



Inhibition of PI3K-AKT-mTOR pathway and modulation of histone deacetylase enzymes reduce the growth of acute myeloid leukemia cells

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Abstract

One of the most widespread forms of blood cancer is known as acute myeloid leukemia (AML) which has an incidence of 80% with poor prognosis. Although there are different treatment methods for AML in clinic, the heterogeneity and complexity of the disease show that new treatments are needed. The aim of this study is to investigate the anticancer effects of inhibition of PI3K and HDAC enzymes on CMK and MOLM-13 AML cells lines. We demonstrated that the combination of LY294002 with SAHA and Tubastatin A significantly decreased the cell viability of both cell lines. In contrast, the LY294002 and PCI-34051 combination did not show a significant difference compared to the single LY294002 administration. The combination treatment of LY294002 and HDAC inhibitors did not induce apoptosis significantly. However, LY294002 + SAHA and LY294002 + PCI-34051 resulted in G0/G1 and G2/M cell cycle arrest in CMK cells, respectively. On the other hand, compared to control cells, LY294002 + SAHA and LY294002 + PCI-34051 led to G0/G1 phase arrest in MOLM-13. Furthermore, the LY294002 + PCI-34051 combination elevated the expression rate of LC3BII/I, an autophagy marker, in CMK cells by 2.5-fold. Our study revealed that the combinations of PI3K inhibitor and HDAC inhibitors showed a synergistic effect and caused a reduction in cell viability and increased cell cycle arrest on MOLM-13 and CMK cell lines. In addition, the expression of LC3BII was elevated in the CMK cell line. In conclusion, although more mechanistic studies are required, a combinational inhibition of PI3K and HDAC could be a promising approach for AML.

Keywords Acute myeloid leukemia · PI3K pathway · Histone deacetylase enzymes · Cell cycle · Combination therapy

Abbreviations

AML	Acute myeloid leukemia
PI3K	Phosphoinositide 3-kinase
mTOR	Mammalian Target of Rapamycin
HDAC	Histone deacetylase
IC20	The 20% inhibitory concentration
IC50	The 50% inhibitory concentration
LC3B	Microtubule-associated protein light chain 3

Background

Acute myeloid leukemia (AML) is a highly complex malignancy that a variety of mutations and chromosomal aberrations play a role in adults [1, 2]. The American Cancer Society estimates that there will be nearly 20,380 new cases of AML, of which 11,310 will result in death in the US in 2023 [3]. Since AML is one of the most common types of leukemia in adults, new treatment methods are always needed in the clinic [1, 4]. The importance of deregulated signaling pathways in the pathogenesis of AML has been also demonstrated by many studies [2, 5–8]. Among these pathways, the mammalian target (mTOR) signaling pathway of Phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR protein complex is one of the most important intracellular pathways that regulate cell growth and proliferation, cell survival, cell metabolism and angiogenesis [9, 10]. PI3K/Akt/mTOR inhibitors, used alone or in combination with other drugs, have been shown to downregulate cell viability and induce apoptotic cell death mechanism in many studies both

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in vitro or in vivo [8, 10–15]. Besides, the alteration of gene expression by epigenetic dysregulation revealed that it is the hallmark of hematopoietic malignancies [16–20]. Abnormal expression of histone deacetylase (HDAC) enzymes, which makes DNA less accessible to transcription factors by the removal of the acetyl group from histone proteins, is observed in many types of cancer and associated with disease progression and correlated with poor patient prognosis. [17, 20–22]. HDAC inhibitors (HDACis) exhibit promising anticancer characteristics against solid and hematological malignancies with relative resistance in normal cells. HDACis exert their effect by triggering both mitochondria-mediated apoptosis and caspase-independent autophagic cell death [23]. Several HDACis are undergoing clinical trials and some have FDA approval, such as Vorinostat (SAHA), Romidepsin, Belinostat, and Panobinostat [24]. Recent studies have reported that combination treatment of SAHA and some chemotherapeutic agents such as cytarabine, etoposide, decitabine has a synergistic effect on AML cells and inhibits leukemic growth by reducing cell proliferation [25, 26]. Among HDACis, Tubastatin A, a selective HDAC6 inhibitor, is an HDAC inhibitor that has shown promising effects for cancer treatment in various studies. Tubastatin A led to an important reduction in cell migration and decreased the colony-forming capacity of cells in glioblastoma cells with a reversing effect of epithelial-mesenchymal transition [27]. Moreover, inhibition of HDAC6 by Tubastatin A in melanoma cells suppressed cell cycle and cell proliferation [28]. HDAC8 is another enzyme that has been reported to be highly expressed in an AML subtype carrying the inversion of chromosome 16 [inv (16)] and in different hematopoietic malignancies [29]. The HDAC8-specific inhibitor PCI-34051 has been shown to induce apoptosis in T-cell lymphomas [30]. While single-agent therapy has limited applicability in cancer management, combination therapy is highly successful in the treatment of many cancer types. The HDAC enzymes represent important targets considering that many epigenetic mechanisms have a crucial role in AML pathogenesis and are potential targets for AML, given the effects of HDAC enzymes on both AML and different cancers. Targeting the PI3K/AKT/mTOR pathway is a very important way to prevent leukemic growth in AML. However, there is a possibility that inhibition of PI3K alone would not be successful, because of the complexity of this pathway and its intersection with other pathways. In this framework, we investigated the combinatorial effect of PI3K and HDAC inhibition on CMK and MOLM-13 AML cell lines. We reported the anti-cancer effect of PI3K inhibitor, LY294002, and different HDAC inhibitors such as SAHA, Tubastatin A, and PCI-34051 inhibitors. The results show that the combination of LY294002 and HDACis exhibited a synergistic effect on both cell lines. The administration of drug combinations induced mild apoptotic cell death at

low drug doses of IC₂₀ values. The LY294002 + SAHA and LY294002 + PCI-34051 combinations resulted in G₀/G₁ and G₂/M cell cycle arrest in CMK, respectively, while LY294002 combination with all three HDACis that are used in this study led to G₀/G₁ phase arrest in MOLM-13 cell lines when compared to untreated control cells. Besides the LY294002 + PCI-34051 combination treatment increased the expression rate of LC3BII/I by 2.5-fold in CMK cell lines. In conclusion, our study demonstrated that combining LY294002, a specific PI3K inhibitor, with HDAC inhibitors, SAHA, Tubastatin A and PCI-34051, inhibited cell proliferation, and induced cell cycle arrest on AML cell lines.

Materials and methods

Chemicals

RPMI-1640, Fetal Bovine Serum (FBS), penicillin/streptomycin, and phosphate-buffered saline (PBS) were purchased from Serox. The drugs that were used in this study (LY294002, SAHA, Tubastatin A, and PCI-34051) were obtained from Sigma-Aldrich. The inhibitors were prepared as 5 mM stock solutions in DMSO (dimethylsulfoxide) according to the recommendations of the supplier and the main stocks were stored at -20 °C.

Cell maintenance

The CMK and MOLM-13 AML cell lines were obtained from the German National Biological Materials Resource Center (DSMZ) and cultured under the recommended conditions which are using RPMI-1640 supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. The cells were incubated at 37 °C in 5% CO₂ environment. CMK and MOLM-13 cells were seeded in 5 × 10⁶ cells/10 mL/96 mm × 21 mm tissue culture plates. Cells were passaged every 2–3 days when they were 60–70% confluent. Collected cells were centrifuged at 700 rpm for 5 min. Then, the supernatant was removed, and the cell pellet was dissolved with a fresh medium.

