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Original Research

Oridonin Synergistically Enhances the Pro-Apoptotic Effect of Venetoclax on Acute Myeloid Leukemia Cells by Inhibiting AKT Signaling

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Abstract

Background: Acute myeloid leukemia (AML) is a recurrence-prone hematologic malignancy. The advent of molecularly targeted therapies provides new opportunities to enhance the effectiveness of AML treatments. Venetoclax, a selective inhibitor of the anti-apoptotic protein Bcl-2, has shown promising results; however, resistance often arises due to elevated expression of the Mcl-1 protein, among other factors. Overcoming this resistance to improve therapeutic outcomes is a pressing issue that requires further investigation. Studies have demonstrated that oridonin, by inhibiting AKT signaling that regulates Mcl-1 expression, can effectively suppress tumor cell growth. This study aims to investigate whether oridonin can synergistically enhance the anti-leukemic effects of venetoclax and explore the underlying mechanisms behind this effect. **Methods**: *In vitro* experiments were performed to evaluate the effects of the combination of oridonin and venetoclax on AML cell proliferation, apoptosis, cell cycle distribution, and mitochondrial membrane potential. Transcriptome sequencing was used to elucidate the molecular mechanisms underlying the synergistic induction of AML cell apoptosis by the combination therapy. Western blotting and reverse transcription quantitative polymerase chain reaction (RT-qPCR) techniques were used to validate the findings. Additionally, an AML mouse model was established to observe the synergistic anti-AML effects of venetoclax combined with oridonin *in vivo*. **Results**: Both venetoclax and oridonin individually exhibited inhibitory effects on AML cell proliferation, resulted in cell cycle arrest, and induced cell apoptosis. Moreover, combination of the two drugs resulted in a synergistic effect. We also observed that oridonin inhibited AKT phosphorylation, upregulated the expression of Bim and Bax proteins, facilitated Mcl-1 degradation, and enhanced the apoptotic effects of venetoclax in AML cells. Finally, *in vivo* experiments demonstrated that the combination of oridonin and venetoclax effectively inhibited the growth of AML xenograft tumors in mice and prolonged the survival time of tumor-bearing mice. **Conclusions**: Oridonin and venetoclax synergistically promote AML cell apoptosis by inhibiting AKT signaling.

Keywords: oridonin; venetoclax; AKT signaling pathway; acute myeloid leukemia (AML); apoptosis

1. Introduction

Acute myeloid leukemia (AML) is a malignant neoplasm of the hematopoietic system characterized by abnormal function, excessive proliferation, and impaired apoptosis of hematopoietic stem cells during their differentiation and maturation within human bone marrow. Until recently, chemotherapy and hematopoietic stem cell transplantation were mainstay treatments for AML [1]. However, with the emergence of targeted small molecule drugs in recent years, new opportunities for the treatment of elderly AML patients, patients unable to tolerate standard treatment, and individuals with relapsed or refractory AML have been made possible. Among these drugs, the Bcl-2 inhibitor venetoclax has gained significant attention due to its ability to induce high rates of complete remission (CR) when used in combination with hypomethylating agents, despite the absence of specific mutational targets.

Venetoclax, an orally administered Bcl-2 inhibitor, has been used in the induction and consolidation therapy of AML patients who are unable to tolerate standard chemotherapy. Although the single-agent CR rate for venetoclax is only 20%, when combined with hypomethylating agents or low-dose cytarabine, the composite CR rate (CR + CRi, complete remission with incomplete blood count recovery) increases to 66.4% [2]. In relapsed/refractory AML, the CR + CRi rate is 42% [3]. Despite these promising outcomes, approximately 30% of patients remain resistant to venetoclax, and studies have shown that this resistance is associated with elevated intracellular expression of Mcl-1 [4]. Additionally, other mechanisms such as dysregulation of Bcl-2 family members and apoptotic regulators have also been implicated in venetoclax resistance. Overcoming such venetoclax resistance represents an urgent clinical challenge.

Oridonin, a tetracyclic diterpenoid compound extracted from the Chinese medicinal herb *Isodon rubescens*, has demonstrated anti-inflammatory and anticancer effects [5–7]. Previous studies determined that oridonin exerts its

antitumor activity by inhibiting tumor cell growth through the suppression of AKT signaling [5,8,9]. Additionally, it has been reported that the expression of Mcl-1, a protein associated with venetoclax resistance, is regulated by AKT signaling [10]. Based on these findings, we propose a hypothesis that the combined treatment of oridonin and venetoclax holds promise in overcoming venetoclax resistance.

The aim of this study was to investigate the synergistic pro-apoptotic effects of the combination of oridonin and venetoclax on AML using both *in vitro* and *in vivo* experimental models, and to explore the underlying mechanisms behind any observed effects. Results demonstrated that oridonin enhanced apoptosis effects induced by venetoclax in AML cells by inhibiting the AKT signaling. Additionally, in an AML mouse model, the combination of oridonin and venetoclax reduced tumor burden and significantly prolonged survival. These findings provide a new strategy to overcome venetoclax resistance in clinical settings, and also establish a theoretical basis for the combination therapy of oridonin and venetoclax in treating AML patients.

2. Materials and Methods

2.1 Materials

Monoclonal antibodies against Bcl-2 (#4223), Mcl-1 (#94296), Bim (#2819), Bax (#5023), caspase3 (#14220), PARP (#9532), γ-H2AX (#9718), AKT (#9272), phospho-AKT (#13038), p21 (#2947), c-Myc (#18583), CDK2 (#2546), phospho-CDK2 (#2561), and β-actin (#4970) were purchased from Cell Signaling Technology (Danvers, MA, USA). HRP-labeled goat anti-rabbit secondary antibody (ZB-5301) was purchased from Zhongshan Golden Bridge Company (Beijing, China). Venetoclax was purchased from Selleck (Houston, TX, USA) and dissolved in Dimethylsulfoxide (DMSO) at a final concentration of 10 mM. Oridonin was purchased from MedChemExpress (Monmouth Junction, NJ, USA) and also dissolved in DMSO at a final concentration of 10 mM.

2.2 AML Cell Lines and Normal Human Hematopoietic Stem Cells

AML cell lines used in the study were as follows: The THP-1 cell line was derived from human acute monocytic leukemia cells; OCI-AML3 cells were derived from human acute myeloid leukemia cells with DNMT3A and NPM1 mutations; U-937 is a cell line exhibiting monocyte morphology that was derived from histiocytic lymphoma; MOLM13 cells were derived from human acute monocytic leukemia cells with a Flt3-ITD mutation.

Cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and authenticated by short tandem repeat (STR) profiling and cytochrome c oxidase gene analysis. All cell lines underwent mycoplasma testing. OCI-AML3, MV4-11, and MOLM13 cells were cultured in complete growth medium consisting of RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. THP-1 cells were grown in RPMI-1640 without calcium nitrate, and with 0.05 mM β-mercaptoethanol, 10% fetal bovine serum, and 1% penicillin-streptomycin.

Normal human hematopoietic stem cells were obtained from peripheral blood collected from healthy donors after mobilization with 5 µg/kg granulocyte colony-stimulating factor (G-CSF). $CD34^+$ cells, representing hematopoietic stem cells, were obtained by magnetic bead sorting.

2.3 Cell viability Assay

Cells were seeded in 96-well plates (5×10^3–1×10^4 cells/well) and treated with indicated concentrations of venetoclax or oridonin for 12, 24, or 48 h. After incubation, 10 µL of CCK-8 reagent (#KGA317-1, KegGen-BioTECH, Nanjing, China) was added to each well and incubated for an additional 2–4 h. Absorbance at 450 nm was measured using a microplate reader, and the proliferation inhibition rate of venetoclax or oridonin on indicated cell lines at different time points (12, 24, and 48 h) was calculated. The concentration range of venetoclax was 2.5 nM to 160 nM for MOLM13 cells and 0.25 µM to 32 µM for the remaining cell lines. The concentration range of oridonin was 0.25 µM to 32 µM for all cell lines. This experiment was independently repeated three times. The cell proliferation inhibition rate percentage was calculated as follows: (1 – (A_experiment – A_blank)/(A_control – A_blank)) × 100%.

2.4 Flow Cytometry Analyses

2.4.1 Apoptosis

Apoptosis detection was performed using a commercial apoptosis assay kit (#KGA1014, Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Venetoclax or oridonin, was used alone or in combination to treat AML cell lines for 24h. A blank (untreated) control group was run in parallel. Cells were collected, centrifuged at 300 gravity (g) for 5 minutes at room temperature, washed twice with phosphate buffer saline (PBS), and cell pellets were collected after removing the supernatant. Each experimental group had 3 replicate wells with 2×10^5 cells per replicate well. Using a pipette, 500 µL of Binding Buffer solution was applied to each sample, followed by 5 µL of Annexin V-FITC and 5 µL of Propidium Iodide (PI) and cells were gently mixed by tapping. The samples were incubated in the dark at room temperature for 10 minutes and cell apoptosis was measured within 1 hour. Experiments were independently repeated 3 times. Combination index (CI) values were calculated using the CompuSyn software (Version 1.0, ComboSyn, Inc. Paramus, NJ, USA), where CI = 1 indicates an effect in the absence of synergism or antagonism, CI <1 indicates synergism, and CI >1 indicates antagonism.

