Pathol. Oncol. Res. (2011) 17:645–656
DOI 10.1007/s12253-011-9365-z

RESEARCH

Combined Inhibition of PI3K and mTOR Exerts Synergistic Antiproliferative Effect, but Diminishes Differentiative Properties of Rapamycin in Acute Myeloid Leukemia Cells

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Received: 22 February 2010 / Accepted: 6 January 2011 / Published online: 19 February 2011

Abstract A novel strategy has been suggested to enhance rapamycin-based cancer therapy through combining mammalian target of rapamycin (mTOR)-inhibitors with an inhibitor of the phosphatydilinositol 3-kinase PI3K/Akt or mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway. However, recent study demonstrated the potentiating effect of rapamycin on all-*trans*-retinoic acid (ATRA)-mediated differentiation of acute myelogenous leukemia (AML) cells, prompting us to investigate the effects of longitudinal inhibition of PI3K/Akt/mTOR signaling pathway on both proliferation and differentiative capacity of AML. In NB4, HL-60, U937 and K562 cell lines, rapamycin exerted minimal antiproliferative effects, and combining PI3K inhibitor LY 294002 and rapamycin inhibited proliferation more than LY 294002 alone. Rapamycin potentiated differentiation of ATRA-treated NB4 cells, but the combination of rapamycin and LY 294002 inhibited the expression of CD11b in both ATRA- and phorbol myristate acetate (PMA)-stimulated cells more than PI3K inhibitor alone. These results demonstrate that, although the combination of PI3K inhibitor and rapamycin is more effective in inhibiting proliferation of AML, the concomitant inhibition of PI3K and mTOR by LY 294002 and rapamycin has more inhibitory effects on ATRA-mediated differentiation than the presence of PI3K-inhibitor alone, and diminishes positive effects of rapamycin on leukemia cell differentiation.

Keywords ATRA · Leukemia · PI3K · PMA · Rapamycin

Abbreviations

PI3K	Phosphatydilinositol 3-kinase
mTOR	Mammalian target of rapamycin
AML	Acute myelogenous leukemia
MAPK/ERK	Mitogen-activated protein kinase/extracellular signal-regulated kinase
APL	Acute promyelocytic leukemia
ATRA	*All-trans*-retinoic acid
PMA	Phorbol myristate acetate

Introduction

As constitutive activation of phosphatydilinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway has been demonstrated in various malignancies, including primary samples from patients with acute myelogenous leukemia (AML) [1] and/or myelodysplastic syndrome (MDS) [2], several therapeutic strategies are being developed to modulate this signaling pathway. Wortmannin and LY 294002, pharmacological inhibitors of PI3K, exert strong antiproliferative effects on AML cell

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lines in vitro, but their poor solubility and high toxicity have limited their clinical application [3]. Proapoptotic and chemosensitizing effects of Akt inhibitors perifosine and phosphatidyl ether analogues (PIA) have been proven in vitro [4, 5], but clinical effects of combined therapy including perifosine have been demonstrated only in patients with multiple myeloma [6]. Rapamycin and its derivatives are the most clinically developed inhibitors of the PI3K/Akt/mTOR pathway mostly because they are already in use as efficient immunosupressive agents in the setting of heart and kidney transplantation [7]. Rapamycin targets mTOR kinase, a more distal component of the pathway, which plays critical role in cell growth, proliferation and metabolism [8]. Preclinical studies have shown cytostatic and differentiative effects of mTOR inhibitors on acute myeloid leukemia cells [7, 9, 10]. However, results of the clinical trials using rapamycin derivatives as a single agent have shown response in four out of nine AML patients treated with sirolimus [9], five out of 23 AML treated with deforolimus [11], or no response in nine patients with AML treated with everolimus [12].

Modest efficacy of mTOR inhibitors when used in monotherapy may be partially explained by the feedback activation of proximal components of the pathway, as an increase in the activity and phosphorylation of Akt is commonly observed in tumor cells treated with rapamycin alone [13, 14]. In AML samples, everolimus was found to activate PI3K/Akt by up-regulating insulin-like growth factor-1 receptor signaling, and simultaneous inhibition of two pathway components, PI3K and mTOR, circumvented feedback activation seen with mTOR inhibitor alone [15]. In various tumors, rapamycin has been demonstrated to be more effective when combined with proximal pathway inhibitors; synergistic effects between rapamycin and LY 294002, an upstream inhibitor of PI3K, are commonly observed in vitro [13, 15]. Another possible approach is to combine the PI3K/Akt/mTOR inhibitors with inhibition of a parallel pro-survival signaling pathway such as the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, which was found to be compensatory activated in solid tumor samples from patients treated with rapamycin derivative [16, 17].

