

Combinatorial Antitumor Effect of Vinblastine and the PI3K-Akt-mTOR Pathway Inhibition in AML Cells

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Vinblastine ve PI3K-Akt-mTOR Yolağı İnhibitörünün Kombinasyonunun AML Hücreleri Üzerindeki Antitümör Etkisi

SUMMARY

Acute Myeloid Leukaemia (AML) is a cancer arising from haematopoietic stem and precursor cells. The current study proposed a novel combinatorial treatment of Vinblastine and LY294002. The combination aimed to induce apoptosis and study autophagy by co-targeting microtubules and PI3K/AKT/mTOR signalling pathways. The effects of Vinblastine and LY294002 on cell viability were evaluated using the MTT assay. Vinblastine was applied at nM concentrations, whereas LY294002 was applied at μ M concentrations. Minimising Vinblastine concentrations to around the IC20 and combining with increasing concentrations of LY294002 revealed synergistic effects. The apoptosis and cell cycle effects of compounds alone and in combination were quantified and assessed using Annexin V-FITC/PI double-staining and PI staining, respectively. As observed in the apoptosis results, 20 μ M LY294002 masks the combinatorial effect with Vinblastine in both CMK and MOLM-13. However, co-treatment with 10 μ M vinblastine and a constant vinblastine concentration led to a 1.2-fold increase in MOLM-13 and a 15.3-fold increase in CMK. In the cell cycle results, CMK cells showed an increased accumulation in G0/G1 and S phases following the combinatorial treatment, when no significant change was detected in the MOLM-13 cell cycle pattern. To support the findings and examine Beclin-1 gene expression, PCR and gel electrophoresis were performed. Compared with the negative control, BECLIN-1 gene expression was suppressed in CMK and elevated in MOLM-13 after drug combination. Overall, this study indicates the efficacy of Vinblastine:LY294002 combination and provides preliminary data about the designed treatment strategy.

Keywords: LY294002, vinblastine, apoptosis, autophagy, acute myeloid leukaemia, combination therapy.

ÖZ

Akut Miyeloid Lösemi (AML), hematopoietik kök ve prekürsör hücrelerdeki anomalilerden kaynaklanan bir kanser türüdür. Etkili olduğu bilinen kombinasyon tedavilerine bir yenisini eklemeyi hedefleyen bu çalışma, AML hücrelerinde farklı sinyal yollarını hedefleyebilen Vinblastine ve LY294002 anti-kanser ilaçlarının kombinasyonel etkisini incelemektedir. Amacı apoptozu aktifleştirmek ve muhtemel otofaji aktivasyonunu incelemektir. İlaçların hücre canlılığı üzerindeki etkisini incelemek için MTT testi kullanılmıştır. Vinblastin ile nanomolar (nM) düzeyde gözlemlenen etki, LY294002'nin mikromolar (μ M) düzeyde gösterdiği etkinin üzerindedir. Vinblastin'in nanomolar seviyede gösterdiği sitotoksik etkiyi en aza indirebilmek için Vinblastin'in IC20 konsantrasyonuna yakın bir değer belirlenmiş ve LY294002'nin artan konsantrasyonları kombine edilmiştir. İlaç kombinasyonu sonucunda sinerjistik etki gözlemlenmiştir. Hedeflediğimiz ilaç kombinasyonlarının hücrelerin apoptotik ve hücre döngüleri üzerindeki etkilerini araştırmak için sırasıyla Annexin V-FITC/PI çifti boyama ve PI boyama yöntemleri kullanılmıştır. CMK ve MOLM-13 hücrelerinin apoptoz sonuçlarında gözlemlendiği üzere, 20 μ M LY294002 dozu Vinblastin ile kombine edildiğinde daha baskın gelmiş ve ilaç kombinasyonunda Vinblastin'in etkisini maskeleymiştir. Ancak, 10 μ M ve değişen vinblastin konsantrasyonları ile yapılan kombine tedavi, MOLM-13 hücrelerinde apoptoz oranında 1,2 kat ve CMK hücrelerinde 15,3 kat artışa yol açmıştır. İlaçların hücre döngüsü üzerindeki etkileri hücre bazında farklılık göstermiştir. Benzer şekilde, CMK hücrelerinde kombine tedavi sonrasında G0/G1 ve S fazlarında artmış hücre birikimi gözlenirken, MOLM-13 hücrelerinde hücre döngüsü duraklama paterninde anlamlı bir değişiklik tespit edilmemiştir. Bu sonuçları desteklemek ve BECLIN-1 geninin aktivasyonunu incelemek amacıyla yapılan PCR ve jel elektroforez analizleri sonucunda, CMK'de BECLIN-1 ekspresyonunda azalma ve MOLM-13'te gen ekspresyon seviyesinde artış gözlemlenmiştir. Farklı mutasyonlara sahip AML hücrelerinde sitotoksik etkisi araştırılan ve farklı sinyal yollarını hedefleyen Vinblastine:LY294002 kombinasyonunun verimini vurgulayan ön veri sağlamaktadır.

Anahtar Kelimeler: LY294002, vinblastine, apoptoz, otofaji, akut miyeloid lösemi, kombinasyon terapileri.

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INTRODUCTION

Acute Myeloid Leukaemia (AML) is a malignant bone marrow disease arising from abnormalities in haematopoietic stem cells and their precursors, resulting from the halt of differentiation and leading to clonal expansion (Ayvaz, Yenigül, & Akçok, 2024; DiNardo, Erba, Freeman, & Wei, 2023; Saultz & Tyner, 2022). Mutations occur at the molecular level, and clonal aberrations in chromosome structure form the multiple prognostic markers used to diagnose AML (Ahn & Kim, 2022). Mutations in the FMS-like Tyrosine Kinase-3 (FLT3) receptor are recognised as diagnostic standards and are frequently reported as Internal Tandem Duplications (ITDs) and Tyrosine Kinase Domain (TKD) mutations. Since FLT3 mutations are detected in almost 30-40% of all newly diagnosed cases, they are considered concerning and prevalent (He et al., 2018; Swaminathan & Wang, 2020). Mutations result in functional disruptions of the receptor, leading to hyperactivation of FLT3 and its downstream signalling pathways (e.g., PI3K, MAPK, and JAK2/STAT5) (Darici et al., 2021; He et al., 2018). In addition to the effect of FLT3 mutations on disease formation, AML cases show that more than 70% of patients have a constitutively active PI3K/Akt/mTOR axis (Dhyani et al., 2022; He et al., 2021; Sandhöfer et al., 2014). The phosphoinositide 3-kinase (PI3K)–Akt–Target of Rapamycin (mTOR) axis is responsible for the proliferation, survival, and differentiation of haematopoietic cells, controlling oncogene activation and mutations by reversing the activation of its upstream tyrosine kinase receptor (Nepstad, Hatfield, Grønningsæter, & Reikvam, 2020). Although there are routine treatment protocols (surgery, radiation therapy, and chemotherapy – monotherapy), AML is a heterogeneous disorder that makes patients highly susceptible to developing resistance against applied treatment regimens and results in poor prognosis (Dhyani et al., 2022; He et al., 2018).

