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The Anti-leukemic Effect of the Inhibition of Spleen Tyrosine Kinase and Histone Deacetylase Enzyme on the FLT3-ITD(+) Acute Myeloid Leukemia Cells

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HDAC inhibitors

Abstract: Spleen tyrosine kinase (Syk) engages in crosstalk with several critical signaling pathways, including PI3K, NF-κB, and JAK/STAT, all of which play a pivotal role in the pathogenesis and progression of acute myeloid leukemia (AML). Several studies recorded that deregulated Histone Deacetylase (HDAC) enzymes are involved in the pathogenesis of AML. The study aims to reveal the effect of Syk and HDAC co-inhibition on MOLM-13 and MV4-11 AML cell lines which are harboring the receptor of FMS-like Tyrosine Kinase's (FLT3) Internal Tandem Duplication (ITD). AML cells were incubated using both R406, a Syk inhibitor, and HDAC inhibitors alone and in combination, and increasing concentrations of R406 and HDAC inhibitors revealed a significant reduction of MOLM-13 and MV4-11 cells' viability using MTT cell viability assay. Furthermore, the combination of R406 and Valproic acid (VPA) resulted in a reduction in the proliferation of both cells correlated with the synergistic effect of the two drugs revealed by the combination index (CI). Moreover, investigating apoptosis for the combined administration of drugs resulted in induced apoptosis using Annexin-V/PI double staining. We observed changes in the mRNA expression level of MYC in response to combination treatment via Real-time PCR analysis. Even though further studies are needed, targeting Syk and HDAC enzymes in AML cells could be a promising strategy in the treatment of AML with FLT3 ITD (+) mutation.

Dalak Tirozin Kinaz ve Histon Deasetilaz Enzim İnhibisyonunun FLT3-ITD(+) Akut Miyeloid Lösemi Hücreleri Üzerindeki Anti-lösemik Etkisi

Anahtar Kelimeler
Lösemi,
Dalak tirozin kinazı,
Histon deasetilaz enzimleri,
Kombinasyon tedavisi,
R406,
HDAC inhibitörleri

Öz: Spleen Tirozin Kinaz (Syk); PI3K, NFκB ve JAK/STAT sinyal yolakları gibi akut miyeloid lösemi (AML) ilerlemesinde önemli rol oynayan temel sinyal yolakları ile etkileşime girer. Birçok çalışma, disregule Histon Deasetilaz (HDAC) enzimlerinin AML patogenezinde rol oynadığını kaydetmiştir. Çalışmanın amacı, Syk ve HDAC ko-inhibisyonunun, FMS benzeri tirozin kinaz reseptöründe (FLT3) İnternal Tandem Duplikasyonu (ITD) barındıran MOLM-13 ve MV4-11 AML hücreleri üzerindeki etkisini ortaya koymaktır. AML hücreleri hem Syk inhibitörü olan R406 hem de HDAC inhibitörleri ile tek başına ve kombinasyon halinde inkübe edildi ve inhibitörlerinin artan konsantrasyonlarının, MTT hücre canlılığı testi ile MOLM-13 ve MV4-11 hücrelerinin çoğalmasında önemli bir azalma olduğu ortaya konuldu. Ayrıca, R406 ve Valproik asit (VPA) kombinasyonu, kombinasyon indeksi (CI) tarafından ortaya çıkarılan iki ilacın sinerjistik etkisiyle ilişkili olarak her iki hücrenin proliferasyonunda bir azalmaya neden oldu. Ayrıca, Annexin-V/PI çift boyama yöntemi kombinasyon çalışmasının AML hücrelerinde apoptozu indüklediğini gösterdi. Ayrıca, Gerçek zamanlı PCR analizi ile kombinasyon tedavisinden sonra MYC'nin mRNA ekspresyon seviyesinde değişiklikler gözlendi. Daha fazla çalışmaya ihtiyaç duyulmasına rağmen, AML hücrelerinde Syk ve HDAC enzimlerini hedeflemek, FLT3 ITD (+) mutasyonu olan AML hastalarının tedavisinde umut verici bir strateji olabilir.

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1. Introduction

Different chromosomal abnormalities or dysregulations of key signaling pathways involved in essential cellular processes play a crucial role in the pathogenesis of acute myeloid leukemia (AML), a prevalent hematopoietic malignancy [1, 2]. Internal Tandem Duplication (ITD) occurring in the FMS-like tyrosine kinase receptor (FLT3), receptor is the most common of mutation driving the pathogenesis of AML, accounting for nearly 25% of all AML cases [3]. Considering the high frequency of this mutation in patients, targeting FLT3 is crucial for developing novel, personalized AML treatment approaches. The complex and heterogeneous nature of AML pathogenesis indicates the potential additional need for the development of novel treatment approaches.

Spleen Tyrosine Kinase (Syk) intersects with important signaling pathways involved in the pathogenesis of AML, including PI3K, NFκB and JAK/STAT signaling pathways [4]. Syk is an enzyme that has roles in both the immune system and non-immune biological processes such as cellular survival, differentiation and activation [5]. Phosphorylation and activation of the Syk enzyme are induced by various upstream signaling pathways that are essential for the development of AML, such as integrin signaling and FLT3-ITD and this suggests that Syk is located in the central signaling axis of multiple pathways that cause AML [6]. Therefore, it is essential to demonstrate its role, especially in FLT3-mutated AML cells, to reveal novel targeting approaches. Furthermore, different studies noted that deregulated HDAC enzymes act in the pathogenesis of AML due to the importance of epigenetic regulations [7, 8]. Moreover, numerous studies noted the anti-proliferative and anti-apoptotic efficacy of HDAC inhibitors (HDACi) on AML [8–11]. Valproic acid (VPA), an HDAC inhibitor, is studied in various studies that are investigating its anticancer activity [12–15]. Many studies have shown that VPA often potentiates or synergizes with multiple drugs in AML [16–18]. Panobinostat, another potential HDAC inhibitor, has also been indicated as a significant anti-leukemic agent in early-phase studies [19–21]. Numerous studies have demonstrated that the combination of panobinostat with other therapeutic agents yields greater efficacy than its use as a monotherapy [17, 19].

