Original Article
Chidamide plus decitabine synergistically induces apoptosis of acute myeloid leukemia cells by upregulating *PERP*

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Abstract: Acute myeloid leukemia (AML) is a malignant clonal disease that originates from hematopoietic stem cells. Because AML has a generally unsatisfactory long-term prognosis, new therapeutic options are required. To this end, we explored the effects of chidamide and decitabine alone or in combination on the AML cell lines THP-1, MV4-11, HL60, and Kasumi-1. Notably, the two drugs exhibited a synergistic effect against these cell lines. Similarly, we also found potential synergistic effects in primary cells of relapsed/refractory (r/r) AML. A transcriptome sequencing analysis performed to elucidate the underlying molecular mechanism revealed differentially expressed genes and regulatory pathways, particularly with regard to apoptosis, when comparing cells subjected to single and combination treatments. We identified *PERP* as a downstream target gene of the transcription factors P53 and P63, and it was expressed at considerably higher levels in combination-treated cells relative to monotherapy-treated cells. We further used a lentivirus-mediated small interfering RNA to inhibit the endogenous expression of *PERP* in AML cell lines and observed a significant increase in cell proliferation. Collectively, our results demonstrate, for the first time, the role of *PERP* in the response of AML to a combination drug regimen, providing a new potential treatment protocol and target in this context.

Keywords: Chidamide, decitabine, acute myeloid leukemia, apoptosis, *PERP*

Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal disease characterized by the loss of normal differentiation of hematopoietic progenitor cells. It often presents as a severe infection, bleeding, or organ infiltration [1]. The overall prognosis of AML remains poor [2], and long-term survival is only achieved by approximately 40% and 10% of younger and older patients, respectively. The poor survival outcome is primarily attributed to a high rate of relapse, even after the achievement of complete remission (CR) with standard chemotherapy or allogeneic stem cell transplantation [3]. An improved understanding of AML pathogenesis has led to the development of various targeted inhibitors and other novel drugs, and combinations of these molecularly-targeted drugs with standard chemotherapies have generally yielded higher response rates [4]. For example, Jonas and Pollyea reported that the combina-

tions of hypomethylating agents and new drugs yielded better responses [5].

Chidamide (CS055), a novel oral histone deacetylase inhibitor (HDACI), selectively suppresses the activities of histone deacetylases 1, 2, 3, and 10. Many studies have investigated the therapeutic effects of chidamide for blood diseases and solid tumors [6, 7], and in December 2014, the China Food and Drug Administration approved chidamide for the treatment of peripheral T-cell lymphoma [8]. Decitabine (Dacogen®, 5-aza-2'-deoxycytidine) is a widely used DNA methylation inhibitor. Although its anti-leukemic activity was first reported almost 40 years ago, decitabine was only recently approved as a first-line treatment for elderly (>65 years old) AML patients deemed ineligible for intensive chemotherapy [9]. Here, we focus on the therapeutic and potentially synergistic effects of chidamide plus decitabine against AML cells.

Materials and methods

Cell lines, primary tumor cells, and reagents

The AML cell lines THP-1, MV4-11, HL60, and Kasumi-1 were purchased from the American Type Culture Collection (Manassas, VA, USA). MV4-11 cells were cultured in Iscove's Modified Dulbecco's Media (Hyclone, Logan UT, USA), and all other lines were cultured in Roswell Park Memorial Institute-1640 medium (Hyclone). All cell culture media were supplemented with 10% fetal bovine serum (Gemini Bio-Products, Sacramento, CA, USA). All cell lines were cultured in a 95% humidity incubator at 37.5°C in an atmosphere of 5% CO_2.

Primary AML cells were isolated from peripheral blood containing >50% blasts. These specimens were collected before chemotherapy. Chidamide was received as a gift from Chipscreen Biosciences Co., Ltd. (Shenzhen, China). Decitabine and puromycin 2HCl were obtained from Selleck Chemicals (Houston, TX, USA).

Cell viability assay

An MTT proliferation assay kit (Biosharp Life Sciences, Anhui, China) was used to measure the viability of cells exposed to 0.1-15 μM chidamide and 0.25-20 μM decitabine for 48 h. For combined experiments, the maximum drug concentrations were close to the 50% inhibitory concentration (IC_{50}) values and then diluted in equal proportions. Cells were plated in 96-well plates at a concentration of 2×10^5 cells/mL in 100 μL of a complete medium and treated with different doses of drugs for 48 h. Then, 20 μL of MTT reagent (5 mg/mL) was added to each well and incubated for 4 h at 37.5°C. Finally, the dissolving solution was added, and the optical density was measured at 570 nm the next day. The experiments were repeated in triplicate, and each sample was tested three times.

Flow cytometry analysis

The AML cell lines were incubated with chidamide and decitabine alone or in combination for 48 h. Then, apoptosis was measured using Alexa Fluor 647-conjugated annexin V and propidium iodide (PI) (4A Biotech Co., Ltd., Beijing, China), according to the manufacturer's instructions. Briefly, the cells were harvested, washed twice with phosphate-buffered saline, and

resuspended in 200 μL of binding buffer. Then, the cells were incubated with 2 μL Alexa Fluor 647-conjugated annexin V and 1 μL PI for 10 min. Apoptosis was analyzed using a Navios flow cytometer (Beckman Coulter, Brea CA, USA).

Western blots

The total proteins were extracted from each sample using a universal protein lysate/extraction reagents (Aidlab, Beijing, China) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). The protein concentration was measured using a BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Then, 40 μg of each protein sample was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) and transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, USA). Following blocking, primary antibodies specific for the following proteins were applied: cleaved caspases 3 and 9, caspase 8, BCL-2, BCL-XL, and phospho-P53 (Ser15) (p-P53) (Cell Signaling Technology, Danvers, MA, USA); PERP (Shanghai Abways Biotechnology Co., Ltd., Shanghai, China); AML-ETO (Santa Cruz Biotechnology, Dallas, TX, USA); MLL-AF4 (Bethyl Laboratories, Montgomery, TX, USA); GAPDH (Bioworld Technology, Louis Park, MN, USA); β-actin (4A Biotech); and β-tubulin (Sungene Biotech, Tianjin, China). After three washes with tris-buffered saline plus Tween-20, the membranes were incubated with appropriate secondary antibodies (ZSGB-Bio, Beijing, China). Finally, a chemiluminescent kit (Millipore Corporation, Billerica, USA) was used to visualize the reactive proteins. Conventional western blot protocols were used throughout [10].

ATP-based tumor chemosensitivity assay (ATP-TCA)

An ATP-TCA kit (Huzhou Hichuang Biotech Co., Ltd., Zhejiang, China) was used according to the provided instructions to evaluate cell viability by ATP chemiluminescence. This measured the tumor cell sensitivity to the antitumor drugs [11].

Gene expression profile generation and analysis

After a 48 h incubation with or without the aforementioned drugs, MV4-11 cells were har-

vested and stored in Trizol reagent (Molecular Research Center, Cincinnati, OH, USA) to isolate RNA, which was processed and sequenced by Novogene (Beijing, China) on a HiSeq device (Illumina, San Diego, CA, USA) to yield a transcriptome. Transcriptome sequencing (RNA-seq) libraries were constructed using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). The RNA-seq reads were aligned to a reference genome to generate a precise junction read map using STAR RNA-Seq aligner software (version STAR_2.5.1b). The read numbers mapped to each gene were counted using HTSeq v0.6.0 software. Significant differences in expression were analyzed using DESeq2. Genes with absolute fold changes >0.5 and a Benjamini-Hochberg adjusted *P*-value <0.05 were validated further. clusterProfiler software was used to perform Gene Ontology and DO functional enrichment analyses as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathway enrichment analyses of differentially expressed gene (DEG) sets.

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

The extracted total RNAs were reverse transcribed (Takara, Dalian, China) and subjected to qRT-PCR using Go Taq qPCR Master Mix (Promega, Shanghai, China), according to the manufacturer's instructions. The cycling parameters used were 95°C for 2 min; followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min, and then 65°C to determine the dissociation curve. The relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method [12]. *GAPDH* expression was used as an internal control for normalization. The primer sequences for *PERP* were 5'-AGATGCTTGTCT-TCCTGAGAGTGAT-3' (forward) and 5'-GATGTAA-GTGACAGCAGGGTTGG-3' (reverse), and those for *GAPDH* were 5'-CGCTGAGTACGTCGTGGAG-TC-3' (forward) and 5'-GCTGATGATCTTGAGGC-TGTTGTC-3' (reverse).

Lentivirus construction and infection

The process of RNA interference (RNAi) lentiviral packaging is roughly as follows: *PERP*-RNAi contains three specific constructs, encoding small interfering RNA (siRNA) designed to knock down *PERP* expression and a control RNAi lentiviral-based plasmid that includes a siRNA construct encoding a known scrambled sequ-

ence that does not result in specific degradation of any cellular mRNA. We selected lentiviral vector GV248, which contains a puromycin-resistance gene and a green fluorescent protein reporter gene. For lentiviral packaging, 293T cells were co-transfected with *PERP* siRNA or negative control siRNA and two helper plasmids. The supernatant containing packaged lentivirus from the cells was collected, concentrated by centrifugation, and purified by filtration. THP-1, MV4-11, and HL60 cells were infected with these three lentiviruses and the control lentivirus, according to the manufacturer's protocol.

Mononuclear cell separation

Peripheral blood samples were collected from 35 AML patients (non-acute promyelocytic leukemia) and 20 healthy individuals. The peripheral blood mononuclear cells (PBMCs) were separated using Ficoll solution (TBD Science, Tianjin, China), according to the manufacturer's protocol.

Statistical analysis

Data were expressed as the mean of several independent experiments ± standard deviation or standard error of the mean. The apoptosis rate of cells and the expression level of *PERP* mRNA were analyzed by *t*-tests, and the other results were analyzed using two-way analysis of variance (Prism 7.0; Graph Pad Inc., San Diego, CA, USA). Significant differences were considered at $*P<0.05$, $**P<0.01$, $***P<0.001$, and $****P<0.0001$. The IC_{50} was calculated using SPSS v26.0 software (IBM Corp., Armonk, NY, USA). Combination indices (CIs) were calculated using Calcusyn v2.0 (Biosoft, Cambridge, UK).