Differentiation therapy of human leukemia is an alternative approach that has been successfully used for the targeted treatment of acute promyelocytic leukemia (APL) carrying a typical t(15;17) translocation. *All-trans*-retinoic acid (ATRA)-based therapy of APL provides the most successful example of differentiation therapy of malignancies as complete remission is induced in over 90% of patient treated with ATRA [18]. The effect of pharmacological doses of ATRA is generally attributed to the effects of pharmacological doses of ligand on the fusion protein promyelocytic leukemia (PML)/retinoic acid receptor α (RARα) which is encoded by t(15;17); therefore, ATRA-therapy is currently being restricted only to APL-patients. However, many recent studies suggest some therapeutic potential of ATRA in non-APL AML, especially in combination with drugs that target proliferative pathways [19]. Our previous study demonstrated that Akt, a component of PI3K/Akt/mTOR pathway, has a role in ATRA-mediated differentiation of HL-60 and NB4 leukemia cell lines and showed that commercial PI3K and Akt inhibitors affect not only proliferation, but also the differentiative property of leukemia cells [20]. Recent study demonstrated that rapamycin, which inhibits more distal component of PI3K/Akt/mTOR pathway, potentiates ATRA-mediated effects on growth arrest and differentiation of leukemia cells [21]. Therefore, the present study was undertaken in order to determine the effects of synergistic inhibition of PI3K and mTOR not only on the proliferation, but also on the differentiative capacity of various leukemia cells in vitro.

Materials and Methods

Chemicals

Phorbol myristate acetate (PMA) was purchased from Sigma (St. Louis, MO, USA), and dissolved in 100% DMSO to a stock concentration of 500 μM. ATRA was purchased from Calbiochem (San Diego, CA, USA), and dissolved in 100% DMSO to a stock concentration of 1 mM. MEK inhibitor PD 98059, PI3K inhibitor LY 294002, and mTOR inhibitor rapamycin were obtained from Calbiochem (San Diego, CA, USA), and dissolved in 100% DMSO to a stock concentration of 50 mM (PD 98059), 25 mM (LY 294002) and 50 μM (rapamycin). Propidium iodide, RNAse and Igepal were purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal antibodies to phospho-specific (phospho-Thr^{202}/Tyr^{204}) and total p44/42 MAP kinase, and secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Cell Signaling Technology (Beverly, MA, USA). FITC-conjugated monoclonal antibodies (MoAbs) to CD11b (IM0530) and FITC-conjugated isotypic control (IM0639) were purchased from Immunotech Beckman Coulter (Marseille, France). Annexin V-FITC Kit (IM3546) was purchased from Immunotech Beckman Coulter (Marseille, France).

Cell Culture and Cell Differentiation

HL-60 cells (ECCACC no. 88112501) were obtained from the European Collection of Animal Cell Cultures (PHLS, Porton, Salisbury, UK). NB4 and U937 cells were kindly

provided by Dr M. Golemovic, and K562 cells by Prof D. Batinic (Clinical Hospital Zagreb, Croatia). The cells were grown exponentially in RPMI 1640 medium (EuroClone, Italy) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco/Invitrogen, Grand Island, NY, USA) in 5% CO_2 humidified atmosphere at 37°C.

For cell differentiation, cells were harvested, resuspended in fresh medium containing 10% FBS, and seeded at density of 0.2×10^6/mL in 6-well plates (Nunc, Roskilde, Denmark). The cells were pretreated with inhibitors (PD 98059, LY 294002, rapamycin and their combination), and differentiated in the presence of 500 nM PMA or 1 μM ATRA at the times and concentrations indicated in the figure legends. At the end of incubation, Versene (Gibco/Invitrogen, Grand Island, NY, USA) was used to collect PMA-differentiated and control cells. The number of viable cells was determined by hemocytometer and trypan dye exclusion.