2.4.2 Mitochondrial Membrane Potential

Treatment with venetoclax or oridonin alone, or a combination of the two, was applied to AML cells for 24 h. An untreated control group was also included in these analyses. JC-1 solution was prepared according to the instructions provided with the kit (#KGA604) from KeyGen Biotech Co., Ltd. (Nanjing, China). Cells were centrifuged at 300 g for 5 minutes at room temperature, and the original culture medium was removed. Each group had three replicate wells, and each well contained 2×10^5 cells. The experiment was repeated three times. Cells were washed once with 500 µL of the prepared JC-1 staining buffer, and then the buffer was removed after centrifugation. The cells were next resuspended in 250 µL of culture medium, 250 µL of JC-1 staining working solution was added, and the cells were incubated in a CO_2 incubator in the dark for 20 minutes. After incubation, the cells were centrifuged at 300 g for 5 minutes at room temperature to remove the JC-1 staining working solution, and then washed twice with 1 mL of JC-1 staining buffer. Finally, cells were resuspended in 200 µL of JC-1 staining buffer and the changes in mitochondrial membrane potential in each group were measured using flow cytometry as instructed by the manufacturer.

2.4.3 Cell Cycle

Cells were collected by centrifugation at 300 g for 5 minutes in a pre-cooled 4 °C centrifuge and washed with PBS. Each experimental group was conducted in 3 replicate wells. 1 mL of pre-cooled 75% ethanol solution were added to each well and cells were fixed overnight at –20 °C. Following this, cells were collected by centrifugation and the ethanol fixative was washed from the cells. A working staining solution was prepared using a ratio of Rnase A:PI of 1:9. 300 µL of staining working solution was added to each sample and the cells incubated at room temperature in the dark for 30–60 minutes. Staining intensity was recorded using a spectrophotometer at a wavelength of 488 nm.

2.5 RNA Sequencing Analysis

10 mL of mobilized human peripheral blood was decanted into a 50 mL sterile centrifuge tube. $CD34^+$ hematopoietic stem cells from healthy individuals were obtained by using a magnetic bead sorting kit (Miltenyi Biotec, Bergesch Gladbach, Germany). Log-phase MOLM13 and OCI-AML3 cells were washed twice with PBS and cell density of logarithmically growing cells were adjusted to 5×10^5/mL, and 10 mL of cells were seeded in a 10 cm culture dish. Venetoclax, oridonin, and their combination were used to subsequently treat the cells for 24 h. After this incubation period, the cells were washed twice with PBS and collected. Total RNA was isolated suing the Trizol Reagent (#15596026, Invitrogen Life Technologies, Carlsbad, CA, USA), after which the concentration, quality and integrity were determined using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Three micrograms of RNA were used as input material for the RNA sample preparations. The sequencing library was then sequenced on NovaSeq 6000 platform (Illumina, San Diego, CA, USA) by Shanghai Personal Biotechnology Cp., Ltd. (Shanghai, China). Based on these alignment results, we used HTSeq (0.9.1) statistics to compare the Read Count values on each gene as the original expression of the gene, and then used FPKM to standardize the expression. Then difference expression of genes was analyzed by DESeq (1.30.0) with screened conditions as follows: expression difference multiple $|\log2\text{FoldChange}| > 1$, significant p-value < 0.05. At the same time, we used R language Pheatmap (1.0.8) software package to perform bidirectional clustering analysis of all different genes of samples. We got heatmap according to the expression level of the same gene in different samples and the expression patterns of different genes in the same sample with Euclidean method to calculate the distance and Complete Linkage method to cluster. We mapped all the genes to Terms in the Gene Ontology (GO) database and calculated the numbers of differentially enriched genes in each Term. Using topGO to perform GO enrichment analysis on the differential genes, calculate p-value by hypergeometric distribution method (the standard of significant enrichment is p-value < 0.05. Differentially expressed genes were further validated using reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

2.6 qRT-PCR Assays

Cells in logarithmic growth phase were seeded at a density of 3×10^5/mL in 6-well plates and treated with venetoclax, oridonin, or a combination of both, an untreated (blank) control group was run in parallel. Cells were harvested after 24h and total RNA was extracted using Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA purity and concentration were measured using a NanoDrop 2000 spectrophotometer, and sample integrity was checked by electrophoresis. cDNA was synthesized using a reverse transcription kit (#RR037A, TaKaRa, Kyoto, Japan) and PCR was performed using an RT-PCR kit (#RR820A, TaKara, Kyoto, Japan) on ABI 7500 Real-Time PCR System (Thermo Scientific, Waltham, MA, USA). Thermocycling was carried out in three steps: 95 °C for 30 seconds for denaturation, cycling at 95 °C for 10 seconds and 60 °C for 30 seconds, and reactions were held 60 °C for 60 seconds following thermocycling. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method with β-actin as the internal reference standard. The sequences of the primers used for qRT-PCR are given in Table 1.

2.7 Western Blot Analysis

Logarithmically growing cells were seeded at a density of 3×10^5/mL in 6-well plates and treated with oridonin alone, venetoclax alone, or a combination of both. Blank (untreated) controls were also run in parallel. After 24 h of

Table 1. Primer sequences for qRT-PCR.

Gene	Primer	Sequence
Bcl-2	forward	5′-CGACTTCGCCGAGATGTCCAG-3′
	reverse	5′-CGGTTCAGGTACTCAGTCATCCAC-3′
Mcl-1	forward	5′-GCCGCTGACGCCATCATGTC-3′
	reverse	5′-CAACTCGTCCTCCTCCTCCTVTG-3′
Bim	forward	5′-GCCCCTACCTCCCTACAGAC-3′
	reverse	5′-CAGGTTCCTCCTGAGACTGC-3′
Bcl-xl	forward	5′-GTGCGTGGAGAGCGTAGACAAG-3′
	reverse	5′-AGAGCGAGCCCAGCAGAACC-3′
b-actin	forward	5′-CGCTGCGCTGGTCGTCGACA-3′
	reverse	5′-TGCACGCACGATTTCCCGCT-3′

treatment, the cells were collected, washed twice with PBS, and lysed using pre-cooled cell lysis buffer. The lysates were centrifuged at 1400 g for 15 minutes at 4 °C, and the resulting supernatants were collected as whole cell protein lysates. Protein concentration was determined using a BCA assay kit, and equal concentrations of protein were loaded onto SDS-PAGE gels for electrophoresis. The proteins were subsequently transferred to PVDF membranes (Millipore, Billerica, MA, USA), membranes were subsequently blocked with 5% skim milk for 1 hour at 4 °C, and finally incubated with primary antibodies overnight. After washing with TBST buffer three times, the membranes were incubated with secondary antibodies for 1 hour at room temperature. The membranes were washed three times with TBST, and chemiluminescent reagents were used for detection. Protein bands were visualized using enhanced chemiluminescence (ECL) and recorded using X-ray film and an Amersham Imager 600 imaging system (GE Healthcare, Chicago, IL, USA). Grayscale values were measured using Image J software (version 1.46R, National Institutes of Health, Bethesda, MD, USA).

2.8 In Vivo Experiments

Female immunodeficient NTG mice (NOD/ShiLtJGpt-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Gpt) (5–6 weeks old) were purchased from Sibeifu Biotechnology Co., Ltd (Beijing, China). 5×10^6 OCI-AML3 cells stably expressing luciferase were injected into the tail vein of NTG mice, and *in vivo* imaging was performed on the fourth day to confirm successful establishment of the AML xenografts. Following this, mice were randomly divided into four groups of nine, including a blank (untreated) control group, a venetoclax monotherapy group, an oridonin monotherapy group, and a combination therapy group. Venetoclax was administered orally at a dose of 100 mg/kg body weight once daily, and oridonin was administered orally at a dose of 40 mg/kg body weight once daily. In the combination group, the doses of venetoclax and oridonin were the same as those used in the monotherapy groups. *In vivo* imaging was performed twice a week to observe tumor burden and fluorescence intensity in the affected organs before and after drug treatment, and the mouse weight and survival were recorded. On the 11th day, three mice from each group were sacrificed and mononuclear cells were obtained by grinding the affected spleen. Bone marrow cells were obtained by flushing mouse femurs. CD33(#366608)/CD45(#304026) antibodies (Biolegend, San Diego, CA, USA) were subsequently used to label the cells, and flow cytometry was used to analyze the leukemia cell proportion in the bone marrow and spleen of the mice in each group. Remaining mice were monitored for weight and survival, and survival curves were plotted. These animal experiments were conducted in accordance with the project permit (No. 20200322) granted by the Zhengzhou University Committee on Life Science Ethics, and complied with the Zhengzhou University guidelines for animal care and use.

