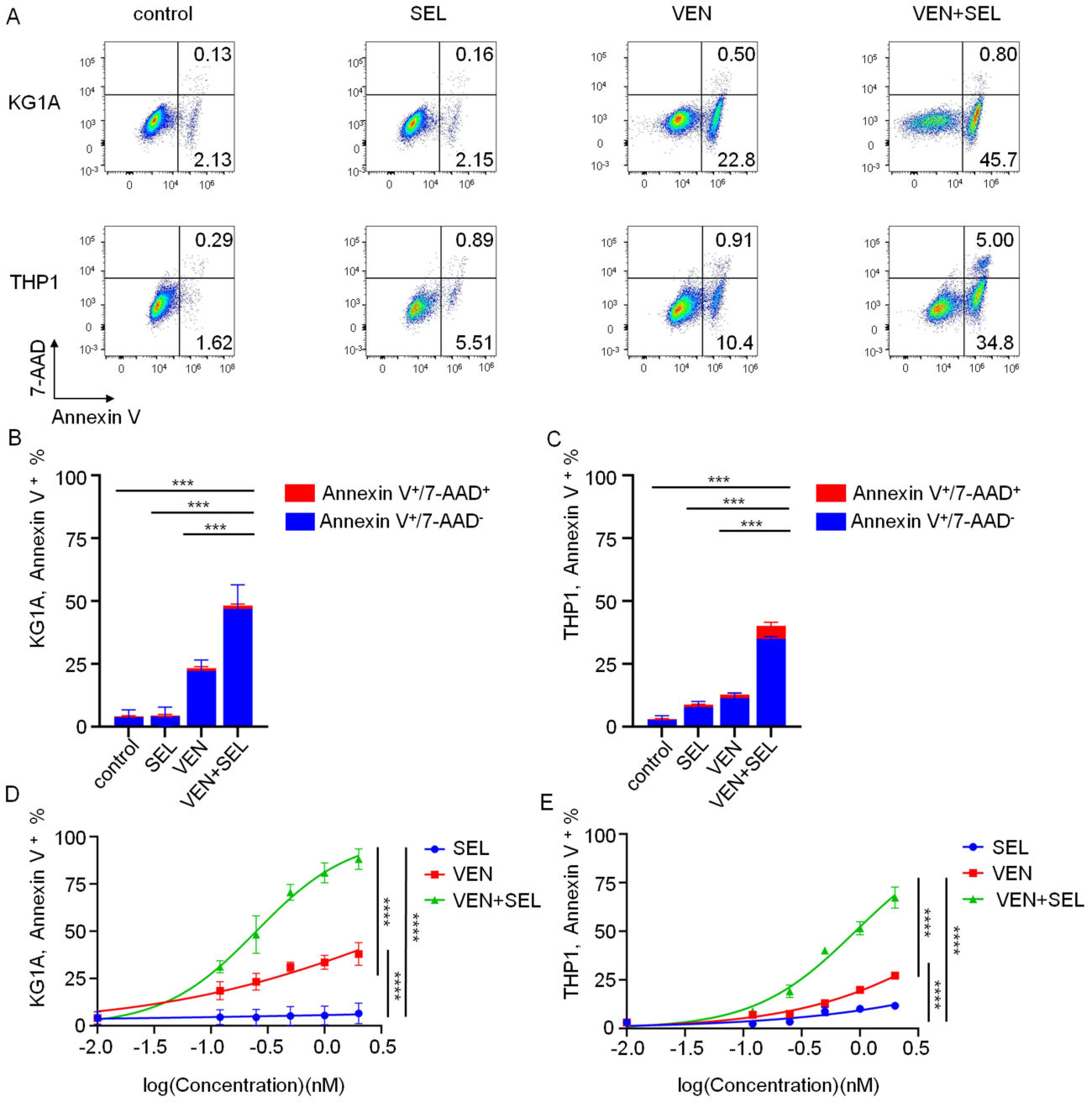


**Figure 1** Selinexor synergizes with venetoclax to enhance apoptosis of AML cell lines in vitro. (**A**) Flow cytometric analysis of apoptosis in KG-1A and THP-1 cells. Cells were stained with annexin-V and 7-AAD. (**B** and **C**) Statistical differences were calculated on the basis of the total number of Annexin $V^+$ cells for KG-1A cells (**B**) (treated with 250 nM selinexor, venetoclax or their combination) and THP-1 cells (**C**) (treated with 500 nM selinexor, venetoclax or their combination). (**D** and **E**) Determination of IC50 values for KG-1A (250 nm) (**D**) and THP-1 (950 nm) (**E**). The data are shown as the mean±SD. ***p< 0.001, ****p< 0.0001.