The Expression of CD11b Surface Marker

The surface expression of CD11b, or binding of FITC-conjugated mouse IgG1 was determined by flow cytometric analysis as previously described [20]. Briefly, cells were washed with cold PBS and incubated with 5 μl of anti-CD11b or an appropriate isotypic control (mouse IgG1) in a total volume of 100 μL. After 20 min of incubation in dark at room temperature, cells were washed and analyzed using FACSCalibur system (Becton Dickinson). A total of 15,000 events were collected from the gated area. Data were analyzed using CellQuest software (Becton Dickinson). On a single FL-histogram, cursor of the control samples was set to include up to 1.0% of the fluorescence in the positive region. The results are presented as either percentage of positive cells or mean fluorescence intensity (MFI) values.

The Analysis of the Cell Cycle and Annexin V-FITC—Staining

The analysis of the cell cycle was performed as previously described [22]. Briefly, an aliquot of cells was washed with ice-cold PBS and stained directly with propidium iodide (PI) solution (10 mM Tris, pH 8.0, 10 mM NaCl, 10 μg/ml RNAse, 50 μg/ml PI, 0.1% Igepal) for 30 min. DNA analysis was performed using FACSCalibur system and Cell Quest software (Becton Dickinson). The percentage of cells in the particular phase of the cell cycle was determined by using ModFit software (Becton Dickinson).

The annexin V-FITC—staining was performed as suggested by manufacturer. Briefly, cells were harvested, washed twice with ice-cold PBS and incubated on ice with annexin V-FITC solution and PI for 15 min. Analysis was performed using FACSCalibur system. For each sample, 10,000 events were collected to eliminate cell debris. The percentage of FITC—positive cells within gated population was determined using Cell Quest software (Becton Dickinson).

Isolation of Total Cell Lysates

Cells were collected, washed twice in ice-cold PBS and incubated on ice in cell lysis buffer containing: 20 mM Tris–HCl (pH 7.5), 1 mM Na_2EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM beta-glycerophosphate, 1 μg/ml leupeptin, and 1 mM PMSF. After 10 min, the cells were sheared by seven passages through a 23-gauge needle. The lysates were incubated on ice for additional 5 min, and then centrifuged at 14,000×*g* for 10 min. The resultant supernatants were collected, and the sample protein concentration was determined using the Coomasie dye-binding assay from Bio Rad (Munchen, Germany).

Western Blot Analysis

The equal amount of lysates (75 μg of proteins) were boiled for 5 min in SDS polyacrylamide gel electrophoresis (PAGE) sample buffer, and loaded on two parallel 8% SDS-polyacrilamide gels. Electrophoresis was carried out using a Bio-Rad Minigel apparatus, and proteins were transferred to nitrocellulose membranes using a Bio-Rad wet-blotting system. Nonspecific binding was inhibited by incubation in TBS-Tween buffer (25 mM Tris, 150 mM NaCl, 0.1% (*v*/*v*) Tween 20) containing 5% (*w*/*v*) non-fat dried milk for 30 min. After blocking, membranes were washed three times in TBS-Tween and incubated overnight at +4°C with primary antibodies diluted (1:1000) in TBS-Tween containing 5% (*w*/*v*) BSA. After incubation, membranes were washed three times and incubated at room temperatures for 2 h with HRP-conjugated secondary antibody diluted (1:2000) in TBS-Tween containing 5% (*w*/*v*) non-fat dried milk. Bands were visualized using ECL kit (Pierce, Rockford, IL, USA).

Statistical Analysis

The data are shown as means±S. E. M. and analyzed using two-tailed Student's *t*-test. Values with $p<0.05$ were considered significant.