Results

Chidamide plus decitabine acted synergistically on AML cell lines

We first calculated the IC_{50} values for chidamide and decitabine alone when applied to AML cell lines for 48 h. The IC_{50} values were lower for chidamide in all four cell lines (**Figure 1A**). Chidamide plus decitabine inhibited AML cell proliferation in a concentration-dependent manner, and the combination had a stronger inhibitory effect than that of either drug alone (**Figure 1B**). Next, we calculated the CIs to
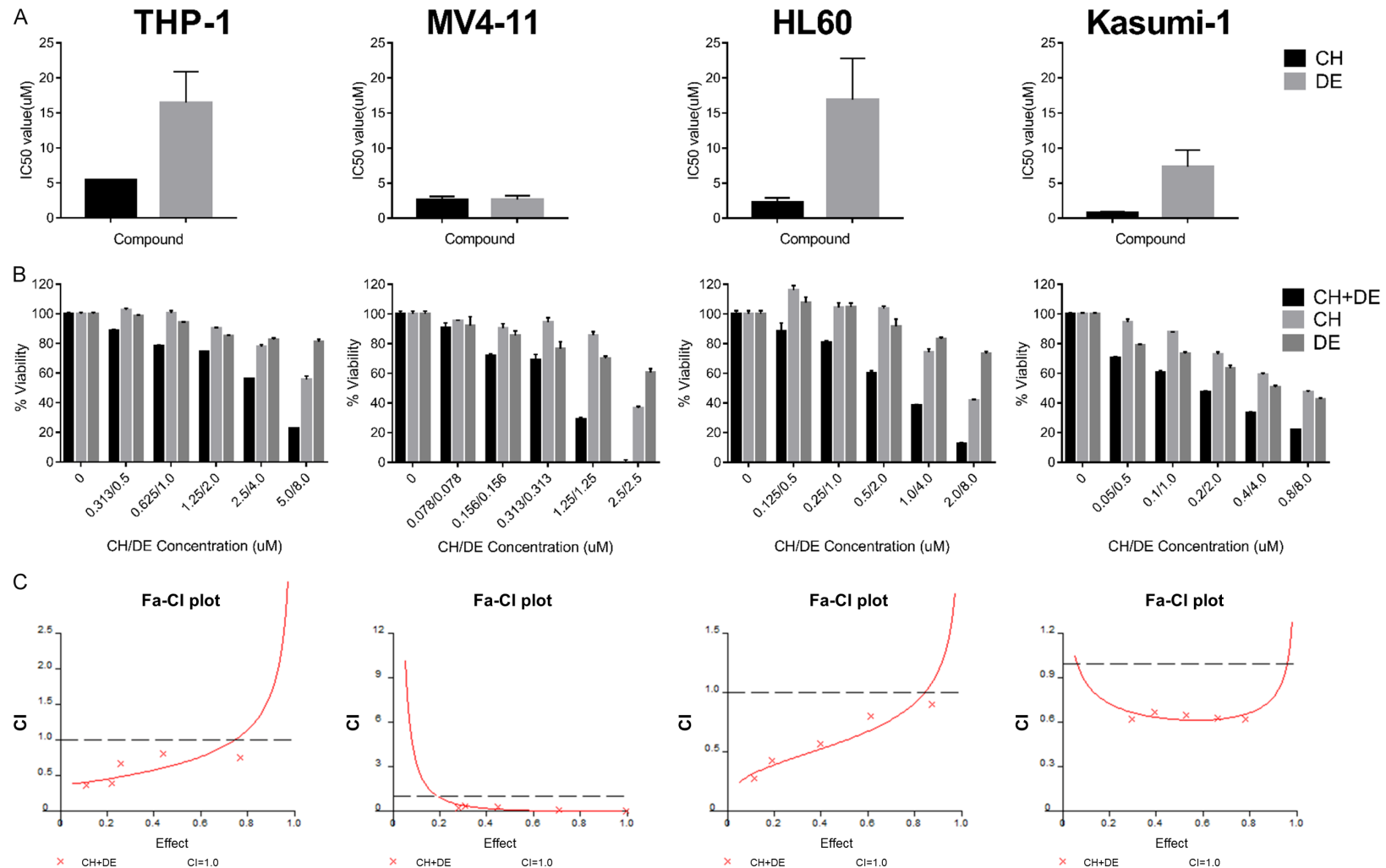
Figure 1. Chidamide plus decitabine synergistically inhibits the proliferation of acute myeloid leukemia (AML) cell lines. A. The 50% inhibitory concentration (IC$_{50}$) values in THP-1, MV4-11, HL60, and Kasumi-1 cells. After treating the AML cell lines with chidamide or decitabine for 48 h, an MTT assay was performed to determine the cell survival rate, and SPSS 26.0 software (IBM Corp., Armonk, NY, USA) was used to calculate the IC$_{50}$ value in THP-1, MV4-11, HL60, and Kasumi-1 cells. B. Viability of AML cell lines determined by MTT assay after treatment with chidamide and decitabine alone or in combination for 48 h. Values represent means expressed as percentages compared with the untreated control. Error bars represent standard deviations. C. Combination indices (CIs) for the two drugs explored in the THP-1, MV4-11, HL60, and Kasumi-1 cell lines using Calcusyn 2.0 software (Biosoft, Cambridge, UK). The horizontal axis represents the inhibitory effect achieved when the two drugs are combined, and the vertical axis represents the CIs. A CI of <1, 1, or >1 indicates a synergistic, additive, or antagonistic effect, respectively. Abbreviations: chidamide (CH), decitabine (DE), chidamide plus decitabine (CH+DE), Fa-CI, fraction affected-combination index.

Table 1. Combination index values of THP-1 acute myeloid leukemia cell line

CH (μM)	DE (μM)	CI values
0.3125	0.5	0.369
0.625	1	0.395
1.25	2	0.678
2.5	4	0.817
5	8	0.756

CH: chidamide; DE: decitabine; CI, combination index.

Table 3. Combination index values of HL60 acute myeloid leukemia cell line

CH (μM)	DE (μM)	CI values
0.125	0.5	0.275
0.25	1	0.431
0.5	2	0.570
1	4	0.803
2	8	0.902

CH: chidamide; DE: decitabine; CI, combination index.

Table 2. Combination index values of MV4-11 acute myeloid leukemia cell line

CH (μM)	DE (μM)	CI values
0.15625	0.15625	0.242
0.3125	0.15625	0.385
0.625	0.625	0.278
1.25	1.25	0.089
2.5	2.5	6.16e-011

CH: chidamide; DE: decitabine; CI, combination index.

Table 4. Combination index values of Kasumi-1 acute myeloid leukemia cell line

CH (μM)	DE (μM)	CI values
0.05	0.5	0.623
0.1	1	0.672
0.2	2	0.652
0.4	4	0.628
0.8	8	0.625

CH: chidamide; DE: decitabine; CI, combination index.

determine whether the two-drug combination had a synergistic effect. The CIs are shown in **Tables 1-4**, and the fraction affected-CI plot is shown in **Figure 1C**. The CIs of all concentrations were <1 in all four cell lines, indicating that the two-drug combination had a synergistic effect on AML cells.

Chidamide plus decitabine significantly induced apoptosis, inhibited the expression of AML fusion proteins, and upregulated p-P53 in AML cells

We performed flow cytometry analysis and western blot assays to determine the ability of the drugs to induce apoptosis in AML cells. Notably, treatment with chidamide plus decitabine induced higher rates of apoptosis than single treatment with either drug. Furthermore, chidamide alone and in combination induced a significantly higher apoptosis rate relative to the control (*P*<0.05; **Figure 2A**). The western blot assays showed that all AML cell lines exhibited increased levels of pro-apoptotic proteins and decreased levels of anti-apoptotic proteins in response to the combination treatment relative to the single-drug treatments (**Figure 2B**). We showed the original pictures of the western blot assays (Figures S1, S2, S3 and S4).

We further examined the effects of both drugs on fusion proteins, which are expressed in so-

me studied cell lines. Surprisingly, chidamide inhibited fusion proteins MLL-AF4 and AML-ETO, both alone and in combination with decitabine (**Figure 2C** and **2D**). We showed the original pictures of the western blot assays (Figure S5). Given the importance of *TP53* in the apoptotic pathway, we tested the effect of the drugs on p-P53 (ser15), and the results showed that chidamide plus decitabine upregulated *TP53* in both *TP53* mutant cells (MV4-11) and in *TP53* wild-type cells (Kasumi-1) (**Figure 2E**). We showed the original pictures of the western blot assays (Figure S6).

Primary AML cells were more sensitive to combination drugs

We included five cases of relapsed/refractory (r/r) AML in an ATP-TCA experiment to explore the effects of the studied drugs on primary AML cells. Notably, the combination of chidamide plus decitabine exerted synergistic effects on cells in four cases (**Figure 3A-D**). The clinical features of the five r/r AML cases are listed in **Table 5**.

