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ORIGINAL ARTICLE

Rapamycin and Niacin combination induces apoptosis and cell cycle arrest through autophagy activation on acute myeloid leukemia cells

Lale Beril Subay[1] · Emel Başak Gencer Akçok[2] · İsmail Akçok[1]

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Abstract

Background Acute myeloid leukemia (AML) is a heterogeneous hematological malignancy caused by disorders in stem cell differentiation and excessive proliferation resulting in clonal expansion of dysfunctional cells called myeloid blasts. The combination of chemotherapeutic agents with natural product-based molecules is promising in the treatment of AML. In this study, we aim to investigate the anti-cancer effect of Rapamycin and Niacin combination on THP-1 and NB4 AML cell lines.

Methods and Results The anti-proliferative effects of Rapamycin and Niacin were determined by MTT cell viability assay in a dose- and time-dependent manner. The combination indexes were calculated by isobologram analysis. Furthermore, apoptosis was investigated by Annexin-V/Propidium Iodide(PI) double staining and cell cycle distribution was measured by PI staining. The expression levels of autophagy-related proteins were detected by western blotting. The combination of Rapamycin and Niacin synergistically decreased cell viability of AML cell lines. The combination treatment induced the apoptotic cell population of THP-1 and NB4 by 4.9-fold and 7.3-fold, respectively. In THP-1 cells, the cell cycle was arrested at the G2/M phase by 10% whereas the NB4 cells were accumulated at the G0/G1 phase. The combination treatment decreased Akt and p-Akt expression. Besides, the ATG7 expression was reduced by combination treatment on THP-1 cells. Similarly, the ATG5 level was downregulated in NB4 cells. The level of LC3B-II/LC3B-I, which is an indicator of autophagy flux, was upregulated in THP-1 and NB4 cells.

Conclusion Although further studies are required, the combination of Rapamycin and Niacin combats cell proliferation by inducing cellular apoptosis, cell cycle arrest and autophagy activation.

Keywords Cancer · Rapamycin · Niacin · Combination therapy · Acute myeloid leukemia · Autophagy

Introduction

Acute myeloid leukemia (AML) is a life-threatening malignant clonal disease that prevents the expansion and differentiation of cells due to genetic changes occurring in hematopoietic progenitor cells, and thus interfering with normal hematopoiesis [1–3]. AML is one of the most common types of leukemia in adults. The American Cancer Society estimates nearly 20,800 new cases of AML and about 11,220 deaths from AML in the United States in 2024 [4]. The deaths caused by AML are over 80,000 worldwide, and this number is expected to double in the next decades [5]. Due to the challenging process of AML treatment and highly increased incidences and deaths, new treatment strategies are required. Chemotherapy is one of the most effective treatments for AML. However, the adverse effects and drug resistance are associated with reducing the effectiveness of chemotherapeutic drugs. Combination therapy shows promise in increasing the effectiveness of chemotherapy drugs in comparison to single therapy. It also has the potential to minimize side effects by lowering the dosages of individual drugs.

Autophagy is a catabolic process at the basal level stimulated by stress, where intracellular substances such as damaged organelles and proteins are transported to lysosomes for breakdown, and therefore maintain the cellular homeostasis

✉ İsmail Akçok
ismail.akcok@agu.edu.tr

[1] Faculty of Life and Natural Sciences, Department of Bioengineering, Abdullah Gül University, Sumer Campus, Kayseri 38080, Turkey

[2] Faculty of Life and Natural Sciences, Department of Molecular Biology and Genetics, Abdullah Gül University, Kayseri 38080, Turkey

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[6, 7]. Targeting AML treatment via autophagy activation may be a promising avenue. The link between autophagy and recurrent genetic mutations in AML has revealed the potential to use autophagy modulation as a therapeutic intervention for various AML subtypes. This approach is promising in increasing the effectiveness of treatment and overcoming drug resistance [8]. AML treatment can be achieved by activating the autophagy process. Induction of autophagy may occur through mTOR inhibition [9]. mTOR regulates the fundamental cellular activities and mTOR dysfunction promotes cellular growth, which is associated with cancer [10]. In this study, Rapamycin (RAPA), an immunosuppressant and anti-proliferative agent, which potently inhibits mTOR activity by binding fk506-binding protein 12 kDa (FKBP12) to form a molecular complex, was used to target AML cells.

Natural products are important resources used in the treatment of diseases and also play an important role in drug development. Although chemotherapy is one of the most effective cancer treatments, its serious side effects and possible drug resistance make treatment difficult. Natural products with many pharmacological activities are promising in terms of creating synergy, reducing drug resistance, and reducing side effects when combined with chemotherapy drugs [11]. Vitamin B3, also known as nicotinic acid or niacin, is a water-soluble vitamin with a carboxyl group (-COOH) at the 3-position of pyridine found mostly in black beans and rice. Niacin has been reported to regulate NRF-2/HO-1 signaling through activation of GPR109A receptor and promote autophagy through NRF-2 signaling through activation of GPR109A [12]. Considering the cellular effects of Rapamycin and Niacin, their combination may be effective on AML cells.

The single treatment of Rapamycin and Niacin significantly reduced the cell proliferation of THP-1 and NB4 cells in a dose- and time-dependent manner, most importantly, the combination of Rapamycin and Niacin synergistically decreased the cell viability of both THP-1 and NB4 cells which was assessed by isobologram analysis. The co-administration of Rapamycin and Niacin induced apoptotic cell death and cell cycle arrest on for both cell lines used in this study. Following these, the autophagy-related protein expressions showed that the combination therapy induced autophagy activation. In conclusion, our study reveals for the first time that combination of Rapamycin and Niacin exhibits anti-proliferative and apoptotic effects on cells although further analysis is required to unveil the molecular mechanisms behind it.

Materials and methods

Chemicals

RPMI-1640, Fetal Bovine Serum (FBS), penicillin/streptomycin, and phosphate-buffered saline (PBS) were purchased from Serox. Rapamycin and Niacin were purchased from Sigma-Aldrich. The inhibitors were dissolved in DMSO (dimethyl sulfoxide) and ddH_2O, respectively. The main stocks were prepared according to the descriptions of the manufacturers and stored at -20 °C.

Cell maintenance

THP-1 and NB4, two AML cell lines utilized in this investigation, cell lines were obtained from the German National Biological Materials Resource Center (DSMZ). The cells were grown in RPMI 1640 medium that contained 100 U/mL of penicillin and streptomycin and 10% FBS as supplements. The cells were maintained in a 5% CO_2 environment at 37 °C.