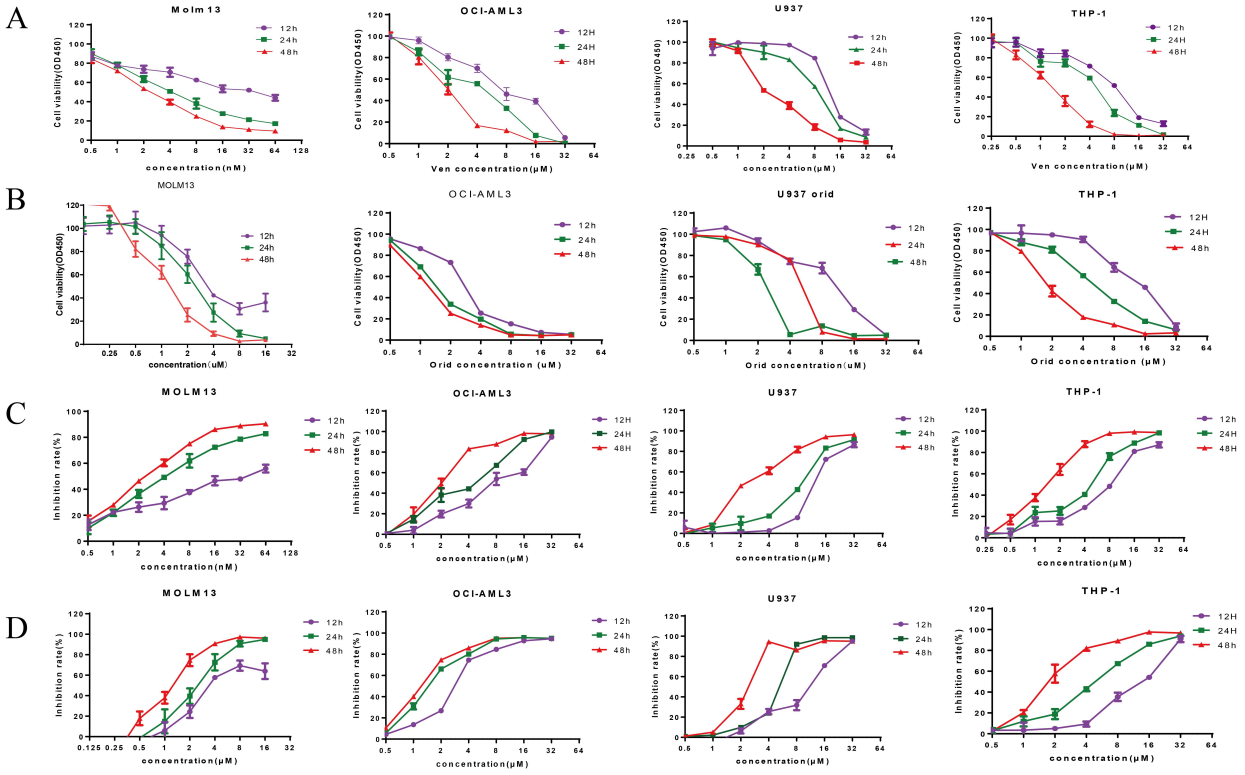
3. Results

3.1 Venetoclax and Oridonin Synergistically Inhibit Cell Proliferation

To investigate the effects of venetoclax and oridonin on AML cell proliferation, we initially used the CCK-8 assay to quantify the effects of different concentrations of venetoclax (Fig. 1A,C) and oridonin (Fig. 1B,D) on the proliferation of MOLM13, OCI-AML3, U937, and THP-1 cell lines at 12 h, 24 h, and 48 h pot-treatment. Results obtained indicate that venetoclax or oridonin alone can inhibit the viability and proliferation of AML cells, exhibiting both time and concentration-dependent effects. The half-maximal inhibitory concentrations (IC50) values were calculated using Graphpad Prism 6 software (GraphPad Software, San Diego, CA, USA) (Fig. 1E) and showed that in these four cell lines, the IC50 value of Venetoclax for the MOLM13 cells at 24 h was 4.7 nM, indicating that MOLM13 cells were significantly sensitive to venetoclax. In contrast, the measured IC50 values of venetoclax for the OCI-AML3, U937, and THP-1 cells at 24 h were 3.9 µM, 5.0 µM, and 4.3 µM, respectively. These findings indicate resistance to venetoclax in these 3 AML cell lines. The IC50 values (95% confidence interval) of oridonin for the four cell lines showed relatively small changes and ranged from 0.5 µM to 5 µM.

3.2 Venetoclax and Oridonin Synergistically Promote Apoptosis in AML Cells

Above results indicate that both venetoclax and oridonin can inhibit the growth of AML cells. To investigate whether the combination of these two drugs has a synergistic effect on promoting AML cell apoptosis, we used an Annexin V-FITC and PI double staining assay to measure apoptosis when AML cells were treated with the two drugs alone or in combination. The results indicated that both venetoclax (Fig. 2A) and oridonin (Fig. 2B) alone induce AML cell apoptosis. After treatment with a combination of the two drugs, the induction of cell apoptosis was further



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AML cells	Ven (μm)			Orid (μm)		
	12 h	24 h	48 h	12 h	24 h	48 h
MOLM13	0.03183 (0.02531~0.04003)	0.004762 (0.004229~0.005362)	0.002584 (0.002390~0.002794)	4.855(4.028~5.851)	2.542(2.325~2.778)	1.300(1.099~1.537)
OCI-AML3	7.811(6.710~9.093)	3.912(3.388~4.517)	2.033(1.875~2.205)	2.827(2.580~3.098)	1.558(1.443~1.683)	1.254(1.172~1.341)
U937	10.010(9.130~11.061)	5.011(4.360~5.716)	2.317(2.012~2.659)	0.9687(0.764~1.174)	0.6865(0.555~0.818)	0.355(0.259~0.451)
THP-1	7.458(6.784~8.199)	4.313(3.897~4.773)	1.48(1.932~1.574)	12.46(11.25~13.79)	4.876(4.580~5.192)	1.851(1.705~2.008)

Fig. 1. The inhibitory effects of venetoclax and oridonin on acute myeloid leukemia (AML) cell line proliferation using a CCK8 assay. MOLM13, OCI-AML3, U937, THP-1 cells were treated with venetoclax (concentrations ranging from 0.5 nM to 64 μM) or oridonin (concentrations ranging from 0.125 μM to 64 μM). (A) Cell viability of AML cells after treatment with different concentrations of venetoclax for 12 h, 24 h, and 48 h. (B) Cell viability of AML cells after treatment with different concentrations of oridonin for 12 h, 24 h, and 48 h. (C) Proliferation inhibitory rate of AML cells after treatment with different concentrations of venetoclax. (D) Proliferation inhibitory rate of AML cells after treatment with different concentrations of oridonin. (E) IC50 (95% confidence interval) values of venetoclax and oridonin on AML cells after 12 h, 24 h, and 48 h of treatment. Each experiment was repeated independently three times. Con, control; Ven, venetoclax; Orid, oridonin; Comb, combination of Ven and Orid.

enhanced. Specifically, the combination index (CI) values, calculated using Compusyn software, indicated a CI of less than 1, indicating a synergistic effect on promoting apoptosis (Fig. 2C). The CI theorem of Chou-Talalay offers a quantitative definition for additive effect (CI = 1), synergism (CI <1), and antagonism (CI >1) in drug combinations [11].

Based on the synergistic induction of cell apoptosis by the two drugs, we selected 2.5 nM venetoclax and/or 2 μM oridonin for single or combined treatment of MOLM13 cells, and 4 μM venetoclax and/or 4 μM oridonin for single or combined treatment of OCI-AML3 cells. Further, we used RT-qPCR to detect changes in expression of Bcl-2 family-related genes in drug-treated cells. The results showed that there was no significant effect of venetoclax on the transcription levels of Bcl-2 and Mcl-1 in MOLM13 cells, but that venetoclax induced an increase in Mcl-1 transcript abundance in OCI-AML3 cells. However, when combined with oridonin, the transcription level of Mcl-1 decreased significantly in OCI-AML3 cells. The gene expression levels were represented as Mean ± SD. The statistical analysis was conducted using *t*-test to assess the significance of the expression differences (Fig. 2D).

Western blotting was also used to detect changes in expression of apoptosis-related proteins and Bcl-2 family regulatory proteins following drug treatment. Results obtained indicate that venetoclax alone increased the levels of various markers of apoptosis such as cleaved (cf) Caspase3, cf-PARP, and γ-H2AX proteins in MOLM13 and OCI-AML3 cells. Of note, a more significant effect of venetoclax on MOLM13 was noted, and oridonin further enhanced this effect. This indicates that venetoclax can cause DNA damage, induce cell apoptosis, and this effect is further enhanced when combined with oridonin (Fig. 2E).