Cell proliferation assay

Cell proliferation assay was used to determine the anti-cancer effect of LY294002 and HDACis (SAHA, Tubastatin A and PCI-34051) on AML cell lines. The proliferation of CMK and MOLM-13 cells was measured by MTT Cell Viability Assay (Sigma Aldrich) after inhibitor administration. For this purpose, first, the relevant inhibitors were added to 10,000 cells/well of a 96-well plate and incubated for 48 h. Following the incubation, 10 µl of MTT reagent was added to each well and incubated for 2 h at 37 °C. Then, 96-well

plate was centrifuged at 1800 rpm for 10 min and the formed formazan crystals were dissolved with 100 μ l of DMSO. To homogenize the formazan crystals, DMSO was added to the crystals and then the plates were incubated on a shaker for 15 min at room temperature. Then the absorbance of the formazan was measured in the spectrophotometer with a Varioskan™ LUX multi-mode microplate reader (Thermo Scientific) at 570 nm. The cell proliferation plot was created according to the spectrophotometric results and the IC₂₀ values (the drug concentration that inhibits cell growth by 20%) were calculated for each indicated inhibitors.

The combination index (CI) calculation by isobologram analysis

Combination analysis was performed by using the CompuSyn software (Biosoft, Cambridge, United Kingdom). The CI values were calculated by the program. The effects of the drug combination that was used in this study were evaluated using the CI based on Chou-Talalay's multidrug effect equation. A CI of < 1, = 1, or > 1 is indicative of synergistic, additive, or antagonistic effects, respectively [31].

Cell cycle analysis

The cells were seeded into the 6-well plates at a density of 1×10^6 /well and treated with drugs alone or in combination for 48 h. Afterward, the cells were centrifuged at 260 g for 10 min at 4 °C. The pellet was washed twice with PBS (pH 7.4) and then 4 ml of 70% ethanol was added to the cells. Then, the cells were kept at - 20 °C for at least 24 h for fixation. Next, the cells were centrifuged for 10 min, and the pellet was resuspended with 1 ml of cold PBS. After centrifugation, the obtained cell pellet was dissolved with 1 ml 0.1% Triton-X (in PBS), and 100 μ l of RNAase (200 μ g/ml, Sigma Aldrich) was added. The cells were incubated at 37 °C for 30 min. Following the incubation, 100 μ l propidium iodide (1 mg/ml, Sigma Aldrich) was added and the cells were incubated at room temperature for 15 min. Cell cycle analysis was measured by BD LSRFortessa (Becton Dickinson) flow cytometry.

Annexin V/PI double staining

The amounts and localization of phosphatidylserine were determined by Annexin V/Propidium iodide dual staining method in CMK and MOLM-13 cells by flow cytometry as described previously [32]. For this purpose, 1×10^6 cells were incubated with LY294002 alone or in combination with SAHA, PCI-34051 and Tubastatin A in triplicates using DMSO as control for 48 h. After 48 h of drug incubation, cells were harvested and then centrifuged for 5 min at 1700 rpm at 4 °C. In the following steps, the cells were

washed twice with 1XPBS and centrifuged at each wash. After the last wash, PBS was removed, and the cell pellet was dissolved in 200 μ l of 1X Annexin V binding solution (diluted 1:10 with ultrapure water). Then, 2 μ l of Annexin V and 4 μ l of propidium iodide were added to the mixture and incubated for 15 min at room temperature in the dark. Lastly, cells were analyzed in BD LSRFortessa (Becton Dickinson) flow cytometry.

Western blotting

The CMK and MOLM-13 cells were seeded into 6-well plates with a density of 5×10^6 cells/ml and treated alone or in combination with the above-mentioned drugs for 48 h. Then, cells were harvested and lysed in 1X RIPA lysis buffer including the protease inhibitors. Total protein concentration was determined by the DC Protein quantification Kit (BioRad). The cell lysates were loaded at a protein concentration of 20 μ g for each well, and then separated by SDS-Gel electrophoresis. The Trans-Blot Turbo Transfer System (BioRad) was used for the transfer of gel to the PVDF membrane for 5 min. The membrane was blocked by using 5% dried milk in $1 \times$ TBST buffer (0.15 M NaCl, 0.02 M Tris pH 7.6 and 0.1% Tween20) for 1 h at room temperature. After, the membrane was incubated with primary antibodies overnight at 4 °C followed by the conjugation with secondary antibodies for 1 h at room temperature. The primary antibodies that were used are as follows; LC3B (Cell Signaling) and GAPDH (Cell Signaling). The signal was detected by Pierce™ ECL Western Blotting Substrate with Chemi-Doc™ (BioRad). Protein quantification was analyzed by using Image Lab Software (BioRad).

Statistical Analysis

The statistical analysis was performed by GraphPad Prism 8. The comparisons of two experiment sets were done by two-way ANOVA with Dunnet's Multiple Comparison test and the level of significance was set $P < 0.05$.

Results

The PI3K inhibitor, LY294002, reduces the cell viability of AML cells

LY294002 is a specific PI3K inhibitor and acts by binding to the ATP binding site of the kinase enzyme [33]. As a result of this binding, AKT phosphorylation, which is present on the PI3K downstream pathway, is inhibited. The inhibition of the PI3K/AKT pathway, which is a regulatory pathway in cell proliferation, is critical to overcome cellular growth in cancer, especially in hematopoietic

malignancies [33]. Therefore, we treated MOLM-13 and CMK cell lines with LY294002 in a dose-dependent manner between 1 and 20 μM to investigate the anti-cancer efficacy through the inhibition of the PI3K pathway (Fig. 1). A significant decrease in cell viability was observed when LY294002 was administered at the low micromolar concentration on both cell lines. The sensitivity of both cell lines to the inhibitor was found to be similar. The IC₂₀ values of LY294002 inhibitor were calculated from single treatment for CMK (1 μM) and MOLM (2.8 μM) cell lines (Fig. 1a and b). SAHA (0.07 μM), Tubastatin A (1 μM) and PCI-34051 (1.7 μM) for CMK cell line (Fig. 1a) and LY294002 (2.8 μM), SAHA (0.05 μM), Tubastatin A (0.3 μM) and PCI-34051 (5.5 μM) for MOLM-13 cell line (Fig. 1b). In conclusion, we suggest that targeting PI3K with LY294002 could prevent proliferation of leukemia cells via inhibition of the PI3K/AKT pathway.

Inhibitory effect of HDAC inhibitors on the cell viability of AML cell lines

There are various HDAC inhibitors that target different classes of HDAC enzymes [18, 20]. The abnormal activity of HDACs can result in cancer pathogenesis by suppressing genes mainly involved in the regulation of angiogenesis, proliferation, migration, and metastasis [19, 20, 34]. In this study, we aim to use various HDAC inhibitors targeting different HDAC enzymes and evaluate the most effective HDAC enzyme on CMK and MOLM-13 cell lines. Among them, SAHA is an FDA-approved inhibitor for treatment of cutaneous T cell lymphoma in 2006, and it has been studied in many types of cancer and shown to be effective [35]. Therefore, this inhibitor was used as a control drug in our study. There is accumulating evidence shows that AML cells