Cell proliferation assay

MTT cell viability assay was performed to determine the anti-proliferative activity of Rapamycin and Niacin on THP-1 and NB4 cell lines as described previously [13]. Briefly, THP-1 and NB4 cell lines were seeded into 96-well plates as 10,000 cells/100µL per well in triplicates. Then, the cells were applied varying concentrations of Rapamycin and Niacin both alone and in combination for a duration of 24 h, 48 h and 72 h at 37 °C in a 5% CO_2 incubator. Following the incubation process, 10 µL of MTT solution was added to each well and the cells were incubated between two to four hours at 37 °C in a 5% CO_2 incubator. The 96-well plate was centrifuged at 1800 rpm for 10 min. Next, 100 µl of DMSO solution was added to each well to dissolve the formed formazan crystals. The plates were placed onto a waving rotator for 15 min. The absorbance was measured with a Varioskan LUX multimode microplate reader (Thermo Scientific) at 570 nm. Cell viability graphs were plotted and IC_{50} values were calculated for both Rapamycin and Niacin.

Combination index (CI) calculation by isobologram analysis

Isobologram analysis was performed using the Calcusyn 2.0 program (CompuSyn software, Biosoft, Cambridge, United Kingdom). The effects of the used drug combination were evaluated by using CI based on Chou-Talalay's multidrug

effect equation. CI < 1, CI = 1 or CI > 1 is indicative of synergistic, additive, or antagonistic effects, respectively.

Annexin-V FITC/propidium iodide dual staining

The apoptotic cell death was assessed for THP-1 and NB4 cells treated with Rapamycin alone, Niacin alone and combination of both. The amounts and location of phosphatidyl serine were determined by flow cytometry using the Annexin V/Propidium iodide double staining method. 1×10^6 cells were seeded in a 6-well plate and treated with single and combined doses for 48 h. After incubation at 37ºC and 5% CO_2, cells were collected and centrifuged at 1700 rpm for 5 min at 4ºC. The supernatant was discarded, and the cells were washed twice with 1X PBS. The cell pellet was resuspended with 200 µL of 1X Annexin binding solution. 2 µL Annexin V-FITC was added to Annexin positive tubes and incubated for 15 min at room temperature in the dark. Then, 5µL Propidium iodide was added to the PI-positive tubes. The negative control tube was not stained, but the positive control tube was stained with the same amounts of Annexin V-FITC and PI. Cells were then analyzed on a BD LSRFortessa (Becton Dickinson) flow cytometer [14].

Cell cycle analysis

The effect of single and combination doses of Rapamycin and Niacin on the cell cycle of THP-1 and NB4 cells was analyzed by flow cytometry. 1×10^6 cells were seeded in a 6-well plate and treated with Rapamycin and Niacin alone and in combination. Then the cells were incubated for 48 h. Following the incubation, the cells were collected and centrifuged at 260 G and 4ºC for 10 min. The cell pellet was washed twice with 1 mL of 1X cold PBS. Then, the cell pellet was dissolved with 1 mL of 1X PBS and 4 mL of ethanol was added. The mixture was incubated overnight at -20ºC to fix the cells. After incubation, cells were centrifuged. The cell pellet was washed once with 1 mL 1X cold PBS and then centrifuged. The cell pellet was homogenized with 1 mL of 0.1% Triton-X and 100 µL of RNAase. The mixture was incubated at 37ºC in the dark for 30 min. Cells were stained with 25 µL of PI and incubated for 15 min in the dark at room temperature [15]. Samples were analyzed with a BD Biosciences LSRFORTESS Cell Analyzer flow cytometer.

Western blot analysis

5×10^6 cells were treated with Rapamycin, Niacin and in combination for 48 h. After the incubation, the cells were lysed in RIPA buffer (50 mM Tris–HCl pH:8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS, protease, and phosphatase inhibitors). The protein concentration was measured using the RC DC protein assay kit (Bio-Rad, USA). 20 µg/well total protein was separated by 10% SDS-PAGE and transferred to PVDF membranes. Akt (1:1000, Cell Signaling, USA), p-Akt (1:1000, Cell Signaling, USA), ATG5 (1:500, Santa Cruz), ATG7 (1:250, Santa Cruz), LC3B (1:1000, Cell Signaling, USA) and GAPDH (1:2000, Proteintech) expression levels were checked. The membranes were blotted with primary antibodies overnight at 4ºC and then, incubated with appropriate secondary antibodies [peroxidase affiniPure goat anti-rabbit IgG (1:10,000) peroxidase affiniPure goat anti-mouse IgG (1:10,000)] at room temperature. The signals of membranes were detected using the Pierce ECL western blotting substrate kit (Thermo Scientific, USA).

Statistical analysis

GraphPad software (8.0.2, San Diego, CA) was used to analyze data. The results are presented as mean ± standard deviation (SD). The statistical significance was calculated using a one-way and two-way analysis of variance (ANOVA) for Dunnett's assay compared to the untreated controls. A value of $p < 0.05$ was statistically significant and a value of $p < 0.0001$ was highly statistically significant.

Results

Rapamycin treatment significantly inhibited the cell proliferation of THP-1 and NB4 cells

THP-1 and NB4 cells were treated with Rapamycin alone (0.1 µM- 25 µM) for 24, 48 and 72 h. Rapamycin treatment significantly reduced the cell growth in both cell lines in a dose- and time-dependent manner. The IC_{50} concentrations of Rapamycin for THP-1 cells are determined as 18.5, 4.9 and 5.5 µM for 24, 48 and 72 h, respectively (Fig. 1a). 10 µM Rapamycin treatment reduced the cell viability of THP-1 as 35%, 58% and 62% at 24, 48 and 72 h, respectively. The time-dependent Rapamycin treatment of THP-1 cells showed that the growth inhibitory effect of Rapamycin increases with increasing periods of time (Fig. 1c, left). For the NB4 cell line, the IC_{50} values were calculated as 16.7, 4 and 5.9 µM for 24, 48 and 72 h, respectively (Fig. 1b). In NB4 cells, the cell viability was reduced after 10 µM Rapamycin treatment as 22%, 74% and 67% at 24, 48 and 72 h, respectively (Fig. 1c, right). As a result of our findings, Rapamycin treatment significantly reduced cell proliferation on THP-1 and NB4 cells at low micromolar concentrations.