The expression levels of Bcl-2 family proteins were examined using western blotting. Results obtained are shown in Fig. 2F. In regards to Mcl-1, treatment with venetoclax alone increased the expression of Mcl-1, with a lower increase in sensitive MOLM13 cells compared to more resistant OCI-AML3 cells. Various Mcl-1 phosphorylation sites can alter the funcrion of this protein. For example, Mcl-1 phosphorylation at Ser159 (Mcl-1s) makes the Mcl-1 protein unstable and easily degraded, while phosphorylation at Thr163 (Mcl-1t) increases Mcl-1 stability and promotes an anti-apoptotic role [12]. In this study, after treatment with venetoclax alone, the abundance of Mcl-1t decreased in MOLM13 cells, while the abundance of Mcl-1s increased. In OCI-AML3 cells, Mcl-1s did not exhibit a significant change, but Mcl-1t was observed to be increased. When venetoclax was combined with oridonin, both cell lines showed an increase in Mcl-1s and a decrease in Mcl-1t abundance. We also observed that after 24 h of treatment with venetoclax, the abundance of the anti-apoptotic protein Bcl-xl decreased, and this effect was further enhanced when combined with oridonin. When we studied the pro-apoptotic protein Bax we noted that treatment with venetoclax or oridonin alone increases Bax abundance, and Bax abundance levels further increased when these two drugs were combined. Similarly, the pro-apoptotic protein Bak showed that with oridonin alone an increase in Bak abundance was noted. Taken together, these findings indicate that oridonin can synergize with venetoclax to promote AML cell apoptosis by, in part, downregulating anti-apoptotic proteins.

3.3 Venetoclax and Oridonin Synergistically Decrease the Mitochondrial Membrane Potential

Mitochondria play an important role in the activation of apoptosis. In the early stages of apoptosis, the permeability of the mitochondrial membrane changes and the opening of permeability transition pores cause a decrease in the mitochondrial transmembrane potential. In normal cells, the mitochondrial membrane potential is maintained at a relatively high level, resulting in the accumulation of the dye JC-1 in the mitochondrial matrix and the formation of JC-1 aggregates that produces red fluorescence that can be detected by flow cytometry. During early apoptosis, the mitochondrial membrane potential decreases, preventing the accumulation and aggregation of JC-1 in the mitochondrial matrix. As a result, JC-1 exists as monomers, leading to the production of green fluorescence [13]. We sought to detected the effects of venetoclax and oridonin on the mitochondrial membrane potential of AML cells. The results (Fig. 3A,B) showed that when venetoclax was combined with oridonin, there was a significant decrease in mitochondrial membrane potential in the MOLM13, OCI-AML3, U937, and THP-1 cell lines, and the differences were statistically significant ($p < 0.05$). The values were represented as Mean $\pm$ SD. The statistical analysis was conducted using *t*-test.

3.4 Transcriptome Sequencing Reveals the Molecular Mechanism of Oridonin and Venetoclax Synergism in Inducing Apoptosis in AML Cells

We next conducted transcriptome sequencing to explore the molecular mechanism that governs the synergistic effect of oridonin and venetoclax in promoting apoptosis in AML cells. Previous results determined that different AML cell lines have different sensitivities to venetoclax, with MOLM13 being sensitive and OCI-AML3, U937, and THP-1 showing resistance. Our findings indicate that oridonin can synergize with venetoclax to induce apoptosis in AML cells; thus, to investigate the mechanism that controls this sensitivity to venetoclax, we selected venetoclax-sensitive MOLM13 cells, venetoclax-resistant OCI-AML3 cells, and normal peripheral blood $CD34^+$ hematopoietic stem cells as controls. We subsequently performed transcriptomic sequencing analysis to explore this mechanism. Transcriptomic sequencing indicates that both MOLM13 and OCI-AML3 cells have many differentially expressed genes when compared to normal hematopoietic stem cells, and there are also differential gene expressions between the two AML cell lines (Fig. 4A). Through GO functional enrichment analysis, we uncovered functional differences in MAPK signaling pathway regulation, cell cycle arrest, and p53-associated apoptosis regulation between OCI-AML3 and MOLM13 cells (Fig. 4B,C).

To investigate which of these differences contribute to drug resistance, we treated OCI-AML3 cells with 4 µM venetoclax, 4 µM oridonin, or a combination of both, and explored their molecular mechanisms through transcriptome sequencing. The results showed that the quantitative gene expression changes induced by venetoclax were less than those induced by oridonin in OCI-AML3 cells after drug treatment (Fig. 4D). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that compared with the venetoclax single-drug group, the combination therapy group also contained differentially expressed genes in the p53 and MAPK signaling pathways as well as cell cycle regulation (Fig. 4E,F). These transcriptome sequencing results suggested that the PI3K/AKT and p53 signaling may affect the sensitivity of AML cells to venetoclax.

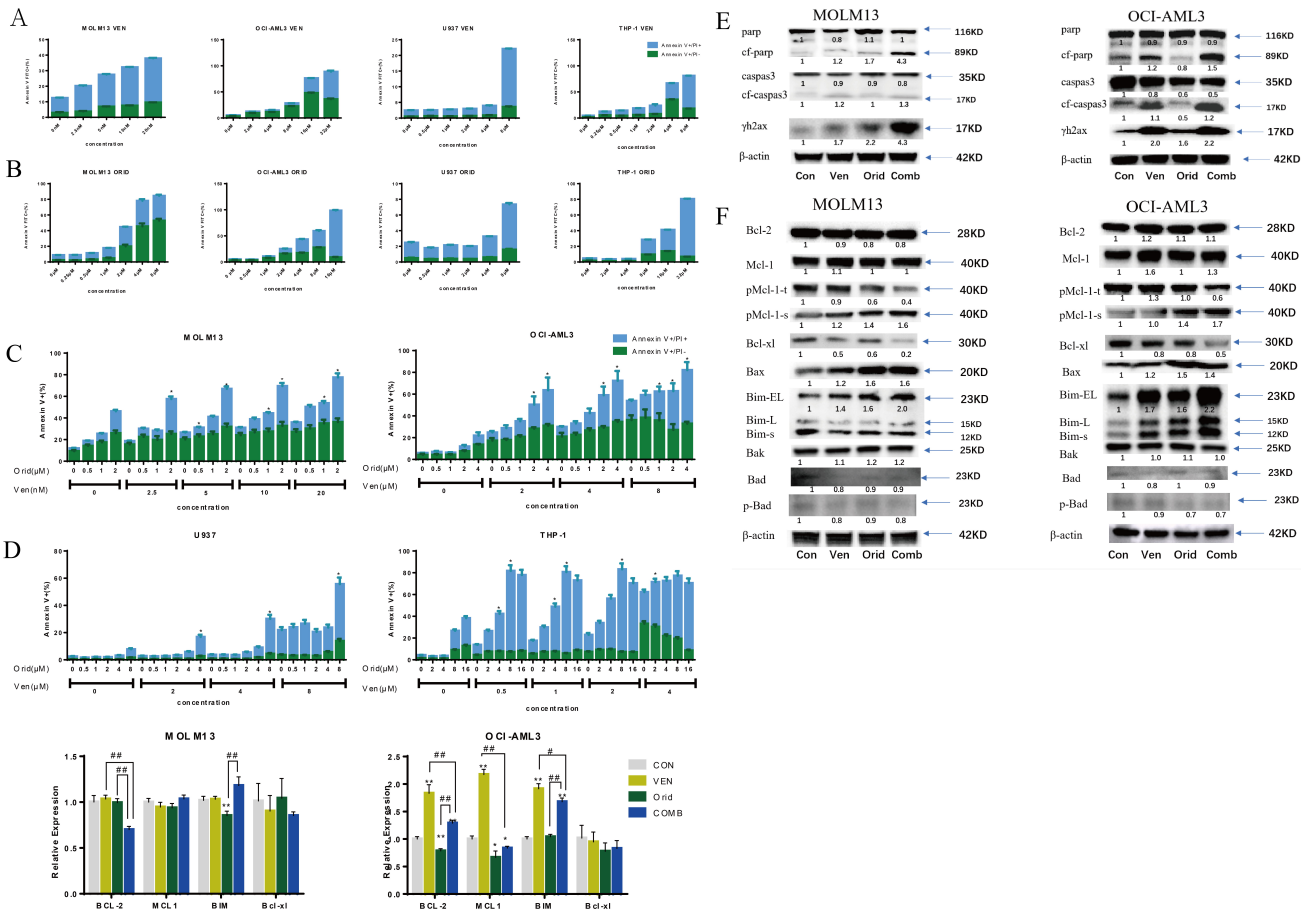

Fig. 2. Annexin V-FITC and Propidium Iodide (PI) double staining assay was used to quantify apoptosis in AML cells treated with different concentrations of venetoclax and oridonin. (A) Apoptosis rate of AML cells treated with venetoclax (0~32 μM) for 24 h. (B) Apoptosis rate of AML cells treated with oridonin (0 μM~32 μM) for 24 h. (C) Changes in AML cell apoptosis following treatment with different concentrations of venetoclax and oridonin for 24 h, and the combination index (CI) was calculated. * indicates CI <1. (D) Changes in mRNA levels of indicated Bcl-2 family members in AML cells treated with venetoclax and oridonin for 24 h (Mean ± SD; single */#: $p < 0.05$; double **/##: $p < 0.01$; */**: Ven/Orid/Comb vs Con; #/##: Comb vs Ven/Orid). (E,F) MOLM13 cells were treated with 2.5 nM venetoclax and OCI-AML3 cells were treated with 4 μM venetoclax, while MOLM13 cells were treated with 2 μM oridonin and OCI-AML3 cells were treated with 4 μM oridonin. Subsequently, apoptosis-related proteins and Bcl-2 family members were detected by western blotting 24 h after treatment. Con, control; Ven, venetoclax; Orid, oridonin; Comb, combination of Ven and Orid.