hours to analyze the effects of glycolysis and mitochondrial OXPHOS on the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR). The ECAR results demonstrated that the VEN+SEL combination significantly inhibited glycolytic function in KG-1A (Figure 3A) and THP-1 (Figure 3B) cells, as evidenced by decreased glycolysis and glycolytic capacity compared with their single treatment regimens (Figure 3C and D). However, the OCR results from the comparison group indicated that VEN+SEL treatment did not impact mitochondrial OXPHOS in KG-1A (Figure 3E) and THP-1 (Figure 3F) cells compared with treatment with selinexor and venetoclax alone. There was no significant induction of basal respiration, maximal respiration or ATP production after treatment with selinexor or venetoclax alone or their combination after 24 h in KG-1A (Figure 3G) and THP-1 (Figure 3H) cells.

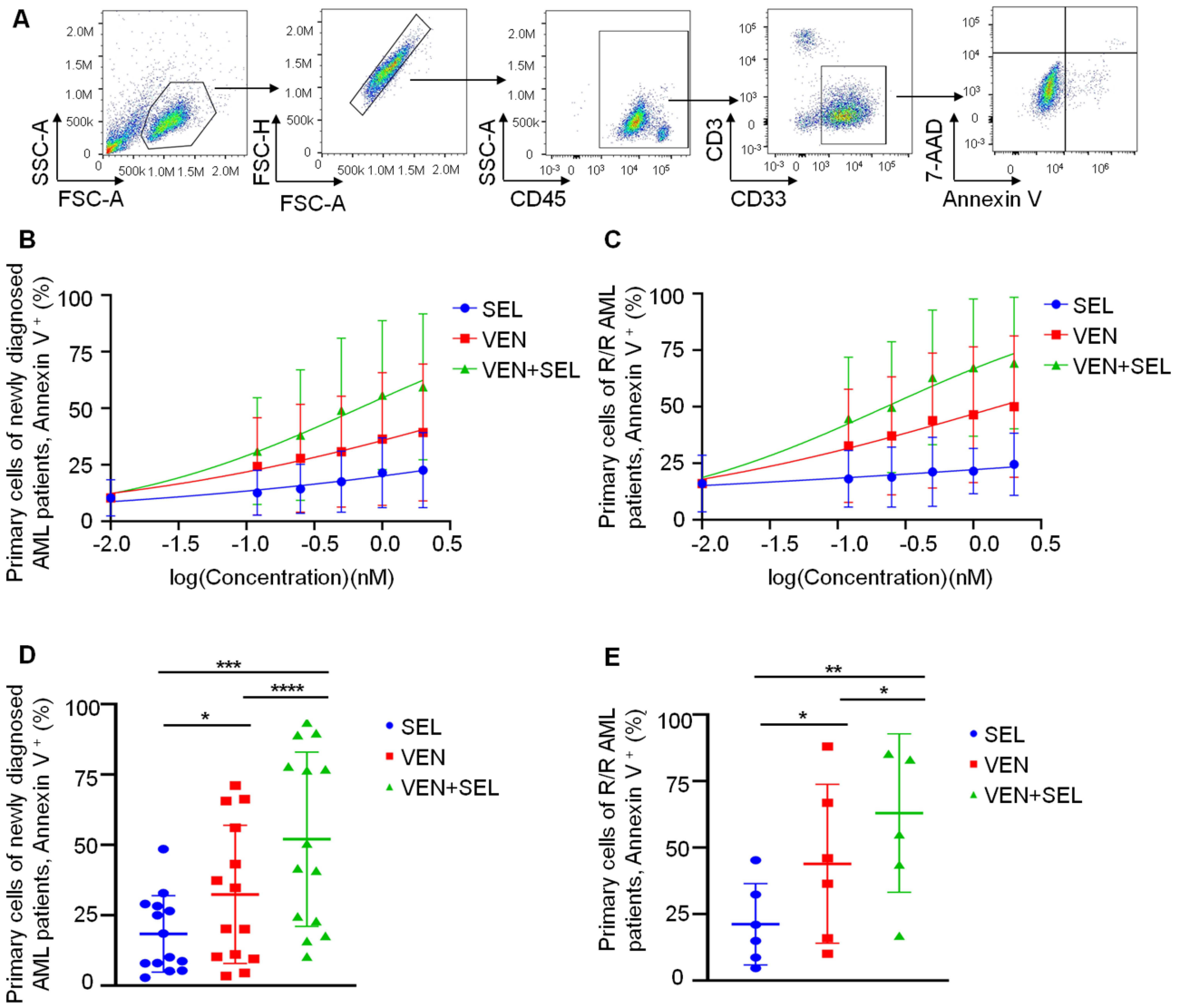


**Figure 2** Selinexor synergizes with venetoclax to increase apoptosis of primary AML blasts. (**A**) In the flow gating strategy, primary AML cells were first gated by forward scattering (FSC) and side scattering (SSC), and then cells were stained for CD45, CD3 and CD33 simultaneously. (**B** and **C**) Apoptosis determination of primary cells of newly diagnosed AML (**B**) and R/R AML (**C**) treated with different concentrations of selinexor, venetoclax or their combination. (**D** and **E**) Statistical differences were calculated on the basis of the total number of Annexin $V^+$ cells for primary cells of newly diagnosed AML (**D**) and R/R AML (**E**) (treated with 500 nM selinexor, venetoclax or their combination). The data are shown as the mean ± SD; *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001.

## The Selinexor and Venetoclax Combination Does Not Promote NK Cell Killing Capability

Whether there is a synergistic effect by NK cells after selinexor or venetoclax pretreatment of AML cells is unknown. To further explore the synergistic potential between the VEN+SEL combination and NK cells, KG-1A and THP-1 cells were pretreated with selinexor alone, venetoclax alone or their combination at different concentrations for 24 hours and then incubated with NK cells at an E:T ratio of 2.5:1 for 6 hours. Then, NK cell killing capacity was calculated (Figure 4A). The toxicity of NK cells to KG-1A (Figure 4B and C) and THP-1 (Figure 4D and E) cells pretreated with selinexor, venetoclax and their combination was investigated by cytotoxicity assays, and the results suggested that VEN+SEL combination pretreatment did not promote NK cell-mediated cytolysis of KG-1A or THP-1 cells compared to the single-regimen pretreatments.

## DNA Replication-Related Genes are Downregulated with Selinexor and Venetoclax Cotreatment

To explore the potential mechanisms of the VEN+SEL combination in AML cells, RNA sequencing of the THP-1 cell line was carried out. The results demonstrated that there were multiple differences in gene expression between the four