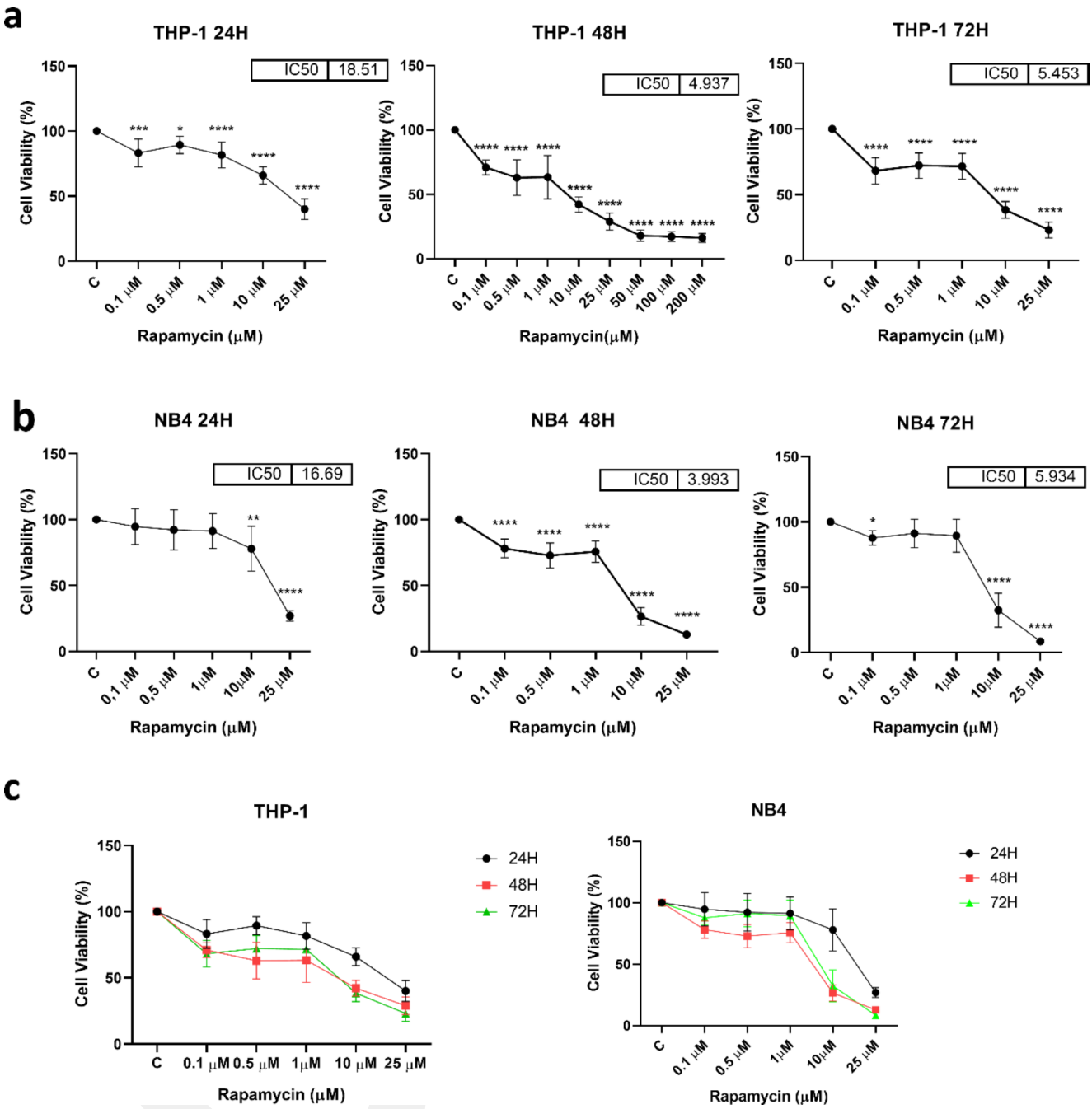

Fig. 1 The cell viability of Rapamycin treatment in time- and dose-dependent manner for THP-1 (**a**), NB4 (**b**), the comparison graphs for time-dependent treatment of Rapamycin in THP-1 and NB4 (**c**). The results were the average of experiments performed in triplicates with three independent experiments ($n=3$). All data are presented as mean ± S.D. (ns = $P>0.05$, * $P\leq0.05$, ** $P\leq0.01$, *** $P\leq0.001$, **** $P\leq0.0001$)

Niacin reduces the cell viability of THP-1 and NB4 cells

The anti-proliferative effect of Niacin was determined on THP-1 and NB4 cells in a time-dependent and dose-dependent manner. Firstly, low concentrations of Niacin (5-500 μM) were tested on the cells, however, there was no significant growth inhibition in lower concentrations of Niacin on both cell lines (data not shown). Then, millimolar range (0.1–40 mM) concentrations were tested for each cell. In THP-1 cells, the IC_{50} concentrations were determined as 28.9, 14.1, and 14.8 mM for 24, 48 and 72 h, respectively (Fig. 2a). 20 mM of Niacin treatment decreased the cell growth by 47%, 75% and 76% at 24, 48 and 72 h,