3.5 Venetoclax and Oridonin Synergistically Induce Cell Cycle Arrest

Based on the results of our sequencing, we used flow cytometry to detect changes in cell cycle distribution after drug treatment in MOLM13 and OCI-AML3 cells. The effects of these drugs on cell cycle dynamics were different in the various AML cell lines used. We observed that venetoclax had no significant effect on cell cycle distribution. In contrast, oridonin arrested MOLM13 cells in the G1 phase and OCI-AML3 cells in the G2 phase of the cell cycle. In addition, when the two drugs were used in combination, the cell cycle arrest effect observed with oridonin treatment alone was further enhanced in both MOLM13 cells (arrested in the G1 phase) and OCI-AML3 cells (arrested in the G2 phase) ($p < 0.01$). Although oridonin also arrested U937 cells in S phase ($p < 0.01$), this effect was weakened when the two drugs were used together. In THP-1 cells, oridonin arrested cells in the G2 phase, and the addition of venetoclax did not significantly alter this effect. These results indicate that the combination of venetoclax and oridonin can result in a significant arrest of the cell cycle of AML cells.

Furthermore, we used western blotting to detect changes in the expression of several cell cycle-related proteins. Namely, we examined p21, CDK2, phospho-CDK2, and c-Myc in MOLM13 and OCI-AML3 cells after drug treatment. The results showed that the expression of p21 was down-regulated after venetoclax alone, most notably in MOLM13 cells. Oridonin was found to up-regulate p21 when used alone, and the combination of the two drugs

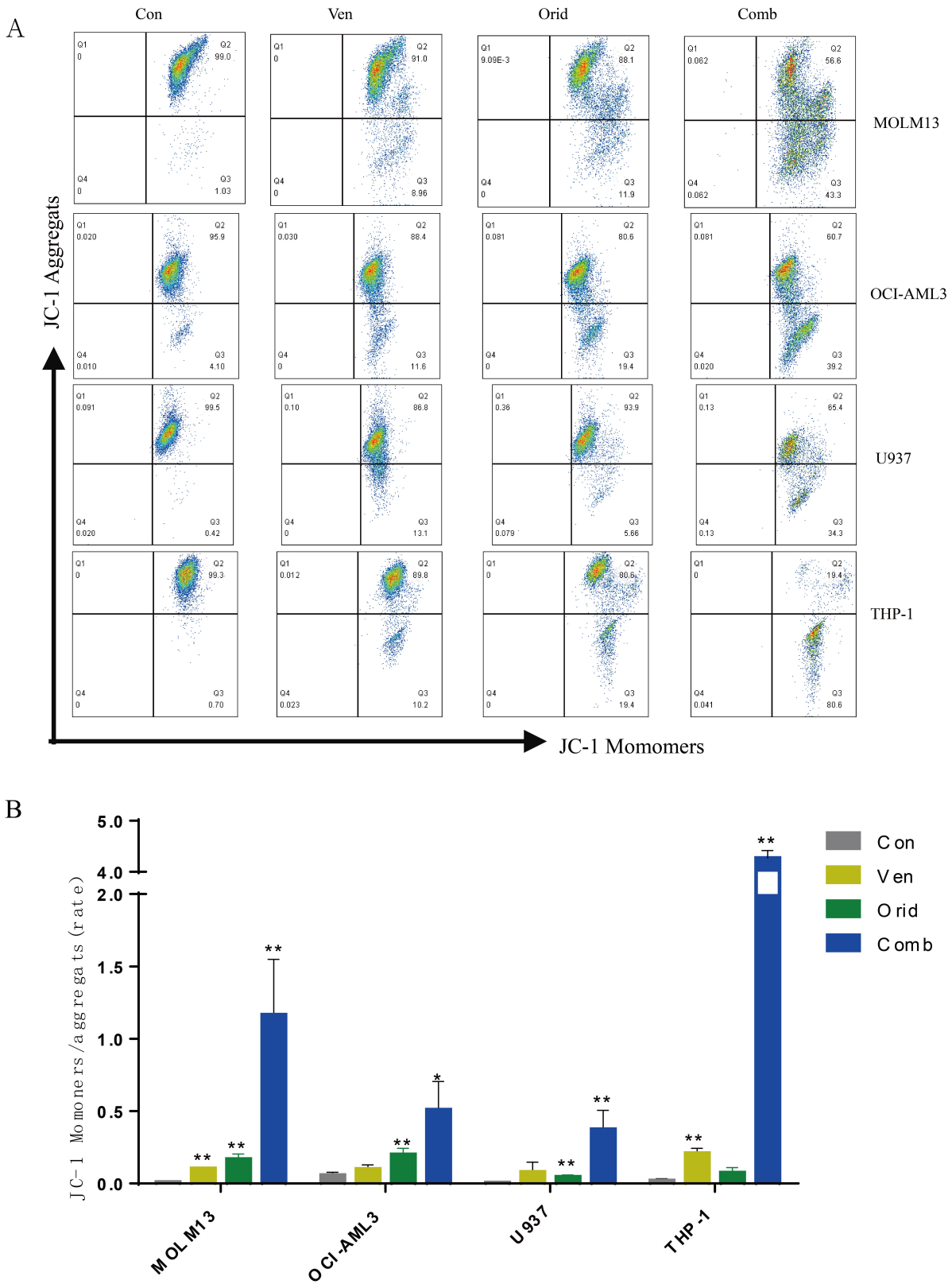

Fig. 3. Changes in mitochondrial membrane potential of AML cells treated with venetoclax or oridonin alone, or in combination. (A) Venetoclax and Oridonin were applied to four cell lines, including MOLM13, OCI-AML3, U937, and THP-1 for 24 h (the concentration of venetoclax was 2.5 nM, 4 μM, 8 μM, 1 μM in MOLM13, OCI-AML3, U937, and THP-1 cells, respectively; the concentration of oridonin was 2 μM, 4 μM, 8 μM, 4 μM, respectively), and the ratio of JC-1 monomers/polymers was detected by flow cytometry. (B) Statistical differences between experimental groups and control groups were compared using *t*-test (*: $p < 0.05$, **: $p < 0.01$).