Results

To test for the effects of synergistic inhibition of PI3K and mTOR on leukemia cell proliferation, LY 294002, a PI3K

inhibitor, and mTOR inhibitor rapamycin were used at doses that have been previously used to show synergistic effects of inhibitors in leukemic T-cell lines [23, 24]. Myeloblastic (AML-M2) HL-60, promyelocytic (AML-M3) NB4, monocytic (AML-M5) U937 and erythro-megakaryocytic K562 cell lines were seeded at a density of 0.2×10^6/ml and grown for 24–96 h in the presence of inhibitors or their combination. As shown in Fig. 1, rapamycin alone exerted minimal inhibitory effect on the number of viable cells, LY 294002 at a concentration of 10 μM was effective in AML cell lines, and combining LY 294002 and rapamycin resulted in significantly more inhibition of proliferation at all doses tested. In addition, the effects of inhibitors and their combination on the cell cycle distribution were determined in all leukemia cell lines tested. As shown in Table 1, both LY 294002 and rapamycin induced an increase in the proportion of cells in G_0/G_1-phase of the cell cycle. However, rapamycin alone had no significant effects on the percentage of cells within the sub-G_1 fraction of the cell cycle. An increase in the percentage of sub-G_1 was detected in cells treated with high dose of LY 294002 (10 μM) or the combination of inhibitors.

To further investigate the effects of the combined inhibition of PI3K and mTOR on differentiative property of leukemia cells, HL-60 and NB4 cells were differentiated in the presence of phorbol miristate acetate (PMA), which potently induces differentiation of both cell lines into macrophage-like phenotype. After 24 h, PMA induced adherence of both cell types (data not shown), so that cells were collected using EDTA and analyzed for the presence of CD11b differentiation marker by flow cytometry (Fig. 2). As previously described, both the percentage of CD11b-positive cells and mean florescence intensity (MFI) were higher in control NB4 cells in comparison to HL-60 [20]. PMA induced a significant increase in the level of CD11b in both cell lines, rapamycin (20 nM) alone had no significant effects on PMA-induced increase in the percentage of positive cells, LY 294002 (10 μM) alone significantly inhibited PMA-effects in NB4 cell line, and the increase in CD11b-expression was significantly more inhibited in both cell