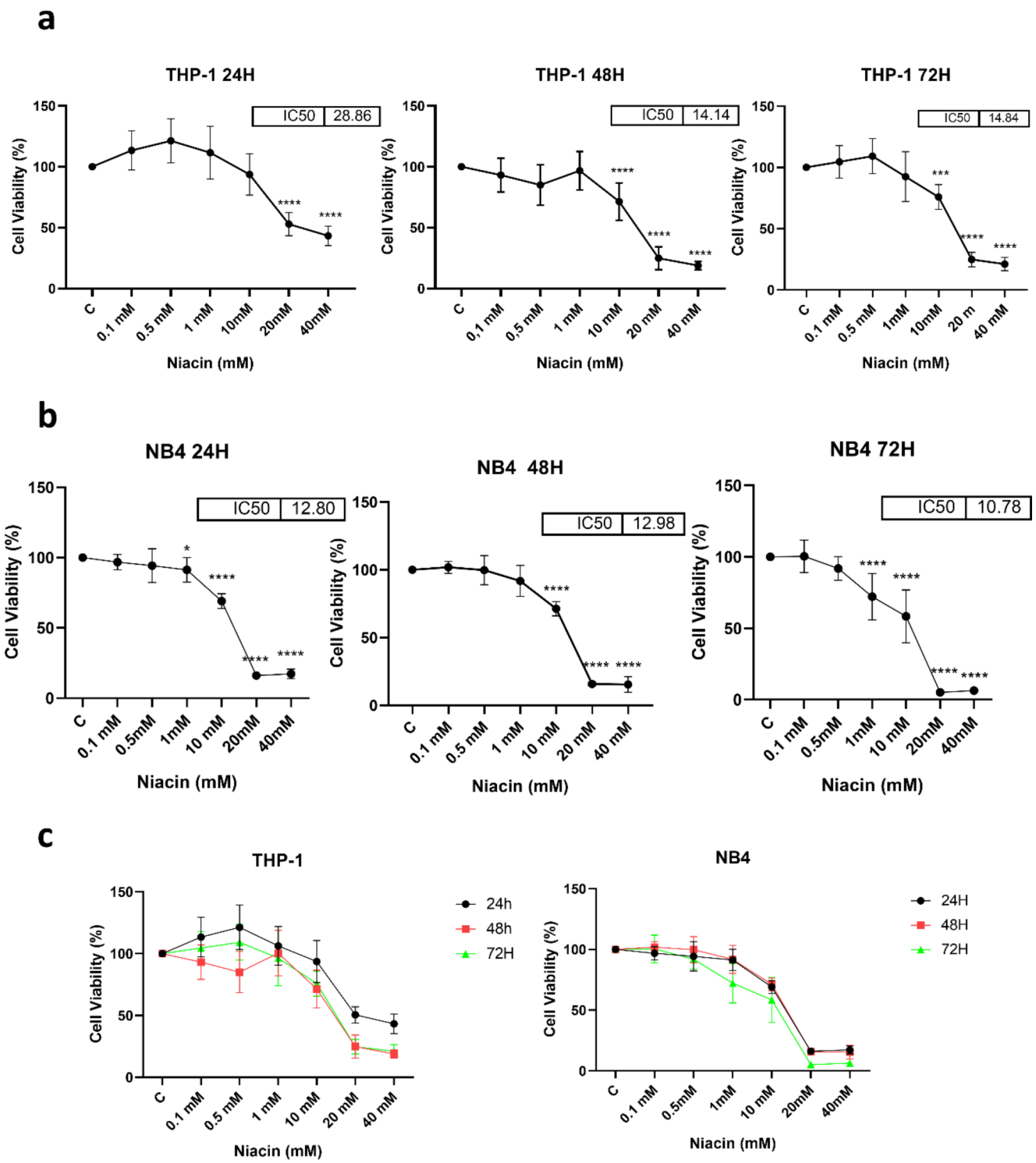

Fig. 2 The cell viability of Niacin treatment in time- and dose-dependent manner for THP-1 (**a**), NB4 (**b**), the comparison graphs for time-dependent treatment of Rapamycin in THP-1 and NB4 (**c**). The results were the average of experiments performed in triplicates with three independent experiments ($n = 3$). All data are presented as mean ± S.D. (ns = $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$)

respectively (Fig. 2c, left). 10 mM Niacin treatment reduced the cell growth on THP-1 as 7%, 29% and 24% at 24, 48 and 72 h, respectively (Fig. 2c, left). The IC_{50} values for NB4 were calculated as 12.8, 12.9 and 10.8 mM after 24,48 and 72 h, respectively (Fig. 2b). 10 mM Niacin treatment reduced the cell growth on NB4 as 28%, 31% and 42% at 24, 48 and 72 h, respectively (Fig. 2c, right). Our results demonstrated that the cell viability of NB4 cells was significantly reduced in high concentrations of Niacin.

The combination of Rapamycin and Niacin induced an anti-proliferative effect

To determine the anti-proliferative effect of drugs in combinational treatment, firstly, the IC_{50} concentration of Rapamycin and increasing concentrations of Niacin (1, 5, 15, 20 mM) were co-administered on THP-1 and NB4 cells. Rapamycin and Niacin combination showed that the cell population was significantly reduced relevant to untreated control and single administration of Rapamycin (Fig. 3). THP-1 cells were treated with low-dose Rapamycin (0.1 and 0.5 µM) and increasing concentrations of Niacin. The results revealed that the cell proliferation of THP-1 was decreased by 62% and 78%, when treated with 0.1 µM Rapa + 20 mM Niacin doses, relevant to 0.1 µM Rapamycin and untreated control, respectively (Fig. 3a). For THP-1 cells, high-dose Rapamycin (1, 5 and 10 µM), 5 µM Rapa + 20 mM Niacin concentration reduced the cell growth by 37% and 78% when compared to 5 µM Rapamycin and control cells, respectively (Fig. 3b). Whilst NB4 cells treated with 5 µM Rapamycin reduced the cell viability by 25% compared to control, 5 µM Rapa + 15 mM Niacin showed a better anticancer effect by inhibiting the cell growth by 88% and 48% compared to control, and 5 µM Rapamycin treatment, respectively (Fig. 3c). Overall, the results showed that the co-administration of Rapamycin and Niacin significantly reduced the THP-1 and NB4 cell populations when compared to single treatments of each combination set.

Rapamycin and Niacin combination exhibited synergistic anti-proliferative effects

Investigation of synergistic effects of Rapamycin and Niacin combination was displayed by isobologram analysis. CI values of Rapamycin and Niacin combinations are shown in Table 1. In THP-1 cells, 1 µM Rapa + 20 mM Niacin and 5 µM Rapa + 20 mM Niacin combinations showed a synergistic effect. However, 10 µM Rapa + 20 mM Niacin showed less synergistic effect than the low-dose Rapamycin combinations with a CI value of 0.94756. Similarly, 5 µM Rapa + 15 mM Niacin and 10 µM Rapa + 1 mM Niacin treatments synergistically inhibited the cell growth of NB4 cells. The synergistic effect was also determined for THP-1 treated with lower doses of Rapa (0.1 µM and 0.5 µM) and increasing doses of Niacin. The results revealed that 1 µM Rapa + 1 mM Niacin combination concentration showed a high synergistic effect on THP-1 cells. Therefore, these concentrations were used in further analysis; apoptosis and cell cycle assays.

Rapamycin and Niacin combination induced apoptotic cell death of THP-1 and NB4 cells

To investigate the mechanisms underlying the anti-proliferative effect of the combination treatments, apoptotic cell death was examined. The findings of the experiment indicated that apoptotic cell death was induced for THP-1 cells treated with the combination of Rapamycin and Niacin. In the single treatment of 5 µM Rapamycin and 20 mM Niacin, the apoptotic cell death was increased by 1.2 and 2.4-fold, respectively, for THP-1 cells. Moreover, when 5 µM Rapa + 20 mM Niacin combination was applied, the apoptotic cell population of THP-1 was increased significantly by 4.9-fold (Fig. 4a). The majority of the apoptotic cell population was late apoptotic cells as such the late apoptotic cell population of THP-1 was increased after combination treatment by 4.5-fold compared to control. Regarding the low-dose Rapamycin combination, the apoptotic cell death of THP-1 treated with 0.1 µM Rapa + 1 mM Niacin combination was also investigated; however, no significant apoptotic cell death was observed (data not shown). The apoptotic cell population of NB4 was increased by 1.8- and 1.7-fold after 5 µM Rapamycin and 15 mM Niacin treatment alone. However, when 5 µM Rapa + 15 mM Niacin combination was applied, the apoptotic cell population was increased significantly by 6-fold, when compared to untreated cells (Fig. 4b). Apoptosis was induced by 6.5-fold in response to 10 µM Rapamycin alone and when the 5 µM Rapamycin was combined with 15 mM Niacin, the late apoptotic cell death was increased by 7.3-fold. To sum up, the results of the apoptosis assay showed that the co-administration of Rapa and Niacin induced the apoptotic cell death of both cell lines but to a better extent for THP-1 cells.