are subject to dysregulated epigenetic modifications. Hence, in the current study, two different HDAC inhibitors, Tubastatin A and PCI-34051, were used together with the control drug, SAHA. First, MOLM-13 and CMK cell lines were treated with Tubastatin A, an HDAC 6 inhibitor, and PCI-34051, an HDAC8 inhibitor, alone to target specific HDAC enzymes (Fig. 2). Tubastatin A resulted in a dose-dependent reduction of cell viability by approximately 90% with the highest dose administered (10 μM) (Fig. 2a). Similarly, our results showed an 80% reduction in cell viability after 1 μM SAHA treatment for both AML cell lines when compared to untreated control (Fig. 2b). The sensitivity of both cell lines was similar in response to SAHA and Tubastatin A treatment. However, the response of two cell lines against PCI-34051 was different, such that CMK cells were more sensitive to PCI-34051 than MOLM-13 cells. Treatment with PCI-34051 caused a 90% cell death when 10 μM concentration was applied to the CMK cell line, while the same concentration resulted in a 50% reduction in the MOLM-13 cell line compared to untreated control at 48 h (Fig. 2c). Although the two cell lines represent AML model, CMK and MOLM-13 cell lines have different FLT3 expression profiles. CMK cells are FLT3-wt cells while MOLM-13 cells are dependent on FLT3-ITD mutation. This varying genetic background could cause different responses to inhibitors. The IC₂₀ values for the HDACis were calculated for SAHA (0.07 μM and 0.05 μM), Tubastatin A (1 μM and 0.3 μM) and PCI-34051 (1.7 μM and 5.5 μM) for CMK and MOLM-13 cell lines (Fig. 2). Taken together, we concluded that the administration of different HDAC inhibitors could have a different impact on the cell viability of different subtypes of AML cell lines.

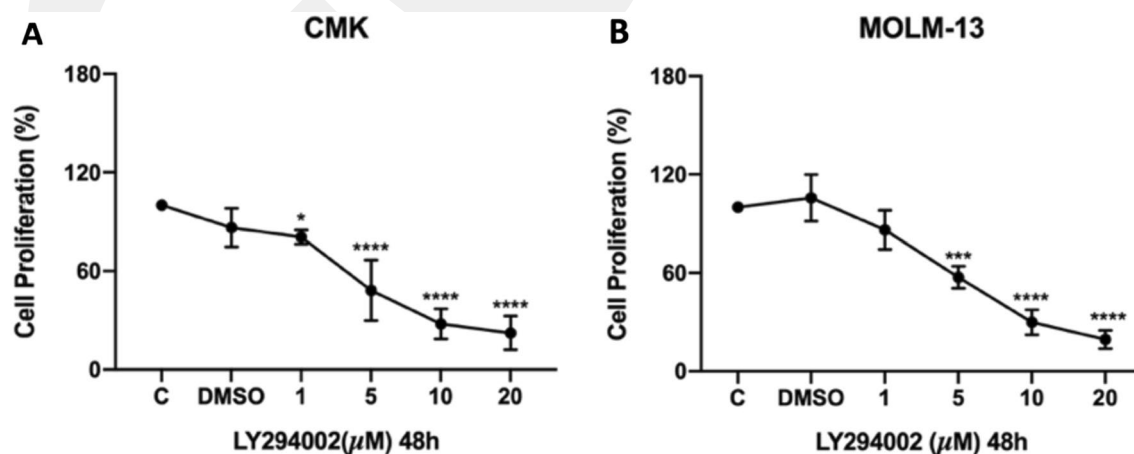


Fig. 1 The cell viability of LY294002, PI3K inhibitor, on CMK (a) and MOLM-13 (b) cells for 48 h. Experiments were performed in triplicates and were repeated in three independent experiments

(n=3). Statistical analyzes were achieved with two-way ANOVA. All data are presented as mean \pm S.D. (ns = $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$)

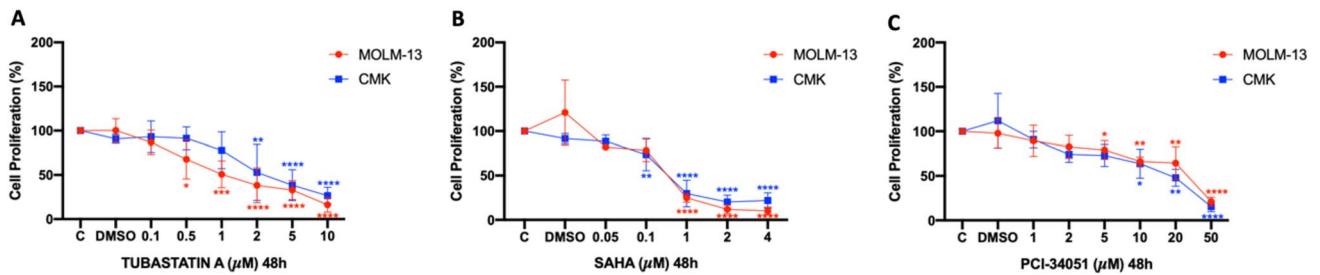


Fig. 2 The effects of HDAC inhibitors, Tubastatin A (a), SAHA (b) and PCI-34051 (c), on the proliferation of CMK and MOLM-13 cells represented by blue and red colored lines, respectively. Experiments were performed in triplicates and were repeated in three independent

experiments (n=3). Statistical analyzes were achieved with two-way ANOVA. All data are presented as mean ± S.D. (ns=P>0.05, *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.00001)

The anti-cancer activity of the combination of LY294002 and HDACis on AML cell lines

When the heterogeneous and complex disease progress of AML is considered, there is always a need for possible additional treatment strategies. Studies in the literature have shown that simultaneous inhibition of PI3K and HDAC enzymes results in success in different types of cancer [36, 37]. Here, we aimed to examine targeting of two important drivers of AML in two cell lines with different

genetic backgrounds using combinatorial approaches. For this purpose, the IC20 values of LY294002 were combined with IC20 of SAHA, Tubastatin A and PCI-34051. The LY294002 + Tubastatin A combination reduced cell proliferation by up to 43.6% and 56.3% in untreated control CMK and MOLM-13 cells, respectively (Fig. 3a, d). Similarly, the cell proliferation was reduced by 47.1% in MOLM-13, while CMK cell line showed a 42.4% reduction after the combination treatment with LY294002 + SAHA (Fig. 3b, e). The treatment with LY294002 + PCI-34051