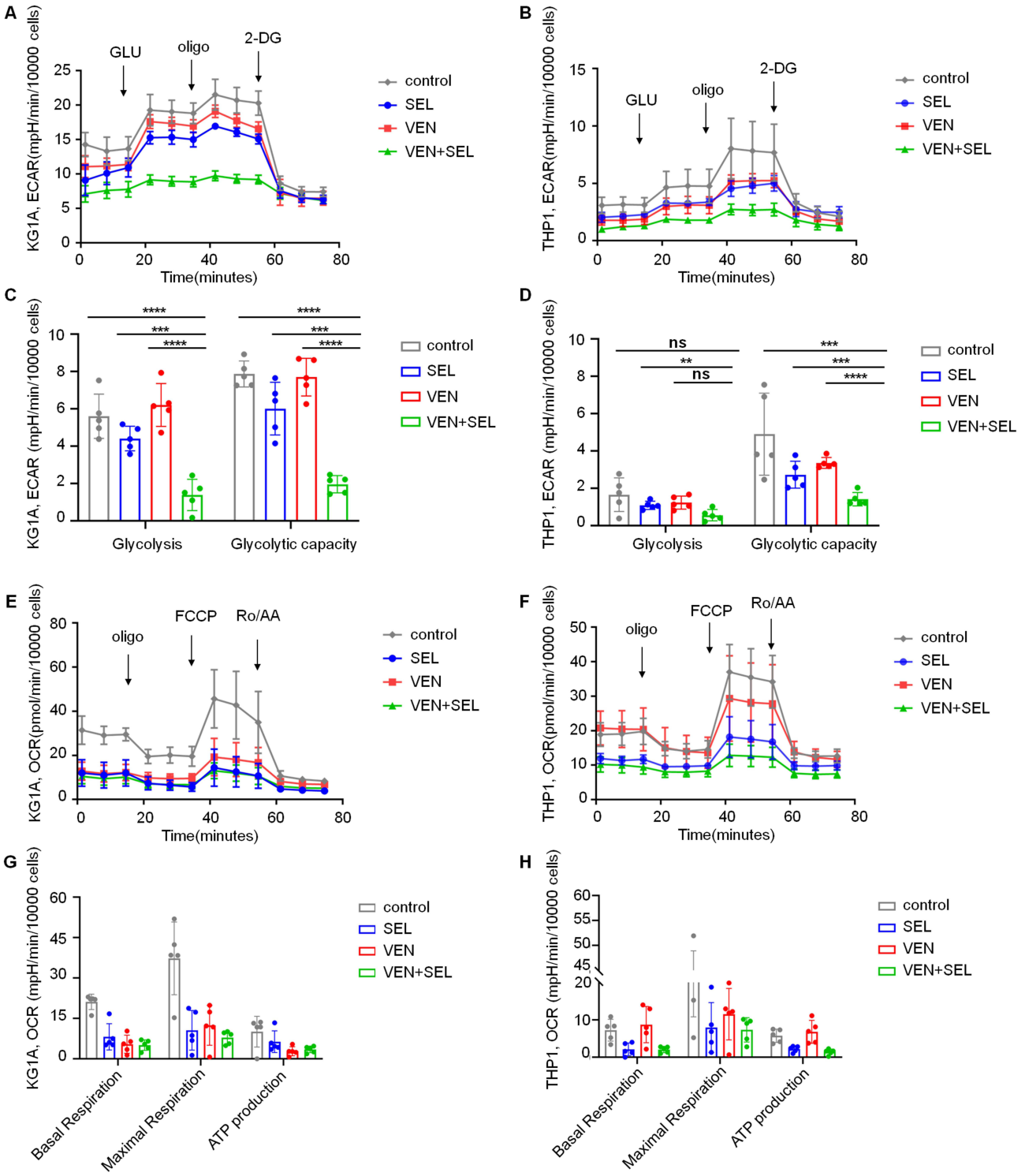


**Figure 3** The venetoclax-selinexor combination decreases the glycolytic function of AML cell lines in vitro. (**A** and **B**) Representative Seahorse plots of the Glyco Stress Test of KG-1A (**A**) and THP-1 (**B**) cells (treated with IC50 concentrations of regimens) obtained by recording ECAR from five independent tests are shown. (**C** and **D**) The values of the indicated respiratory parameters were calculated from the Glyco Stress Test and are shown as the mean ± SD from five independent tests of KG-1A (**C**) and THP-1 (**D**) cells. (**E-F**) Representative Seahorse plots of the Mito Stress test of KG-1A (**E**) and THP-1 (**F**) cells (treated with IC50 concentrations of regimens) assessed by recording the OCR from five independent tests are shown. (**G** and **H**) The values of the indicated respiratory parameters were calculated from the XF Cell Mito Stress Test and are shown as the mean ± SD from five independent tests of KG-1A (**G**) and THP-1 (**H**) cells. Statistical analysis was performed using Student's *t* test; **p < 0.01, ***p < 0.001, ****p< 0.0001.