The effect of Rapamycin and Niacin combination on cell cycle distribution of THP-1 and NB4 cells

To further investigate the molecular mechanisms behind the decreased cell viability in response to the combination treatment, the cell cycle distribution was investigated. The results demonstrated that when THP-1 cells were treated with single Niacin, the cells were accumulated at the S phase by 10% (Fig. 5a). The combination treatment of THP-1 cells induced the cell distribution towards the G2/M

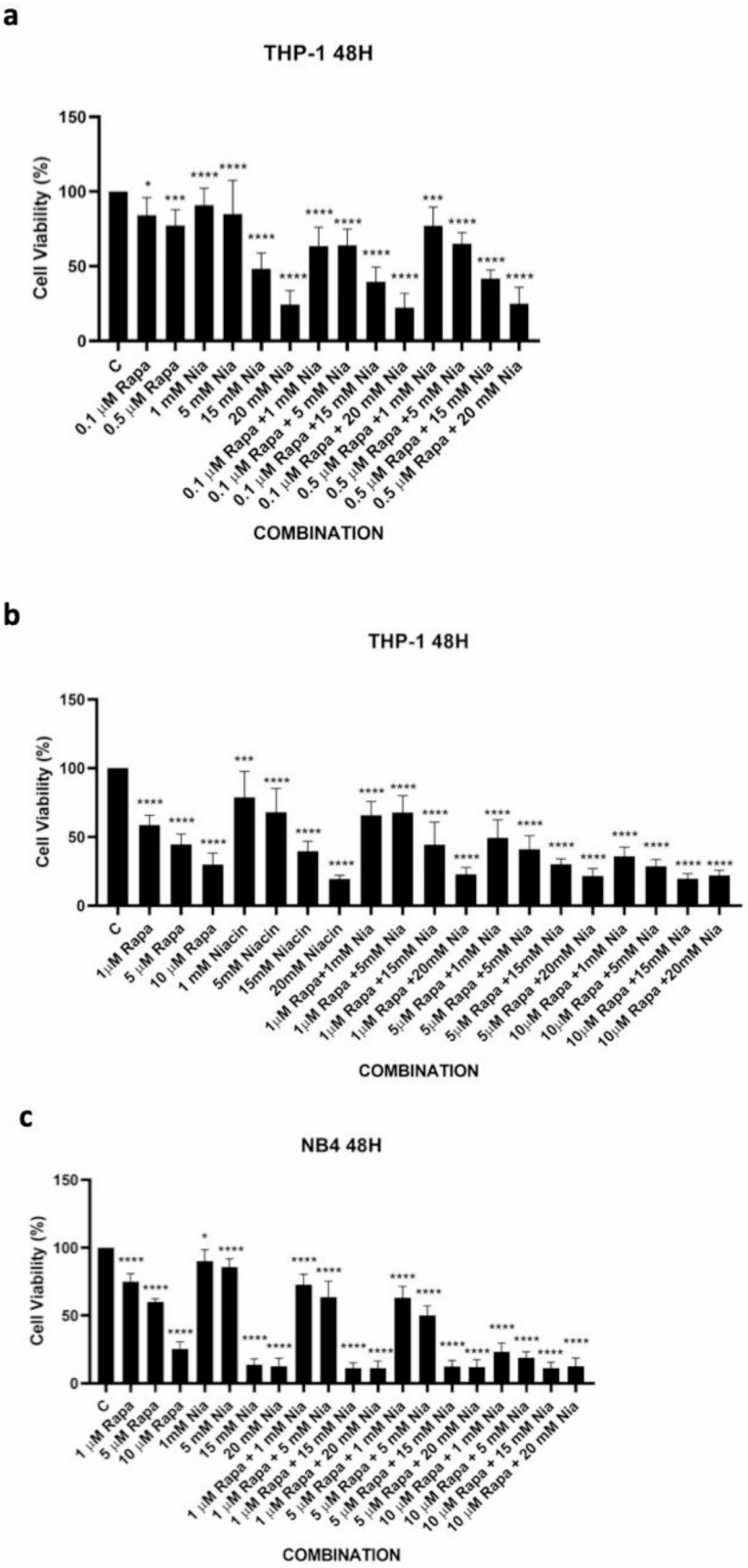

Fig. 3 The inhibitory effects of Rapamycin and Niacin combinations on THP-1 in lower concentrations of Rapamycin (0.1 and 0.5 μM) (**a**), at higher concentrations of Rapamycin (1, 5 and 10 μM) on THP-1 (**b**), and NB4 (**c**) for 48 h. The results were the average of experiments performed in triplicate with three independent experiments ($n = 3$). All data are presented as mean ± S.D. (ns = $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$)

Table 1 CI values of the Rapa and Niacin combination on THP-1 and NB4 cells

Cell lines	Rapamycin	Niacin	CI value
THP-1	1 µM	1 mM	2.04985
		5 mM	4.18145
		15 mM	2.07329
		20 mM	0.66971
	5 µM	1 mM	2.23262
		5 mM	1.57067
		15 mM	1.18902
		20 mM	0.74853
	10 µM	1 mM	1.42895
		5 mM	0.95244
		15 mM	0.61829
		20 mM	0.94756
NB4	1 µM	1 mM	0.99777
		5 mM	1.33672
		15 mM	0.67635
		20 mM	0.83266
	5 µM	1 mM	2.14287
		5 mM	1.83642
		15 mM	0.79085
		20 mM	0.94953
	10 µM	1 mM	0.55528
		5 mM	0.67286
		15 mM	0.85105
		20 mM	1.08210

phase by 10%. Similarly, NB4 cells treated with 5 mM and 15 mM Niacin were arrested at the S phase by 13% and 5.5%, respectively (Fig. 5b). However, the NB4 cells were arrested in the G0/G1 phase by 10% in response to the 10µM Rapa + 5 mM Niacin combination. The result now provides evidence that after combination treatment, both THP-1 and NB4 cells were accumulated at the G2/M phase and G0/G1 phase, respectively.

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

The expression levels of p-AKT, AKT, ATG5, ATG7 and LC3B were determined to examine the induction of autophagy. THP-1 cells were treated with 5µM Rapa + 20 mM Niacin and NB4 cells were treated with 5 µM Rapa + 15 mM Niacin in this experimental setup. In THP-1 cells, the expression of p-AKT and AKT were decreased by 0.3- and 0.7-fold compared to control (Fig. 6a). Similarly, the AKT level was decreased in NB4 cells, however, the p-AKT level was not changed. After combination treatment, the ATG7 expression was mildly reduced in THP-1 cells. Moreover, the expression level of ATG5 was decreased in NB4 cells by 0.4-fold (Fig. 6b). The LC3B-II/LC3B-I was significantly increased in both cells. Interestingly, Niacin treatment on THP-1 cells increased the LC3B-II/LC3B-I ratio by 22.6-fold, while the combination had a 16.3-fold impact on the LC3B ratio. According to our findings, the combination of Rapa and Niacin induced autophagy in AML cells.