Gene expression analysis

We performed gene expression profiling to explore the mechanism underlying the efficacy of combination therapy. Principal component analysis revealed that 89.37% of the cumulative variance could be explained by three
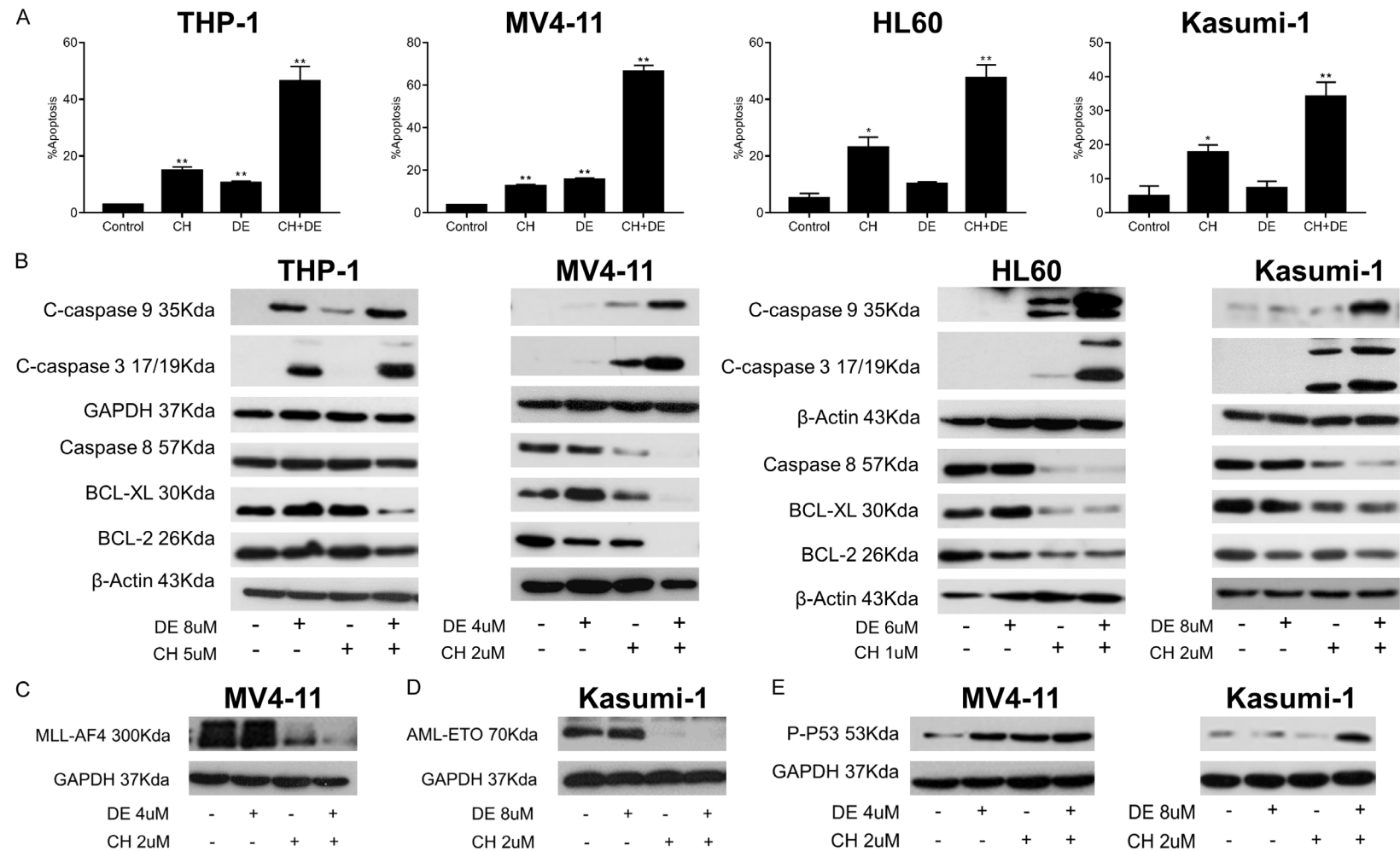
Figure 2. Chidamide plus decitabine significantly induced apoptosis, inhibited the expression of fusion protein, and upregulated phospho-P53 (ser15) in acute myeloid leukemia (AML) cell lines. A. The percentage of apoptosis in AML cell lines treated with chidamide and decitabine alone or in combination for 48 h, as determined by flow cytometry. Data are presented as the mean ± standard deviation of two independent experiments. (*t*-tests, *P<0.05 and **P<0.01 vs. control). B. Western blot analysis of the levels of cleaved caspase-3, cleaved caspase-9, caspase-8, BCL-XL, and BCL-2 proteins in THP-1, MV4-11, HL60, and Kasumi-1 cells after exposure to chidamide and decitabine alone or in combination for 24 or 48 h. GAPDH or β-actin was used as the loading control. C, D. Western blot analysis of the effects of chidamide and decitabine on the levels of AML-specific fusion proteins. The MV4-11 cells were treated with the drugs for 48 h, and the Kasumi-1 cells were treated with the drugs for 72 h. E. Western blot analysis of the effect of chidamide and decitabine on the level of phosphorylated P53 (ser15) in cells after 16 h of treatment alone or in combination. GAPDH was used as the loading control. Abbreviations: chidamide (CH), decitabine (DE), chidamide plus decitabine (CH+DE).
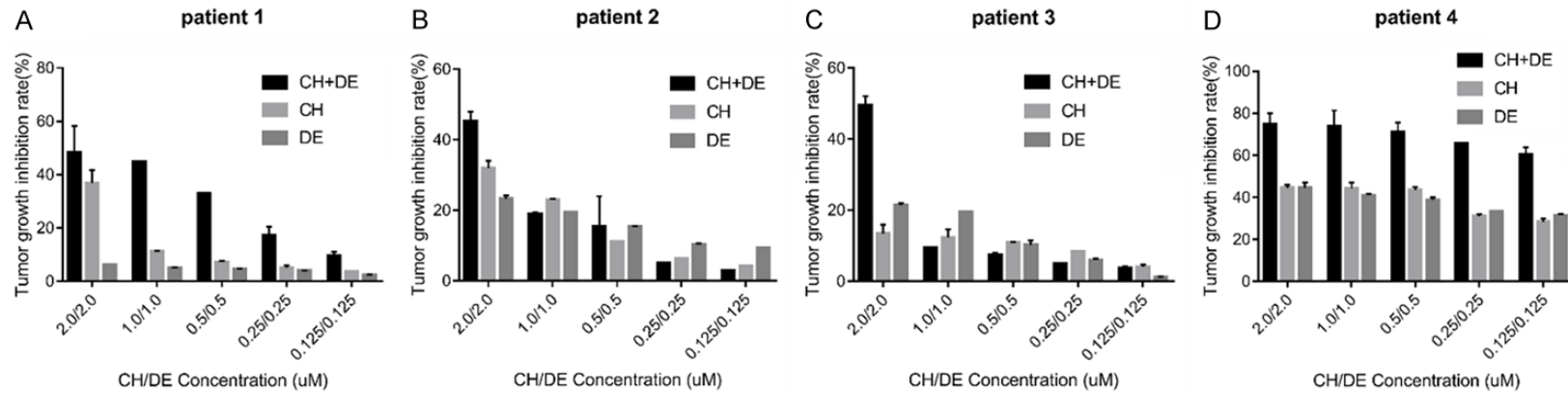
Figure 3. Sensitivity of relapsed/refractory (r/r) primary acute myeloid leukemia (AML) cell lines to chidamide plus decitabine as detected by an ATP-based tumor chemosensitivity assay (ATP-TCA). A-D. Inhibition rate of primary cells of r/r AML cells by chidamide plus decitabine. Values represent as percentages compared with the untreated control. Error bars represent the coefficient of variation. Abbreviations: chidamide (CH), decitabine (DE), chidamide plus decitabine (CH+DE).

Table 5. Clinical features of five patients with relapsed/refractory acute myeloid leukemia

Clinical features	Patients				
	P1	P2	P3	P4	P5
Gender	Female	Male	Male	Female	Female
Age (years)	61	71	73	50	72
FAB AML classification	AML-M1	AML-M2	AML with myelodysplasia-related changes	AML-M2	AML-M1
Genetic mutation/fusion gene	FLT3-ITD+, NPM1+, TET2+	AML1-ETO+, TET2+, ASXL1+	TP53+	TP53+	AML1-ETO+
Karyotype	46,XX,i(7)(q10)	46,XY,t(8;21)(q22;q22)	44,XY,del(5)(q13q33),del(7)(q22),dic(9;13)(q34;p11),del(12)(q11),-13.dic(13;19)(p11;q13),-16,inc(cp20)	45-47,XX,del(1)(p13). Del(3)(q12),+8,add(17)(p11),inc[cp20]	46,XX,t(8;21)(q22;q22)
Chemotherapy regimen	Induction: IDA × 1 Consolidate: ID-MA × 4 Relapse Reinduction: CLAG	Induction: DA × 1 Consolidate: IDA, DA, ID-M-A Relapse Reinduction: Di-priming	Induction: decitabine + CAG Reinduction: decitabine + arsenic acid + cytarabine	Induction: D-HAG Reinduction: D + DA No remission BCL-2 inhibitor + decitabine	Induction: DA No remission

FAB: French-American-British; AML, acute myeloid leukemia.

main components and demonstrated the experimental consistency and specificity of the therapeutic effect (**Figure 4A**). A hierarchical clustering heat map of the DEGs confirmed drug exposure-related differences in gene expression in the four MV4-11 cell subgroups (**Figure 4B**). A Venn diagram was plotted to show the overlap of the DEGs in each treatment group relative to the control (**Figure 4C**), and volcano maps of the DEGs were constructed to depict the upregulated and downregulated genes in response to combination treatment relative to single treatment (**Figure 4D** and **4E**). A KEGG enrichment analysis indicated that combination therapy induced genetic changes in multiple pathways, including those involved in the cell cycle, spliceosome, and lysosome, and these differed strongly from the genetic changes induced by single therapies (**Figure 4F** and **4G**). The DEG expression patterns included the significant (*P*<0.05) upregulation of some apoptosis-related genes, such as *PERP*, *AVEN*, and *BCL2L11*. Discrete changes in these gene pools likely provide a distinct molecular explanation for the synergistic effects of the drug combination in vitro.

Verification of RNA-seq results and lentivirus infection efficiency

The RNA-seq analysis revealed the significantly higher upregulation of *PERP*, a potential downstream target of P53 and P63, in response to combination treatment compared with the repose to a single treatment. Subsequent qRT-PCR (**Figure 5A**) and western blot (**Figure 5B**) findings confirmed the RNA-seq data. We showed the original pictures of the western blot assays (Figure S7).

We further explored the mechanism whereby chidamide and decitabine promote apoptosis and the role of *PERP* in AML cells by infecting THP-1, MV4-11, and HL60 cells with *PERP*-specific siRNA-encoding lentiviruses. Then, we used qRT-PCR and western blots to detect *PERP* mRNA (**Figure 5C**) and protein (**Figure 5D**), respectively. We showed the original pictures of the western blot assays (Figure S8). qRT-PCR demonstrated that *PERP* was most strongly knocked down by LV-*PERP*-RNAi-3 (66241-1; *P*<0.05), while PERP was undetectable by western blot in all infected cells. Thus, we selected LV-*PERP*-RNAi-3 (66241-1) for further experiments.

PERP downregulation strongly promoted proliferation and induced chemotherapeutic drug resistance in AML cells

Lentivirus-infected cells were subjected to an MTT assay to explore the role of *PERP* in AML cell proliferation. Notably, the AML cell lines infected with LV-*PERP*-RNAi-3 exhibited significantly increased proliferation rates relative to those infected with LV-SC-RNAi (control; **Figure 6A**). Next, we elucidated the role of *PERP* in response to combination drug treatment in stable lentivirus-infected cell lines. An MTT proliferation assay revealed that *PERP* knockdown was associated with significantly reduced toxicity in response to the combination treatment when compared with the control (scrambled siRNA) group (**Figure 6B**). Flow cytometry revealed that *PERP* knockdown attenuated drug-induced apoptosis (**Figure 6C**). Western blot revealed that *PERP* knockdown reduced the ability of the drugs to induce pro-apoptotic protein expression (**Figure 6D**). We showed the original pictures of the western blot assays (Figures S9 and S10). In summary, *PERP* knockdown strongly promoted proliferation and decreased the responsiveness of AML cells to the treatment with chidamide plus decitabine.