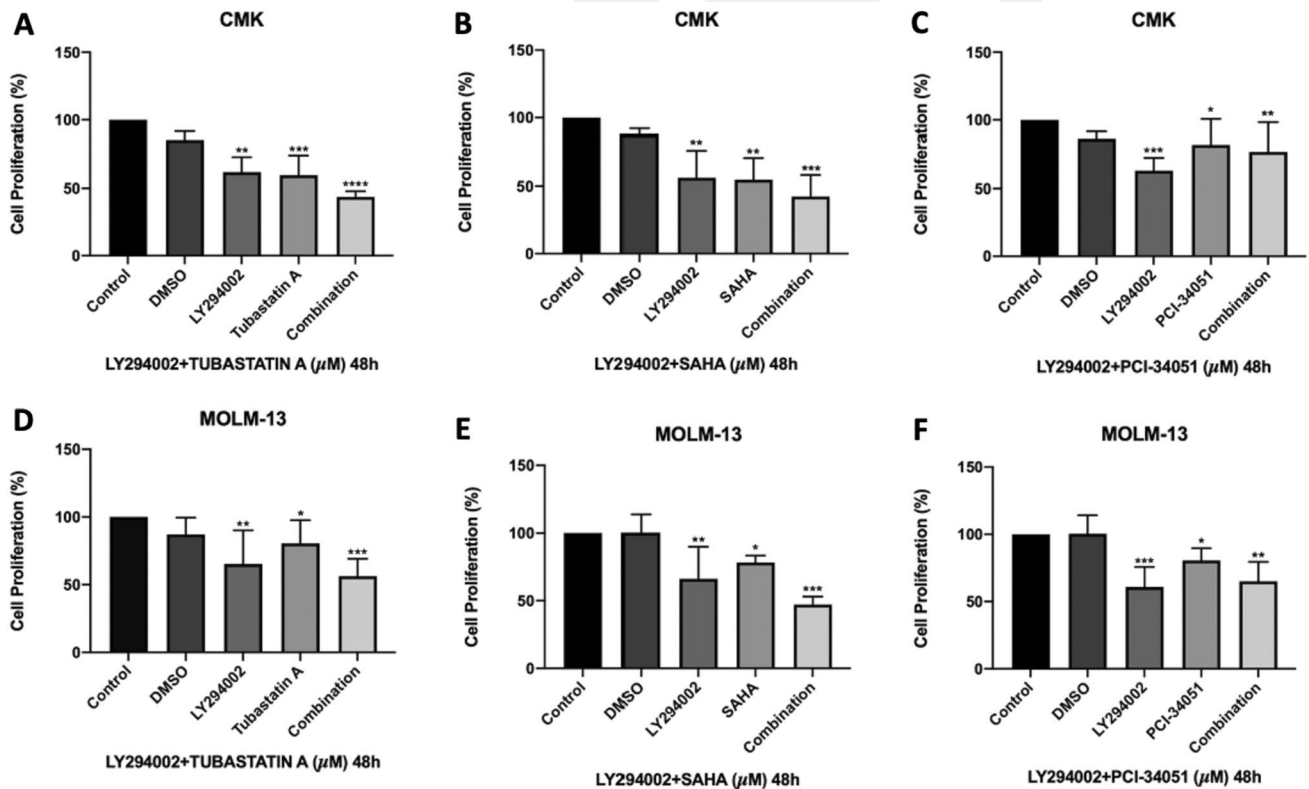


Fig. 3 The effect of LY294002 and HDACis combination on proliferation of CMK (a–c) and MOLM-13 (d–e) cells at constant IC20 concentration. Experiments were performed in triplicates and

were repeated in three independent experiments (n=3). All data are presented as mean ± S.D. (ns=P>0.05, *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.00001)

Table 1 The combination index values of CMK and MOLM-13 cells treated with the combination of LY294002 with Tubastatin A, SAHA and PCI-34051, were calculated and isobolograms were determined by CompuSyn software

	Dose				Combination index (CI) value		
	Tubastatin A	SAHA	PCI-34051	LY294002	Tubastatin A + LY294002	SAHA + LY294002	PCI-34051 + LY294002
CMK	1 μ M	0.07 μ M	1.7 μ M	1 μ M	0.19185	0.84438	0.40968
MOLM-13	0.3 μ M	0.05 μ M	5.5 μ M	2.8 μ M	1.03254	0.42750	1.22049

A CI of < 1 , $= 1$, or > 1 is indicative of synergistic, additive, or antagonistic effects, respectively

resulted in a decreased proliferation by 77.2% and 65%, for CMK and MOLM-13 cells, respectively (Fig. 3c, f). The results of the combination study showed a significant decrease in both cell lines when the LY294002 + Tubastatin A and the LY294002 + SAHA combination applied to both untreated control and the single treatments. On the other hand, no better antiproliferative effect was observed than single administration of LY294002 in the combination of LY294002 + PCI-34051 (Fig. 3c, f). Taken together, the results of the combination treatment of LY294002 with SAHA and Tubastatin A revealed a better synergistic inhibitory effect on the proliferation of both AML cells, while the combination with PCI-34051 was not significantly different compared to the single treatments and untreated control for both cells. The isobologram test reveals that the combination treatments demonstrated a synergistic effect when LY294002 was combined with all HDAC inhibitors for the CMK cell line (Table 1). However, for the MOLM-13 cell line, the LY294002 + SAHA was found to be synergistic while the Tubastatin A and PCI-34051 combinations were additive and mildly antagonistic, respectively. These results showed that the combination of LY294002 and HDACis on AML cell lines was mostly synergistic.

The effect of combination treatment on apoptotic cell death on AML cell lines

To further investigate the effect of HDAC and PI3K inhibition, we sought to examine the effect of the combination treatment on apoptotic cell death. We treated CMK and MOLM-13 cell lines with the above-mentioned combinations for 48 h and assessed apoptotic cell death using Annexin-V double staining analyzed by flow cytometer. Based on our findings, no significant difference was observed in total apoptotic cell death in all combinations in the CMK cell line compared to the untreated control (Fig. 4). We found that the combination with LY294002 and Tubastatin A did not affect the apoptotic cell death in CMK cells (Fig. 4a). On the other hand, we observed an increase of 15.8% and 16.4% in late apoptotic cell population in the combinations of LY294002 + SAHA and LY294002 + PCI-34051 compared to the untreated control in CMK cells (Fig. 4b, c). In the MOLM-13 cell line, LY294002 + SAHA combination

revealed 1.17-fold increased apoptosis compared to control cells, while LY294002 + Tubastatin A combination resulted in a 0.52-fold increase in apoptotic cell death (Fig. 4d, e). In addition, in the combination of LY294002 + Tubastatin A, a 0.48-fold increase in late apoptotic cells was observed in the MOLM-13 cell compared to the untreated control. There was a 1.38-fold increase in late apoptotic cell death in MOLM-13 cell in the LY294002 + SAHA combination compared to the untreated control (Fig. 4e). For the early apoptotic cell population, this ratio resulted in 0.12 times compared to the control in LY294002 + SAHA combination. LY294002 + PCI-34051 did not result in a significant increase in apoptosis in MOLM-13 cells (Fig. 4f). We conclude that the reduction in cell proliferation may not be driven by apoptosis as our results did not reveal a significant change in apoptotic cell death.

The combination of LY294002 and HDACis induce cell cycle arrest on CMK and MOLM-13 cell lines

HDACs are involved in promoting cell cycle progression and proliferation through inhibition of the expression level of the key regulators of the cell cycle, such as p53, pRb, E2F and p21, resulting in increased proliferation [38]. Thus, we wanted to explore the effect of the combination regime on the cell cycle distribution of MOLM-13 and CMK cell lines. The results revealed that IC20 values of LY294002 + Tubastatin A combination treatment showed G0/G1 phase arrest in CMK cells compared to the control (Fig. 5a) with the increase in G0/G1 phase cells from 57.5 to 67.3%. CMK cells treated with combinations of LY294002 + SAHA and LY294002 + PCI-34051 were arrested in the G0/G1 (74.4%) and G2/M (65.5%) phases, respectively (Fig. 5b, c). In CMK cell line, treatment of the cells with single and combination treatment have shown G0/G1 arrest percentages as 68.3%, 68.1% and 67.3% after only LY294002, Tubastatin A and LY294002 + Tubastatin A combination treatment. On the other hand, for LY294002 + SAHA combination treatment, G0/G1 arrest percentages were as 71.3%, 75% and 74.4% after only LY294002, SAHA, and LY294002 + SAHA combination treatment. For LY294002 and PCI-34051 combination treatment, G0/G1 arrest percentages were as 74%, 72.6% and 65.5% after only LY294002, PCI-34051 and