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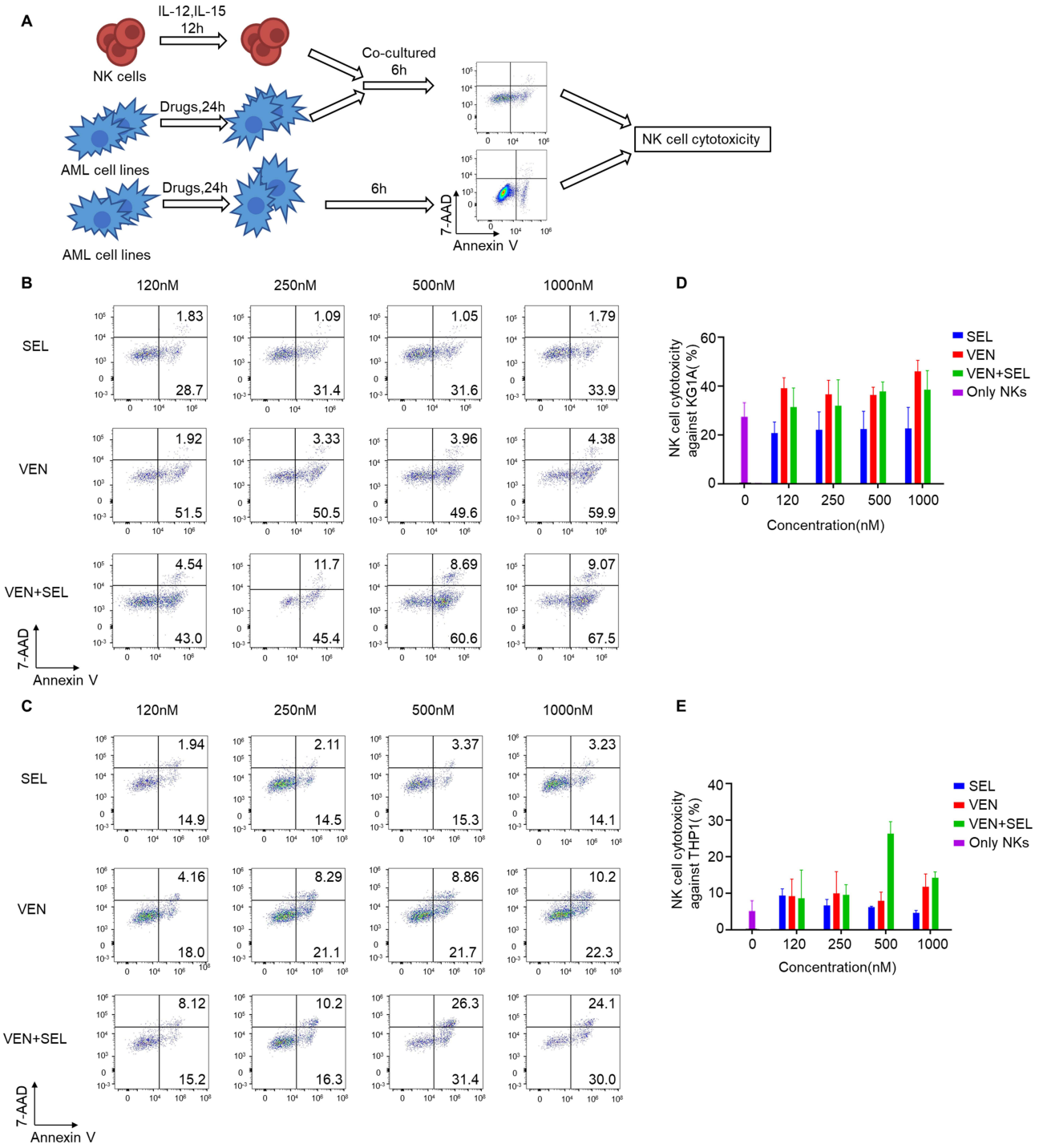


**Figure 4** Flow cytometric analyses of NK cell cytotoxicity against pretreated AML cell lines. A scheme of NK cytotoxicity acquisition is shown in (**A**). The cytotoxicity of NK cells against KG-1A (**B** and **C**) and THP-1 (**D** and **E**) cells pretreated with selinexor, venetoclax or their combination was investigated. Compared to single-regimen pretreatment, venetoclax-selinexor combination pretreatment did not result in greater NK cell-mediated cytolysis of KG-1A and THP-1 cells.

sets of samples by principal component analysis (PCA) (Figure 5A). The VEN+SEL combination set (green star) was separated from the other groups, and the distributions of the VEN set (orange quadrate) and SEL set (blue triangle) were separated from the control set (gray circle) (Figure 5A). The differential gene volcano map showed that there were 2537 significantly differentially expressed genes in the VEN+SEL set compared with the single VEN set (Figure 5B), suggesting that selinexor plays an auxiliary role in enhancing the proapoptotic effect of venetoclax during AML