Primary AML cells exhibit reduced PERP expression relative to normal PBMCs

A qRT-PCR analysis of isolated PBMCs from 35 AML patients and 20 healthy individuals confirmed the significantly lower levels of *PERP* mRNA in the samples from the AML patients (**Figure 7**).

Discussion

Because AML is a biologically and clinically heterogeneous disease [13], the resultant limited treatment selectivity is associated with very high rates of r/r disease after standard chemotherapy. R/r AML is associated with a dismal prognosis, with 3-year overall survival rates of ≤10% [14, 15]. Accordingly, new and effective treatments are a matter of urgency.

Previous reports have described the great potential of chidamide for the treatment of hematological diseases [16, 17]. Accordingly, we explored the effects of chidamide and decitabine alone or in combination on AML cells. Furthermore, an exploration of drug toxicity
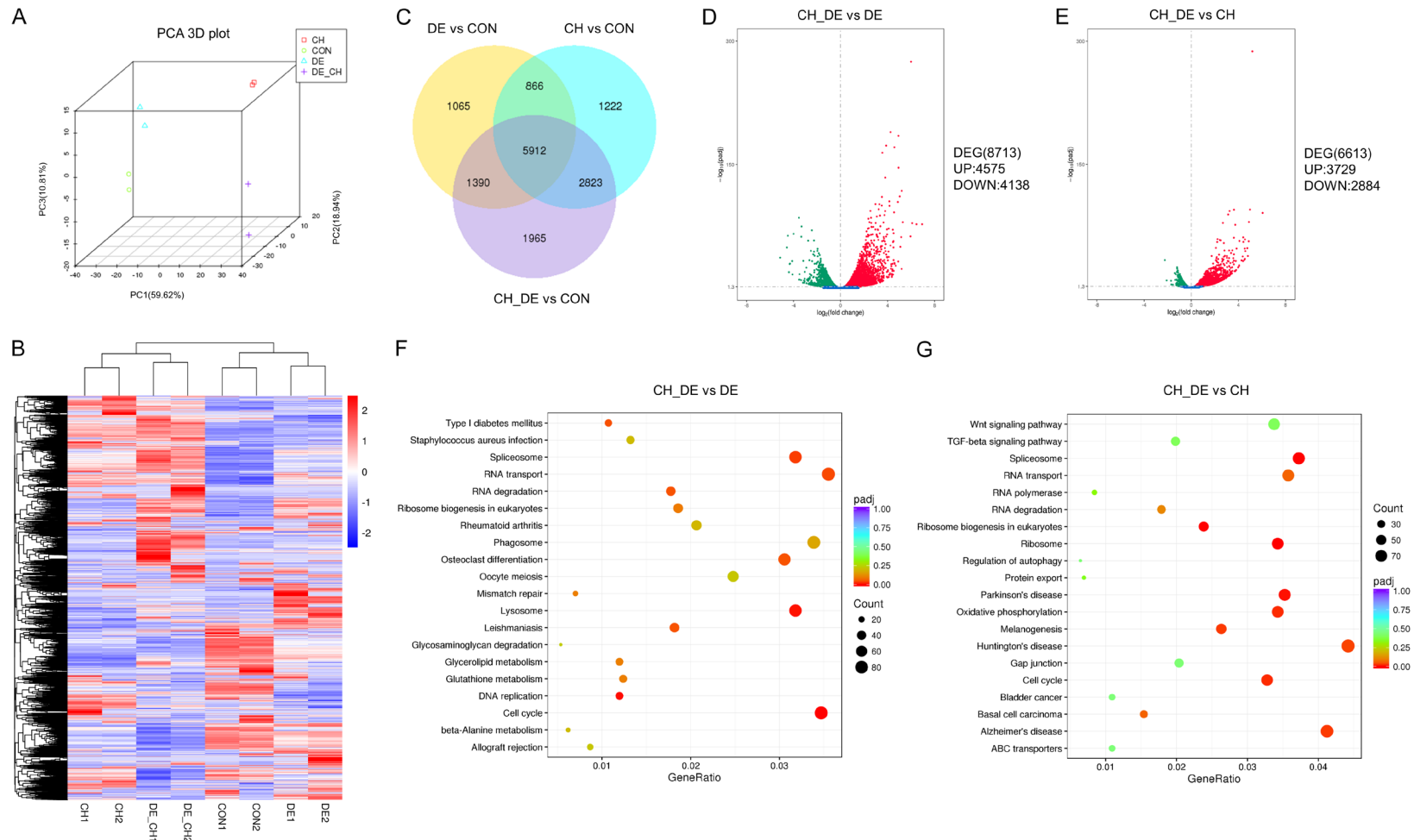
Figure 4. Gene expression analysis of MV4-11 cells. A. Principal component analysis (PCA) revealed obvious differences between treatments. B. A hierarchical clustering heat map of the differentially expressed genes (DEGs) shows the four MV4-11 cell line subgroups distinguished by treatment type. Each row and column in the matrix represents a gene and sample, respectively. The color bar indicates changes in the relative levels of gene expression, which were normalized by standard deviations (0= average expression level of a given gene). Red and blue indicate the upregulation and downregulation of genes in response to the drug treatments, respectively; a darker color indicates a more significant change. C. Venn diagram of the relationships among the genes induced by the three treatments. Overall, 5912 genes were regulated by all treatments, whereas 1222, 1065, and 1965 were affected by chidamide only, decitabine only, or a combination thereof, respectively. D, E. Volcano mapping analysis of the DEGs upregulated and downregulated by the drug combination versus monotherapy. Red and green depict upregulated and downregulated DEGs, respectively, in response to combination drug therapy. F, G. Scatter plot of Kyoto Encyclopedia of Genes and Genomes enrichment analysis. The dot size indicates the number of enriched genes in the indicated pathway. The dot color indicates the degree of difference in the enriched gene. Abbreviations: chidamide (CH), decitabine (DE), chidamide plus decitabine (CH+DE), CON, control, UP, upregulated, DOWN, downregulated.