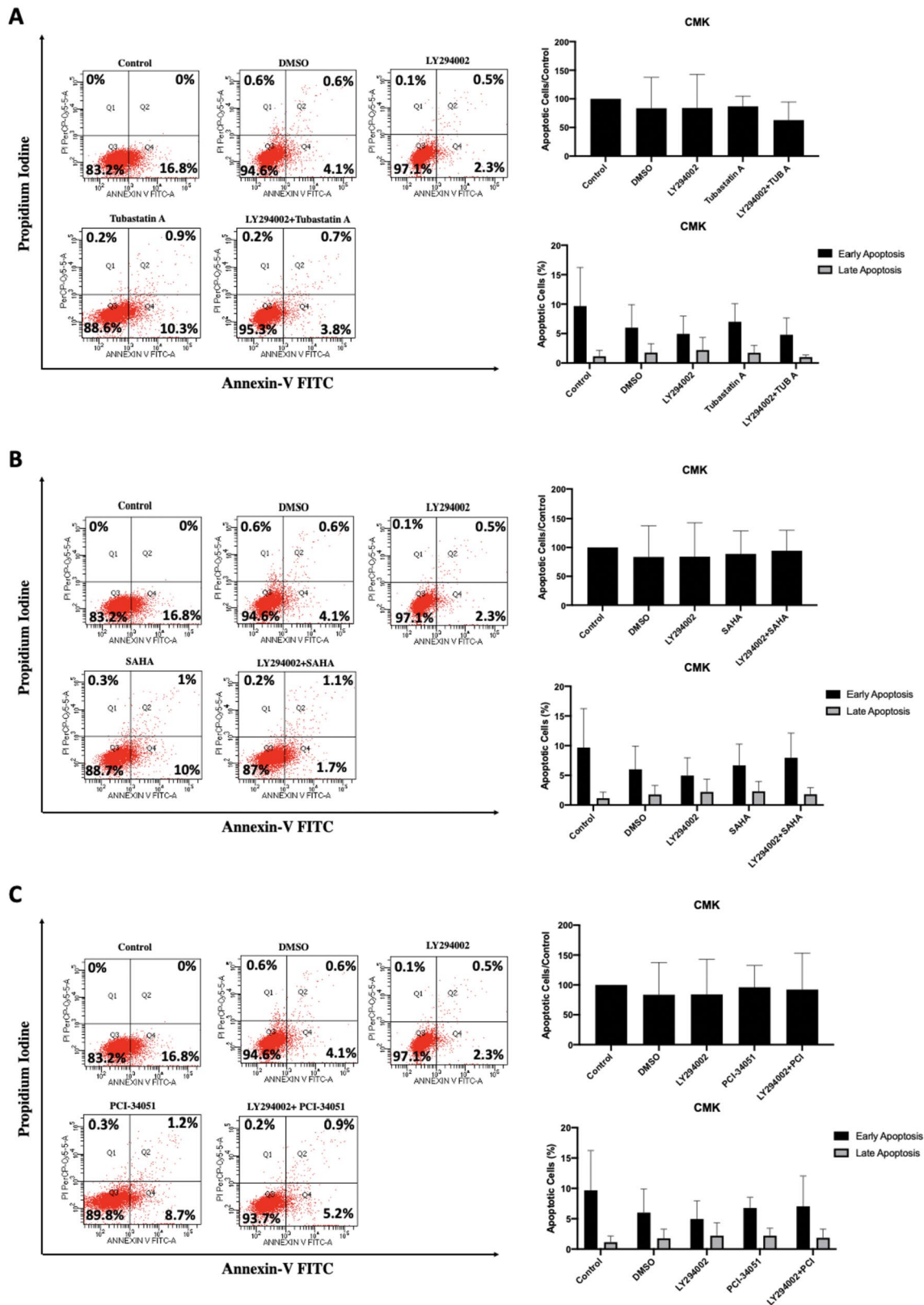


Fig. 4 Efficacy of the combinations on apoptotic cell death in CMK (a–c) and MOLM-13 (d–f) cells. PI-stained cells were analyzed by flow cytometry to determine the percentage of apoptotic cells. Representative histograms and cell percentages are shown. Cells in the lower right (Q4; Annexin V-FITC+/PI–) and upper right (Q2;

Annexin V-FITC+/PI+) quadrants show early and late apoptosis, respectively. The graphs in the right panel showed the percentages of apoptotic cells of three biological replicates. The left graph shows the total number of apoptotic cells (Q2 + Q4), and the right graph shows the early (Q4) and late (Q2) apoptotic cell populations separately

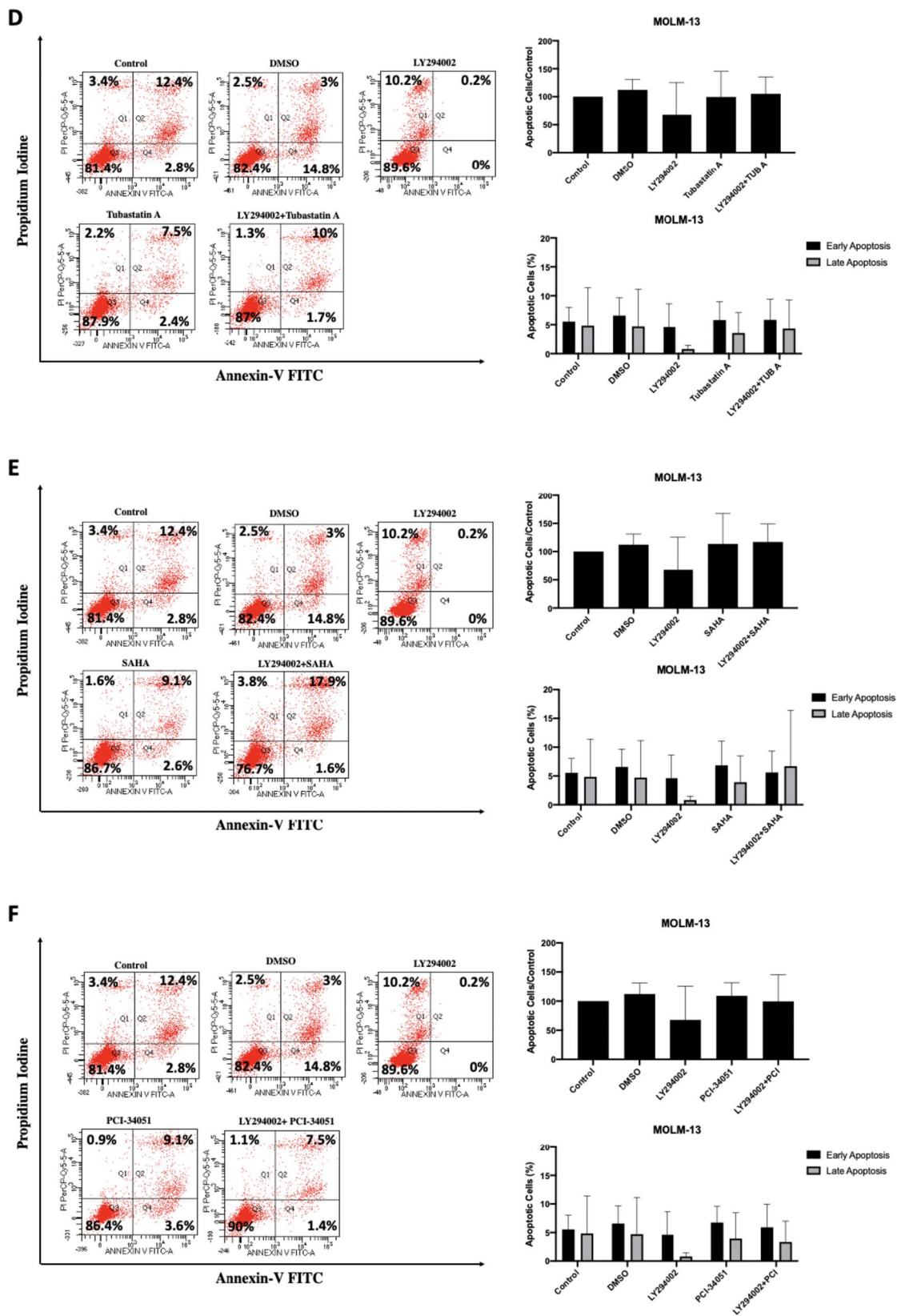


Fig. 4 (continued)