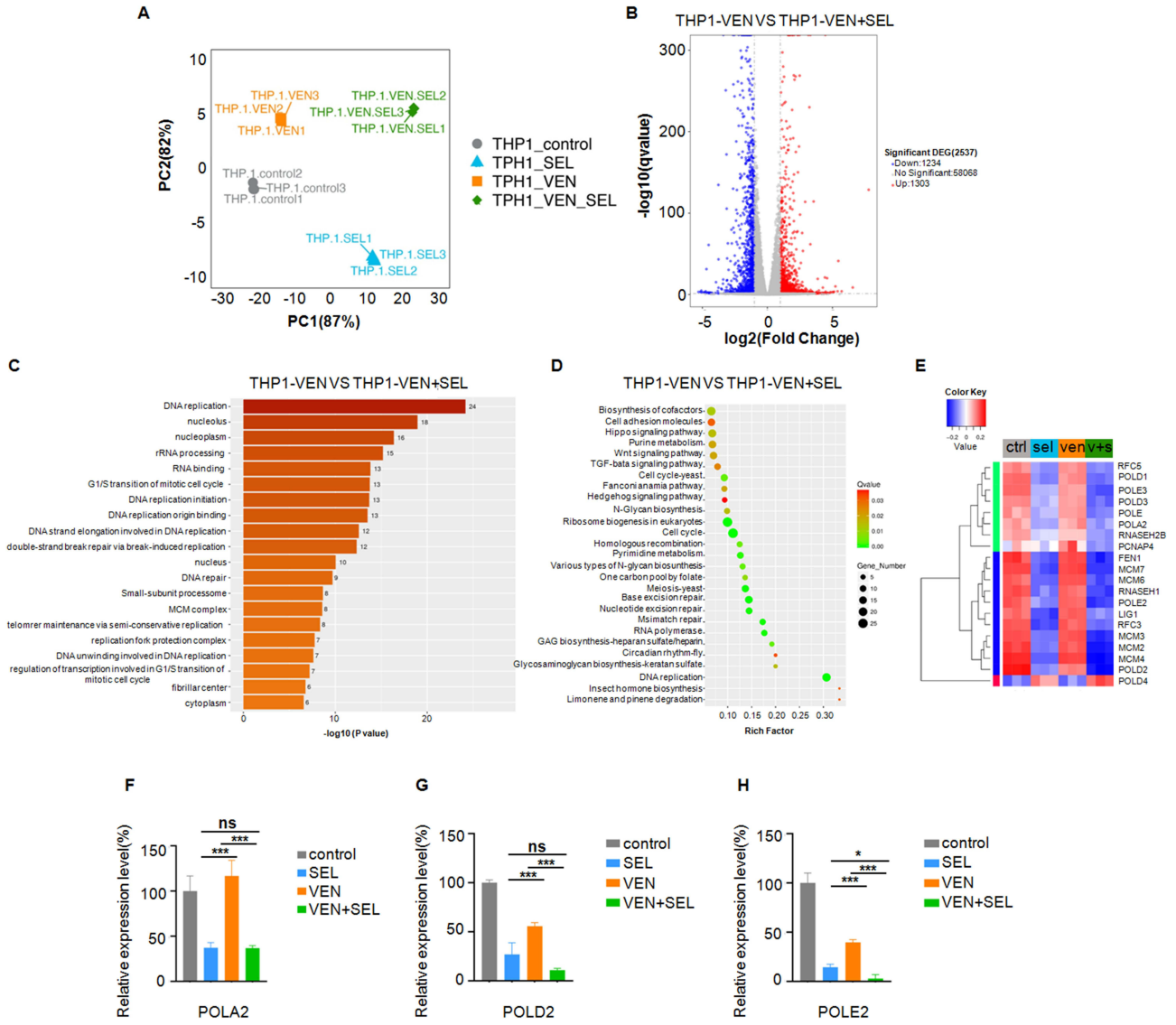


**Figure 5** DNA replication-related genes are downregulated with venetoclax-selinexor combination treatment in THP-1 cells. (**A**) PCA plot of THP-1 cells in four groups treated without or with venetoclax (VEN), selinexor (SEL) or the venetoclax-selinexor (VEN+SEL) combination (control set, SEL set, VEN set and VEN+SEL set) (n=3). (**B**) Volcano plot of differentially expressed genes in THP-1 cells treated with VEN+SEL compared with those treated with VEN alone. (**C**) GO enrichment analysis of differentially expressed genes between the VEN set and the VEN+SEL set. (**D**) KEGG bubble plots showing enriched pathways of downregulated genes between the VEN set and the VEN+SEL set. (**E**) Heatmap visualizing the transcriptional response of the DNA replication pathway in KEGG of THP-1 cells treated with selinexor, venetoclax or the VEN+SEL combination. Experiments were performed in biological replicates (n=3). (**F-H**) Validation of downregulated genes using RT−qPCR. Statistical analysis was performed using Student's *t* test; *p < 0.05, ***p < 0.001.

treatment. Next, according to GO and KEGG analyses, we found that the DEGs in DNA replication pathways were significantly downregulated in the VEN+SEL combination set compared with the VEN set. According to the GO pathway enrichment analyses, 337 BP terms, 603 CC terms and 48 MF terms were significantly enriched in the DEGs, and the genes in the DNA replication pathway were the most significantly downregulated (Figure 5C). Based on the KEGG annotations, DNA replication was significantly downregulated (Figure 5D). The heatmap indicated that the DNA replication-related genes among the DEGs of the VEN+SEL set were significantly inhibited compared with those of the VEN set (Figure 5E). To verify the downregulation of genes from RNA-seq in the VEN+SEL set, three differentially expressed genes (POLD2, POLA2 and POLE2) were selected from the heatmap, and RT–qPCR and Western blot were applied to detect their expression patterns. The results of RT–qPCR (Figure 5F-H) and western bolt (Supplementary Figure 2) were consistent with the RNA-seq results.

## Discussion

Different combinations of venetoclax are commonly applied in clinical trials in AML patients.[3,9] However, these venetoclax-based regimens are not sufficient to persistently provide an effective cure because most AML patients will relapse after treatment.[23] Functional studies have demonstrated that gain of FLT3-ITD mutation or loss of TP53 confers resistance to venetoclax-based therapies.[24] Therefore, it is still necessary to explore more effective venetoclax combination regimens for clinical application to treat AML patients. In this study, the experimental results suggested that selinexor can synergistically promote the antileukemia activity of venetoclax in AML cells in vitro, suggesting that the combination of venetoclax and selinexor might be used in clinical practice for R/R AML patients.