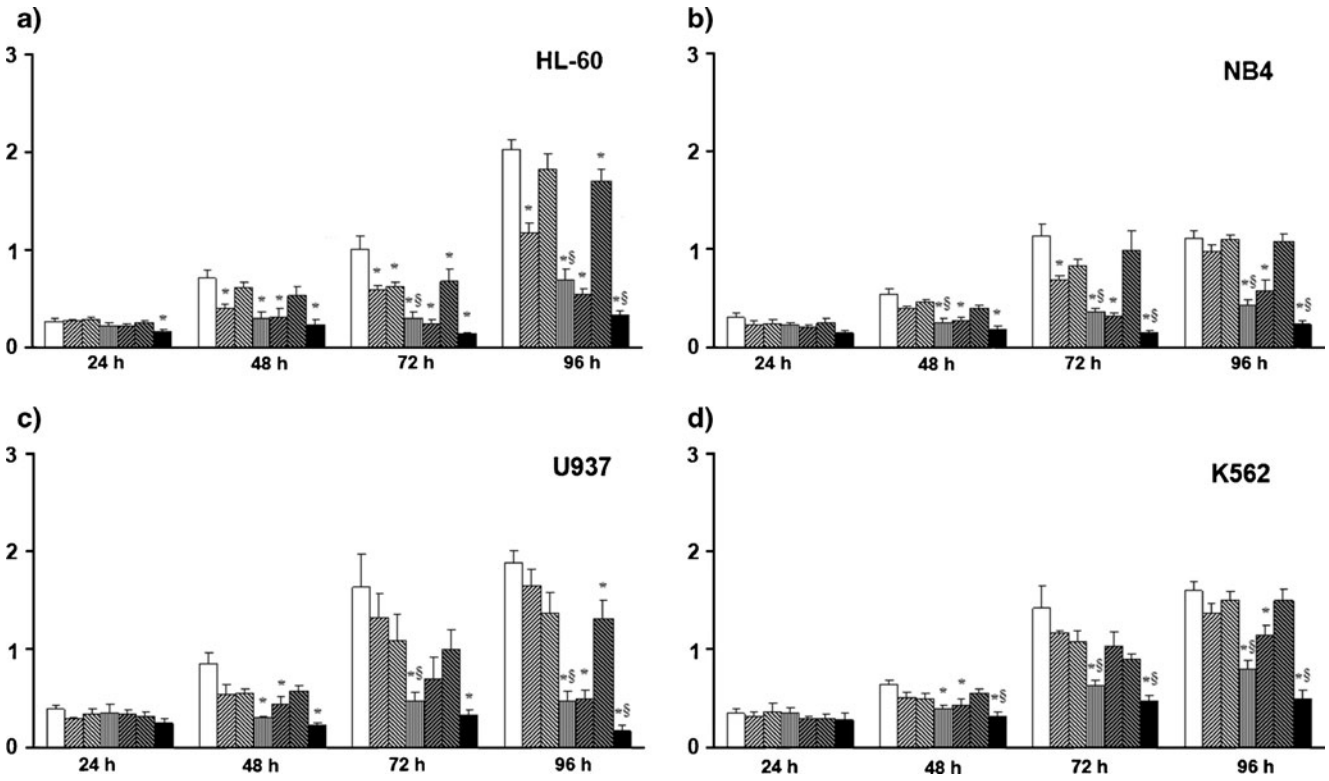

Fig. 1 The effects of LY 294002 and rapamycin alone or in combination on the growth of leukemia cell lines. HL-60 cells (**a**), NB4 cells (**b**), U937 cells (**c**), and K562 cells (**d**) were seeded at an initial concentration of 0.2×10^6/mL. The cells were incubated in the presence of following agents (columns from *left* to *right*): vehicle alone (control), LY 294002 (5 μM), rapamycin (5 nM), the combination of LY 294002 (5 μM) and rapamycin (5 nM), LY 294002 (10 μM), rapamycin (20 nM), and the combination of LY 294002 (10 μM) and rapamycin (20 nM) for 24–96 h. At the time indicated, the number of viable cells was determined by trypan exclusion and hemocytometer. The results are the mean±SEM of at least three independent experiments. *$P<0.05$ (Student's *t*-test) with respect to the control. §$P<0.05$ (Student's *t*-test) with respect to the cells treated with the same concentration of LY 294002 or rapamycin alone

Table 1 The effects of LY 294002 and rapamycin alone or in combination on the cell cycle distribution of leukemia cell lines. HL-60, NB4, U937 and K562 cells were seeded at an initial concentration of 0.2×10^6/mL and incubated in the presence of different concentrations of LY 294002 (5 and 10 μM), rapamycin (5 nM and 20 nM) or the combination of inhibitors. The cells were harvested after 24–96 h, stained directly with propidium iodide and analyzed using flow cytometry. The results are the mean±SEM of at least three independent experiments. *$P<0.05$ (Student's *t*-test) with respect to the control. **$P<0.05$ (Student's *t*-test) with respect to the cells treated with the same concentration of LY 294002 or rapamycin alone