Discussion

Acute myeloid leukemia (AML) is a fatal blood cancer characterized by excessive proliferation of malignant stem cells [16]. Combination therapy is a more effective treatment method in some cases due to the side effects caused by using high drug concentrations in a single therapy. The present study aimed to combine Rapamycin and Niacin to achieve a synergistic anti-proliferative effect on AML cells. Together with this, the molecular mechanism behind the anti-proliferative effect was investigated in terms of apoptotic cell death, cell cycle distribution and autophagy induction.

Literature studies show that the combination of Rapamycin and natural compounds exerts a stronger anticancer effect than single treatments. A study reported that the combination of Rapamycin and resveratrol elicited stronger cytotoxicity than single treatment, and the combination of resveratrol and Rapamycin had a significant synergistic antitumor effect in multiple myeloma cells [17]. In another study, it was reported that the combination of Rapamycin and Chloroquine inhibited cell viability in liposarcoma to a better extent than single drug treatment and caused extensive apoptosis by increasing the number of autophagosomes [18]. Autophagy is the process that facilitates the intracellular degradation of damaged organelles using endogenous lysosomes. Targeting AML via autophagy activation may be a promising avenue. This approach is promising in increasing the effectiveness of treatment and overcoming drug resistance [8]. Rapamycin, an immunosuppressant and anti-proliferative agent, potently inhibits mTOR activity [19]. In line with our results for NB4 but not THP-1 cells, mTOR inhibition by Rapamycin promotes the inhibitory effect, induces apoptosis, and leads to the accumulation of cells at the G1 phase on AML cells [20, 21]. mTOR inhibition has an important role in the activation of autophagy [9].

Niacin is the precursor of the coenzymes NAD (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide-phosphate) and it has very critical importance for various cellular processes and metabolism. Besides, it has an impact on the process of carcinogenesis [22], neuroprotection [23], cardiovascular diseases [24] and skin health [25]. It has been shown that Niacin has a protective role on rumen epithelial cells from butyrate-induced apoptosis which may reduce oxidative damage, and induction of DNA repair at low millimolar concentrations [26]. A study performed in the South Australian population suggested low dietary intake of preformed nicotinic acid

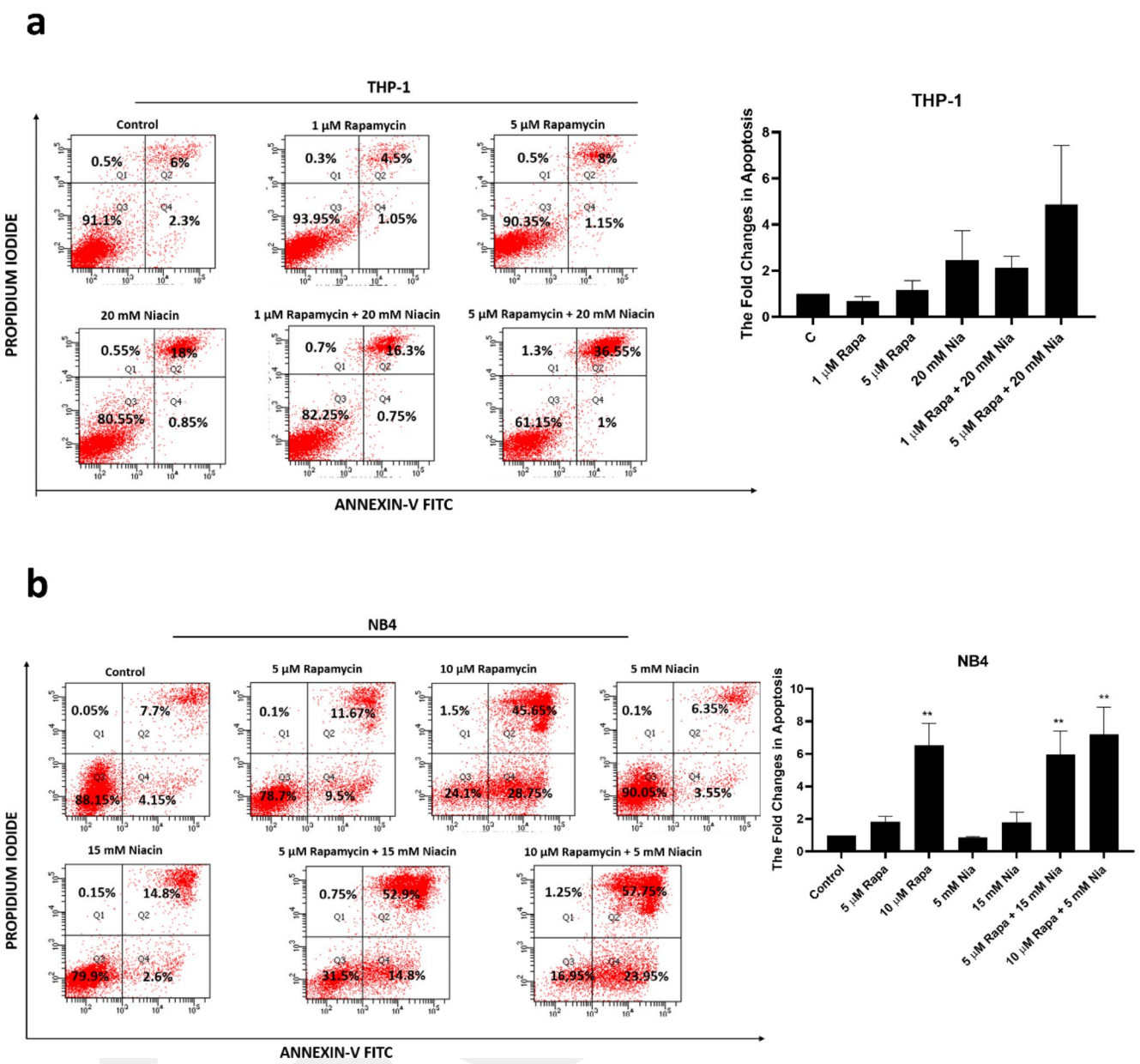

Fig. 4 Rapamycin and Niacin combination promotes cellular apoptosis on THP-1 (**a**), and NB4 (**b**) for 48 h. Cells in the lower right (Q4; Annexin V-FITC+/PI−) and upper right (Q2; Annexin V-FITC+/PI+) quadrants show early and late apoptosis, respectively. Graphs in the right panel show the fold changes of total apoptotic cells. The experiment was repeated twice in biological replicates. All data are presented as mean ± S.D. (ns = $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$)