Managing disease progression with drug combinations and selecting plant-based compounds

are the ongoing promising strategies in AML treatment (Dhyani et al., 2022; Mirzaie et al., 2024). Vinblastine, a plant-derived chemotherapeutic agent extracted from *Catharanthus roseus*, acts as an antimetabolic agent by binding tubulin, preventing microtubule assembly, and arresting the cell cycle at the M-phase (Patil, Sarkate, Nirmal, & Sakhale, 2023; Prasad, Gupta, & Aggarwal, 2016). Another anti-cancer agent LY294002 hydrochloride (2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride) is a flavonoid derivative responsible for inactivating the PI3K/Akt signalling pathway. The compound competitively inhibits PI3K and acts reversibly. Inhibiting PI3K/Akt signalling results in G1-phase arrest and induces apoptosis (Jiang, Fan, Zhou, Li, & Deng, 2010; West, Castillo, & Dennis, 2002).

Autophagy and apoptosis are cell-fate-determining mechanisms, with apoptosis directly associated with cell death. Autophagy is a microenvironment-dependent process that may help cells evade apoptosis. While apoptosis degrades the entire cellular structure, autophagy degrades misfolded or damaged macromolecules and organelles (Elkon & Oberst, 2019; Glick, Barth, & Macleod, 2010). Since the BECLIN-1 gene has a regulatory role in the PI3K pathway and modulates autophagosome formation (Köchli, Hu, Chan, & Tooze, 2005; Pang et al., 2018), the current study evaluates the anticancer effect of Vinblastine and LY294002 by targeting tubulins and the PI3K pathway, and assessing preliminary information on BECLIN-1 gene expression to delineate the apoptosis-autophagy interconnection. Concurrently, the study aims to combine Vinblastine and LY294002 to explore synergistic effects and to attenuate excessive drug exposure.

MATERIALS AND METHODS

Chemicals

LY294002 hydrochloride was purchased from Cayman Chemical, and a 5 mM stock solution in DMSO was prepared and stored at -20 °C. A 10 mM stock solution of Vinblastine (Sigma) was prepared in DMSO and stored at -20 °C. MTT

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) was purchased from Sigma. RPMI-1640 and Fetal Bovine Serum (FBS) were purchased from Gibco. Penicillin/streptomycin was purchased from Capricorn.

Cell culture and maintenance

CMK and MOLM-13 were acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cells were cultured in RPMI 1640 medium supplemented with 100U/mL penicillin-streptomycin and 10% FBS. Cell maintenance conditions were set to 5% CO₂ at 37 °C. The procedure was performed by centrifugation of cells at 700 rpm for 5 minutes. After centrifugation, the supernatant was discarded. The cell pellet was resuspended in fresh medium and seeded into a 100mm Petri dish at a density of 1×10^6 cells/ml. The cells were subcultured at a 1:2 ratio every 48 hours.

Cell viability assay

The MTT assay was used to assess the cytotoxic effects of Vinblastine and LY294002. 1×10^4 cells were seeded in 96-well plates, each containing 200 µL of RPMI medium. The incubation time for the combinatorial experiment was set to 48 hours. After drug incubation, 10 µL of MTT was added to the cells for an additional 2-4 hours to allow formazan crystal formation. The formed formazan crystals were centrifuged at 1800 rpm for 10 minutes. The sedimented crystals were dissolved in 100 µL of DMSO and incubated in the dark for 15 minutes. The intensity of formazan crystals was measured at 570 nm using a Varioskan™ LUX multimode microplate reader (Thermo Scientific™). GraphPad Prism 8.0.2 was used to visualise the results and calculate the Inhibition Concentration (IC) values.

Combination index (CI) analysis - isobologram test

To comprehend the pharmacodynamics and efficacy of co-treatments involving Vinblastine and LY294002, an analysis was conducted using CalcuSyn,

a Windows software program developed by Biosoft in Cambridge, United Kingdom. The evaluation was derived from Chou-Talalay's multidrug effect equation, employing the Combination Index (CI) (Chou, 2010). The calculated experimental data were evaluated based on their position above, on, or below the reference line used to define antagonism, additivity, and synergism, respectively. If the CI value is smaller than 1, the combinatorial efficacy of drugs is strongly synergistic; if it equals 1, the drugs reveal an additive effect; if it is higher than 1, the combination shows an antagonistic effect (the range of 3.3 - 10 indicates strong antagonism; a value higher than 10 signifies extreme antagonism).

Apoptosis assay: annexin V/FITC-propidium iodide (PI) double staining

Apoptotic cell death was assessed by dual staining using the Annexin V-FITC Apoptosis Kit (Elabscience Bionovation Inc.). 1×10^6 cells/well were treated with the determined Vinblastine concentration (1.5nM for CMK and 0.75nM for MOLM-13) in combination with 10 µM and 20 µM LY294002 for 48 h in a 6-well plate. After incubation, cells were collected, centrifuged at $300 \times g$ for 5 min at 4 °C, washed twice with cold PBS, and resuspended in 100 µL of 1X Annexin binding buffer. Each sample suspension was dyed with 2.5 µL Annexin V-FITC and 2.5 µL PI, and gently vortexed to mix the dyes. Following incubation at room temperature for 15 minutes in the dark, 400 µL of 1X Annexin binding buffer was added to each suspension. Apoptotic cell detection was performed via BD FACSAria III Cell Analyser.