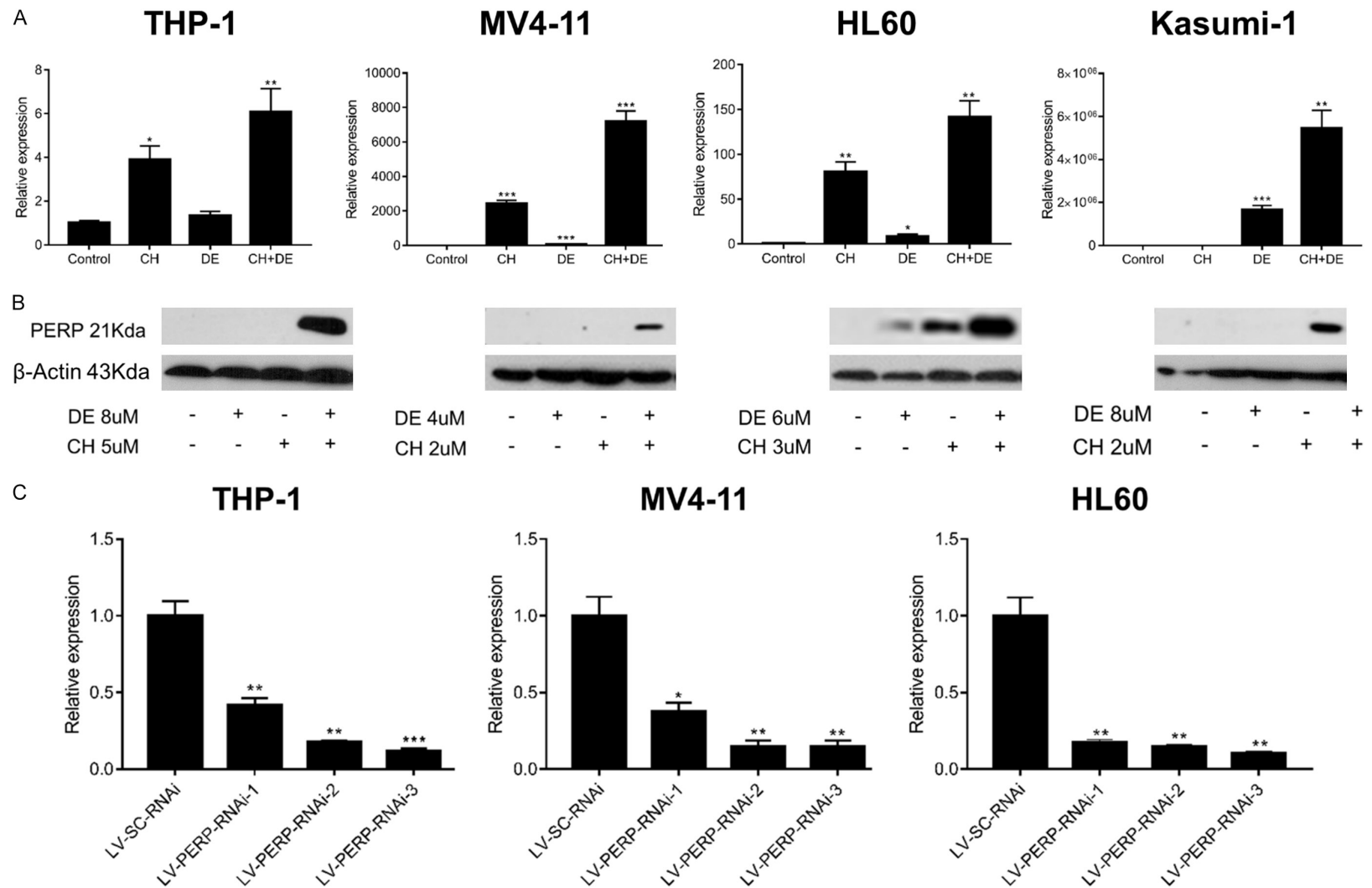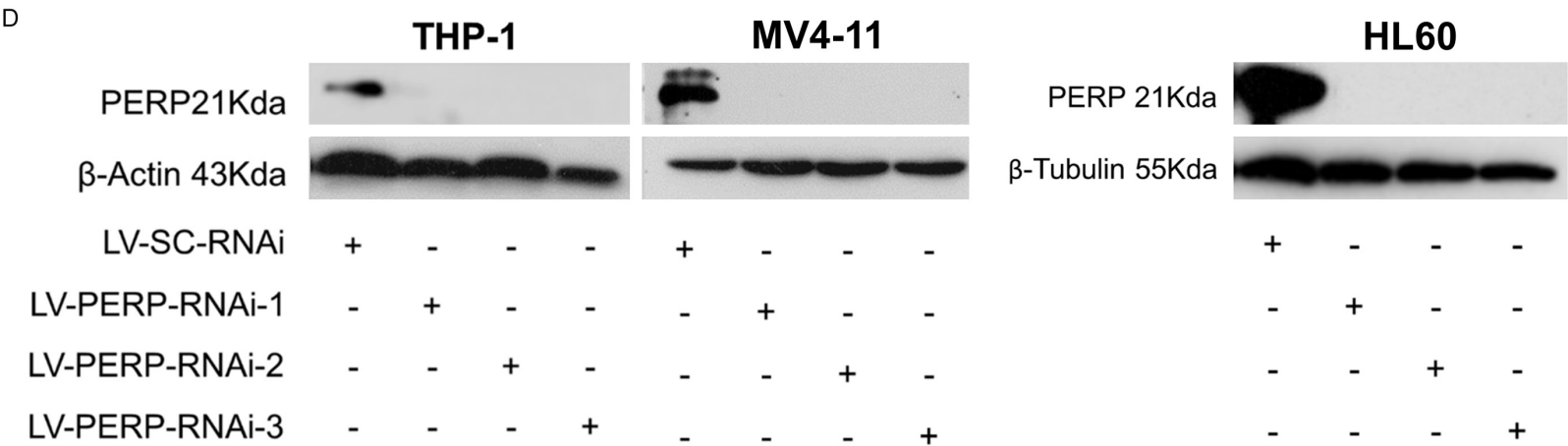
Figure 5. Verification of the transcriptome sequencing results and lentivirus infection efficiency in acute myeloid leukemia (AML) cell lines. (A) The levels of *PERP* mRNA levels were detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in drug-treated cells after 24 h. Data are presented as the mean ± standard error of the mean (SEM) of three independent experiments. GAPDH was used as an internal control. (B) PERP protein levels were detected by western blot after 24 h (THP-1, MV4-11, and HL60) or 48 h (Kasumi-1) of drug treatment. β-Actin was used as an internal control. *PERP* mRNA expression (C) and protein levels (D) in lentivirus-infected AML cell lines were detected using qRT-PCR and western blots, respectively. Data are presented as the mean ± SEM of three independent experiments. β-Actin or β-tubulin was used as an internal control. (*t*-tests, *P<0.05, **P<0.01, and ***P<0.001 vs. control). Abbreviations: chidamide (CH), decitabine (DE), chidamide plus decitabine (CH+DE).
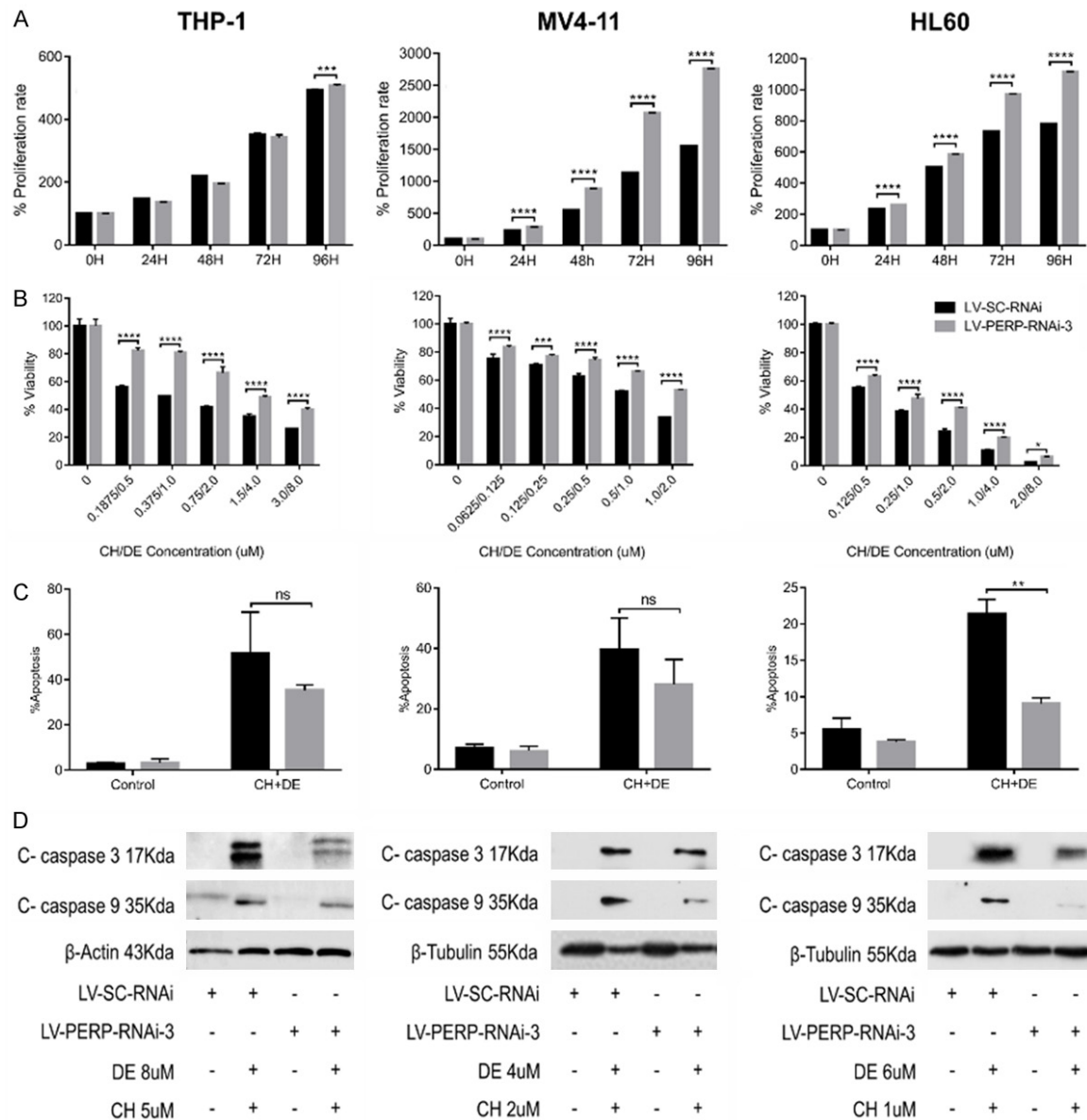
Figure 6. *PERP* knockdown promoted proliferation and induced resistance to combination drug therapy in acute myeloid leukemia (AML) cell lines. A. The effect of PERP knockdown on AML cell proliferation as detected by MTT assay. B. Histograms of MTT assay results demonstrate the negative effect of *PERP* knockdown on the synergistic effects of combination therapy. Values represent means expressed as percentages compared with the control. Error bars represent standard deviations (SDs) (analysis of variance [ANOVA], *$P<0.05$, ***$P<0.01$, and ****$P<0.0001$ vs. the LV-SC-RNAi group). C. Flow cytometry analysis of the effect of *PERP* knockdown on the drug-induced apoptosis of AML cells. Data are presented as the mean ± SD of two independent experiments. (ANOVA, ns: not significant, **$P<0.01$ vs. the LV-SC-RNAi group). D. Western blot analysis of the effect of *PERP* knockdown on the drug-induced expression of pro-apoptotic proteins. β-Actin or β-tubulin was used as the loading control.