This study aims to target Syk and HDAC enzymes in AML, especially in FLT3 ITD (+) cells, with a combinatorial treatment approach. Previous studies have shown that combined inhibition of Syk and other signaling pathways or enzymes has been promising as a treatment strategy for AML. Additionally, the applicability of monotherapy in cancer treatment is limited, whereas dual inhibition strategy involving two drugs has shown considerable promise in the treatment of many tumors and hematopoietic malignancies. Considering that many epigenetic mechanisms have a role in the pathogenesis of AML, especially the dysregulation of HDAC enzymes, inhibition of HDACs is a demonstrating potential approach for AML. Altogether, this work aimed to search for the effect of inhibition of Syk enzyme and HDACs on FLT3 ITD (+) cells and reveal the underlying molecular mechanism. In the present study, we demonstrated the efficacy of Syk inhibitor, R406, and two HDAC inhibitors, including valproic acid (VPA) and panobinostat, in two FLT3 ITD (+) mutated AML cell lines, MOLM-13 and MV4-11. The results indicated that both R406 and VPA treatment synergistically inhibit the leukemic growth in both cell lines. Interestingly, while co-administration of R406 and panobinostat was strongly synergistic only at low doses of panobinostat (0.5 nM), at higher doses of panobinostat the result was additive or slightly antagonistic in both AML cells. Besides, our results demonstrated a significant increase in the total apoptotic cell number following the combined IC30 concentration of R406 and 0.5 nM panobinostat treatment compared to both single-agent and untreated control cells on MOLM-13 cells. Besides, the IC30 of R406 and 2.5 mM VPA combination resulted in a 2.5-fold increase in the total apoptotic cells on the MV4-11 cells compared to the untreated control. Moreover, our observation has shown a significant decrease in MYC mRNA expression level after combination treatments in the MV4-11 cell line. In conclusion, our work revealed that combining R406, a specific Syk inhibitor, with HDAC inhibitors, VPA, and panobinostat, decreased cell viability and induced apoptosis by changing MYC mRNA expression in AML cells.

2. Materials and Method

2.1. Cell lines and Chemicals

Phosphate-buffered saline (PBS), RPMI-1640, Fetal Bovine Serum (FBS) and penicillin-streptomycin were purchased from Serox. The inhibitors used in this study, R406 and panobinostat, were purchased from Selleck Chemicals and Valproic acid was obtained from Sigma-Aldrich. VPA was prepared as 1M stock solutions in water, while, panobinostat and R406 were prepared as 5 mM in dimethylsulfoxide (DMSO) according to the supplier recommendations, and the main stocks were stored at -20^{o}C. DMSO concentration in the assay was <0.1%, which had no cytotoxic effect on the cells.

2.2. Cell Maintenance

FLT3 ITD (+) AML cells (MV4-11 and MOLM-13) were supplied from the German National Biological Materials Resource Center (DSMZ). The cell culture protocols were performed by considering recommendations of the literature, utilizing 10% FBS and 100 U/mL penicillin/streptomycin supplemented RPMI-1640. The routine maintenance of the cells was previously described [22].

2.3. Cell Proliferation

The proliferation of cells, after inhibitor administration, was assessed by the MTT Cell Viability Assay (Sigma Aldrich) as previously described [22]. Cells were seeded as $1x10^4$ per 96-well plate and treated with the indicated drugs for 48 h. Cell proliferation curves were generated based on spectrophotometric measurements obtained by Varioskan™ LUX multi-mode microplate reader (Thermo Scientific™). The inhibitory concentration IC20, IC30, and IC50 (the drug concentration that reduces the cell growth by 20%, 30% and 50%, respectively) were calculated for the indicated inhibitors using GraphPad Prism 8. Combination analysis was performed and Combination Index (CI) values were calculated by using the CompuSyn software (Biosoft, Cambridge, United Kingdom). The equation of Chou-Talalay's multidrug effect was used to evaluate the efficacy of the drug co-administration and the results were analyzed using the CI values. A CI of <1, =1, or >1 is indicative of synergistic, additive, or antagonistic effects, respectively [23].

2.4. Cell Death Assay

Phosphatidylserine amounts and localization were identified in MOLM-13 and MV4-11 cells with Annexin V/Propidium iodide double staining method. 1×10^6 cells were incubated for 48 h with R406 alone or in combination with VPA and panobinostat, along with an untreated control. The cells were analyzed using BD LSRFortessa (Becton Dickinson) flow cytometry as previously described [22].

2.5. Real-Time Polymerase Chain Reaction (RT-PCR)

$4x10^6$ cells/mL were cultivated as single or combination of drugs for 48 hours in 6-well plates. Total ribonucleic acid (RNA) isolation was carried out by following the supplier's protocol. The quantity and quality of RNAs isolated from different samples were analyzed by a Nanodrop Photospectrometer (Thermo Scientific™). cDNA was generated using High-Capacity cDNA Reverse Transcription Kit and was further used for real-time reverse-transcription PCR (RT-PCR) using DyNAmo Flash SYBR Green qPCR Kit (Thermo Scientific™). As an internal control, the mRNA levels of β-actin were used and the obtained results belonging to MYC gene expression were normalized to β-actin levels. Following primer sequences were used; β-actin: F5′-'GCCGCCAGCTCACCAT−3′; R5′-GATGCCTCTCTTGCTCTGGGG−3′; MYC: F5′- GTCAAGAGGCGAACACACAAC−3′; R5′-TTGGACGGACAGGATGTATGC−3′.

2.6. Statistical Analysis

All statistical analyses were performed with GraphPad Prism 8.0 software. Inhibitory concentrations of each drug were specified using Nonlinear Regression analysis. The comparations of two experiment sets were done by 2-Way ANOVA with Dunnet's Multiple Comparison test and the level of significance was set $P<0.05$. All data are presented as mean ± S.D.