First, we demonstrated that venetoclax and selinexor synergized because their combination enhanced apoptosis of AML cells and primary AML blasts from newly diagnosed and R/R AML patients. By targeting XPO1, selinexor is widely known as an apoptosis-inducing drug for multiple myeloma treatment, but previous studies have indicated that selinexor treatment has limited effects in AML patients.[17] This conclusion is consistent with our statistical analyses of the experiments. We found that selinexor treatment alone does not promote apoptosis in KG-1A and THP-1 cells, even at a very high concentration (2 μM). However, when selinexor was combined with venetoclax, we found that the VEN+SEL combination showed a significant antileukemia effect against moderately VEN-resistant THP-1 cells, VEN-sensitive KG-1A cells and primary AML cells, which indicated that the VEN+SEL combination has very potent antileukemia effects in AML patients.

Second, we investigated the mechanism of the VEN+SEL combination on aerobic and anaerobic respiration in AML cells. By inhibiting binding to BCL-2 and proapoptotic proteins, venetoclax induces cell apoptosis. MCL-1 and BCL-XL appear to be significant factors in inducing resistance to venetoclax in AML cells.[25,26] Luedtke found that VEN+SEL combination therapy reduced the expression levels of these proteins, suggesting that this combination therapy can effectively inhibit the expression of two antiapoptotic molecules that induce venetoclax resistance during AML treatment.[26] However, although the molecular mechanisms involved have been demonstrated in previous reports, there has been no validation of the mechanisms related to energy metabolism in terms of anaerobic glycolysis and aerobic respiration. Until now, energy produced by glycolysis has been widely regarded as a main energy source for tumor cells.[27] In this study, we found that the combination treatment significantly reduced glycolytic function but not mitochondrial respiration in AML cells, suggesting that the VEN+SEL combination promoted apoptosis of AML cells by inhibiting anaerobic glycolysis rather than oxidative phosphorylation (OXPHOS). Therefore, it was speculated that the VEN+SEL combination might exert its apoptotic biological functions in AML by inhibiting glycolysis.