confirmed the potential efficacy of chidamide, and experiments that combined the drugs revealed a significant synergistic effect. Specifically, this synergistic effect was observed in both AML cell lines and primary r/r AML cells in an ATP-TCA. Our findings are consistent with previous reports of the combined efficacy of HDACIs with other drugs for AML therapy [18, 19]. For example, the combination of pracinostat, an oral HDACI, plus azacitidine is well tolerated and effective as a first-line regimen for older patients with AML who cannot tolerate intensive therapy [20].

MLL leukemia is characterized by repeated translocations of *MLL* alleles with various other
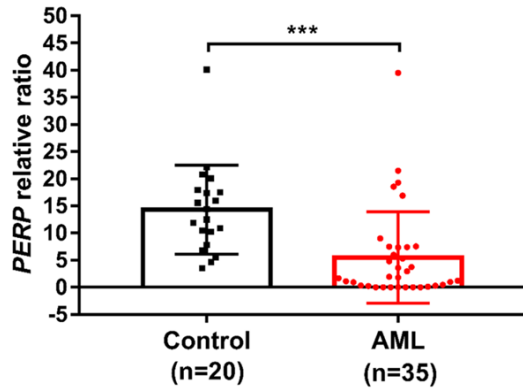
Figure 7. Primary acute myeloid leukemia (AML) cells exhibit reduced *PERP* expression relative to normal peripheral blood mononuclear cells (PBMCs). *PERP* mRNA expression in PBMCs from AML patients and healthy human controls detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Data are presented as the average relative expression levels from three independent experiments versus a single control. *GAPDH* expression was used as the internal control. (*t*-test, ***P<0.001 vs. the control group).

chromosomes [21], and the presence of significant *MLL* rearrangement in a case of AML is an independent poor prognostic factor [22]. HDACIs were shown to exert robust anti-leukemia responses in a mouse model of t(8;21) AML [23], and we previously demonstrated that chidamide-based chemotherapy could induce complete remission (CR) in r/r MLL-AF9-rearranged AML [24]. Consistent with these earlier findings, we showed herein that chidamide, either alone or in combination with decitabine, significantly inhibited the expression of *AML-ETO* and *MLL* fusion genes. This HDACI may, therefore, be an alternative AML treatment option.

Our RNA-seq analysis of the potential synergistic mechanisms exploited by chidamide plus decitabine revealed the effects of this combination on a wide range of genes enriched in pathways involved in apoptosis and the cell cycle. Similarly, the synergistic effect of decitabine plus an HDACI on genome-wide expression in U937 cells was revealed by researchers [25]. Although the transcriptome may be affected by variations in AML cell lines and HDACI subtypes, the data strongly support a synergic effect between these inhibitors and other drugs in the treatment of AML.