3. Results

Syk enzyme inhibition via R406 led to a reduced proliferation of MOLM-13 and MV4-11 AML cells

Syk is a multifunctional protein, whose role varies depending on the cancer type. R406, an active metabolite of Fostamatinib, is the first selective FDA-approved inhibitor of the Syk enzyme. It prevents Syk activation by blocking the ATP binding pocket [24]. To investigate the Syk inhibition effect on FLT3 ITD (+) AML cell lines, we treated cells with R406 for 48 hours. The results indicated that leukemic cell proliferation in both MOLM-13 and MV4-11 cells decreased with increasing doses of R406 (0.1 nM- 500 nM) (Figure 1). Moreover, the highest dosage of R406, which is 500 nM, decreased cell viability nearly 95% compared to untreated control cells. According to the cell proliferation curves, the IC20, IC30 and IC50 values of R406 were calculated (Figure 1a-b). IC20, IC30 and IC50 values were determined for MOLM-13 as 2.031 nM, 4.294 nM and 13.94 nM, respectively. On the other hand, IC20, IC30 and IC50 values of R406 were calculated as 3.214 nM, 6.597 nM and 20.44 nM on MV4-11 AML cells, respectively.

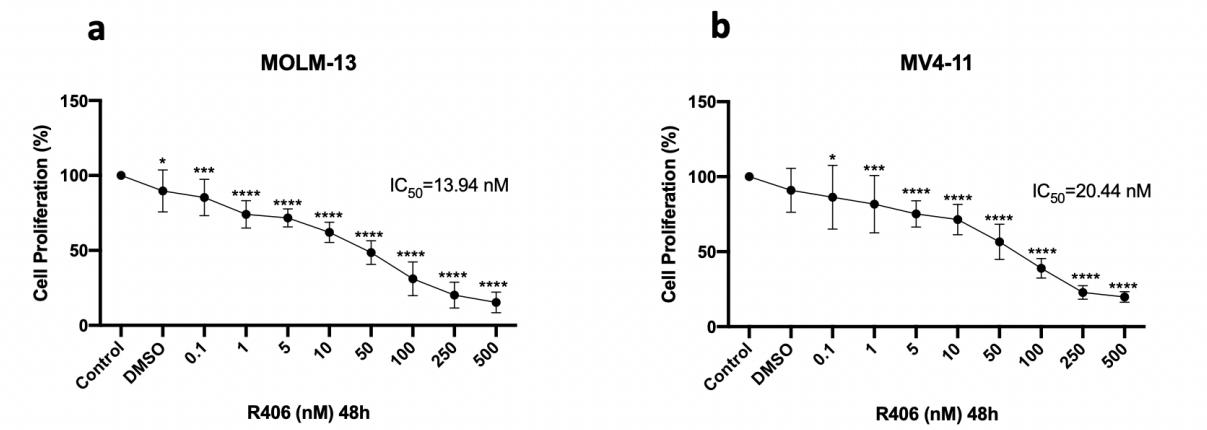


Figure 1. The inhibitory effect of R406 on (a) MOLM-13 and (b) MV4-11 cell lines. These results are representative of data from samples in triplicate in three independent experiments (n = 3). Calculations were made by GraphPad Prism 8 and two-way ANOVA with "Dunnett's multiple comparisons test" with p>0.05 significant value is performed for the data analysis (ns=P > 0.05, *= P≤ 0.05, **= P ≤ 0.01, ***= P ≤ 0.001, ****= P≤ 0.0001).

The Anti-proliferative Effect of HDAC Inhibitors on MOLM-13 and MV4-11 AML cells

Different studies reported that deregulated HDAC enzymes have roles in the pathogenesis of various cancers through the dysregulation of epigenetic mechanisms [7, 8, 25]. Recently, targeting HDACs using small-molecule inhibitors has become one of the promising approaches in cancer treatment. For this purpose, the cytotoxic efficacy of two different HDAC inhibitors, which are valproic acid (VPA) and panobinostat, were investigated. Our results demonstrated that VPA administration resulted in a statistically significant decrease in the cell proliferation of MOLM-13 and MV4-11 cells with increasing dosages at 48h (Figure 2a-b). Besides, administration of panobinostat at increasing concentrations from 0.5 nM to 10 nM led to a notable reduction in cell proliferation of both AML cells after 48 h (Figure 2c-d). From the proliferation curves, IC20, IC30 and IC50 values of each inhibitor were calculated to use for further experiments (Figure 2a-d). Altogether, we can suggest that HDAC enzyme inhibition successfully decreases the proliferation of FLT3 ITD (+) AML cells.