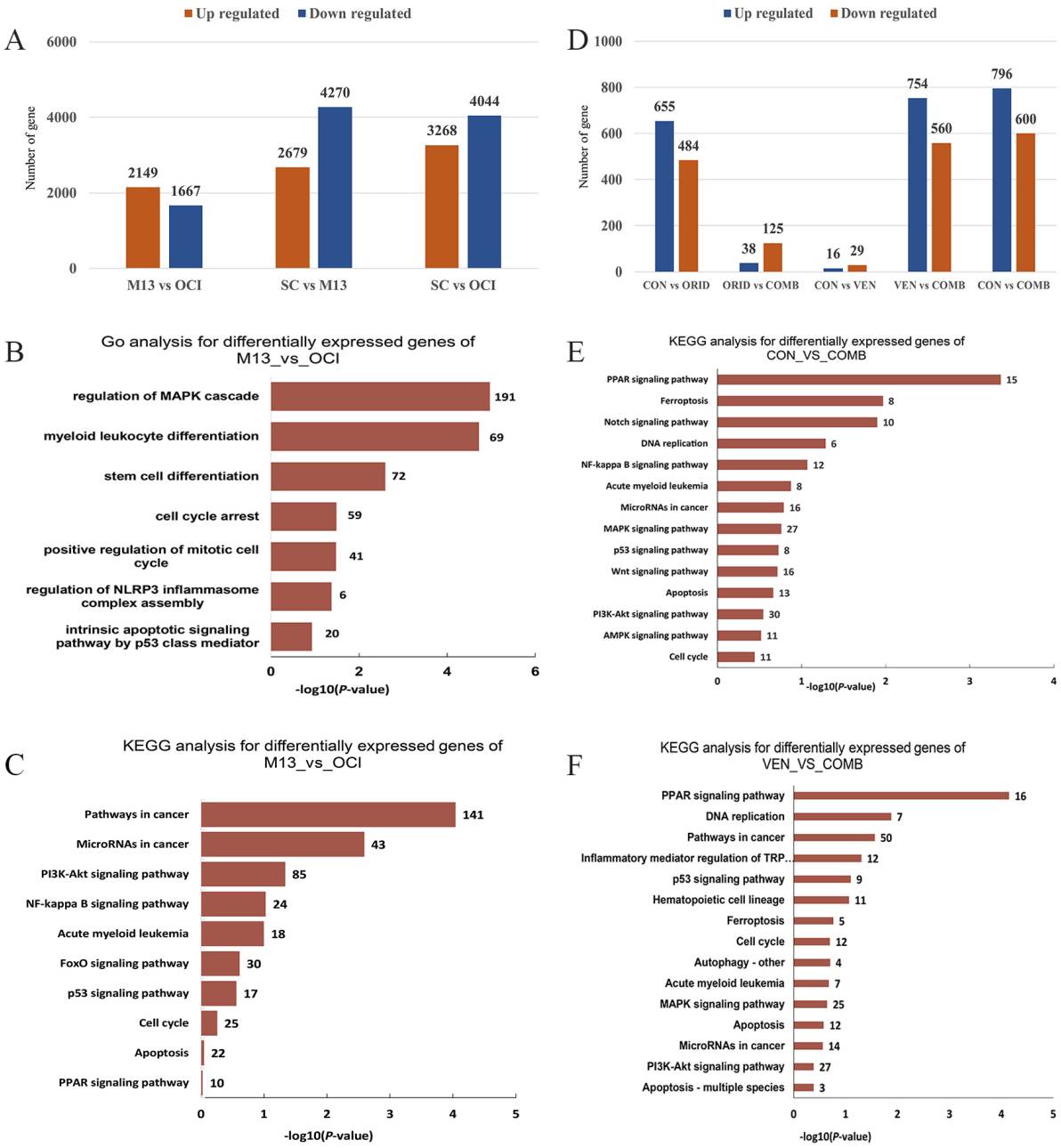

Fig. 4. Differential gene expression analysis and enrichment results of transcriptome sequencing. (A) Differential gene expression in OCI-AML3, MOLM13, and normal hematopoietic stem cells. (B,C) Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes between MOLM13 and OCI-AML3. (D) Differential gene expression before and after drug treatment of OCI-AML3 cells. (E) KEGG enrichment analysis of differential gene expression between combination drug treatment and control groups. (F) KEGG enrichment analysis of differential gene expression between th4 combination drug group and venetoclax monotherapy group. SC, stem cells from healthy person; Con, control; Ven, venetoclax; Orid, oridonin; Comb, combination of Ven and Orid.

increased the expression of p21 compared to venetoclax alone. Oridonin down-regulated the expression of cyclin D1, and both drugs down-regulated the expression of c-Myc. When used together, this effect was further enhanced as the two drugs had no significant effect on the total protein expression of CDK2, but oridonin could inhibit the phosphorylation of CDK2 in MOLM13 cells. This led to a decrease in the level of phospho-CDK2 and G1 cell cycle arrest. In OCI-AML3 cells, oridonin up-regulated phosphorylation of CDK2, leading to an increase in the level of phospho-CDK2. This finding may help explain the different cell cycle arrest stages induced by venetoclax and oridonin in these two cell lines (Fig. 5C).

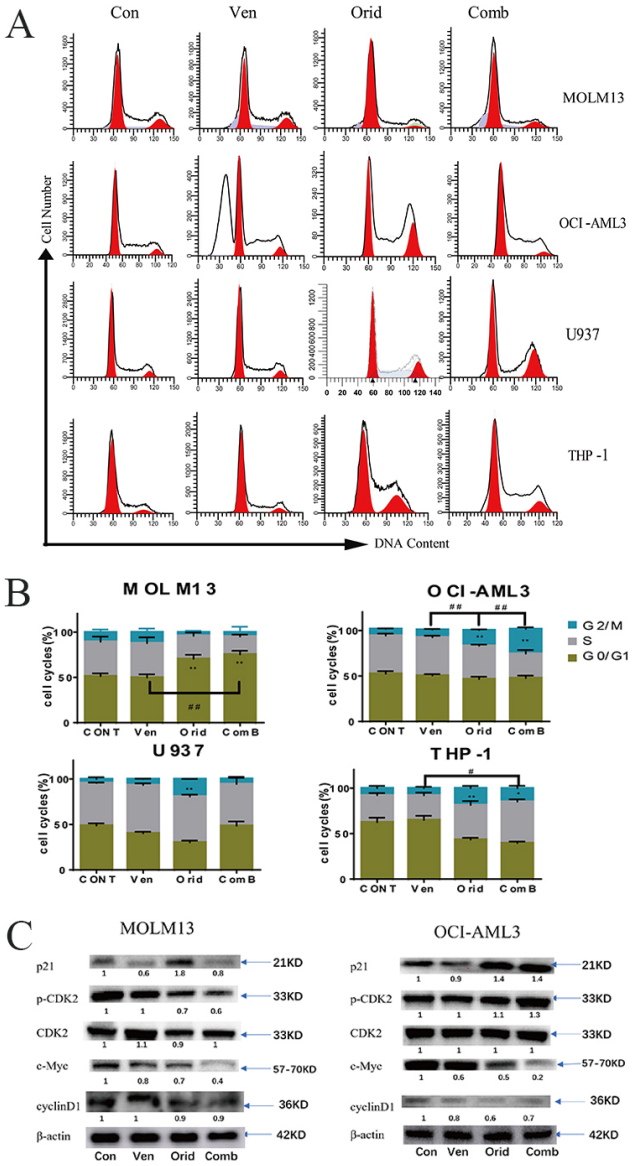

Fig. 5. Cell cycle alterations in AML cells treated with venetoclax or oridonin alone, or in combination. (A) Venetoclax and oridonin were applied to MOLM13, OCI-AML3, U937, and THP-1 for 24 h. The concentration of venetoclax was 2.5 nM, 4 μM, 8 μM, 1 μM in MOLM13, OCI-AML3, U937, and THP-1 cells, respectively. The concentration of oridonin was 2 μM, 4 μM, 8 μM, 4 μM, respectively. Cell cycle dynamics were obtained by flow cytometry. (B) Statistical differences between experimental groups and control groups, as well as between combination groups and single drug groups, in each cell line were compared using *t*-test (* experimental group vs control group; # combination group vs single drug group; */#: $p < 0.05$, **/##: $p < 0.01$). (C) Expression of indicated cell cycle-associated proteins by western blotting. Con, control; Ven, venetoclax; Orid, oridonin; Comb, combination of Ven and Orid.

3.6 Venetoclax and Oridonin Synergistically Promote AML Cell Apoptosis by Inhibiting AKT Signaling

The results of transcriptomic sequencing suggested that the PI3K/AKT and p53 signaling pathways may affect the sensitivity of AML cells to venetoclax. To investigate the mechanism of action, we harvested MOLM13 and OCI-AML3 cells treated with venetoclax, oridonin, or combination for 24h, extracted total protein, and performed western blotting analysis. The results showed that both venetoclax and oridonin can inhibit AKT phosphorylation, decrease phospho-AKT abundance. However, we noted that this effect is enhanced in MOLM13 cells. When the two drugs were used in combination, the inhibitory effect was further increased (Fig. 6A).

To verify the role of the AKT signaling in this process, we added the AKT agonist SC-79 to overcome the inhibitory effect of oridonin on AKT. SC-79 is an AKT agonist that induces phosphorylation of AKT at Thr308 and Ser473 in the cytoplasm and promotes downstream signaling pathway activity. We performed this experiment using the OCI-AML3 cell line which is resistant to venetoclax, and treated cells with 4 μM oridonin alone, 10 μM SC-79 alone, or the combination of the two by pre-treating cells with SC-79 for 1 h before adding oridonin. The level of AKT phosphorylation and cell apoptosis rate were measured 24h later. The results showed that SC-79 alone could increase the abundance of phospho-AKT, and when used in combination with oridonin, it could reverse the inhibitory effect of oridonin on AKT phosphorylation (Fig. 6B). Co-culturing SC-79 with oridonin reduced the apoptotic response induced by oridonin (Fig. 6C).

3.7 The Signaling Pathways in AML Cells Effected by Venetoclax Combined with Oridonin

The signaling pathways in AML cells influenced by oridonin and venetoclax are shown in Fig. 7.

3.8 Synergistic Anti-Leukemia Effect of Venetoclax and Oridonin in Vivo

A total of 5×10^6 OCI-AML3 cells stably expressing luciferase were injected into immunocompromised NTG mice via tail vein injection. On the fourth day, *in vivo* imaging was performed to confirm successful cancer cell engraftment. Subsequently, mice were subjected to drug treatment, and biweekly *in vivo* imaging was conducted to quantify fluorescence intensity. The results indicated that there was no statistically significant difference in fluorescence intensity between the animal groups treated with single-agent venetoclax or oridonin when compared to the control group ($p > 0.05$); however, the difference between the combination therapy group and the control group was statistically significant ($p < 0.01$) (Fig. 8A,B). This finding suggests that venetoclax and oridonin combination therapy could effectively reduce tumor burden in AML xenografted mice. On the eleventh day of treatment, three