Cell cycle assay

Cell cycle analysis was performed using flow cytometry to measure cellular DNA content after Propidium Iodide (PI) staining. Cells were seeded into a 6-well plate, with each well containing 1×10^6 cells. After the drug incubation, cells were collected and centrifuged at $260 \times g$ for 10 minutes at 4 °C. The pellets were washed twice with cold PBS. Later, the sample pellets were dissolved in 1 mL cold PBS, and

4 mL of 70% cold ethanol was added to each sample, which was then left at -20 °C overnight. Afterwards, samples were centrifuged at $260 \times g$ for 10 minutes at 4 °C, and the centrifugation was repeated by washing the samples with cold PBS. The supernatant was discarded, and each pellet was dissolved in 0.5 mL staining solution (0.1% Triton-X 100 içeren PBS, RNase A çözeltisi ve PI reaktifi). Before the analysis, cells were incubated with staining solution for 30 minutes at 37 °C. The analysis was performed using a BD LSRFortessa (Becton, Dickinson) flow cytometer. PI fluorescence signal was optimised by adjusting the photomultiplier tube (PMT) voltages using the untreated control sample, and all subsequent samples were acquired under identical instrument settings without further adjustment. Cells were first selected on an FSC/SSC plot, where debris, dead cells, and non-cellular events were excluded, leaving the main intact cell population. Singlet discrimination was then performed using FSC-A versus FSC-H analysis, and doublets were excluded based on a diagonal, tightly distributed population. The singlet population was confirmed by PI-A versus PI-H analysis. Following singlet gating, DNA content histograms were generated from PI fluorescence intensity within the selected population. Based on the DNA histogram of the untreated control sample, the positions of the G0/G1 and G2/M peaks were defined, and the S phase was determined as the DNA content distribution between these peaks.

RNA isolation and polymerase chain reaction for BECLIN-1 expression

Cells were seeded into 6-well plates at a density of 10^6 cells per well. The RNA was isolated from drug-treated cells using NucleoZOL reagent. To lyse cells, 200 µL NucleoZOL was used. Then, cDNA synthesis was performed (ABM OneScript® Plus cDNA Synthesis Kit). The BECLIN-1 gene expression level was determined by PCR using the EasyTaq® DNA Polymerase kit from TransGen Biotech. The band intensities were measured using

Image Lab Software 6.1 (Bio-Rad Laboratories). Two different housekeeping genes, 18S RNA and β -Actin, were empirically assigned to each cell line due to methodological constraints. While the targeted internal baseline for CMK was 18S RNA, β -Actin was the reference standard for MOLM-13. The forward and reverse primer sequences for BECLIN-1, β -Actin (Akyürek et al., 2024), and 18S RNA are indicated below:

> Beclin-1

(F: 5' CTCCCGAGGTGAAGAGCATC 3'; R: 5' TTTCCAAGGGTCTCGCTGAC 3')

> 18S RNA

(F: 5' AAACGGCTACCACATCCAAG 3'; R: 5' CCTCCAATGGATCCTCGTTA 3')

> β -Actin

(F: 5' GCCGCCAGCTCACCAT 3'; R: 5' GATGCCTCTCTTGCTCTGGG 3')

RESULTS

Evaluation of LY294002 and vinblastine effects on AML cell viability

Vinblastine and LY294002 were primarily tested alone in AML cells to assess their effects on cell viability and to further optimise appropriate drug concentrations for downstream experiments. For LY294002 treatment, the incubation time and drug concentrations (1-20 µM) were set according to a previously published study (Şansaçar, Sağır, & Akçok, 2023). The cell viability response of CMK and MOLM-13 cells to the higher concentrations (10µM - 20µM) of LY294002 was comparable with the current study. However, the sensitivity of cell lines treated with lower concentrations (1 µM - 5 µM) showed slight differences among the cell lines. The 1 µM LY294002 reduced CMK cell viability by 23.1%, while the same concentration in MOLM-13 cells reduced it by 40.6%, a difference of almost 20%. Likewise, cell viability was 43.4% in the 5µM LY294002-treated CMK cells, whereas it was 68.7% in MOLM-13 cells (Figure 1a. and 1b.).

Vinblastine alone was administered to CMK and MOLM-13 cells for 48h at concentrations ranging from 0.5 to 10 nM. The distinct responses of cell lines to the Vinblastine treatment were apparent. Compared with CMK, MOLM-13 showed a more pronounced response to Vinblastine treatment. Vinblastine reduced cell viability of MOLM-13 by 64.1% at 1 nM. At the same concentration, the reduction in cell viability for CMK was 17.98% (Figure 1c. and 1d.).

Although the results of both compound-alone applications were significant, Vinblastine revealed notable reductions in cell viability at nanomolar concentrations. 10 nM Vinblastine reduced viability by over 90% and yielded results nearly equivalent to those of the 20 μ M LY294002 experimental group in both cell lines.

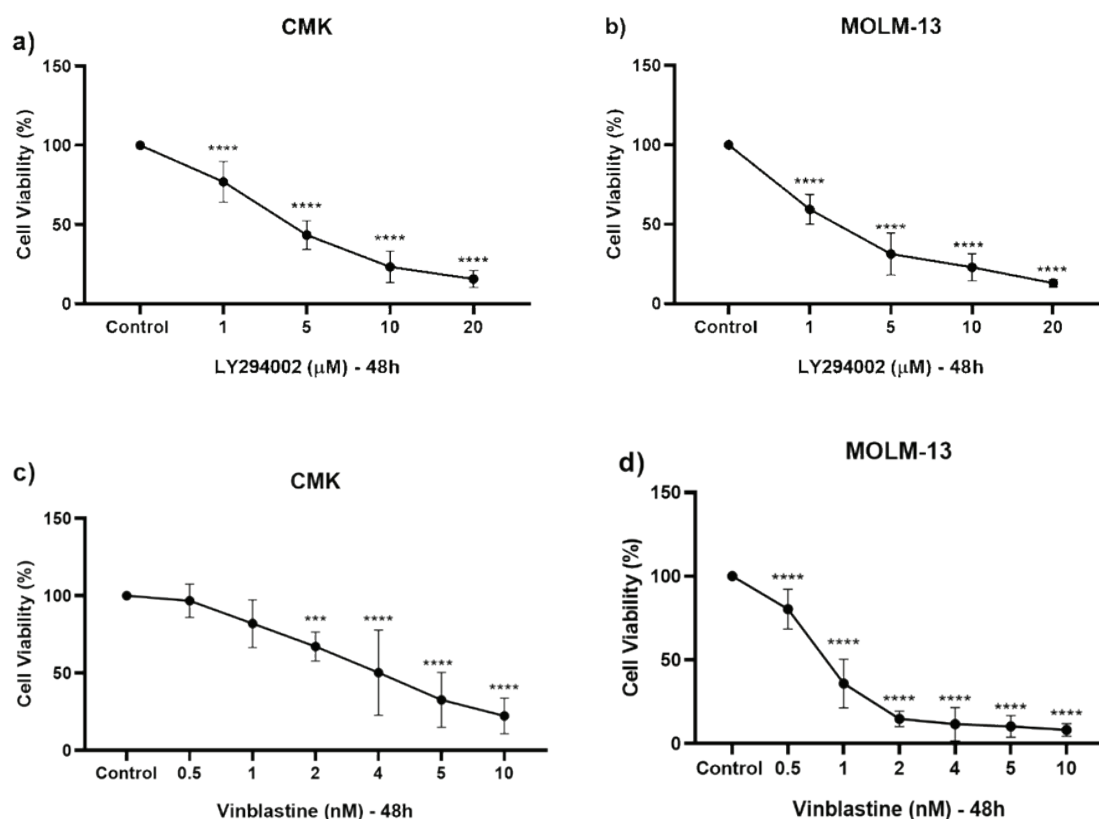


Figure 1. Cell viability effects of single administration of Vinblastine and LY294002 on CMK and MOLM-13 cells. Figure 1a (for CMK) and Figure 1b (for MOLM-13) show the effects of LY294002 on cell viability after 48 hours of incubation. The cell viability assessments of Vinblastine after 48 hours of incubation are shown in Figure 1c and Figure 1d for CMK and MOLM-13 cells, respectively. Experiments are shown as means $\pm$ SD of three independent experimental setups in triplicate ($n=3$) (* $P<0.0001$)