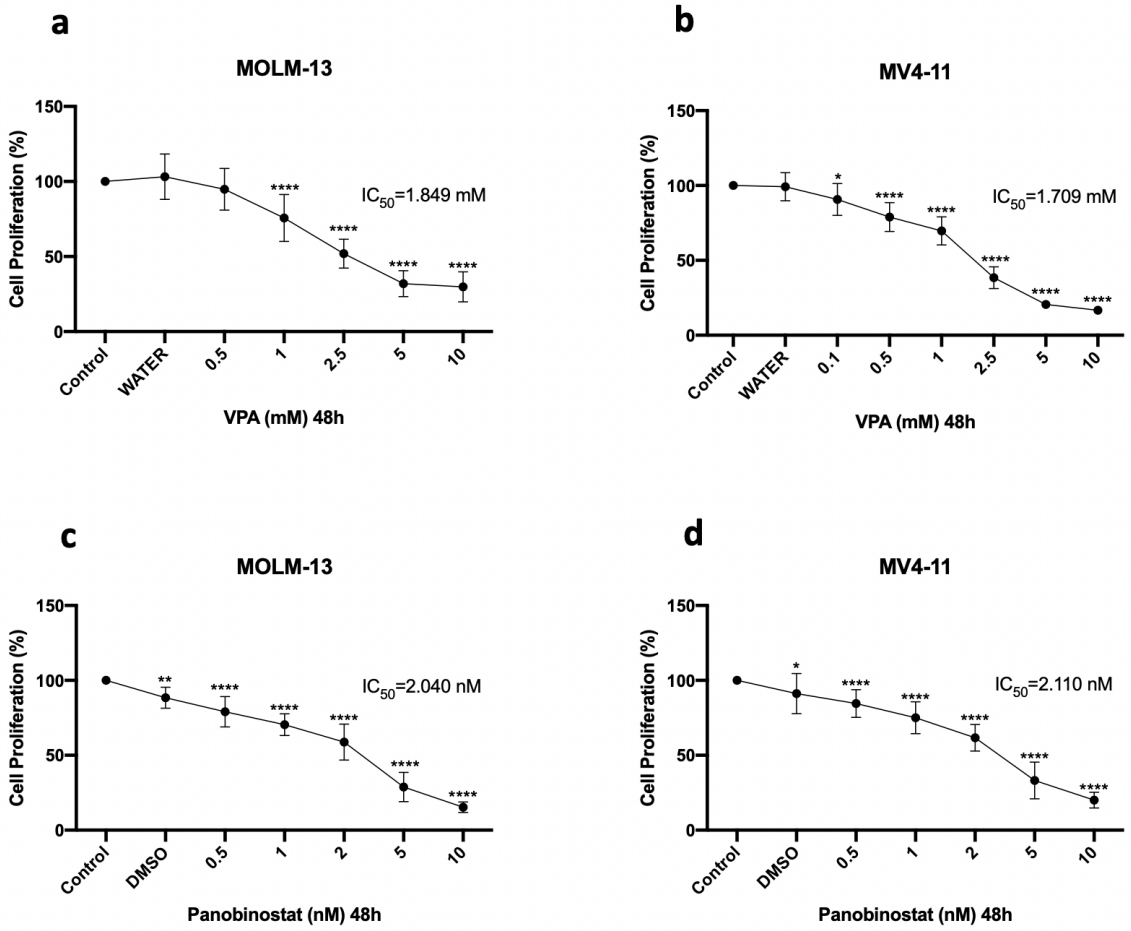


Figure 2. Anti-proliferative Effect of HDAC Inhibitors on FLT3 ITD (+) AML cells (a-d). Each treatment was carried out in triplicate and was repeated in three independent experiments (n=3). Statistical analyses were accomplished using two-way ANOVA. (ns=P > 0.05, *= P≤ 0.05, **= P ≤ 0.01, ***= P ≤ 0.001, ****= P≤ 0.0001).

The Effect of Co-administration of the Syk and HDAC Enzyme Inhibitors on FLT3 ITD (+) AML cells

To indicate the combined action of R406 and HDAC inhibitors, the IC30 value of R406 and increasing concentration of VPA and panobinostat were combined for 48 hours. Our data indicated that R406 combination with increasing concentrations VPA resulted in significant decrease of MOLM-13 (0.5-10 mM) (Figure 3a) and MV4-11 (0.1-10 mM) (Figure 3c) cell viability compared to both untreated control and single drug applications. Furthermore, the application of 4.3 nM R406 and increasing the dosage of VPA (from 0.5 mM to 10 mM) led to a significant reduction in cell proliferation of MOLM-13 cells compared to a single 4.3 nM R406 administration. Similar results were observed for MV4-11 cells after combination with R406 and increasing dosages of VPA. On the other hand, the usage of different concentrations of panobinostat with the IC30 value of R406 led to a profound reduction in cell viability of both cell lines. Interestingly, when compared to the single IC30 value of R406 and its combinations, it was appeared to be effective only when combined with high dosages of panobinostat (5 nM and 10 nM) and significantly reduced the cell viability of MOLM-13 (Figure 3b) and MV4-11 cells (Figure 3d). Consistent with the proliferation curves, our combination index (CI) results support that the combination of VPA and R406 that is strongly synergistic in MOLM-13 and MV4-11 cells (Table 1). However, the combination of IC30 R406 and increasing dosages of panobinostat was additive and mildly antagonistic, except for IC30 R406+0.5 nM panobinostat in both cell lines. These results indicated that the co-treatment of R406 and VPA was strongly synergistic in FLT3 ITD (+) AML cell lines, while the combination of R406 and panobinostat was synergistic only when a low dose of panobinostat (0.5 nM) was used.