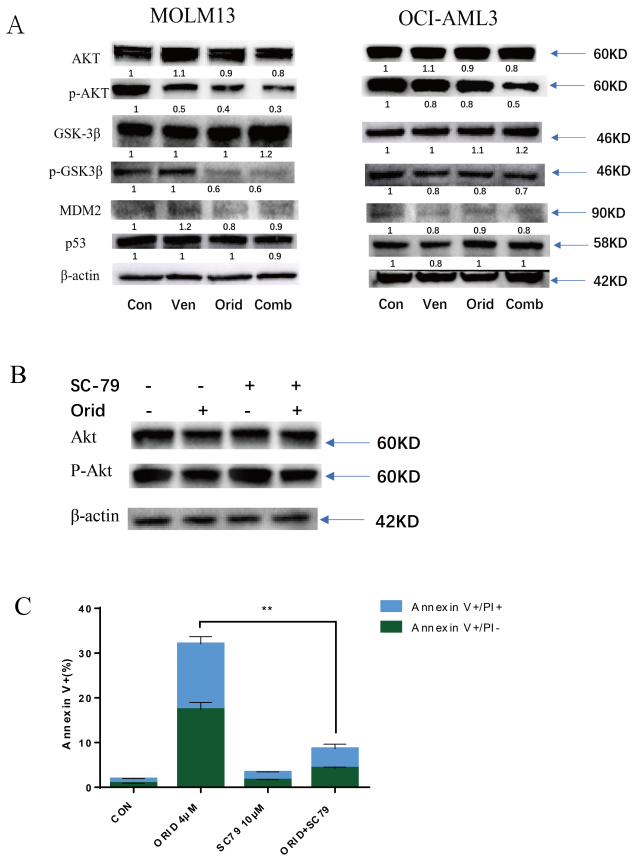

Fig. 6. Expression of key molecules in the MAPK/AKT and p53 pathways. (A) The effects of venetoclax and oridonin on indicated protein abundance in AML cells was investigated by western blotting. (B) Western blotting was used to examine AKT phosphorylation following 4 μM oridonin. Note this effect was reversed by pre-treatment with 10 μM SC-79 for 1 h. (C) Flow cytometry was used to detect that oridonin could induce apoptosis in OCI-AML3 cells, but this effect was abrogated by pre-treatment with SC-79 for 1 h (**: $p < 0.01$).

mice from each group were euthanized, and the size of the spleen was measured. We observed that the spleens of mice in the treatment groups showed enlargement due to infiltration of AML cells. Nevertheless, after receiving the combination drug treatment, a noticeable reduction in tumor burden was observed, accompanied by a shrinkage in spleen size (Fig. 8C). To assess the changes in the proportion of myeloid progenitor cells, bone marrow cells, and spleen grinding cells, cells were labeled with CD33/CD45 antibodies and subsequently incubated. The flow cytometry analysis revealed an increase in the proportion of CD33/CD45 double-positive cells, indicating myeloid progenitor cells was higher in the spleen compared to the bone marrow. In the femoral bone marrow, venetoclax monotherapy group exhibited a significant decrease in the proportion of CD33/CD45 double-positive cells compared to controls ($p < 0.05$). However, there was no statistically significant difference observed between the oridonin monotherapy group and the control group. The combination therapy group demonstrated a further decrease in CD33/CD45 double-positive cells was noted compared to the control group and this was a significant difference ($p < 0.01$). In the spleen, no statistically significant difference was observed in the proportion of CD33/CD45 double-positive cells between either of the monotherapy groups and the control group ($p > 0.05$). However, after receiving combination therapy, a statistically significant decrease in the proportion of CD33/CD45 double-positive cells was observed when compared to either the control group or the monotherapy groups ($p < 0.05$) (Fig. 8D,E). The lungs, liver, and kidneys of the mice were dissected and immunohistochemistry staining was performed using a human-specific (hCD45, #CY5311, Abways, Shanghai, China) antibody. The expression of hCD45 in the combination therapy group was lower than that in the control group (Fig. 8G). Survival time of the mice was recorded, and the results demonstrated a significant extension in the survival time of the combination therapy group compared to controls ($p < 0.01$). However, there was no statistically significant differences observed in animal survival time between the combination and the monotherapy groups ($p > 0.05$) (Fig. 8F).

4. Discussion

Acute myeloid leukemia (AML) is a malignant tumor of hematopoietic stem progenitor cells, and the sustained proliferation of leukemia cells is related to dysregulated maturation and differentiation functions [14]. Venetoclax is an orally administered, highly selective Bcl-2 inhibitor that is currently widely used in the treatment of AML patients who cannot tolerate high-intensity chemotherapy. These patients display a remission rate of approximately 70% in newly diagnosed patients.

The Bcl-2 protein is an important member of the Bcl-2 family of apoptosis modulators. When Bcl-2 and Bax form heterodimers, they inhibit cell apoptosis and thus exhibit an anti-apoptotic role. In contrast, when Bax forms homodimers, it induces cells to undergo apoptosis [15]. When Bim binds to Bcl-2 it blocks the apoptosis process, but when it dissociates from Bcl-2, it can induce cells to undergo apoptosis. Bad can also bind to Bcl-2 to promote Bim dissociation, while Mcl-1 can bind to dissociated Bim to promote an anti-apoptotic role [4,16]. Studies have shown that venetoclax resistance is related to Mcl-1 overexpression and that the activity of Mcl-1 is related to its phosphorylation site [12]. Phosphorylation of Ser159 residue of Mcl-1 (Mcl-1s) promotes Mcl-1 ubiquitination and degradation leading to decreased Mcl-1 abundance and promotion of cell death. In contrast, phosphorylation of the Thr163 residue of Mcl-1 (Mcl-1t) increases its stability and cellular abundance, thereby exerting an anti-apoptotic effect.

This study found that the combination of venetoclax and oridonin has an inhibitory effect on AML cell prolif-

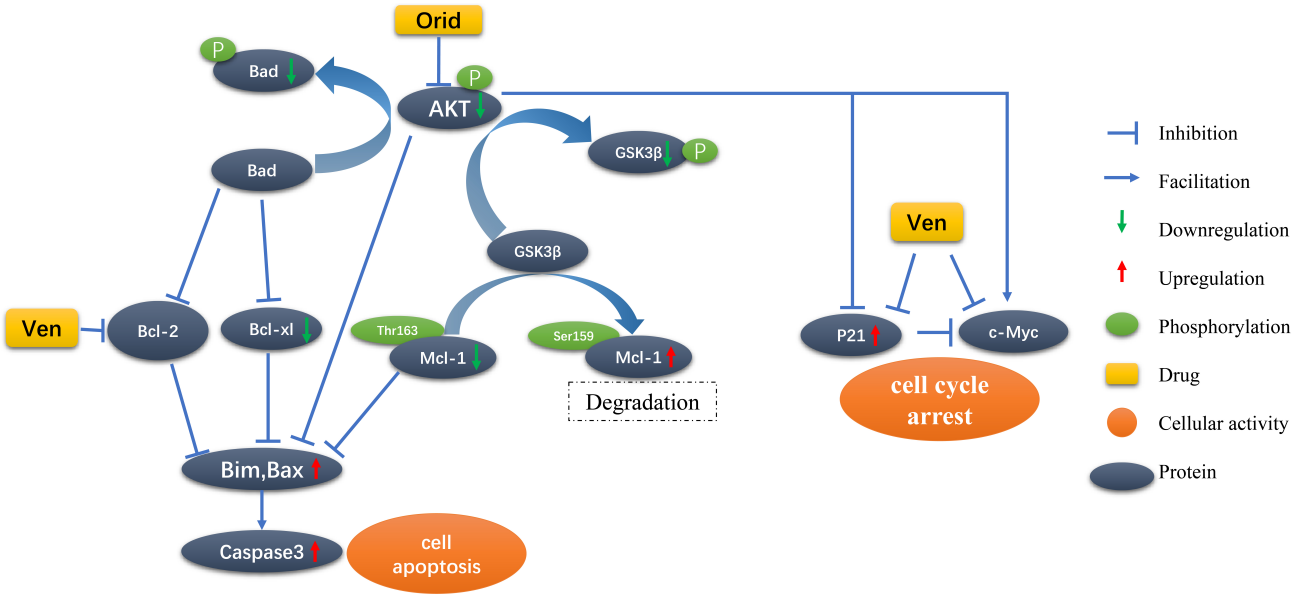

Fig. 7. The signaling pathways impacted by combination treatment with venetoclax and oridonin in AML cells. Orid, oridonin; Ven, venetoclax.

eration and induces an apoptotic response. However, the degree to which it inhibits AML cell proliferation varies is dependent upon the cell line used. After treatment with venetoclax for 24h, the abundance of Mcl-1s increased and the abundance of Mcl-1t decreased in venetoclax-sensitive MOLM13 cells. This results in Mcl-1 being readily degraded, which may be responsible, in part, to this cell line's sensitivity to venetoclax. In contrast, OCI-AML3 cells, which are resistant to venetoclax, showed the opposite effect with an increase in Mcl-1t abundance, a decrease in Mcl-1s abundance, increased in Mcl-1 stability, and an enhanced anti-apoptotic effect. Oridonin can inhibit the generation of Mcl-1t, reduce the resistance of OCI-AML3 cells to venetoclax, and synergistically enhance its anti-apoptotic effect. Oridonin functions by inhibiting AKT phosphorylation and this results in decreased abundance of activated phospho-AKT. Activated phospho-AKT phosphorylates GSK-3β resulting in inactivation of GSK-3β function. Therefore, oridonin can reduce GSK-3β phosphorylation, resulting in an increase its non-phosphorylated state, and causing GSK-3β to phosphorylate Mcl-1 at Ser159. This leads, in turn, to Mcl-1 being easily ubiquitinated and degraded, and weakening its anti-apoptotic effect. As a result, factors affecting venetoclax resistance mediated by Mcl-1 in cells are reduced, and the cells become sensitive to venetoclax once again. Co-culturing OCI-AML3 cells with the AKT activator SC-79 and oridonin can reverse the decrease in phospho-AKT abundance caused by oridonin, and reduce the proportion of OCI-AML3 cell apoptosis caused by oridonin. This finding suggests that AKT plays a critical role in the synergistic induction of cell apoptosis by venetoclax and oridonin.