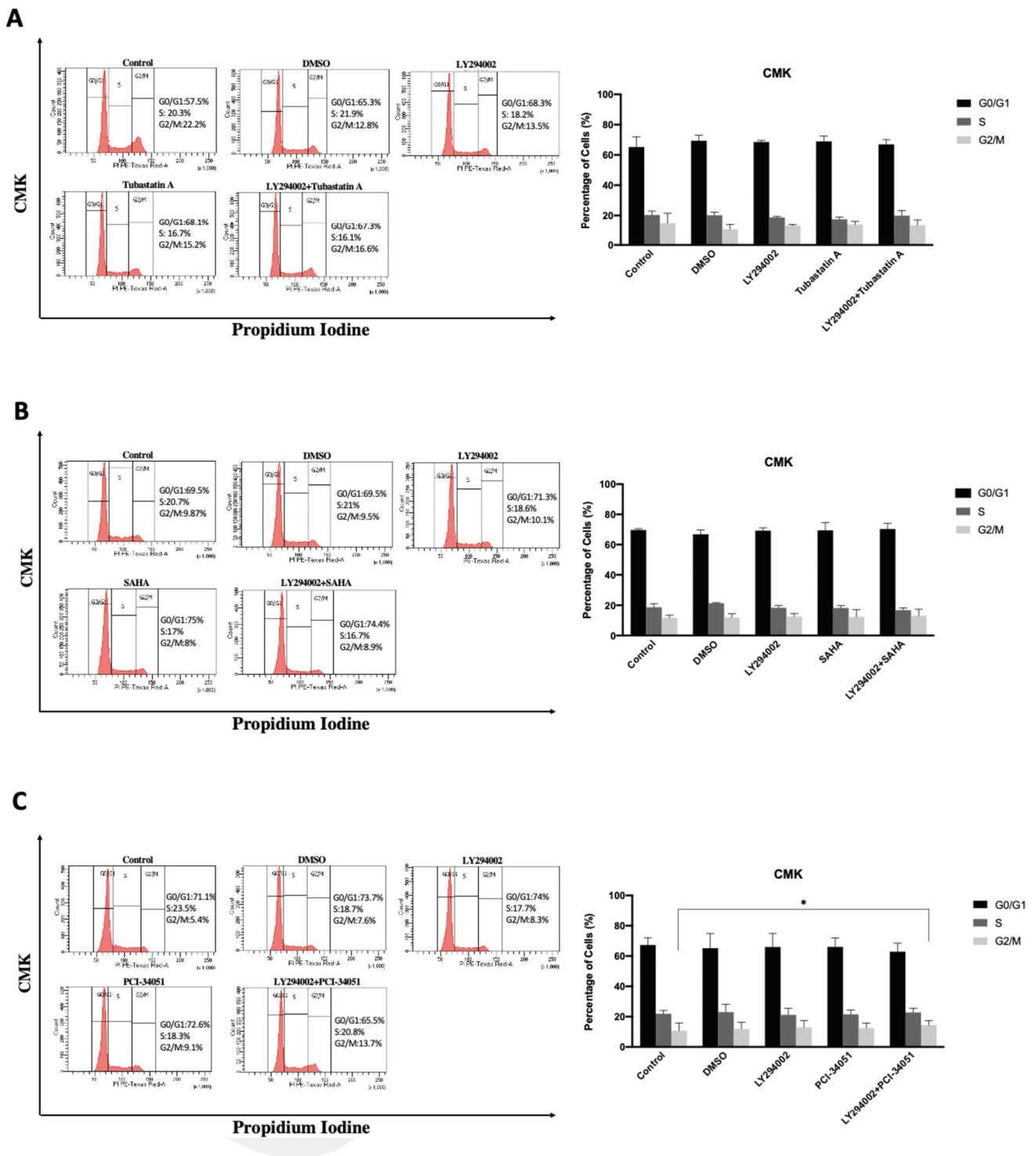


Fig. 5 The effect of drug combination on cell cycle progression for CMK (a-c) and MOLM-13 (d-f). IC20 values of HDAC inhibitors and LY294002 were administered to parental CMK and MOLM-13 cell lines for 48 h, alone and in combination. Three independ-

ent experiments were performed (n=3). Each data is presented as mean ± S.D. (ns = P > 0.05, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.00001)

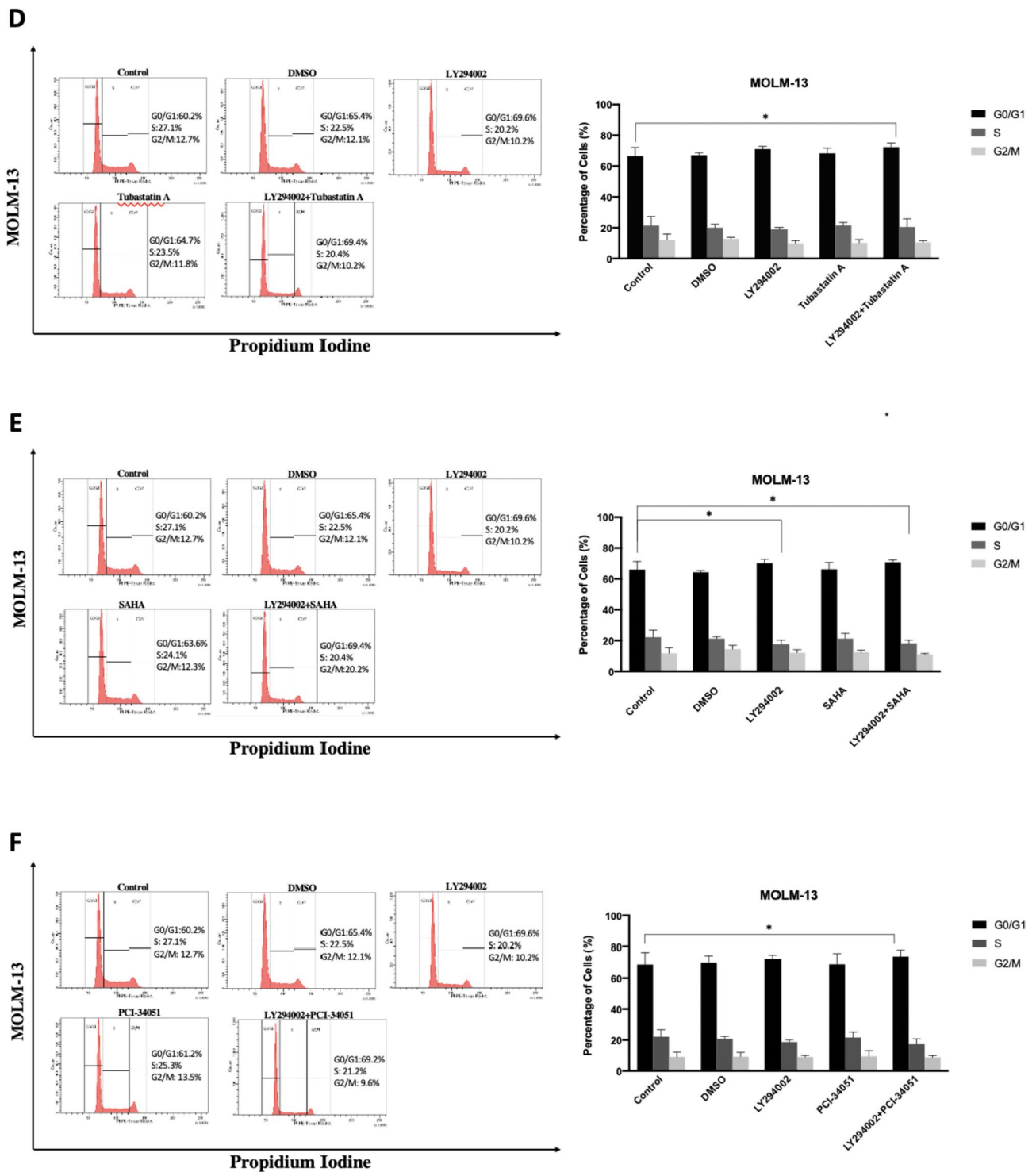


Fig. 5 (continued)