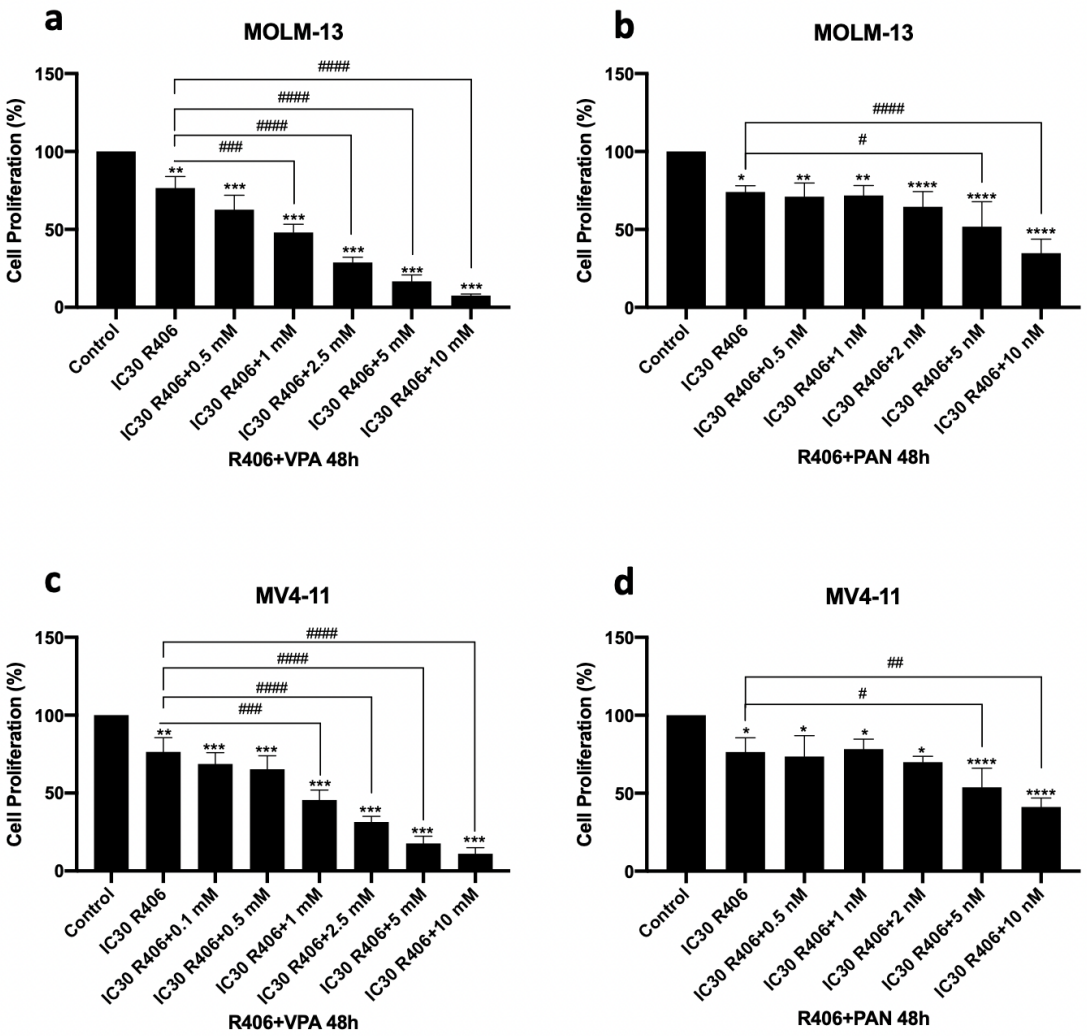


Figure 3. The efficacy of the combination with R406 and VPA on the viability of MOLM-13 (a) and MV4-11 (c) cells. The effect of the combination with R406 and PAN on the viability of MOLM-13 (b) and MV4-11 (d) cells. Each treatment was carried out in triplicate and was repeated in three independent experiments (n=3). Statistical analyses were accomplished using two-way ANOVA (*ns=P > 0.05, */#= P≤ 0.05, **/##= P ≤ 0.01, ***/###= P ≤ 0.001, ****/####= P≤ 0.0001*). *PAN: Panobinostat.*

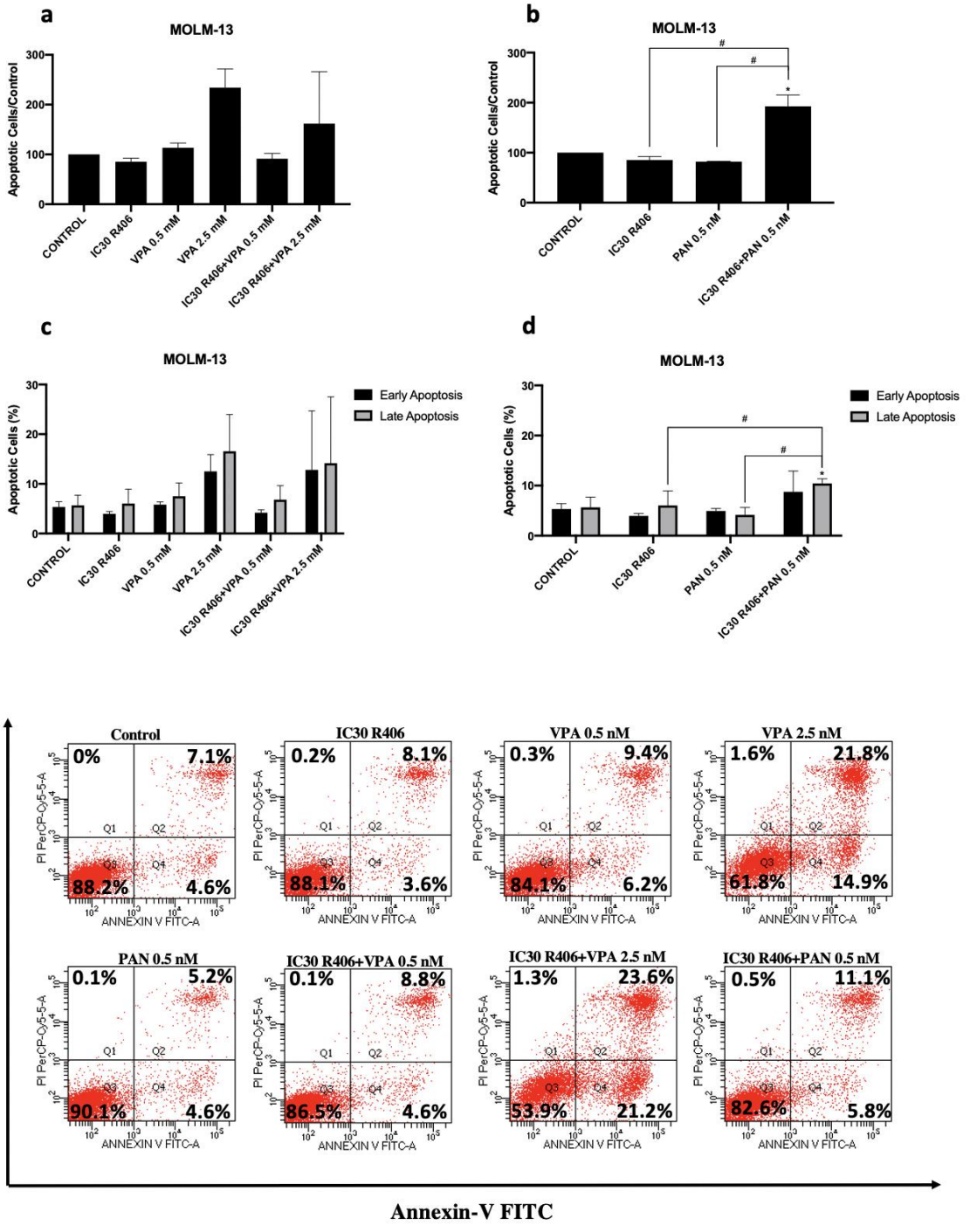
Table 1. The combination index (CI) values of MOLM-13 and MV4-11 cells treated with the combination of R406 and HDAC inhibitors were calculated and isobolograms were determined by CompuSyn software

MOLM-13			**MV4-11**		
R406 (nM)	Valproic Acid (mM)	R406+VPA	R406 (nM)	Valproic Acid (mM)	R406+VPA
Dose		**CI**	**Dose**		**CI**
4.3	0.5	0.23331	6.5	0.1	0.16965
	1.0	0.28921		0.5	0.58815
	2.5	0.38537		1.0	0.49968
	5.0	0.45038		2.5	0.66888
	10.0	0.45721		5.0	0.60696
				10.0	0.68640
R406 (nM)	Panobinostat (nM)	R406+ PAN	R406 (nM)	Panobinostat(nM)	R406+ PAN
Dose		**CI**	**Dose**		**CI**