Combinatorial treatment of LY294002 and vinblastine reveals a synergistic effect on AML cells

We determined the combinatorial concentrations corresponding to the cytotoxicity results of single-

drug compound treatment. Because Vinblastine showed a stronger anticancer effect at nM concentrations than LY294002, we primarily based our experimental design on Vinblastine. The respective Vinblastine concentrations were determined using

the pre-calculated Inhibition Concentration values in GraphPad Prism. Since we aim to combine compounds to successfully reduce cell viability with lower concentrations, 1.5nM (CMK) and 0.75nM (MOLM-13) Vinblastine concentrations were chosen and combined with escalating doses of LY294002. The primary expectation of the combinatorial strategy was to enhance compound activity at lower concentrations and reduce cell viability, as observed with single-compound administrations at higher concentrations. While a single Vinblastine administration at 1.5nM caused an 18.4% reduction, the combination of Vinblastine 1.5nM + 10 μ M/20 μ M LY294002 resulted in reductions in CMK cell viability

of 79.2% (4.3-fold higher) and 85.6% (4.65-fold higher), respectively (Figure 2a. and 2b.). The effects of the drug combinations were almost equivalent to administration of 10nM Vinblastine-alone, which was 77.7%. Overall, the designed combination resulted in a significant reduction in cell viability compared to 1.5nM Vinblastine alone.

As in the case of MOLM-13, the cell viability effect of the combinatorial treatment was nearly on par with that of single 10nM Vinblastine-treated cells. When single 10nM Vinblastine led to a 91.8% reduction in viability, the combinatorial treatment of 0.75nM Vinblastine with 10 μ M and 20 μ M LY294002 resulted in reductions of 87.03% and 89.6%, respectively.

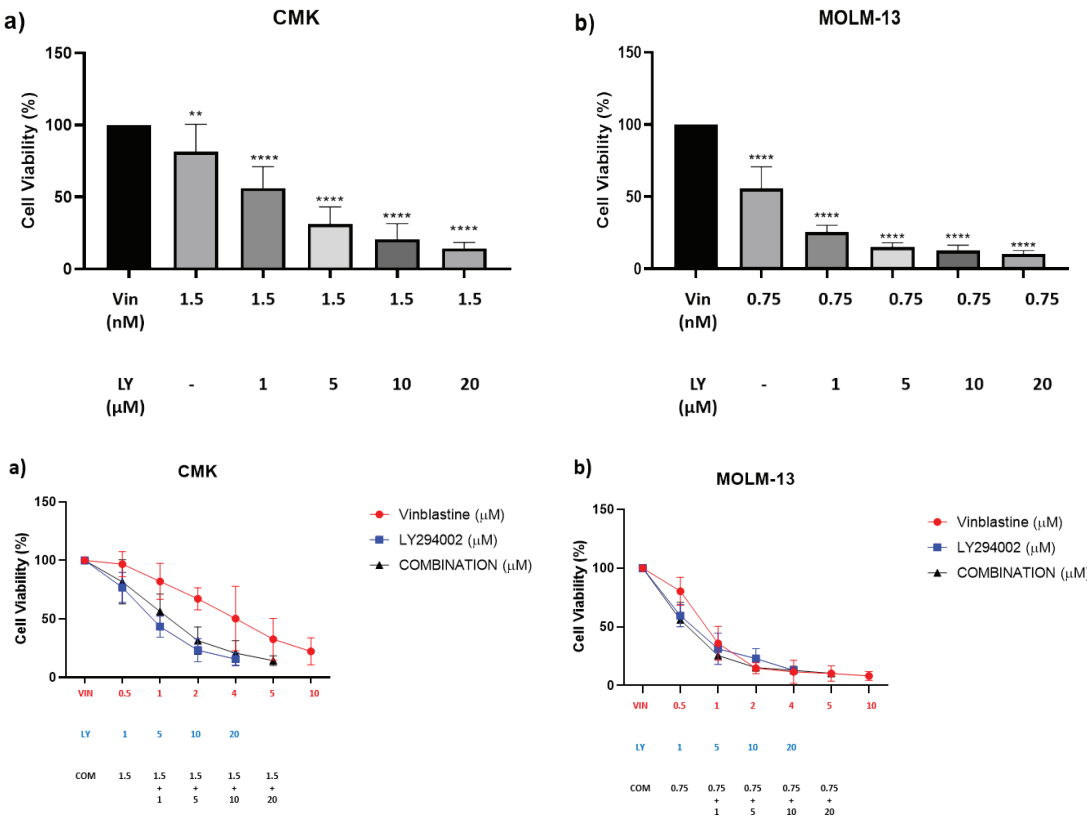


Figure 2. Cell viability effect of Vinblastine:LY294002 co-administration on CMK and MOLM-13 cells. The combinatorial cell viability assessments of Vinblastine at 1.5nM (CMK) and 0.75nM (MOLM-13) with ascending concentrations of LY294002 (calculated and applied in μ M, as shown in Figure 2). Vin represents Vinblastine, and LY represents LY294002. Figure 2a depicts the combinatorial effect of 1.5nM Vinblastine and increasing doses of LY294002 on CMK cells. Similarly, Figure 2b shows the inhibitory effect of 0.75nM Vinblastine and escalating concentrations of LY294002 on MOLM-13 cells. Experiments are shown as means $\pm$ SD from three independent experimental setups, each performed in triplicate (n=3) (*P<0.0001)

Following the combinatorial treatment results, the obtained data were analysed to determine whether the Vinblastine:LY294002 combination exhibited a synergistic effect (Table 1.). All Combination Index (CI) values were below 1 (the threshold value; please refer to Figure S1 and Table 1, Vin:LY Combination

Column), indicating that all applied concentrations were synergistic. Collectively, these results revealed that the Vinblastine:LY294002 combination can effectively reduce AML cell viability at lower concentrations than either compound alone.