LY294002 + PCI-34051 combination treatment. The LY294002 + Tubastatin A, LY294002 + SAHA and LY294002 + PCI-34051 resulted in G0/G1 phase arrest in MOLM-13 cells compared to control cells (Fig. 5d, e, f). In MOLM-13 cell line, treatment of the cells with single

and combination treatment have shown that, for LY294002 and Tubastatin A combination treatment, G0/G1 arrest percentages were as 69.6%, 64.7% and 69.4% after LY294002, Tubastatin A, and LY294002 + Tubastatin A combination treatment (Fig. 5d). For LY294002 and SAHA combination

treatment, G0/G1 arrest percentages were as 69.6%, 63.6% and 69.4% after LY294002, SAHA and LY294002 + SAHA combination treatment. For LY294002 and PCI-34051 combination treatment, G0/G1 arrest percentages were as 69.6%, 61.2% and 69.2% after LY294002, PCI-34051 and LY294002 + PCI-34051 combination treatment. These data suggested that inhibition of PI3K and HDAC may have a significant effect on cell cycle arrest for MOLM-13 and CMK cells. Notably, combination studies resulted in G0/G1 arrest for both cell lines, excluding LY294002 + PCI-34051 for CMK.

The effect of the combination treatment on autophagy on AML cell lines

Our results indicated that apoptotic cell death was not significantly induced in response to the drug treatment, therefore autophagy induction was examined to reveal the mechanism behind the decrease in cell viability. For this purpose, we determined the endogenous microtubule-associated protein light chain 3 (LC3) I to LC3 II turnover by western blotting. Literature studies have shown that inhibition of HDAC8 with selective inhibitors leads to induction of autophagy by measuring turnover of LC3B-I to LC3B-II that is because we focused on HDAC8 inhibition [21, 22, 39]. In order to verify the effect of selective HDAC8 inhibitor PCI-34051, we treated CMK cell lines with SAHA and PCI-34051 combined them with LY294002. In our study, LY294002 treatment mildly increased the LC3B-II/I ratio in this combination compared to untreated control cells. However, when LY294002 was co-administered with SAHA, there was no significant difference in this ratio compared to untreated control cells (Fig. 6a). In contrast to this, when LY294002 + PCI-34051 combination was applied to the

CMK cells, the LC3B-II/I ratio increased approximately 2.5-fold compared to control cells (Fig. 6b). These results showed that the LY294002 + PCI-34051 combination exhibited more LC3-II/I which is an indicator of the induction of autophagy.

Discussion

AML is a cancer of myeloid blood cells that mediates the abnormal rapid growth of the cells that form the blood and bone marrow, eventually resulting in hematopoietic malignancy [1]. In recent years, the emergence of next-generation sequencing has resulted in reform of the treatment of AML, which has opened the door to a better understanding of the pathophysiology of AML and, thus, the discovery of new mutations such as Fms-related tyrosine kinase 3 (FLT3), nucleophosmin (NPM1), or DNA methyltransferase 3A (DNMT3A). Especially, inhibitors selectively targeting these mutations with specific inhibitors are widely applied in the clinic [2, 5, 7]. Although a 40% improved rate is observed in younger patients, the emergence of drug resistance often leads to treatment failure, and the prognosis is discouraging, especially for elderly patients of whom most patients' condition results in death within a year of diagnosis. Therefore, there is always a need to seek for novel and more effective treatment methods for AML. In the present study, we intended to eliminate the leukemic growth in AML by targeting and inhibiting HDAC enzymes, which have a role in epigenetic regulation, and PI3K, which contributes to many important cellular processes, with selective inhibitors. There are various cell lines representing AML with different properties depending on their background. For instance, MV4-11, MOLM-13, and MOLM-14 are well-defined FLT3-ITD-dependent AML cell lines while U937, CMK, OCI-AML-2,

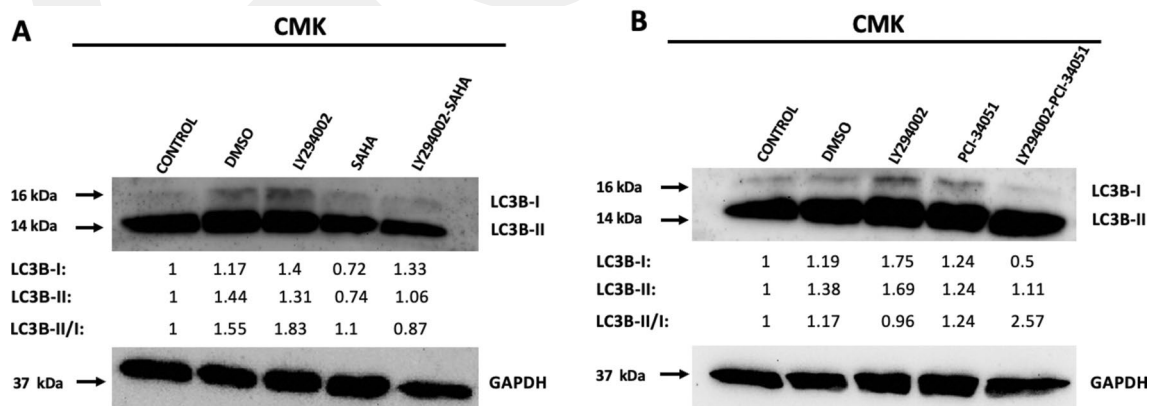


Fig. 6 Immunoblot analysis of LC3B I and II isoforms in CMK cells in response to combination of LY294002 with SAHA (a) and PCI-34051 (b) for 48 h. The relative density of LC3B after normalization

with GAPDH is plotted. The untreated negative control group was taken as 1.0 and the drug-treated groups were compared to the negative control and the fold difference was indicated below the blot

and HL-60 cell lines bear wild-type FLT3 [40]. The FLT3 expression profile is a significant parameter for AML classification. In the current study, MOLM-13 and CMK cell lines have been chosen to perform experiments as these selected cell lines have different FLT3 expression profiles and we aim to compare the responses of these cell lines to the combination treatment.