		HL-60				NB4				U937				K562			
		24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Control	G_0/G_1	46±1	41±0	45±1	49±2	44±1	41±1	44±1	39±3	44±1	53±2	58±2	61±1	32±2	38±1	51±2	47±1
	sub-G_1	3±1	4±1	4±1	2±1	14±3	15±1	24±2	18±7	13±1	8±3	9±5	13±4	12±2	6±1	6±2	5±1
LY 294002 (5 μM)	G_0/G_1	56±2*	48±1*	53±5	47±3	51±2*	48±1*	50±3	46±7	57±5*	55±3	56±2	65±6	34±2	42±3	51±2	50±1*
	sub-G_1	5±1	5±1	8±2	3±1	18±4	21±3	25±1	9±3	14±1	9±3	6±2	7±2	12±3	6±1	6±2	4±1
Rapamycin (5 nM)	G_0/G_1	57±3*	46±2*	52±6	46±3	49±1*	45±1*	47±3	44±4	56±5	56±3	58±1	59±1	41±3*	48±2*	50±3	49±2
	sub-G_1	4±1	5±1	6±2	3±1	16±3	27±6	25±4	9±4	13±2	8±3	6±3	7±3	11±3	6±1	6±2	6±4
LY 294002 (5 μM)+ rapamycin (5 nM)	G_0/G_1	69±2*	57±4**	67±5*	52±4	59±3*	54±5*	55±6	42±4	76±3**	67±6	66±1**	64±2	52±2**	57±2**	56±6	60±4**
	sub-G_1	8±1*	7±2*	13±2*	9±4	19±4	16±2	27±4	21±3	14±3	17±2**	14±5	15±3	12±3	7±2	5±1	4±1
LY 294002 (10 μM)	G_0/G_1	65±4*	59±4*	66±2*	55±3	54±4*	53±7*	53±5	41±1	72±6*	63±6	66±3	67±6	37±3	42±2	46±3	56±3*
	sub-G_1	6±1*	7±1*	10±4	5±2	24±7	18±3	33±1*	17±2	15±2	15±3	9±4	10±2	12±3	8±2	5±1	4±2
Rapamycin (20 nM)	G_0/G_1	56±2*	45±1*	52±5	48±2	50±1*	43±0*	48±2	44±5	57±4*	56±2	57±1	61±1	42±2*	48±1*	51±3	50±1*
	sub-G_1	3±1	5±1	5±2	2±1	15±3	26±6	23±2	7±3	10±2	8±2	5±2	7±2	11±3	5±1	7±3	3±1
LY 294002 (10 μM) + rapamycin (20 nM)	G_0/G_1	76±3*	66±10**	68±3*	53±6	59±3*	59±8*	54±8	47±3	77±4**	74±6*	77±4**	63±9	56±2**	62±1**	59±2**	63±1**
	sub-G_1	8±3	8±2*	14±3*	18±12	28±8	36±4*	44±6*	49±7*	17±3	22±4**	25±8	23±6	13±4	7±3	9±3	6±2