Table 1. Combination Index (CI) values for CMK and MOLM-13 cells treated with constant Vinblastine doses (1.5nM for CMK and 0.75nM for MOLM-13) and increasing concentrations of LY294002 (CI < 1: synergistic; CI = 1.0–1.1: additive; CI > 1.1: antagonistic)

	Vinblastine alone	LY294002 Alone	Vin:LY Combination
	Dose	Dose	CI value
MOLM-13	0.75 nM	1 µM	0.26228
	0.75 nM	5 µM	0.38422
	0.75 nM	10 µM	0.56014
	0.75 nM	20 µM	0.78823
CMK	1.5 nM	1 µM	0.25116
	1.5 nM	5 µM	0.35102
	1.5 nM	10 µM	0.51240
	1.5 nM	20 µM	0.74961

Effects of LY294002 and vinblastine, alone and in combination, on apoptosis in AML cell lines

To assess the effects of the compounds on cell death mechanisms in AML cells, apoptosis was evaluated by Annexin V/FITC double-staining followed by flow cytometric analysis. The 10 µM LY294002-alone treatment induced apoptosis in 37.9% of CMK and 7.8% of MOLM-13 cells. Concurrently, 20 µM LY294002 alone induced apoptosis in CMK cells by approximately 45.6% (Figure 3a – please check the bar graph). The apoptosis induction at the same concentration was 36.7% in MOLM-13 cells (Figure 3b – please check the bar graph). These results highlight the apoptotic impact of single LY294002 administration on AML cells. Additionally, data demonstrate differences in cellular sensitivity to the compound. MOLM-13’s apoptotic pattern did not resemble that of CMK after LY294002 treatment at 10 µM.

Unlike the major induction of apoptosis by LY294002 treatment, Vinblastine alone at 1.5nM (CMK) and 0.75nM (MOLM-13) did not show a notable apoptotic effect in the cell lines. The detected apoptotic bodies were 2.93% for CMK and 2.75% for MOLM-13 (Figure 3 – please consider the bar graphs). Although single Vinblastine at nanomolar concentrations induced limited apoptosis in both cell lines, combining it with LY294002 exerted measurable activity, particularly at 10 µM. Co-administration of 1.5nM Vinblastine with 10 µM LY294002 resulted in an almost 15.3-fold increase in the apoptotic cell population compared to single Vinblastine administration in CMK. The apoptotic cell ratio of CMK cells when combining 1.5nM Vinblastine with 20 µM LY294002 increased by 1.2-fold (Figure 3a – please check the bar graph).

These fold changes at the same LY294002 concentrations with 0.75nM Vinblastine in MOLM-13 were 3.6-fold and 1.3-fold increases in the number of apoptotic cells compared to single-drug treatment (Figure 3b – please check the bar graph).

The apoptotic effect of the drug combination can be clearly observed at 10 μ M LY294002, with constant Vinblastine concentrations, in both cell lines. However, at an elevated LY294002 concentration, the apoptotic profile of both CMK and MOLM-13 cells was overshadowed by 20 μ M LY294002 in drug combination trials. In CMK cells, the increase in the percentage of apoptotic cells after a constant Vinblastine concentration with the 20 μ M LY294002

combination was only 1.1% compared to the effect of 20 μ M LY294002 alone (Figure 3a.). Similarly, a single 20 μ M LY294002 treatment resulted in a 36.65% increase in the population of apoptotic cells in MOLM-13 cells. When Vinblastine was combined with 20 μ M LY294002, the proportion of apoptotic cells was 38.05% (Figure 3b.).

Overall, CMK and MOLM-13 cells were treated with selected dose pairs of Vinblastine and LY294002 to evaluate the cellular apoptotic landscape after the treatment. Given the dominant effect of 20 μ M LY294002, 10 μ M LY294002 was selected as the representative LY294002 dose for subsequent assays.

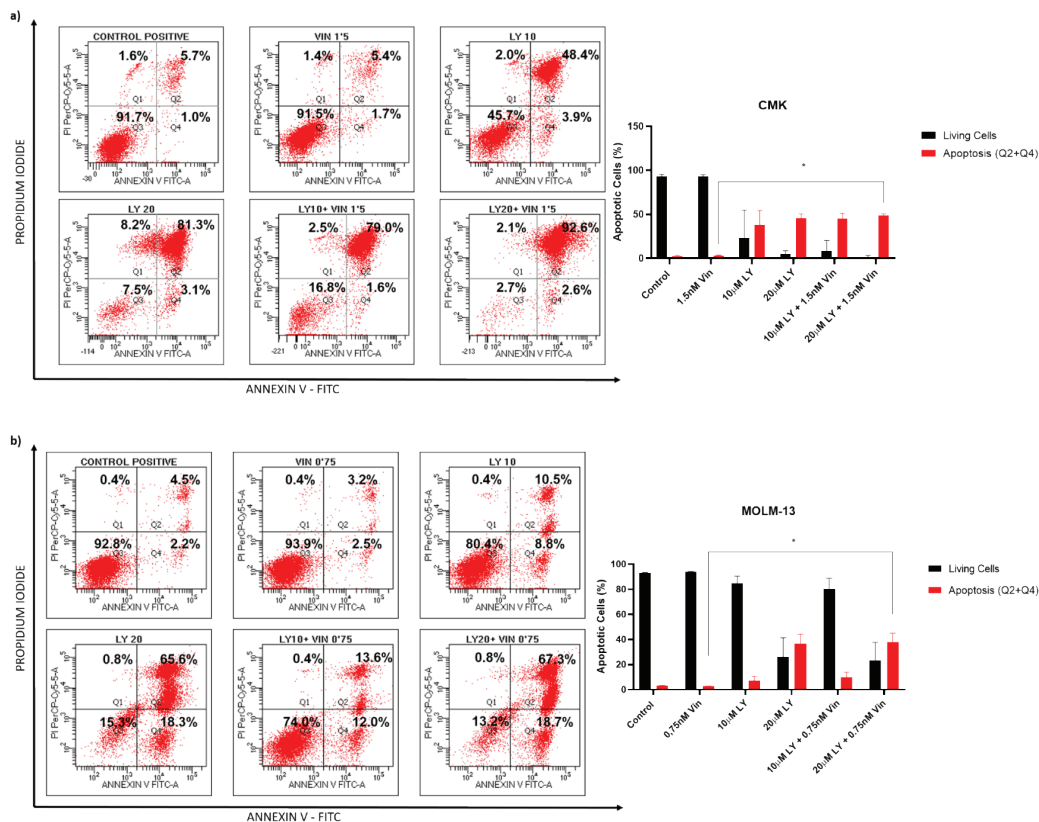


Figure 3. Apoptosis induction in response to single and combined LY294002 and Vinblastine treatments on CMK (a) and MOLM-13 (b) cell lines for 48h. The left panels of Figure 3 depict the flow cytometry histograms for each cell line, with percentages for each biological replicate. The Q1: necrotic cells (Annexin V-FITC-/PI+); the Q2: late apoptosis (Annexin V-FITC+/PI+), the Q3: living cells (Annexin V FITC-/PI-); the Q4: early apoptosis (Annexin V-FITC+/PI-). The sum of the percentages in the Q2 and Q4 quadrants represents the total apoptosis. Graphical results were interpreted according to the average of two biological replicates, and bar chart results were used for analysis (n=2)