Our RNA-seq analysis identified the upregulated expression of *PERP*, which encodes a protein involved in the apoptotic pathway. Traditionally, *PERP* is known as an apoptosis-associated target of P53 [26] and is considered a member of the PMP-22/gas3 family, with involvement in *TP53*-dependent apoptosis in various cell types [27]. These early researches are consistent with our detection of upregulation of p-P53 (ser15) by chidamide plus decitabine in MV4-11 and Kasumi-1 cell lines. However, some scholars believe that *PERP* is also targeted by P63. For instance, Ihrie et al. (2005) believed that *PERP* was regulated by P63 and thus played an important role in epithelial integrity and homeostasis [28]. This earlier observation may explain why we detected *PERP* expression in HL60 (*TP53*-deficient) but not in Kasumi-1 cells (*TP53* wild-type). Our results may suggest that the combination of chidamide plus decitabine induces AML cell apoptosis by upregulating *PERP* independently of the *TP53* status and that the underlying process may be closely related to a downstream target of P53 or may even bypass the classical P53 pathway (e.g., P63) [29]. Although *PERP* downregulation is considered essential for the oncogenic transformation of breast epithelial cells [30], no previous report has discussed the role of *PERP* in AML. Therefore, our study demonstrates, for the first time, that chidamide plus decitabine can upregulate *PERP* expression in AML cell lines and that *PERP* knockdown can promote AML cell proliferation while reducing drug cytotoxicity and inhibiting apoptosis. We further verified our findings by comparing the expression of *PERP* in PBMCs from AML patients and healthy controls. We observed that the expression level of *PERP* in AML samples and from open data base are lower, which may suggest that the low expression level of *PERP* is related to the development and drug resistance of AML.

We note that our study was limited by the use of AML cell lines. Consequently, the biological and genetic heterogeneity of primary AML cells remains unexplored per se. Although we observed enrichment in some metabolic and immune-related pathways that had been previously reported [31-33], we did not explore the potential connections between AML and immunity or metabolism.

In conclusion, our study demonstrates the synergistic pro-apoptotic effects of chidamide plus decitabine on AML cells, and it provides the first evidence indicating the active involvement of *PERP* in this cellular response. Our results show that detecting the expression level of *PERP* can predict the therapeutic effect of dual epigenetic drugs on AML. Although this work requires further functional validation and signaling pathway exploration, our findings demonstrate the potential usefulness of combined decitabine plus chidamide as a treatment for AML.

Acknowledgements

Disclosure of conflict of interest

None.

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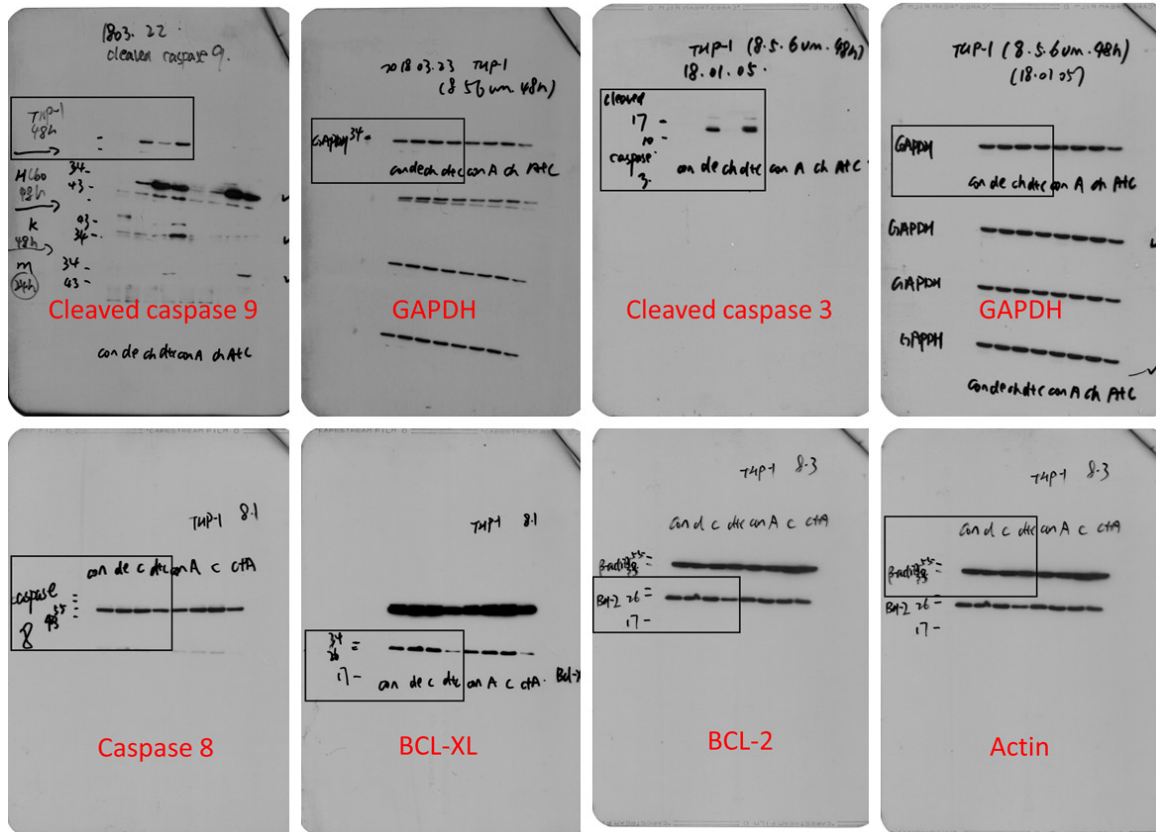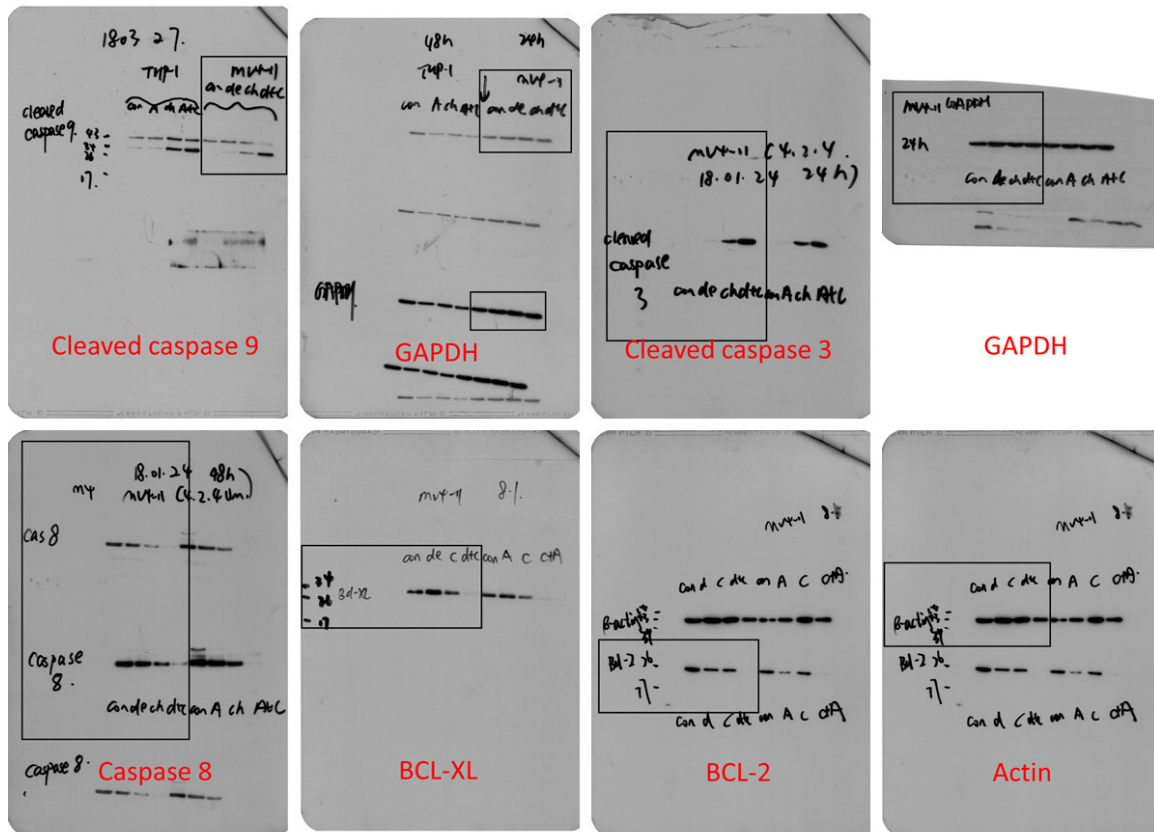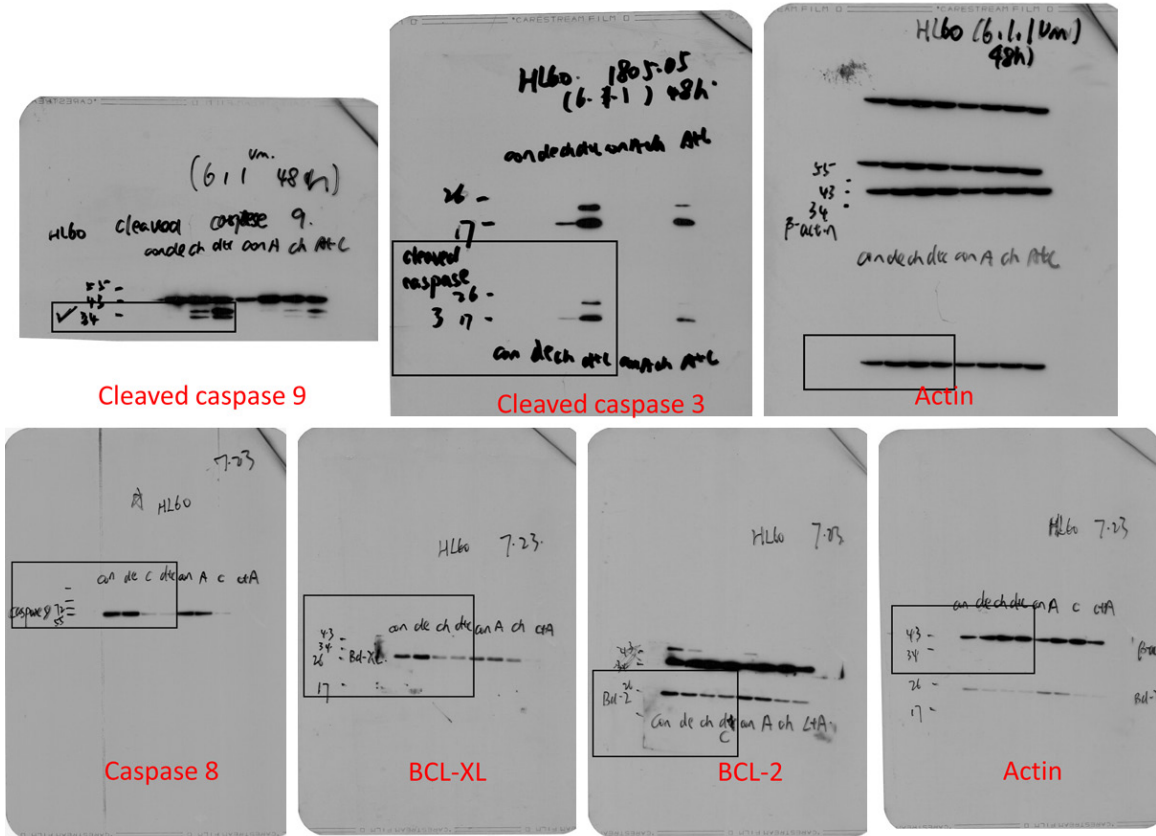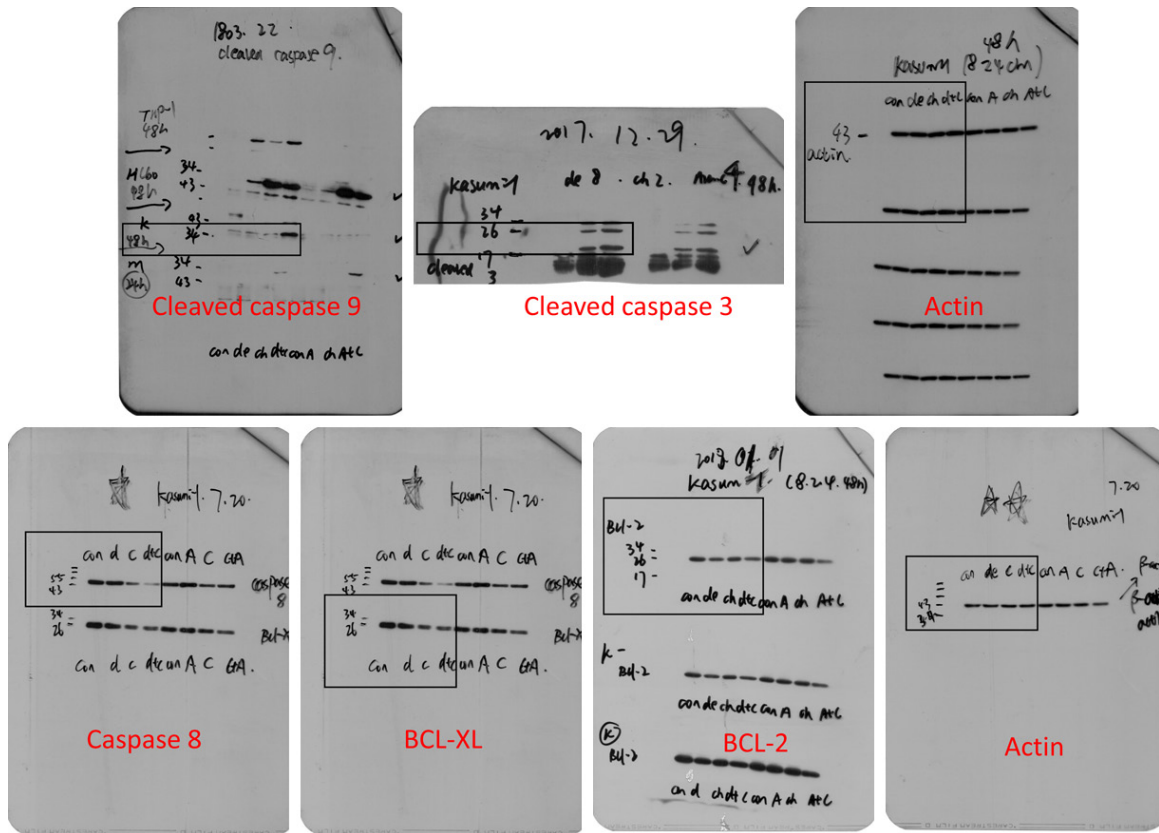
Figure S1. Western blot analysis of the levels of cleaved caspase-3, cleaved caspase-9, caspase-8, BCL-XL, and BCL-2 proteins in THP-1 cell lines after exposure to chidamide and decitabine alone or in combination for 48 h. GAPDH or β-actin was used as the loading control.