(niacin) is related to chromosomal instability, further suggesting the role of niacin in preventing carcinogenesis [22, 27]. Considering the effects of Niacin on cancer cells, this study aimed, for the first time, to show the anti-proliferative effect of the combination with Rapamycin on AML cells, in which induction of apoptosis and arrest of the cell cycle was revealed. Our results revealed that a low micromolar dose of Niacin had no cytotoxic effect on AML cells but cytotoxicity was detected around 10–40 mM concentration. The apoptotic potential and role of niacin were studied in different cancer types [22]. For instance, niacin-related compounds induced apoptosis in HL60 cells at 5–10 mM but not at lower doses, which is in line with our findings [28]. Similarly, for NB4 cells, when 5- and 15-mM Niacin concentrations are compared in terms of apoptosis induction, our results show that 15 mM Niacin is more effective in inducing late apoptosis. Niacin has been reported to induce autophagy through NRF-2 signaling by activation of GPR109A [12]. In HCT116 human colon cancer cells, niacin treatment induced autophagic flux which was shown via LC3B turnover and p62 levels and the study suggested

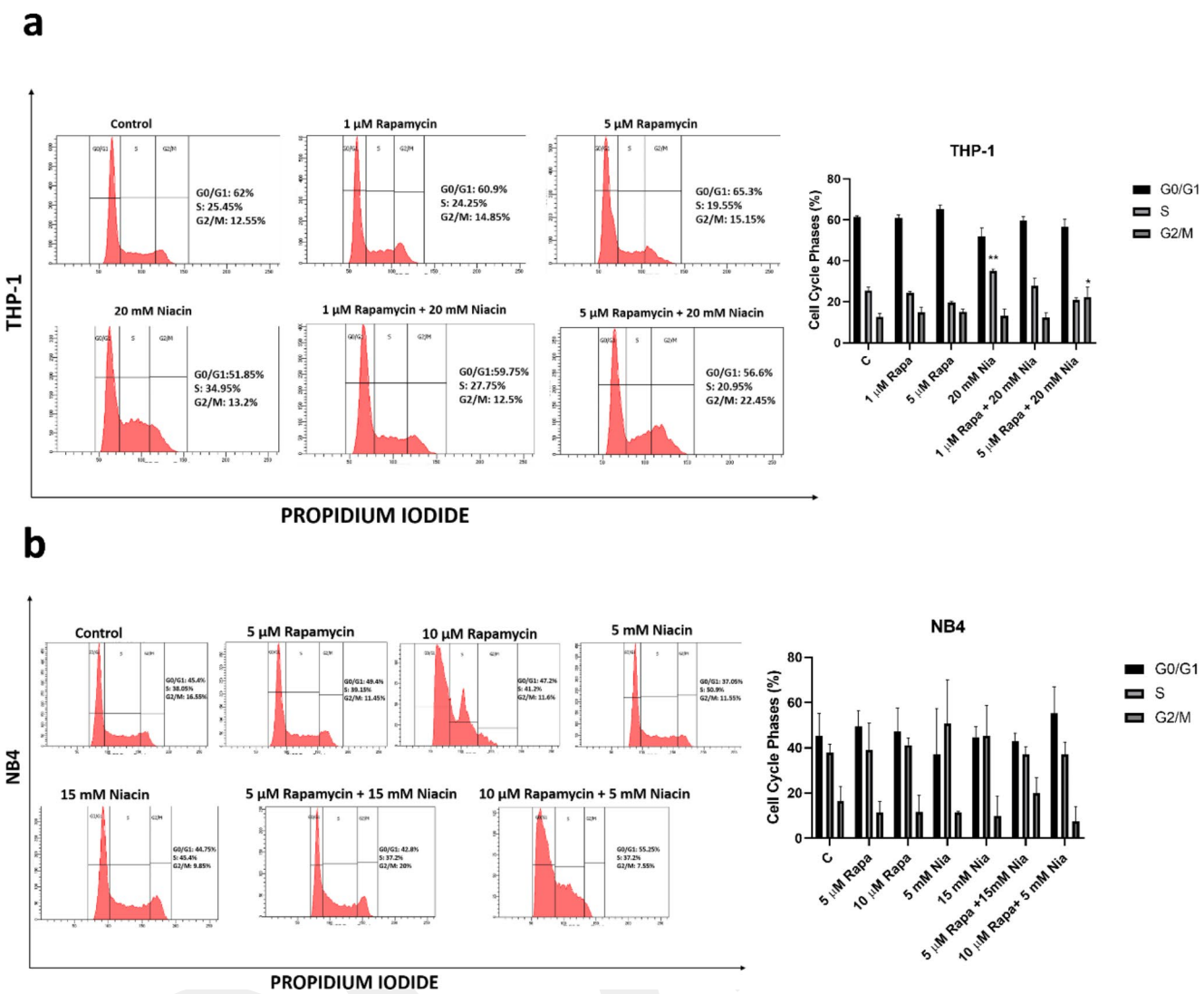

Fig. 5 The cell cycle distributions of THP-1 (**a**), and NB4 (**b**) after treatment of Rapamycin and Niacin for 48 h. The experiment was repeated twice in biological replicates. All data are presented as mean ± S.D. (ns = $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$)

niacin inhibits TRAIL-induced apoptosis through activation of autophagic flux [29].

In this study, Rapamycin and Niacin combinations showed a significant anti-proliferative effect in both cell lines compared to control and single-drug treatments. The synergism was observed when low Rapamycin and high (20 mM) Niacin were combined for THP-1 cells. For NB4 cells, on the other hand, the synergistic effect was observed almost in most of the tested combinations (Table 1). Apoptosis and cell cycle analysis were conducted with the concentrations demonstrating synergistic effects specifically on each cell line. It was determined that 10 nM Rapamycin treatment did not increase the NB4 apoptotic cell population [30]. It was reported that 100 µM Niacin treatment had no apoptotic effect on HCT116 and MCF-7 cells [31]. Similarly, low concentrations of Rapamycin and Niacin did not influence apoptotic cell death, as in our study. After the combination treatment of THP-1 cells, a 10% increase in the G2/M cell population was observed. However, NB4 cells accumulated in the G0/G1 phase by 10% in response to the combination of 10 µM Rapa + 5 mM Niacin. Single Niacin treatment caused a 13% and 10% increase in the cell population in the S phase in THP-1 (20 mM) and NB4 (5 mM) cells, respectively. It was stated in the literature that Niacin treatment increased the S phase in colon cancer cells from 7 to 13% [31]. In a different study, it was reported that Niacin treatment accumulated at G0/G1 phase in EAC (Ehrlich Ascites Carcinoma) cells [32]. Janus et al. showed that HL60 cells treated with nanomolar concentrations of rapamycin have been reported to accumulate in the G1 phase [33]. In our study, the accumulation of THP-1 and NB4 in different stages of the cell cycle could be due to gene regulations