Cell-cycle distribution in CMK and MOLM-13 cells following LY294002 and Vinblastine treatment

After observing apoptotic patterns in cells, we aimed to dissect the effects of the designed treatment strategy on cell lines and to elucidate whether the cell death mechanism is regulated by the cell cycle. For CMK, both single- and combinatorial-compound treatments exhibited consistent cell-cycle distribution patterns. Compared with the untreated control group distributions, the LY294002-alone treatment arrested the majority of the CMK cell population in S-phase; flow cytometry results showed 33.05% and 66.7% accumulation after 10 μ M and 20 μ M LY294002 treatments, respectively. Single 1.5nM Vinblastine treatment increased G2/M phase arrest by 6.85%. Although drug-alone treatment resulted in different cell cycle checkpoint arrest, the combination distribution pattern (compared to the control group), 1.5nM Vinblastine + 10 μ M LY294002, showed an increase in cell accumulation at S-phase of 21.25%. Therefore, combining LY294002 with 1.5nM Vinblastine impaired the S-phase of the cell cycle. The reduction in cell percentage at G0/G1 and G2/M phases was noticeable at this stage. A similar cell cycle progression pattern was observed in the 20 μ M LY294002 + 1.5nM Vinblastine-treated group. The increase in the accumulated cell population at the S-phase was 37.3%, which was only 1.7-fold higher than that observed with the 1.5nM Vinblastine combination and 10 μ M LY294002. Regrettably, the 1.5nM Vinblastine + 20 μ M LY294002 did not show any overall improvement beyond the 20 μ M LY294002-alone treatment, suggesting that cell cycle arrest might not reflect the Vinblastine + 20 μ M

LY294002 combination (Figure 4a.).

The phase distribution of MOLM-13 was mainly detected in the G0/G1 phase. When the majority of the 0.75nM Vinblastine-treated cells were arrested in the G0/G1 phase (approximately 80%), this revealed almost no change compared to the control. The single 10 μ M and 20 μ M LY294002-treated cells showed a slight increase in the accumulated cell population in S- and G2/M-phases. However, the majority of the arrested cell populations were monitored at the G0/G1 phase (Figure 4b.). The LY294002 administration was dominant in combination, albeit less pronounced than in CMK. The combination of Vinblastine 0.75nM with 10 μ M or 20 μ M LY294002 increased the number of accumulated cells in S-phase by 7.2% and 6.1%, respectively (compared to the control group). Regardless of LY294002 concentration, the 0.75nM Vinblastine combination increased the proportion of cells in the G2/M phase. The increase was fairly visible in Figure 4b.

Overall, fewer apoptotic bodies in the Q2-Q4 quadrants and no significant changes in cell cycle phase distribution might result from another cell death mechanism, independent of cell cycle regulation, in MOLM-13. However, blocking the cell cycle mainly at the S phase and triggering apoptosis upon combining 10 and 20 μ M LY294002 with 1.5nM Vinblastine prompted us to interpret the results as a cell cycle-dependent cell death mechanism in CMK. Although the Vinblastine:LY294002 combination was synergistic, efficiently reduced the viability of AML cells, and successfully induced apoptosis in both CMK and MOLM-13 cells, the combination showed a cell line-specific cell cycle distribution pattern in CMK only.

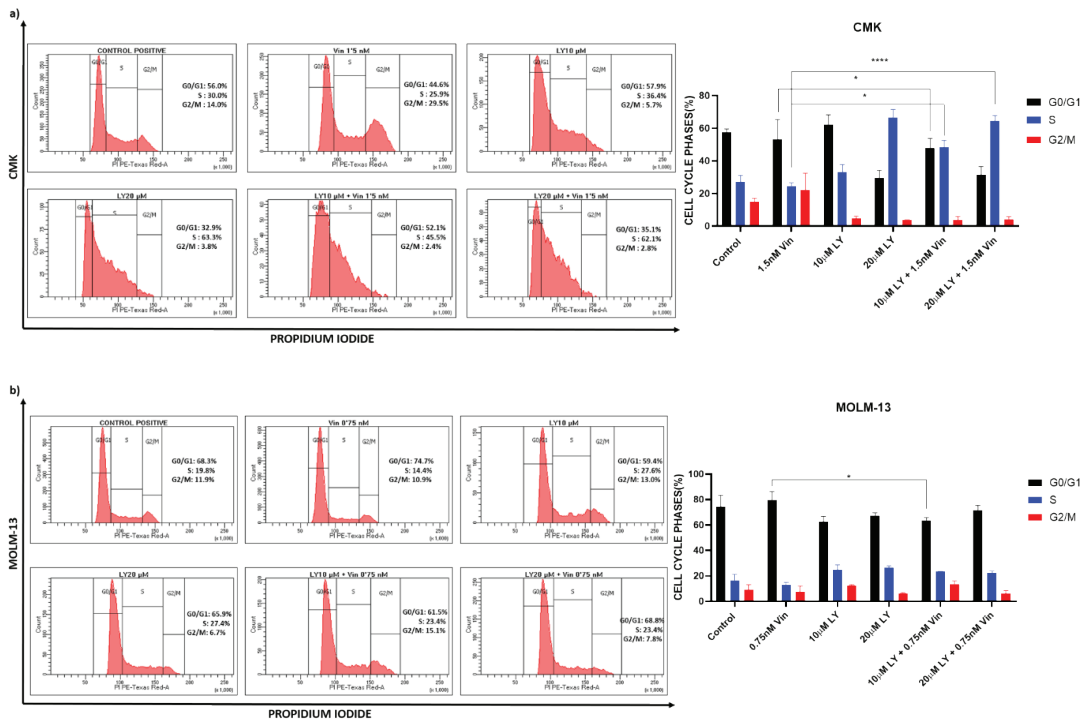


Figure 4. Cell cycle analysis of LY29002 alone or in combination with Vinblastine in AML cell lines: CMK (n=2) and MOLM-13 (n=2). The left panels of Figure 4 depict flow cytometry histograms for each cell line, with percentages relative to one of the two biological replicates (n=2)

Beclin-1 expression in CMK and MOLM-13 cells following LY294002 and Vinblastine treatment

Since Beclin-1 is the primary regulatory gene/protein of the autophagy process (Tran, Fairlie, & Lee, 2021), we examined its gene expression to evaluate and provide preliminary data on the cell death mechanisms in AML cells. Given the suppressive effect of 20 μ M LY294002 on apoptotic cell body detection (Figure 3.), only the co-administration of cell-specific Vinblastine concentrations with 10 μ M LY294002 was used to assess Beclin-1 gene expression.