4.3	0.5	0.68090	6.5	0.5	0.75686
	1.0	1.32715		1.0	3.83020
	2.0	1.73228		2.0	1.72678
	5.0	2.57783		5.0	2.15557
	10.0	2.61656		10.0	2.64271

Investigation of the Combined Inhibition of Syk and HDAC in Cell Death Mechanism

Further, we wanted to reveal the combined effect of R406 and HDAC inhibitors on programmed cell death; apoptosis on MOLM-13 and MV4-11 cells. However, no statistically significant increase in the percentage of apoptotic MOLM-13 cells was observed following treatment with either IC30 of R406+0.5 mM VPA and IC30 of R406+2.5 mM VPA treatment compared to VPA alone(Figure 4a, c). Our results pointed out that the percentage of apoptotic cells for MOLM-13 cells was significantly increased after application of the IC30 value of R406 and 0.5 nM panobinostat combination treatment compared to both the untreated control and single treatments (Figure 4b). Furthermore, we observed that R406 IC30+0.5 nM panobinostat administration led to a significant increase in late apoptotic cells compared to untreated control and single dosage of R406 IC30 and 0.5 nM panobinostat on the MOLM-13 AML cell line (Figure 4d). On the other hand, treatment with R406 IC30 and 2.5 mM VPA resulted in a 2.5-fold increase in total apoptosis in the MV4-11 cell line compared to control cells (Figure 4e). We also observed a slight increase compared to the 2.5 mM VPA single treatment on MV4-11 AML cells (Figure 4e). Similarly, the number of late apoptotic cells was increased after treatment with R406 IC30+2.5 mM VPA on the MV4-11 cells compared to both untreated control and alone application of the drugs (Figure 4g). Interestingly, in the R406 IC30+0.5 nM panobinostat treatment, we determined an increase of total apoptotic cell number on the MV4-11 AML cell line compared to both control and single application of 0.5 nM panobinostat, however, R406 IC30 value alone induced more apoptotic cell death (Figure 4f). In addition, the results indicated that R406 IC30 value alone led to late apoptotic cell death (Figure 4h).


a
MOLM-13
Apoptotic Cells/Control
CONTROL
IC30 R406
VPA 0.5 mM
VPA 2.5 mM
IC30 R406+VPA 0.5 mM
IC30 R406+VPA 2.5 mM
b
MOLM-13
Apoptotic Cells/Control
CONTROL
IC30 R406
PAN 0.5 nM
IC30 R406+PAN 0.5 nM
c
MOLM-13
Apoptotic Cells (%)
Early Apoptosis
Late Apoptosis
d
MOLM-13
Apoptotic Cells (%)
Early Apoptosis
Late Apoptosis
Propidium Iodide
Annexin-V FITC
Control
0%
7.1%
88.2%
4.6%
IC30 R406
0.2%
8.1%
88.1%
3.6%
VPA 0.5 nM
0.3%
9.4%
84.1%
6.2%
VPA 2.5 nM
1.6%
21.8%
61.8%
14.9%
PAN 0.5 nM
0.1%
5.2%
90.1%
4.6%
IC30 R406+VPA 0.5 nM
0.1%
8.8%
86.5%
4.6%
IC30 R406+VPA 2.5 nM
1.3%
23.6%
53.9%
21.2%
IC30 R406+PAN 0.5 nM
0.5%
11.1%
82.6%
5.8%