LY294002 is a specific PI3K inhibitor that competes with ATP at active site of the enzyme. The HDACs exhibit promising anticancer characteristics against solid and hematological malignancies with relative resistance in normal cells [19, 41]. Hence, we used targeted selective HDAC enzyme inhibitors such as SAHA, PCI-34051 and Tubastatin A. A low micromolar IC₅₀ value was determined for LY294002 when for both cell lines and a dose-dependent manner inhibition of cell viability was observed. In line with our findings, in FLT3-ITD mutated and sorafenib-resistant AML cells, this inhibitor reversed the resistance and induced the apoptotic cell death of the resistant cell [33]. In addition, Chen et al. detected synergistic effects of LY294002 inhibitor and As2O₃ inhibitor on AML cells [42]. Furthermore, the combination of Bcl-2 inhibitor, ABT199 with LY294002, showed a synergistic effect on cell cycles of K562, HL60 and KG1a cells [43]. In literature, there are some studies that target PI3K/AKT pathway and HDAC enzymes. In the study of Zhang and his colleagues, the combination treatment of HDAC1/2 inhibition and PI3K/AKT inhibition was performed on Castration-resistant prostate cancer (CRPC) via rationally designed dual inhibitor, fimepinostat, and more effective targeting of each pathway has been achieved compared to the single treatment. Dual HDAC1/2 and PI3K/AKT pathway inhibition by fimepinostat led to robust tumor growth inhibition in different phenotypic subtype models of CRPC. Additionally, combination treatment has shown its effect on inhibition of growth through cell cycle inhibition and apoptosis [44]. In Li's study, an agent for AML, venetoclax, has been used in the combination with the dual PI3K and histone deacetylase inhibitor CUDC-907. The study has shown that CUDC-907 and venetoclax synergistically induced apoptosis in AML cell lines and patient samples [45]. The same group then focused on the CUDC-907 combination with venetoclax against AML. They revealed that the CUDC-907 and venetoclax combination induced metabolic and transcriptomic reprogramming, and suppression of oxidative phosphorylation in AML, which provides additional mechanisms underlying the synergy between the two agents. The result of dual inhibition of PI3K and HDAC demonstrated the crucial role of this combination [46].

Although different combinations of PI3K inhibitors were demonstrated in hematological malignancies or solid tumors, the effect of LY294002 in combination with the HDACs used in this study has not been studied. To address this, our study focused on the combination of LY294002

with different HDAC inhibitors and investigated this combination in point of cell proliferation, cell cycle distribution, cell death mechanism and autophagy modulation. When administered alone, SAHA, Tubastatin A and PCI-34051 treatment resulted IC₅₀ values at low micromolar in both AML cells. Among the HDAC inhibitors used, the combination studies with HDAC8 inhibitor PCI-34051 did not result in a significant difference in cell viability when compared to only LY294002 treatment. In our study, PCI-34051 alone decreased cell viability but did not affect apoptotic cell death or cell cycle arrest. Spreafico et al. tested the PCI-34051 inhibitor on five different AML cells and observed similar findings for some AML cell lines [29]. However, in another study, HDAC8 induced leukemic stem cell (LSC) transformation via p53 deacetylation, and specific HDAC8 inhibition abolishes the leukemia-initiating capacity of both murine and patient-derived LSCs to spread AML [47]. In light of the studies in the literature, we think that the effect of PCI-34051 might be cell-type specific and it was not effective on the cells we used in this study. This effect may be due to the HDAC8 expression levels in cell lines we used. In addition, the combination of SAHA and LY294002 in stromal sarcoma cells showed a strong synergistic effect [48]. Similar results were obtained as the LY294002 + SAHA combination significantly inhibited cell viability in CMK and MOLM-13 cell lines. Contrary to the data in the literature, where 25 μ M LY294002 inhibitor induced the AML blast cells undergoing apoptosis [10], LY294002 did not induce apoptosis in either CMK or MOLM-13 cells in our study. Interestingly, it increased the necrotic cell population in MOLM-13 cells. In MV4-11 cells, which expresses FLT3-ITD, LY294002 at the concentration of 40 and 80 μ M increased the apoptotic cell population by 13.9% and 23.4%, respectively [33]. When we designed our study, we decided to use a subtoxic concentration which is the IC₂₀ value of LY294002 as 1 μ M and 2.8 μ M for CMK and MOLM-13, respectively, in order to observe a synergistic apoptotic effect. The subtoxic concentrations of LY294002 failed to induce apoptosis on its own and we argue that this could be the reason behind the low levels of apoptosis induction when compared the previously mentioned studies in literature.

The study demonstrating the sensitivity of HDAC inhibitors on a number of AML and CML cells showed that CMK cells were not sensitive to Tubastatin A and PCI-34051 inhibitors alone [17]. However, 10 μ M Tubastatin and PCI-34051 used in our study decreased the cell viability by 90%. The drug combination caused no significant induction of apoptosis at the same doses decreased cell viability significantly. Assuming that these results might be dose-dependent, a high apoptosis level could be achieved at higher doses.

Next, we sought to understand the reason behind the reduction in cell viability. We focused on the autophagy process since the PI3K/AKT/mTOR pathway is located

upstream of autophagy which is the cellular process that removes unwanted molecules through lysosome-mediated degradation to promote homeostasis [49]. To investigate autophagy, we examined LC3B protein, also known as microtubule-associated protein 1 light chain 3, is an important protein involved in the autophagy process [50]. The increased LC3B-II level is widely used as a marker of autophagy [39]. The single LY292004 treatment resulted in increased LC3B-II expression in CMK cells compared to the untreated control. The increased expression could be due to a decrease in the expression level of mTOR, which is the negative regulator of autophagy in its downstream pathway, and this causes the activation of autophagy, after the inhibition of PI3K. In this study, although the LC3B ratio was examined to understand the effects of PI3K and HDAC inhibition on autophagy together in the CMK cells, further studies are needed to illuminate the underlying mechanism. Autophagy is an important cellular mechanism in which many different proteins are involved. Therefore, in future studies, the expression level of autophagy-related proteins including ULK-1, ATG5, Beclin-1, p62 can be examined and their potential role in AML can be interpreted more clearly.

Taken together, we demonstrated that two AML cells with different genetic backgrounds could have different responses to the same inhibitors in different biological assays. There was a significant decrease in both cells after a single treatment of both PI3K and HDAC inhibition. On the other hand, combination studies have resulted in different responses that are more plausible in CMK. Furthermore, no significant difference was observed in total apoptotic cell death in all combinations in the CMK cells compared to the control. This suggested that decrease of cell proliferation may not be driven by apoptosis as our results did not reveal a significant change in apoptotic cell death. Interestingly, combination studies resulted in G0/G1 arrest for both MOLM-13 and CMK cells, excluding LY294002 + PCI-34051 for CMK. Moreover, the LY294002 + PCI-34051 combination exhibited increased LC3-II/I ratio which is an indicator of the induction of autophagy in CMK cells. The reason why two AML cells respond differently to the same inhibitors could be because CMK and MOLM-13 AML cells have different genetic backgrounds, epigenetic regulation and metabolic activity due to their different FLT3 expression profiles. Notably, since FLT3 mutation causes activation of signaling pathways downstream of survival and proliferation and is associated with poor prognosis in patients [51]. In particular, the ITD and wild-type FLT3 kinase was demonstrated, in many studies, that they have different activated pathways and different expression and function of myeloid transcription factors. These differences are causing the correlation of FLT3-ITD with increased risk of relapse and impaired overall survival [52]. These factors explain why the FLT3-ITD AML cell line MOLM-13 showed greater resistance and

was less responsive to the combination study. Furthermore, Long et al. noted that the combination of tyrosine kinase and HDAC8 inhibitor led to the upregulation of HDAC8 in FLT3-ITD cells [53]. This further supported for why our results were different in the FLT3-ITD AML cell line MOLM-13 and the FLT3-WT AML cell line CMK.

Conclusion

Our findings indicated that the combination of PI3K pathway inhibition with LY294002 and HDAC inhibitors showed a synergistic effect on MOLM-13 and CMK AML cells in terms of cell proliferation. In conclusion, although these results require further validation, targeting the PI3K pathway and HDAC with selective inhibitors is a notable synergistic combination therapy for AML *in vitro*.

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Author contributions EBGA contributed to the study conception and design. Material preparation, data collection and analysis were performed by MŞ and HS. MŞ and HS analyzed and generated Figs. 1, 2 and 3. MŞ analyzed and generated Figs. 4, 5, and 6. The first draft of the manuscript was written by MŞ and EBGA and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare that they have no competing interests.

Ethical approval Not applicable.

Consent to participate Not applicable.

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