Regarding CMK, single-agent administration of Vinblastine 1.5nM and LY294002 10 μ M slightly upregulated BECLIN-1 gene expression by 1.09- and 1.06-fold, respectively. However, co-administration of drugs resulted in a 22% downregulation of gene expression compared to control. This reduction in gene expression confirms the apoptosis findings presented in Figure 3 and further validates the

mechanism of cell death in CMK cells. Although the BECLIN-1 gene expression pattern in MOLM-13 cells was similar to that in CMK cells after treatment with a single dose of 0.75nM Vinblastine and 10 μ M LY294002 (which increased gene expression by 1.63 and 1.53, respectively), MOLM-13 cells showed an upregulation in BECLIN-1 gene expression after drug co-administration. Diverging from CMK, the drug combination resulted in a 1.18-fold increase, equivalent to 18% upregulation of BECLIN-1 expression in MOLM-13 (Figure 5.). Taken together, an increase in BECLIN-1 expression might suggest modulation of autophagy in MOLM-13 cells.

To conclude, the combinatorial treatment strategy of Vinblastine and LY294002 results in cell viability loss for both cell lines (please refer to Figure 2.); however, the activated cell death mechanisms varied, possibly due to the divergent backgrounds of CMK and MOLM-13 (please refer to cell cycle results and BECLIN-1 expression for CMK cells).

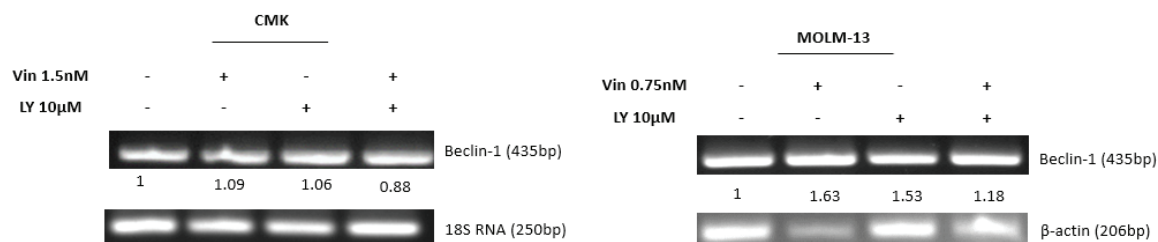


Figure 5. The altered gene expression of BECLIN-1 after 48 h of single and combinatorial treatments with Vinblastine and LY294002 in CMK1 ($n = 2$) and MOLM-13 ($n = 2$) cells, respectively. The 18S RNA gene for CMK and the β -actin gene for MOLM-13 were utilised as housekeeping genes. The changes in gene expression were measured by band intensities using ImageLab 6.1 software (Bio-Rad)

DISCUSSION

In cancer treatment, single-drug therapies often culminate in chemoresistance, leading to a drastic reduction in drug efficacy (Boshuizen & Peeper, 2020; Mason-Osann, Pomeroy, Palmer, & Mettetal, 2024; Pomeroy, Schmidt, Sorger, & Palmer, 2022). Major reasons for monotherapy-related drug resistance are tumour heterogeneity and plasticity. Since the 1950s, the drug combination strategy has revealed promising outcomes in various haematological cancers, including AML (Boshuizen & Peeper, 2020; Pomeroy et al., 2022). However, uncontrolled drug combinations may result in multidrug resistance, leading to unfavourable outcomes (Pomeroy et al., 2022). Therefore, chosen compounds must exhibit anticancer effects, have distinct drug activity, and be used at lower concentrations with the same/similar efficacy as monotherapy (Jin, Wang, & Bernards, 2022; Pomeroy et al., 2022).

Genetic abnormalities, heterogeneity, and regular treatment strategies, including the “7+3 regimen”, cause relapse after complete remission, making AML a therapeutically challenging disorder (Desai, Zandvakili, & Bohlander, 2022; Lin et al., 2024; Tang, Schuh, & Yee, 2021). Understanding AML pathophysiology, common drug resistance, and disease relapse has led researchers to focus on more targeted and combination therapies (Desai et al., 2022; Mason-Osann et al., 2024). Therefore, we sought to investigate the combinatorial effect of the anti-mitotic agent Vinblastine

with the well-known PI3K inhibitor LY294002 on FLT3 wild-type (CMK) and FLT3-ITD+ (MOLM-13) mutated AML cells by targeting autophagy and re-inducing apoptosis.

We first assessed Vinblastine and LY294002 alone, where Vinblastine was highly cytotoxic at nanomolar (nM) concentrations. Vinblastine’s mechanism of action involves targeting the mitotic spindle and preventing cell cycle progression at the M phase, thereby inhibiting aberrant cell proliferation and leading to cell death (Dhyani et al., 2022; Leslie, Koil, & Rayburn, 2005). In our study, we detected a 25.9% cell accumulation in S phase and 29.5% in G2/M phase after treating CMK cells with 1.5nM Vinblastine. As stated in the study by Madoc-Jones & Mauro (1968), if Vinblastine is applied at lower concentrations (e.g., $\mu\text{g/mL}$) within a narrow time window, cells may exhibit sensitivity to the compound and accumulate in the S phase of the cell cycle. So, 1.5nM Vinblastine might have sensitised CMK cells and caused accumulation in S phase. Still, further studies are required for confirmation. Multiple published studies have examined Vinblastine’s anticancer effect on solid and haematological cancers (Leslie et al., 2005; Zhang, Kong, Wang, & Du, 2018). A study conducted in 1984 evaluated the pharmacological activity of Vinblastine across different organisms and cancer types. In the case of AML, 10nM Vinblastine was applied to HL-60 cells, and cell population loss exceeded 95% after 4 hours of incubation with the drug (Ferguson, Phillips,

Selner, & Cass, 1984). Our study also confirmed the anticancer effect of Vinblastine, as we observed a reduction in cell viability of almost 90% in the cell population after 10nM Vinblastine treatment. Another study conducted by Shen et al. (2017) demonstrated that Vinblastine at 100nM was toxic to NCI-H1299 Non-Small Cell Lung Cancer (NSCLC) cells, resulting in more than 30% loss of cell viability compared to the results for Taxol at the same concentration, which showed only approximately 15% loss of cell viability (Shen et al., 2017).