Figure S2. Western blot analysis of the levels of cleaved caspase-3, cleaved caspase-9, caspase-8, BCL-XL, and BCL-2 proteins in MV4-11 cell lines after exposure to chidamide and decitabine alone or in combination for 24 or 48 h. GAPDH or β-actin was used as the loading control.

Figure S3. Western blot analysis of the levels of cleaved caspase-3, cleaved caspase-9, caspase-8, BCL-XL, and BCL-2 proteins in HL60 cell lines after exposure to chidamide and decitabine alone or in combination for 48 h. β-Actin was used as the loading control.

Figure S4. Western blot analysis of the levels of cleaved caspase-3, cleaved caspase-9, caspase-8, BCL-XL, and BCL-2 proteins in Kasumi-1 cell lines after exposure to chidamide and decitabine alone or in combination for 48 h. β-Actin was used as the loading control.
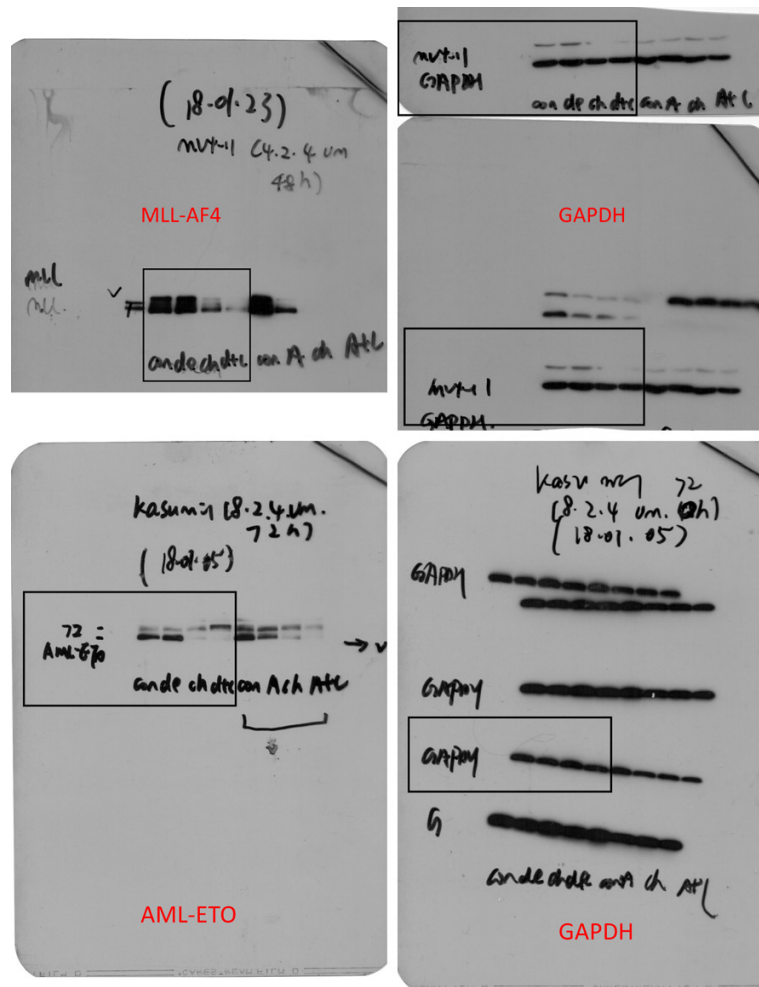
Figure S5. Western blot analysis of the effects of chidamide and decitabine on the levels of AML-specific fusion proteins in MV4-11 and Kasumi-1 cell lines. GAPDH was used as the loading control.
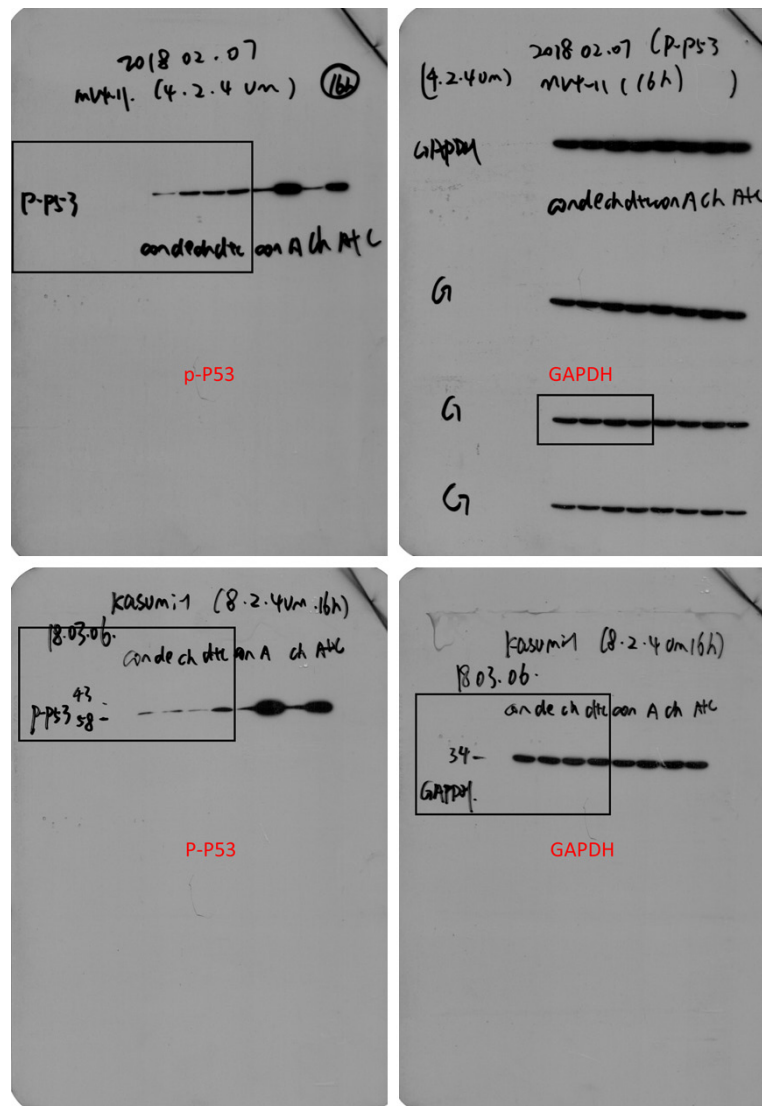
Figure S6. Western blot analysis of the effect of chidamide and decitabine on the level of phosphorylated P53 (ser15) in cells after 16 h of treatment alone or in combination. GAPDH was used as the loading control.
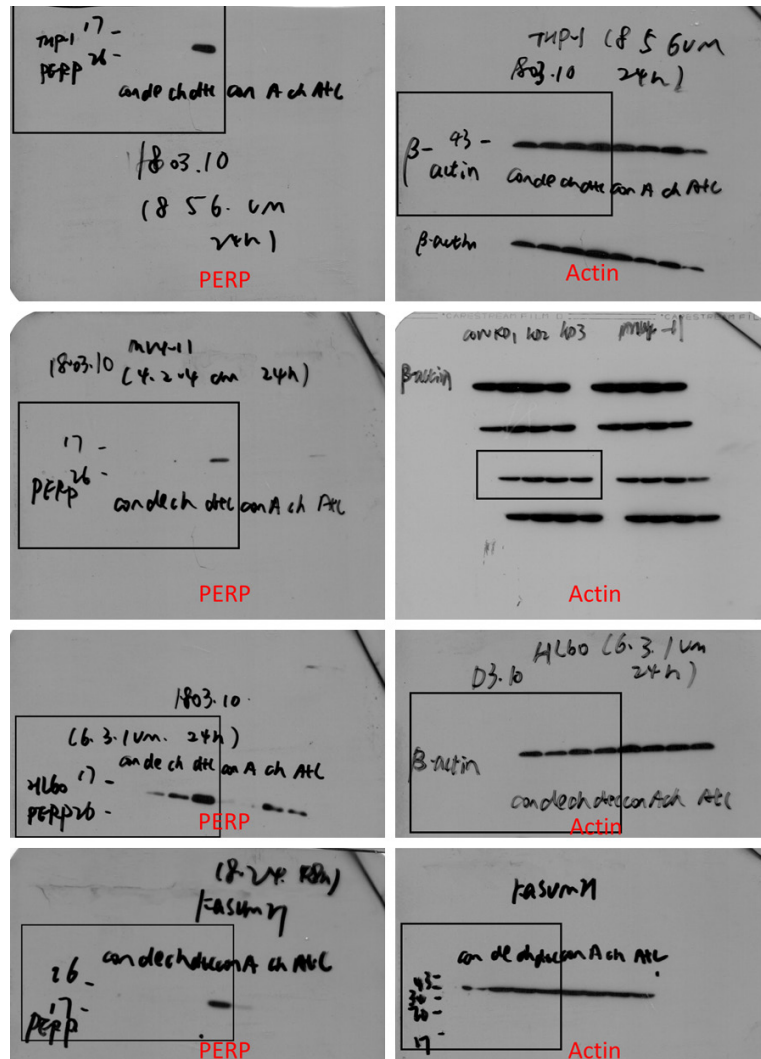
Figure S7. PERP protein levels were detected by western blot after 24 h (THP-1, MV4-11, and HL60) or 48 h (Kasumi-1) of drug treatment. β-Actin was used as an internal control.
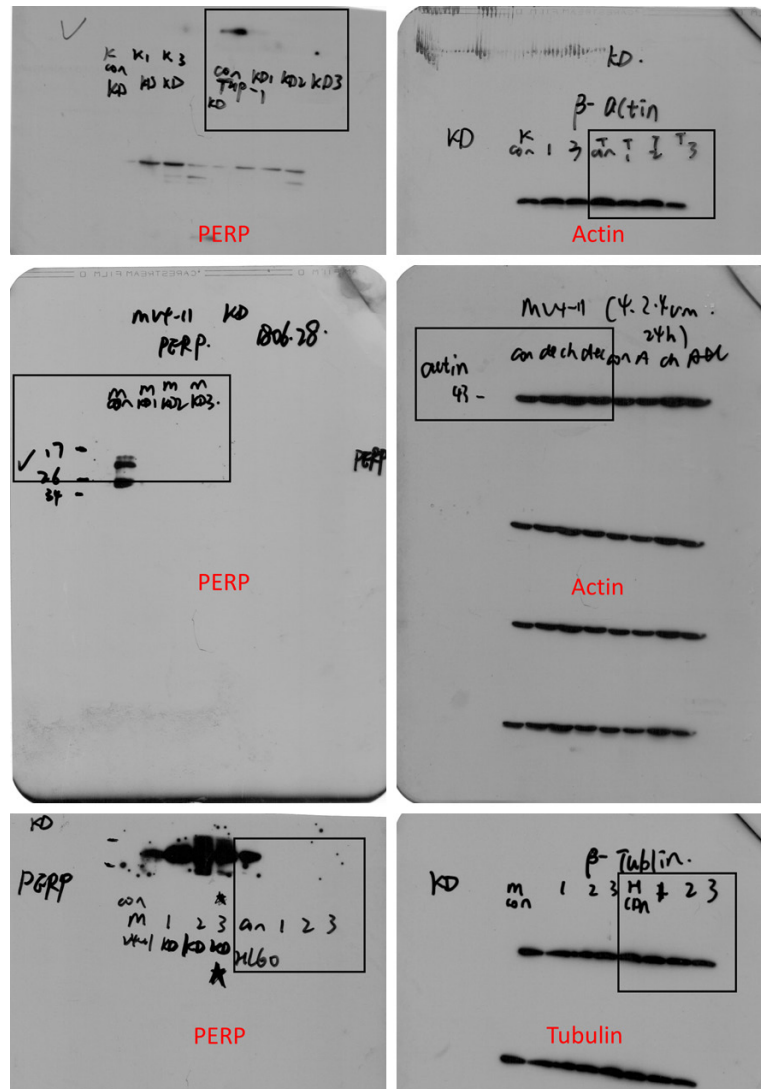
Figure S8. *PERP* protein levels in lentivirus-infected AML cell lines (THP-1, MV4-11 and HL60) were detected using western blots. β-Actin or β-tubulin was used as an internal control.
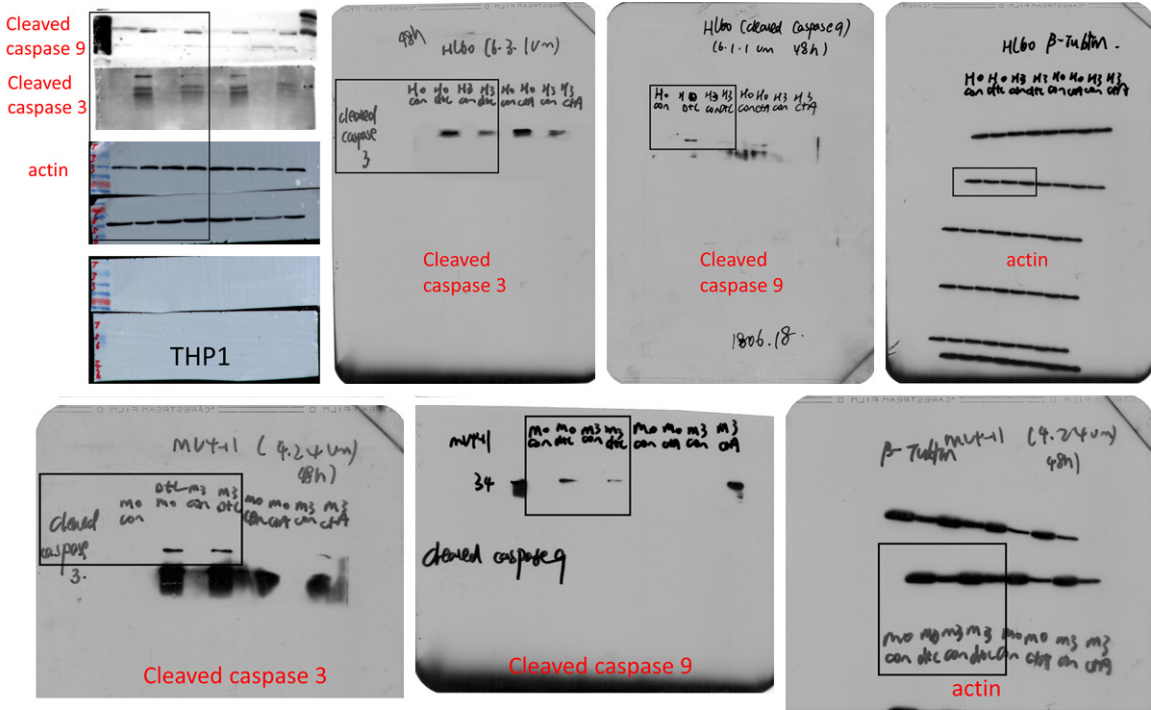
Figure S9. Western blot analysis of the effect of *PERP* knockdown on the drug-induced expression of pro-apoptotic proteins. β-Actin or β-tubulin was used as the loading control.
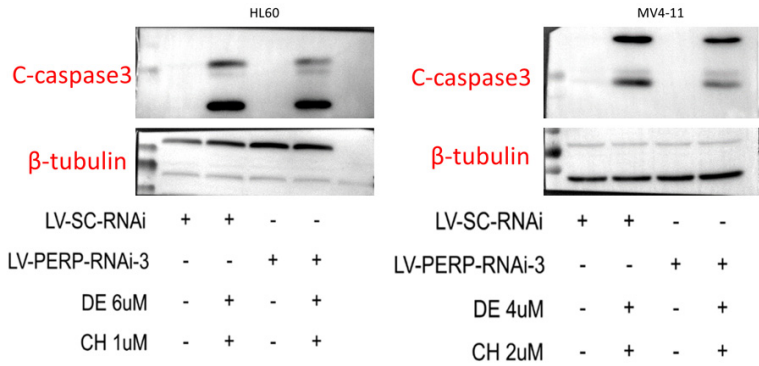

Figure S10. We have redone some of the western experiments, and the results are consistent with those in the manuscript.