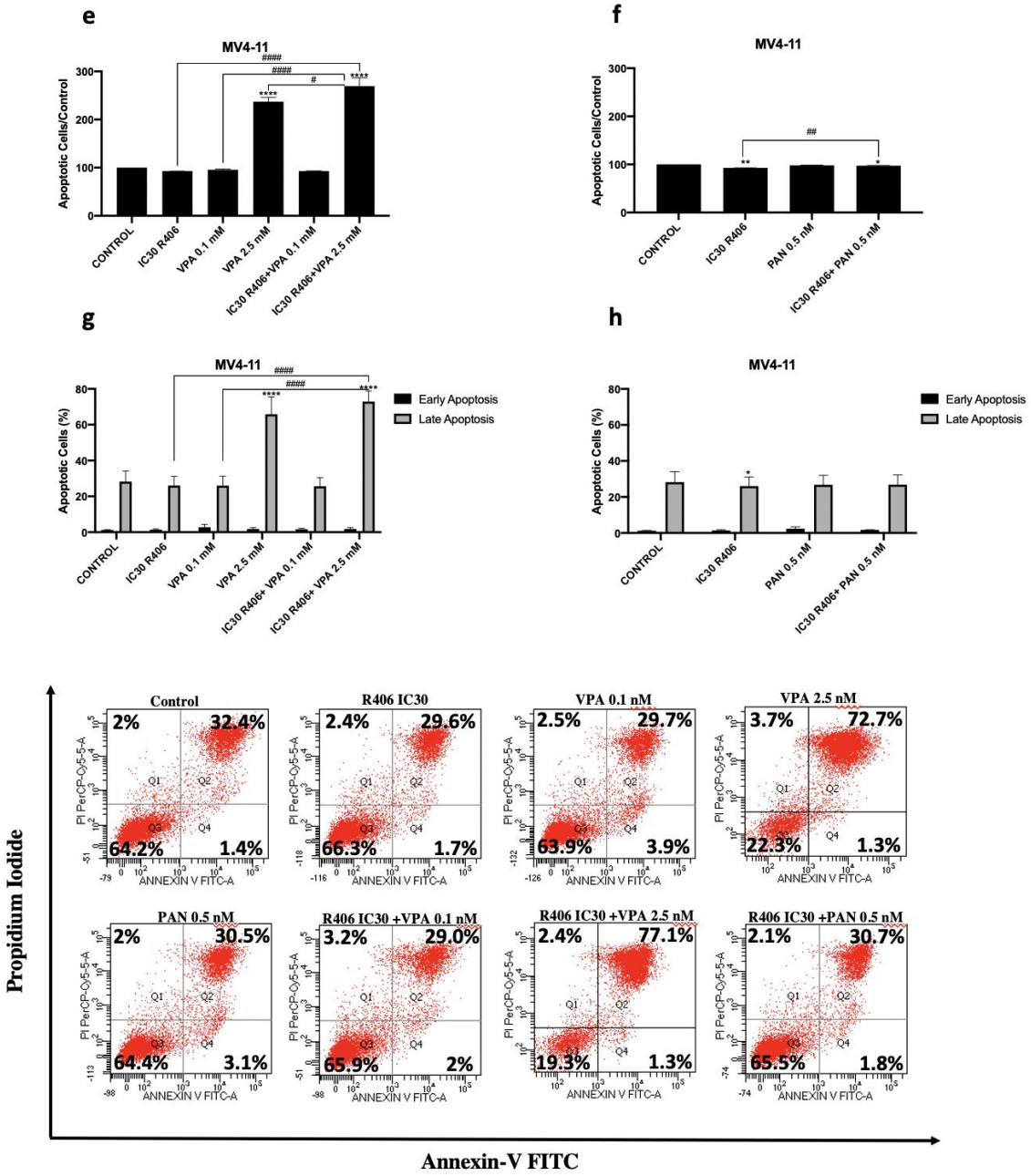


Figure 4. Investigation of R406 and HDAC inhibitor combination on apoptosis for MOLM-13 and MV4-11 cells. The right graph (a, b, e, f) indicates the total number of apoptotic cells (Q2+Q4), and the left graph (c, d, g, h) shows the early (Q4) and late (Q2) apoptotic cell populations separately. The graphs in the panel below show the percentages of apoptotic cells of two biological replicates (n=2). Statistical analyses were accomplished using two-way ANOVA (*ns=P > 0.05, */#= P≤ 0.05, **/##= P ≤ 0.01, ***/###= P ≤ 0.001, ****/####= P≤ 0.0001*). *PAN: Panobinostat.*

Examination of gene expression level of MYC by qPCR

Various studies reported that the MYC oncogene, which plays important roles in the regulation of cellular events such as proliferation, cell cycle progression, differentiation, and apoptosis, is commonly overexpressed in AML and supports leukemogenesis [26, 27]. To further investigate our results, we wanted to reveal whether Syk and HDAC inhibition had any effect on the mRNA expression of MYC in the MOLM-13 and MV4-11 cells. Our data demonstrated that the combination of R406 and VPA resulted in a slight decrease in MYC mRNA expression in both AML cells (Figure 5a). On the other hand, R406+panobinostat resulted in a significant reduction in the MYC expression of MV4-11, but not MOLM-13 cells, compared to a single application of panobinostat (Figure 5b).

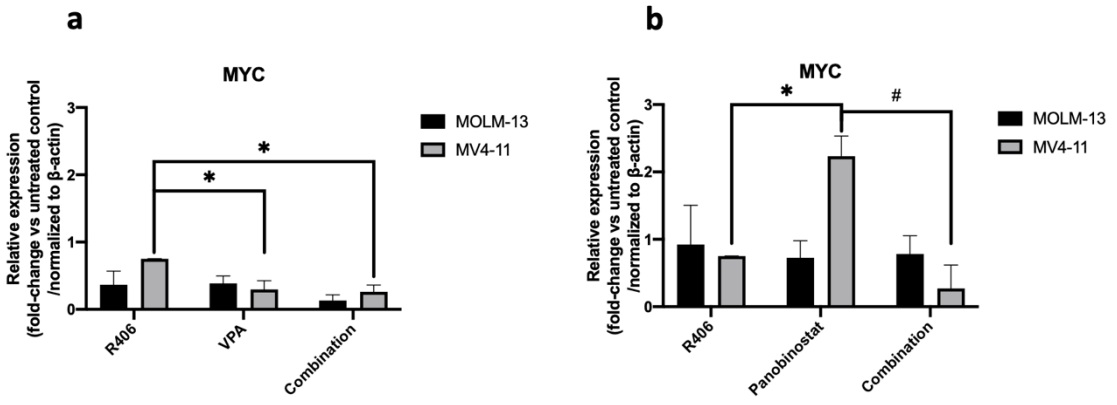


Figure 5. Measurement of the mRNA expression level of MYC after co-administration of Syk and HDAC inhibitors on AML cells (a-b). RT-PCR analysis indicates changes in the relative expression of the MYC gene relative to the housekeeping β-actin gene. Two replicates of the experiment were performed for each sample, and the fold-change of each gene of interest was calculated through Qiagen, Genelglobe (n=2). Statistical analyses were accomplished using two-way ANOVA. (*ns=P > 0.05, */#= P≤ 0.05, **/##= P ≤ 0.01, ***/###= P ≤ 0.001, ****/####= P≤ 0.0001*).

4. Discussion and Conclusion

AML is a heterogenous disease characterized by a complex genetic and molecular landscape [28]. According to the data of the American Cancer Society's AML estimates in the United States for 2024, approximately 22,010 new cases of acute myeloid leukemia (AML) are expected, most of which will be in adults, which in turn are estimated to be resulted in approximately 11,090 deaths [29]. Given the frequency of the FLT3 mutation, which accounts for nearly 25% of all AML cases in patients, targeting FLT3 is crucial for novel, personalized AML treatment options. Thus, it is important to investigate a more efficient treatment strategy for AML disease that has the FLT3-ITD mutation. In the present study, we aimed to address leukemia in FLT3 mutant AML by targeting HDAC enzymes, which are involved in epigenetic regulation, together with the Syk, a kinase which crosstalks with numerous key signaling pathways implicated in the pathogenesis of AML by using the selective inhibitors in two FLT3-ITD (+) AML cell lines, MOLM-13 and MV4-11.

Syk is an essential signaling partner for FLT3 receptor, which is important in the transformation of AML [6]. Therefore, FLT3-ITD is dependent on Syk to transduce oncogenic signals [30]. Specifically, extremely activated Syk is more prevalent in FLT3-ITD mutant AML patients than in those with wild-type FLT3 [6]. The antiproliferative effects of R406 on BaF3 cells stably expressing FLT3-ITD and fusion proteins of Syk, TEL-SYK, a constitutively active Syk, and R406 treatment inhibited the growth of these cells at low nM concentration range [30]. Aberrant activities of HDAC enzymes are observed to prevent myeloid differentiation and thus HDAC inhibition was found to trigger differentiation and proposed as a strategy to induce differentiation [31]. In our study, we target HDAC enzymes by using two inhibitors: VPA and panobinostat. VPA is a promising HDAC inhibitor with many functional effects on AML cells [32]. The single-treatment efficacy of each HDAC inhibitor on both AML cell lines was evaluated and a meaningful reduction in the viability was observed for both cell lines after both VPA and panobinostat treatment compared to control cells.