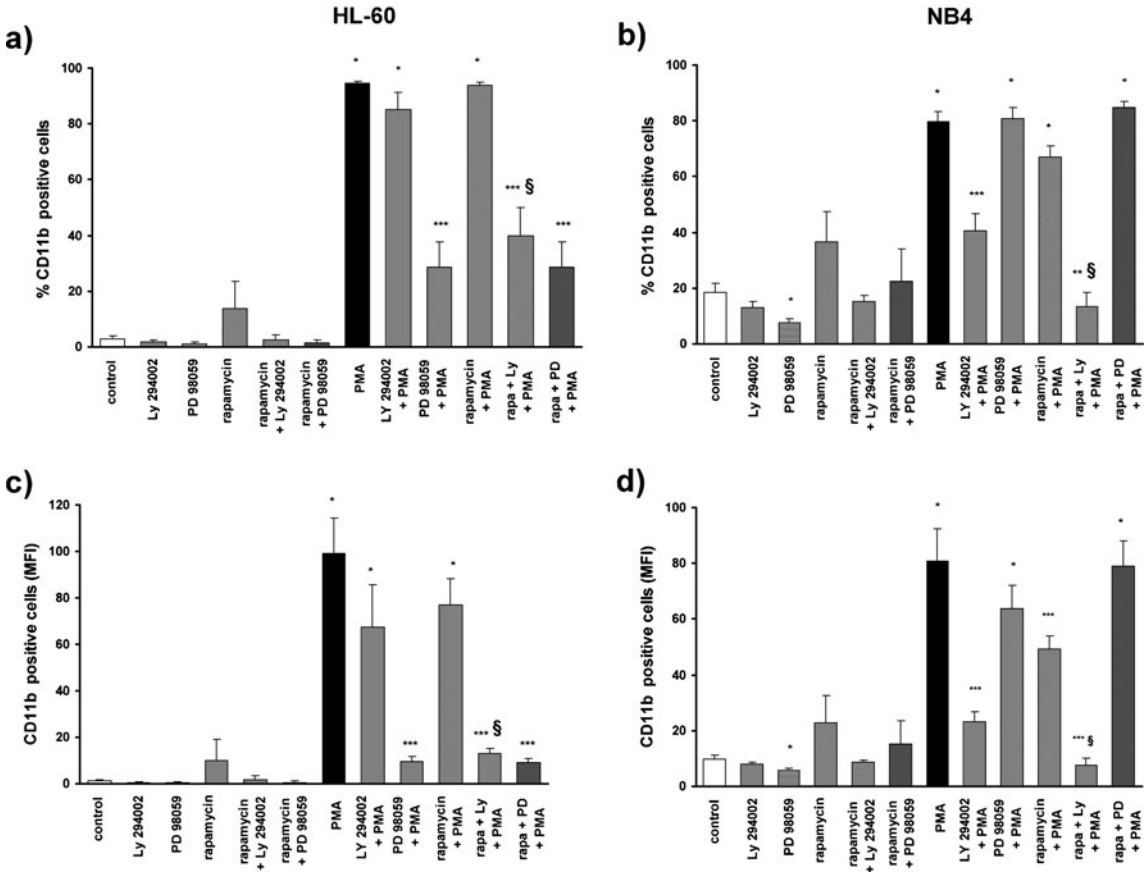

Fig. 2 The effects of inhibitors on CD11b expression in control and PMA-differentiated HL-60 and NB4 cells. HL-60 (**a** and **c**) and NB4 cells (b and d) were seeded at an initial concentration of 0.2×10^6/mL and treated with vehicle alone (control), inhibitors, PMA or the combination of inhibitors and PMA. LY 294002 (10 μM), PD 98059 (20 μM), rapamycin (20 nM), or the combination of inhibitors were added 30 min before the addition of PMA (500 nM) or 0.1% DMSO (vehicle). After 24 h, cells were collected and analyzed for the percentage (**a** and **b**) and MFI (**c** and **d**) of CD11b-positive cells. The results are the mean±SEM of three independent experiments. Student's *t*-test was used to analyze data. **P*<0.05 with respect to the control. ***P*<0.05 in comparison to PMA. ****P*<0.05 in comparison to both control and PMA. §*P*<0.05 with respect to the cells treated with the same concentration of LY 294002 or rapamycin alone

lines when PI3K and mTOR inhibitors were combined together. To test for the effects of the inhibition of a parallel pro-survival pathway, MEK inhibitor PD 98059 was applied at a dose that has been previously shown to inhibit ERK in both cell lines [20, 22]. Although PD 98059 inhibited CD11b-expression in unstimulated NB4 cells, PD 98059 alone had no statistically significant effects on PMA-induced increase in CD11b in NB4 cells. In contrast, MEK inhibitor significantly inhibited PMA-mediated increase in CD11b in HL-60 cells. In both cell lines, combination of rapamycin and MEK-inhibitor did not reduce the PMA-mediated increase in CD11b-expression more than the presence of MEK-inhibitor alone.

HL-60 cells were further labeled with propidium iodide and analyzed for the percentage of cells in particular phase of the cell cycle by flow cytometry. As shown in representative histogram (Fig. 3), 24 h-treatment of HL-60 cells with PMA induced an increase in the proportion of cells in G_0/G_1-phase of the cell cycle. All the inhibitors tested induced a growth arrest in control cells, and PI3K/mTOR inhibitors did not inhibit PMA-mediated effect. In contrast, MEK inhibitor PD 98059 completely prevented PMA-mediated arrest in G_0/G_1, which is probably due to the effect of PD 98059 to inhibit PMA-induced adherence of leukemia cells (data not shown). As summarized in Table 2, the combined inhibition of PI3K and mTOR induced a growth arrest in control cells and did not inhibit PMA-mediated increase in G_0/G_1 in both HL-60 and NB4 cell lines.

The effects of inhibitors and their combination were further tested in HL-60 and NB4 cells differentiated in the presence of ATRA for 24–96 h. As shown in Fig. 4, MEK inhibitor significantly reduced the number of viable control