Third, by conducting RNA-seq analysis, we further investigated the expression of DEGs in AML cells treated with the VEN+SEL combination compared with venetoclax alone. Several studies have found that DNA replication genes (such as POLA2, POLD2 and POLE2) are highly expressed in AML cells and regulate the cell cycle and tumor progression.[28–30] Specifically, previous studies demonstrated that the circular RNA (circRNA) circ_POLA2 was upregulated in AML and promoted cell proliferation,[30] while POLD2 and POLE2 were abnormally overexpressed and enhanced cell viability in several solid tumors.[31–33] In addition, knockdown or inhibition of POLA2/POLD2/POLE2 could inhibit cell viability, enhance sensitivity to cell apoptosis and restrain migration in vitro.[34–36] In our study, we found that genes in the DNA replication pathway were the most significantly downregulated after VEN+SEL combination treatment, which suggested that the downregulation of DNA replication-related genes (eg, POLA2, POLD2, POLE2) might be responsible for the promotion of the apoptosis of AML cell lines and primary cells from AML patients.

In addition, this experiment validated and explored the effects of the VEN+SEL combination against AML cells, but the killing capacity of immune cells against AML cells was not known. Venetoclax or selinexor pretreatment has been verified to enhance the NK cells killing capacity.[37,38] A previous study demonstrated that venetoclax pretreatment promotes the sensitivity of AML cells to NK cell killing, which confirms the synergistic effect between venetoclax and NK cells;[37] another study indicated that selinexor pretreatment significantly enhances the sensitivity of lymphoma cells to NK cell killing, which suggests that selinexor and NK cells have synergistic potential against hematologic malignancies.[38] Whether the VEN+SEL combination has a synergistic effect with NK cell-based immunotherapies remains to be determined. In this study, to further explore the synergistic potential between the VEN+SEL combination and NK cells, KG-1A and THP-1 cells were pretreated with the VEN+SEL combination and then incubated with NK cells. However, in this study, the results after apoptosis evaluations suggested that VEN +SEL combination pretreatment could not enhance the sensitivity of AML cells to NK cell killing.

Two cost-effectiveness analyses of venetoclax plus azacitidine compared with azacitidine alone have been published but have conflicting results;[39,40] and until now, there are no other clinical studies to compare the cost of venetoclax-based treatment with the traditional management of R/R AML patients, including anthracycline-based treatment and the transplant aspects. It is necessary to conduct a prospective clinical trial to evaluate the cost-effectiveness analysis of venetoclax plus selinexor in AML patients who are ineligible for intensive chemotherapy. Although venetoclax-based treatments have been achieved major improvements in the outcomes of AML patients, for most patients the response was of limited duration. Several studies have illustrated that venetoclax-based treatment was successfully used as a bridge to allogeneic hematopoietic stem cell transplantation in R/R AML patients;[41–43] based on these results, we also think that VEL+SEL combination would become a successful bridge therapy to transplant and it should be verified in the future.

## Conclusion

In conclusion, our results suggested that selinexor can promote the proapoptotic effect of venetoclax in AML by modulating the metabolome and transcriptome in AML cells. Based on our experimental results, combining the XPO1 inhibitor selinexor with the BCL-2 inhibitor venetoclax might be an appropriate advanced treatment option for AML patients, especially for patients with R/R AML. However, the patient number in the current cohort is small; it would be interesting to see if there were any differences in the response to this combination therapy with respect to the cytogenetic and molecular genetic risk stratification of AML, and large-scale prospective clinical trials are needed to confirm the potential benefit from venetoclax combined with selinexor. In addition, this study cannot demonstrate whether selinexor has the ability to direct inhibit the expression of DNA replication-related genes and future experiments should be conducted to explore the mechanism and function of DNA replication-related genes in VEN+SEL combination treatment.

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## Disclosure

The authors have no conflicts of interest in this work.

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