The combinations of Syk and HDAC inhibitors have been reported in hematological malignancies. The effect of R406 with the HDAC inhibitors used in this study have not been investigated previously in literature in AML. For this purpose, in this study, we aimed to examine combination approaches using R406 and two HDAC inhibitors, Valproic acid and panobinostat, in terms of cell proliferation, apoptosis, and overexpression of oncogenic genes that have a crucial role in the progression of cancer. Prior to the combination treatment, the cytotoxic effect of a single R406 treatment was investigated on the MOLM-13 and MV4-11 cells. The results noted that R406 significantly decreased leukemic cell proliferation. Firstly, we kept the IC30 of R406 constant and treated both MOLM-13 and MV4-11 separately with increasing doses of both VPA and panobinostat, respectively. The cell viability assay and combination index analysis indicated that the combination with an IC30 value of R406 and VPA strongly decreased the cell viability compared to both untreated control cells, synergistically. Furthermore, the combination results reduced cell proliferation significantly than a single application of R406 IC30 in both cell lines, even when low doses of VPA were used. On the other hand, co-inhibition with IC30 value of R406 and different dosages of panobinostat was found to be synergistic when a low dose of panobinostat (0.5 nM) combination was used in both AML cells, depending on CI analysis. This indicates that panobinostat is more effective when used in lower doses. This study is the first to show that leukemia growth is prevented by using Syk and HDAC enzyme inhibitors together in vitro using selected inhibitors and cell lines. Especially, administration of the HDAC enzyme

inhibitors with other anticancer agents is widely preferred in clinical trials for the treatment of AML [8]. As evidence for this assumption, the co-treatment of hydroxyurea (HU) and VPA has been shown to have remarkable potential in preclinical models of AML. This combination demonstrated that VPA enhanced the ability of HU to slow S-phase progression in p53 wild-type leukemia cells, and this was associated with significantly increased DNA damage. Additionally, in vivo studies showed that combination therapy improved survival in OCI-AML3 and patient-derived xenograft mouse models of AML [33]. Furthermore, a recent study reported that azacitidine and Panobinostat together showed a synergy and sensitized the MV4-11 cells and KMT2A rearranged pediatric patient-derived xenograft lines to cytarabine in multicell co-culture [34]. Moreover, the combination of quizartinib and R406 was reported to be more efficient compared to solely FLT3 inhibition in FLT3-ITD (+) AML models [6]. Here, we hypothesize that both R406 and HDAC inhibitors could be evaluated as more effective combined methods for cancer treatments than monotherapy.

Further validation of the cell proliferation results, cell death assay was conducted to investigate the apoptotic cell death mechanisms and the results demonstrated that the total apoptotic cell number of MOLM-13 cells was significantly increased after treating the cells with the IC30 value of R406 and 0.5 nM panobinostat combination administration compared to both single-agents and untreated control. Interestingly, the IC30 of R406 and VPA treatment (2.5 mM) did not alter total apoptosis in the MOLM-13 cells relative to a single VPA application. However, it increased cell death by almost 2-fold compared to the untreated control. Moreover, treatment with R406 IC30 and 2.5 mM VPA resulted in a 2.5-fold increase in the total apoptotic cells in the MV4-11 cells when compared to the untreated control. Consistent with our results, many studies confirmed that the usage of HDAC inhibitors with other potential anti-cancer drugs synergistically induced apoptosis in different cancer types [25, 35, 36]. Hahn et al. demonstrated a significant increase in apoptosis in response to 100 nM R406 treatment for MOLM-14 cells [37]. The mild apoptosis that we obtained in our study could be due to the relatively low R406 concentration (IC30 values) for cell lines that we used while designing the experiment. However, the effect of R406 could also be because of the differences in cell lines, as Hahn et al. observed that MOLM-14 and KG-1 cells were more sensitive to R406; however, HL-60 cells were the least sensitive to the treatment.

Next, we investigated the efficacy of the designed co-treatment approach on the mRNA expression level of MYC. MYC is frequently activated in AML and has a crucial role in the induction of leukemogenesis [38]. After treating the cells with single and combined drugs, the fold change in MYC expression was measured compared to untreated control cells. We reported that both the R406+VPA and R406+panobinostat combination resulted decrease in the mRNA expression level of MYC. Our results successfully demonstrated the down-regulation of MYC gene expression level in MV4-11 cells. In contrast, we didn't see the same behavior in the MOLM-13 cells. The study by Cheng et al. also supported our results by showing that c-Myc downregulation is crucial for VPA-induced growth arrest and AML myeloid differentiation [39]. In addition to that, a study noted that the transcription of the MYC oncogene could be reduced when Syk was blocked by small molecules such as PRT318 [40]. Altogether, coadministration of HDAC and Syk inhibition can reduce the transcription of the MYC oncogene in AML cells.

In conclusion, our experiments demonstrated that varying biological regulations can lead to distinct responses to the same inhibitors in two AML cells, despite both harboring the same genetic mutation. The interconnected nature of these pathways suggests that simultaneously inhibiting HDAC and Syk may yield a more profound effect than targeting them independently. Despite the absence of dedicated preclinical studies on this specific combination in AML, our research confirms what the literature hints at: combined Syk and HDAC inhibition curtailed proliferation in the FLT3-ITD (+) MOLM-13 and MV4-11 cell lines. In light of all results, we can conclude that despite further investigation being needed, we may suggest that using R406 with selected HDAC inhibitors could be a potential strategy for AML patients who are harboring the FLT3 mutation. Here, even though further studies are needed, targeting Syk and HDAC enzymes in AML cells represents a promising approach to pave the way for a novel therapeutic options for patients suffering from AML with FLT3 ITD (+) mutation.

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