The toxicity of Vinblastine has severe consequences. It can disrupt the normal function of motor neurons and negatively affect the rapid proliferative activity of intestinal epithelial cells and bone marrow cells (Zhang et al., 2018). A recent *in vivo* study found that excessive Vinblastine cytotoxicity disrupts the germ-cell genotype of sperm cells, damages the genetic structure (in addition to disrupting the somatic cell genomic structure), and causes sperm defects (Fahmy, Hassan, Farghaly, & Hassan, 2022). The concept of combining Vinblastine with other anticancer agents, such as Docetaxel or Rapamycin, has shown promising results. Combining Vinblastine with Docetaxel in the treatment of NSCL resulted in a 6-fold reduction in the concentration of Vinblastine IC50 value (Mohseni et al., 2016). In neuroblastoma treatment, replacing a single 250 pM Vinblastine treatment with 50 pM Vinblastine in combination with Rapamycin increases Vinblastine's activity at lower concentrations (Marimpietri et al., 2005). By considering Vinblastine susceptibility of AML cells, setting Vinblastine concentration to a constant value (1.5nM for CMK and 0.75nM for MOLM-13), and combining with LY294002, a noteworthy result was obtained for future studies.

To investigate possible autophagic activity in AML, as proposed in this article's aim, we measured BECLIN-1 gene expression levels. We focused on BECLIN-1 gene expression because it serves as an autophagy marker and can regulate both autophagy

and the PI3K signalling pathway (Kang, Zeh, Lotze, & Tang, 2011). BECLIN-1 gene expression levels after the drug combination differed between the two cell lines. Observing a marked reduction in cell viability, apoptotic patterns, and cell cycle distributions revealed differences between CMK and MOLM-13 cells, which motivated us to explore the mechanisms of activated cell death and their relationship with BECLIN-1.

Autophagy is a bidirectional cell-control mechanism that, depending on the context, can trigger cell death or promote aberrant cell proliferation (Aynur, 2016; Levy & Thorburn, 2019). In our study, reduced BECLIN-1 expression was observed after treating CMK cells with the combination of LY294002 and Vinblastine. However, a rise in BECLIN-1 gene expression in MOLM-13 cells was observed after combinatorial treatment with LY294002 and Vinblastine. So, these outcomes provided us with a new perspective. The initial interpretation we derived from BECLIN-1 gene expression was that the activation of distinct cell death mechanisms in two cell lines might differ due to their distinct FLT3 genetic backgrounds. CMK harbours the wild-type FLT3, whereas MOLM-13 harbours the FLT3-ITD+ mutation (Wang et al., 2020). According to the kinase activity assay results of Yu et al. (2015), only the active kinase BCR-ABL can phosphorylate Beclin-1, and FLT3-ITD+ cells lack active kinases that can phosphorylate Beclin-1 (Yu et al., 2015). Nevertheless, AML with FLT3-ITD+ mutation results in active basal autophagy, which is controlled by ATF4 (Xu et al., 2022; Zalpoor et al., 2022). In addition to ATF4, Beclin-1 is a core target of the PI3K/Akt/mTOR signalling pathway and regulates autophagy. In AML, the PI3K/Akt/mTOR signalling pathway is pathologically upregulated, resulting in abnormal cell proliferation and is associated with adverse prognosis outcomes (Darici et al., 2020; Nepstad et al., 2020). Specifically, disruption of the normal activity of the PI3K/Akt/mTOR signalling pathway directly correlates with FLT3 receptor mutations (Darici et

al., 2020). Of particular interest, when MOG-G-CCM and T98G glioma cell lines were treated with the PI3K inhibitor LY294002 at 10 μ M, MOG-G-CCM cells exhibited autophagic activation, whereas T98G cells were more prone to activating apoptosis than to autophagy (Zajac et al., 2023). Another study performed on breast cancer revealed mechanistically similar results. Single 50 μ M LY294002-treated MCF-7 cells facilitated Beclin-1 expression (Akkoç et al., 2015). In our study, we observed a significant increase in Beclin-1 gene expression in LY294002-treated MOLM-13 cells, consistent with published reports. A possible reason for the slight increase in Beclin-1 gene expression after 0.75nM Vinblastine alone could be disruption of microtubule formation, induction of apoptosis, and indirect activation of autophagy (Köchl et al., 2005; Punnonen & Reunanen, 1990).

Since the BECLIN-1 gene expression level is detected as decreased in untreated FLT3-ITD+ mutated cells (Zare-Abdollahi et al., 2016) and BECLIN-1 might act as a tumour suppressor gene haploinsufficiently in the cells (Wechman et al., 2017), the designed combinatorial treatment of Vinblastine:LY294002 might have led to the activation of autophagy via BECLIN-1 in FLT3-ITD+ mutated cells. This could be one possible explanation for the increase in Beclin-1 levels we observed in MOLM-13.

CONCLUSION

Collectively, the combinatorial treatment of Vinblastine and LY294002 successfully inhibited aberrant cell proliferation in two genetically distinct AML cell lines. Our study also provides general insight into the mechanisms of activated cell death in FLT3 wild-type and FLT3-ITD+ cells. Although the study's experimental design provides limited insight into subcellular mechanisms and is preliminary, the increase in Beclin-1 gene expression may be interpreted as evidence that Beclin-1 is a tumour suppressor gene that promotes autophagy in MOLM-13 cells. In CMK cells, possibly due to the cell's wild-type FLT3 genet-

ic background, a decrease in Beclin-1 expression was observed after drug combination, accompanied by apoptosis as the cell death mechanism.

To address the limitations of this study, one suggestion is to increase the sample size. Additionally, testing the selected dose pairs on non-malignant or normal haematopoietic cells would be advantageous for emphasising the drug's efficacy in non-AML cells and clarifying the importance of dose minimisation. Since the study has examined only the expression level of BECLIN-1 for autophagy activation, further verification is warranted to confirm the findings. For further validation, other core autophagy proteins (e.g., LC3 or Bcl-2) might be analysed. To enhance our understanding of this study, conducting a comprehensive proteomics analysis and examining the relationship among other autophagy- and apoptosis-related proteins may be valuable for future research.

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AUTHOR CONTRIBUTION STATEMENT

Conceptualization & Methodology – E.B.G.A., H.B.A.; Formal analysis & investigation – H.B.A.; Writing & original draft preparation – H.B.A.; Writing, review & editing – H.B.A., E.B.G.A.; Supervision – E.B.G.A.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

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