Activated AKT can also phosphorylate Bad to form phospho-Bad, which cannot bind to Bcl-2 and prevent the release of Bim and Bax. Through this mechanism phospho-Bad serves an anti-apoptotic role [12]. Oridonin reduces the formation of phospho-Bad by inhibiting AKT activation and, in turn, this increases the amount of non-phosphorylated Bad and promotes apoptosis.

Activated AKT can also directly suppress the expression of pro-apoptotic proteins such as Bim and Bax [17]. Oridonin upregulates the cellular abundance of Bim and Bax proteins, downregulates the expression of the anti-apoptotic protein Bcl-xl by inhibiting AKT phosphorylation/activation, and this leads to the promotion of AML apoptosis.

The cell cycle is a complex biochemical process controlled by interaction among various cell cycle regulatory factors. p21, CDK2, c-Myc, and cyclin D1 are closely related to cell cycle control. It has been reported that cyclin D1, which regulates growth through the G1 phase can be suppressed by the AKT/GSK-3β axis [18]. p21, encoded by the *CDKN1A* gene, is an important member of the cyclin-dependent kinase (CDK) inhibitor family that participates in the regulation of the transition from G1 to S phase by inhibiting the activity of the CDK2/cyclin E complex [19]. p21 blocks the G1/S cell cycle transition, and there is ample evidence that the PI3K/AKT signaling pathway is involved in the regulation of p21. AKT activation can promote the expression of MDM2, which binds to p53 and blocks its activity, resulting in increased expression of p21. Moreover, p21 can inhibit the function of CDK2/cyclin E complex, leading to cell cycle arrest [20,21]. In this study, venetoclax and oridonin both downregulated the expression of c-Myc, and when used in combination this effect was further en-

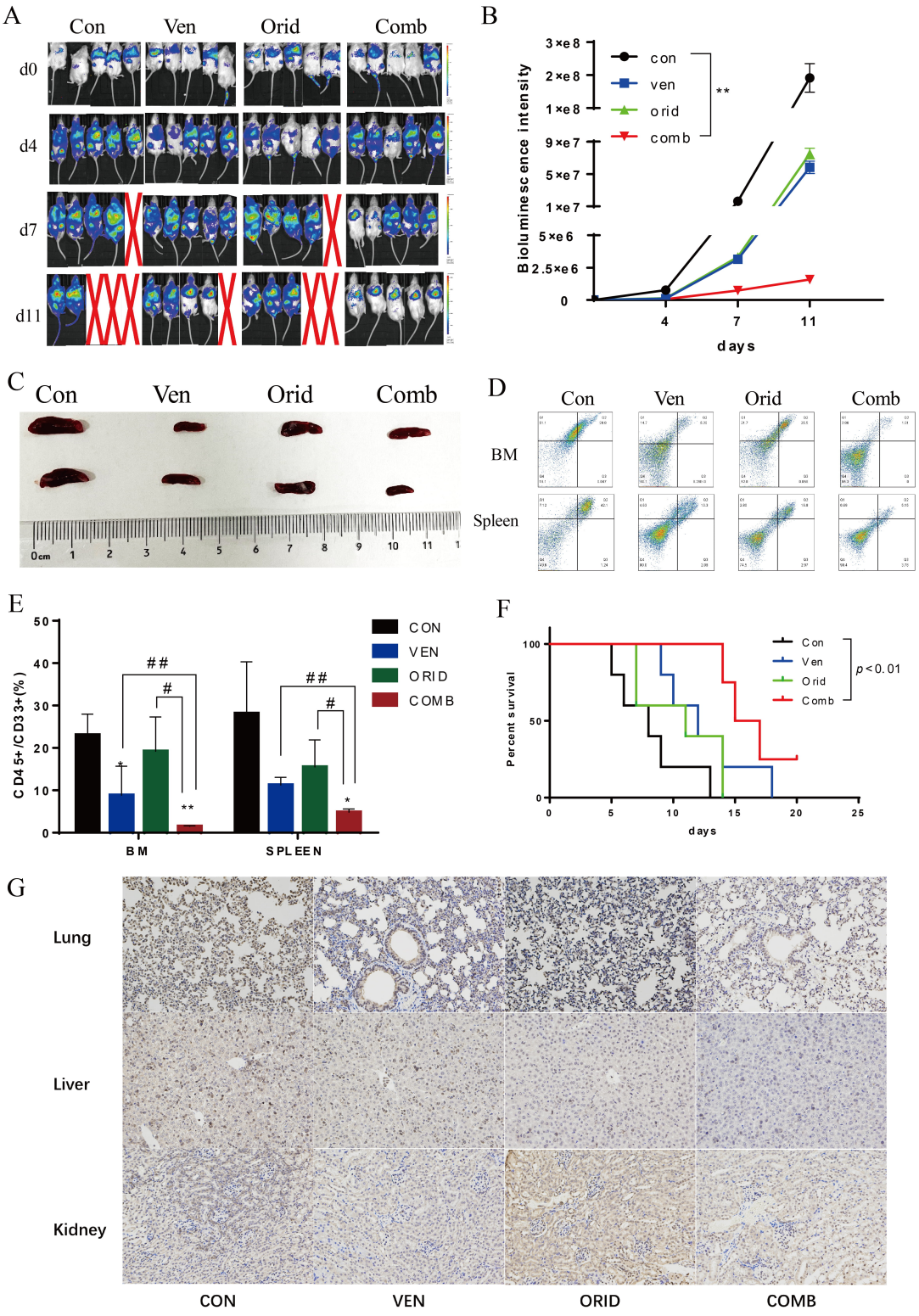

Fig. 8. The effect of venetoclax and oridonin on AML xenografts in mice. (A) *In vivo* imaging of AML-implanted mice treated with venetoclax and oridonin alone, or in combination. (B) Fluorescence intensity values measured in AML-implanted mice. (C) Spleens were obtained after euthanasia on day 11. (D) Flow cytometry scatter plot of $CD33^{+}CD45^{+}$ cells in bone marrow and spleen on day 11. (E) Proportion of $CD33^{+}CD45^{+}$ cells in bone marrow and spleen on day 11 (Mean ± SD, single */#: $p < 0.05$; double **/##: $p < 0.01$; */**: Ven/Orid/Comb vs Con; #/##: Comb vs Ven/Orid). (F) Kaplan-Meier survival curves of AML xenografted mice treated with venetoclax, oridonin, and their combination. Results were analyzed by Graphpad Prism software. (G) hCD45 staining in lungs, elbows, and kidneys of mice on day 11. Con, control; Ven, venetoclax; Orid, oridonin; Comb, combination of Ven and Orid.

hanced. Venetoclax downregulated the expression of p21 in AML cells, while oridonin upregulated its expression, possibly through the inhibition of MDM2. The overall expression level of CDK2 was not significantly affected by either drug. Venetoclax had no significant effect on CDK2 phosphorylation, while the effect of oridonin on phospho-CDK2 varied in different cells, for example, downregulating CDK2 expression in MOLM13 cells and upregulating CDK2 levels in OCI-AML3 cells. This indicates that in addition to regulating the phosphorylation of CDK2 through p21, oridonin conducts this function through modulating other pathways. It is precisely because of the difference in this pathway when the two drugs are used in combination as MOLM13 cells are arrested in G1 while OCI-AML3 cells are arrested in G2.

5. Conclusions

The objective of this study was to explore the mechanism of synergistic anti-leukemia effects of venetoclax and oridonin. Our research yielded three findings. First, oridonin was found to induce apoptosis of AML cells, block the cell cycle progression of AML cells, and synergistically enhance the anti-leukemia activity of venetoclax. Second, oridonin was shown to promote apoptosis by inhibiting AKT phosphorylation, regulating the phosphorylation status of Mcl-1, and promoting its degradation. Third, the combination of venetoclax and oridonin significantly inhibited the progression of AML in xenografted mice and significantly prolonged their survival time. The study suggests that the combination of venetoclax and oridonin may be a promising therapeutic approach for the treatment of AML.

Availability of Data and Materials

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

Author Contributions

LC and XW designed the research study. LC performed the research, analyzed the data and wrote the manuscript. DL and XG provided help and advice on RNA sequencing. CC provided help on performing the research. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Henan Cancer Hospital (approval No. 2019-KY-0017-001). The animal experiment was conducted in accordance with the project permit (No. 20200322) granted by the Zhengzhou University Committee on Life Science Ethics, and complied with the Zhengzhou University guidelines for animal care and use. Informed consent was obtained from all subjects involved in the study.

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Conflict of Interest

The authors declare no conflict of interest.

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