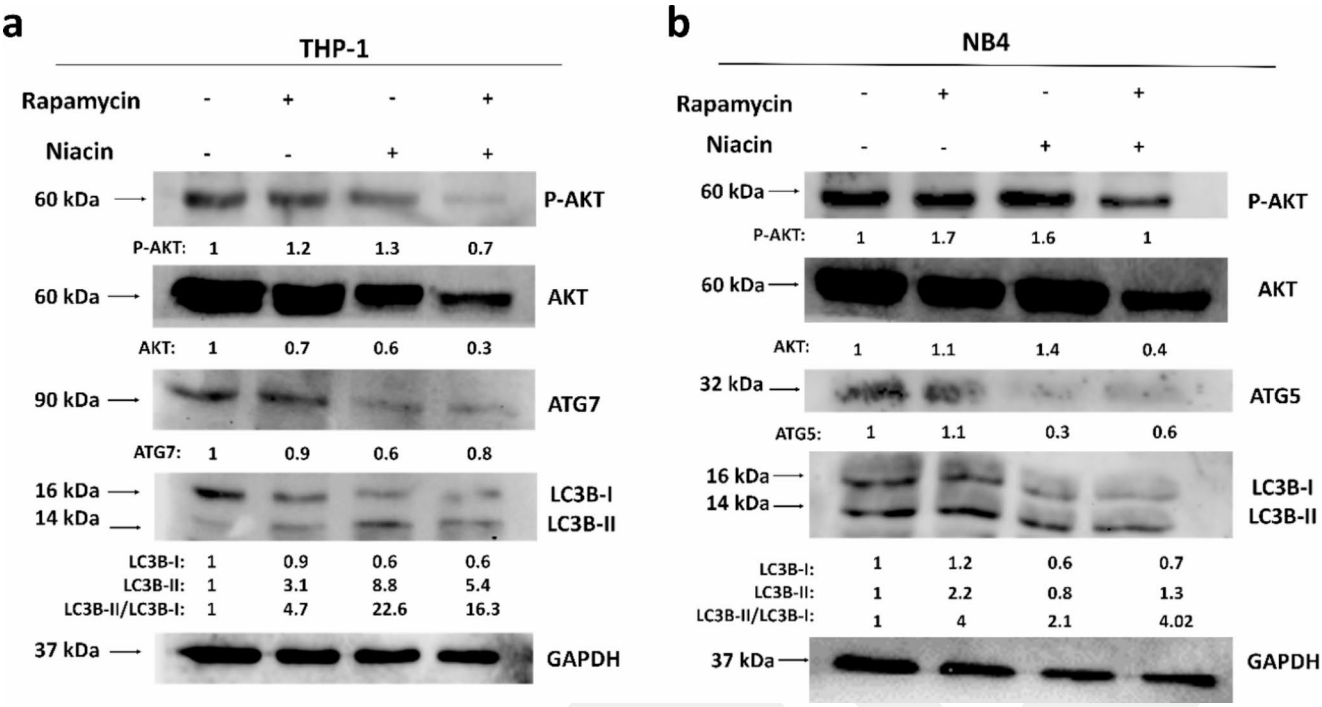

Fig. 6 The expression levels of p-AKT, AKT, ATG7, ATG5, and LC3B on THP-1 (**a**) and NB4 (**b**) after treatment of 48 h. GAPDH was used as a loading control. The overall experiment was done in two biological replicates. Results were normalized according to the control groups, and the protein levels of control groups were indicated as 1

caused by genetic and epigenetic differences, although both cell lines represent AML disease. THP-1 cells carry t(9;11)(p21;q23), leading to the KMT2A-MLLT3 (MLL-MLLT3; MLL-AF9) fusion gene, while NB4 cells carry the t(15;17) PML-RARA fusion gene [34]. These genetic differences may result in different responses to drug treatments.

To further investigate how the Niacin and Rapamycin combination affects the autophagy induction and AKT signaling pathway, we subsequently evaluated the expression levels of related proteins in response to drug treatment. In the study, expressions of Akt, p-Akt, ATG7, ATG5 and LC3B proteins were detected as these proteins are critical players in phagosome expansion. PI3K/Akt pathway is also involved in the autophagy pathway and by phosphorylating various downstream effectors, Akt regulates numerous biological processes such as cell cycle regulation for carcinogenesis, migration, invasion and metastasis, cell survival and cellular senescence [35]. According to the results of our study, Rapamycin and Niacin single treatment increased Akt and p-Akt expression in THP-1 and NB4 cells, while combination treatment reduced Akt and p-Akt protein expressions. Studies have reported that Rapamycin-treated AML cells have elevated levels of Akt phosphorylation [36] and Niacin treatment increases Akt phosphorylation in A431 cells [37]. Similar to our data, it has been reported that 800 µM Niacin treatment increased p-Akt expression in HCT116 colon cancer cells [29]. ATG7 expression, which acts as an autophagy effector enzyme and regulates cell death, apoptosis and autophagosome formation, decreased in THP-1 cells after combination and to a better extent by single Niacin treatment. In line with our result, it has been reported that niacin treatment reduces ATG7 expression in hepatocellular carcinoma cells [38]. Our results showed that the levels of ATG5 protein, which is involved in autophagosome elongation, were reduced in NB4 cells by combination treatment. Microtubule-associated protein 1 light chain 3 (LC3) is an autophagy marker that folds during induction of autophagic flux and is required for autophagosome formation [39]. Both combination therapy and Niacin increased the LC3B-II/I ratio in both cell lines used in this study. Similar to our results, it has been reported that LC3-II expression increases with Niacin treatment in human colon cancer [29]. Upregulation of LC3B-II expression is a marker of autophagy activation. We can conclude that the combination treatment of AML cells led to autophagy induction through upregulating LC3B protein [38–40].

Conclusion

In conclusion, our study suggests, for the first time to our knowledge, that the combination of Rapamycin and Niacin synergistically reduced the cell proliferation of AML cells. Our results demonstrated that Rapamycin and Niacin

combination induced apoptotic cell death and arrest the cell cycle on AML cells. The combination treatment induced autophagy in AML cells. Further extensive investigations are required to gain a better understanding of the mechanisms of these drugs, and it is suggested to elucidate the underlying impact of these pathways on AML. It has been reported that Niacin treatment downregulated the expression of Beclin-1, which acts as the main regulator of autophagy by interacting with other proteins to initiate the formation of autophagosomes, and the level of p62 also increased [38–40]. In light of this information and our findings, the expressions of proteins involved in the apoptosis and autophagy pathways could be determined for a deeper understanding of the mechanisms of drugs as a future aspect.

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Author contributions İ.A. contributed to the study of conception and design. Material preparation, data collection were performed by L.B.S. Analysis and interpretation were performed by L.B.S., E.B.G.A. and İ.A. The first draft of the manuscript was written by L.B.S., E.B.G.A. and İ.A. Supervision of the study was done by E.B.G.A and İ.A. All authors read and approved the final version of the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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