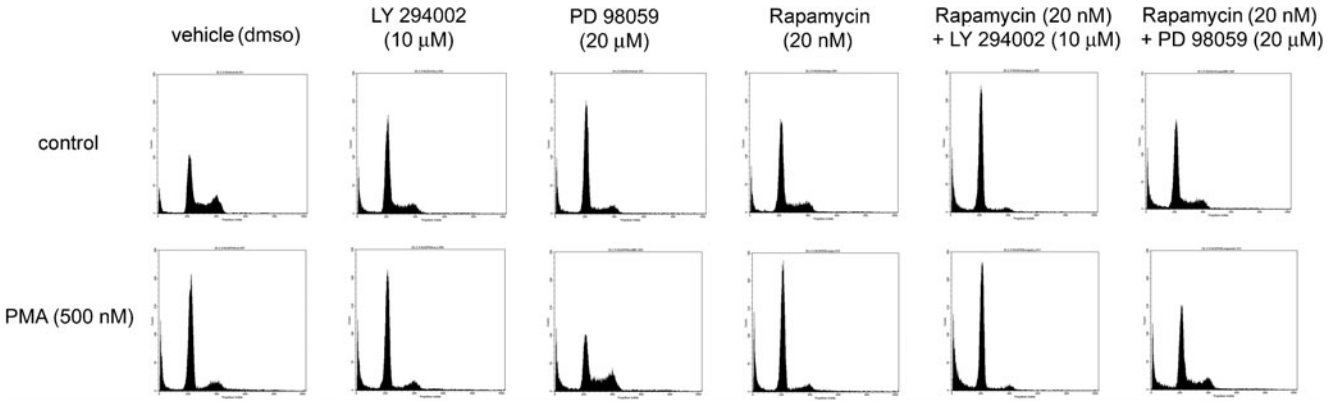

Fig. 3 The representative histogram showing the effects of inhibitors on cell cycle distribution of control and PMA-differentiated HL-60 cells. HL-60 cells were preincubated with inhibitors or their combination and then treated for 24 h with 500 nM PMA or 0.1% DMSO as a vehicle. Cells were harvested, stained directly with propidium iodide and analyzed by flow cytometry

and ATRA-treated cells, and addition of rapamycin had no synergistic effects. As previously described, rapamycin potentiates the effects of ATRA to induce growth arrest of both cell lines [21], and addition of LY 294002 further decreased the number of viable cells. To further investigate the mechanism responsible for the decrease in the number of viable cells, the effects of inhibitors and their combination on cell cycle and annexin V-staining of HL-60 and NB4 cells were determined. As shown in Table 3, all inhibitors tested caused an increase in the proportion of G_0/G_1-phase of both HL-60 and NB4 cells. ATRA, rapamycin and their combination induced an arrest in G_0/G_1-phase, while having no significant effects on percentage of sub-G_1 or annexin V-positive cells. As previously described [25], LY 294002 lead to an increase in annexin V-FITC positive NB4 cells and these effects were reduced during co-treatment with ATRA.

Figure 5 shows the expression of CD11b differentiation marker in NB4 cells, a typical APL line, that were incubated in the presence of ATRA for 48 h. As previously described [20, 21, 25], ATRA induced significant increase in the expression of CD11b, the presence of PI3K or MEK-inhibitors reduced the expression, and rapamycin increased MFI of CD11b-positive cells. However, when LY 294002

Table 2 Cell cycle distribution of HL-60 and NB4 cells after 24-h exposure to the indicated agents. HL-60 and NB4 cells were preincubated for 30 min with 10 μM LY 294002, 20 μM PD 98059, 20 nM rapamycin, or thier combination, and then treated for 24 h with 500 nM PMA or 0.1% DMSO as a control. The cells were harvested, stained directly with propidium iodide and analyzed using flow cytometry. The results are the mean±SEM of three independent experiments. *$P<0.05$ (Student's *t*-test) with respect to the control. **$P<0.05$ in comparison to PMA. ***$P<0.05$ in comparison to both control and PMA.

	HL-60			NB4		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
Control	46±2	39±1	15±1	51±3	38±4	10±2
Control + LY 294002	68±2*	24±2*	8±1	63±2	30±2	7±1
Control + PD 98059	76±2*	16±1*	8±2	59±1	34±3	9±3
Control + rapamycin	61±6*	32±5	8±1	66±6*	30±6	5±2
Control + rapamycin + LY 294002	85±2*	13±1*	2±2*	67±2*	26±1	7±2
Control + rapamycin + PD 98059	77±3*	17±2*	6±2*	63±1	32±2	6±1
PMA	90±3*	5±1*	5±3*	87±2*	12±2*	2±0
PMA + LY 294002	88±1*	5±1*	7±1*	81±3*	13±3*	5±1
PMA + PD 98059	49±3**	34±5**	17±1**	69±3***	27±3*	9±2
PMA + rapamycin	93±1*	4±1*	3±1*	83±1*	13±1*	2±1
PMA + rapamycin + LY 294002	93±1*	3±1*	4±1*	74±1*	27±4*	6±3
PMA + rapamycin + PD 98059	66±3***	24±4***	10±1	74±2***	23±2*	7±1

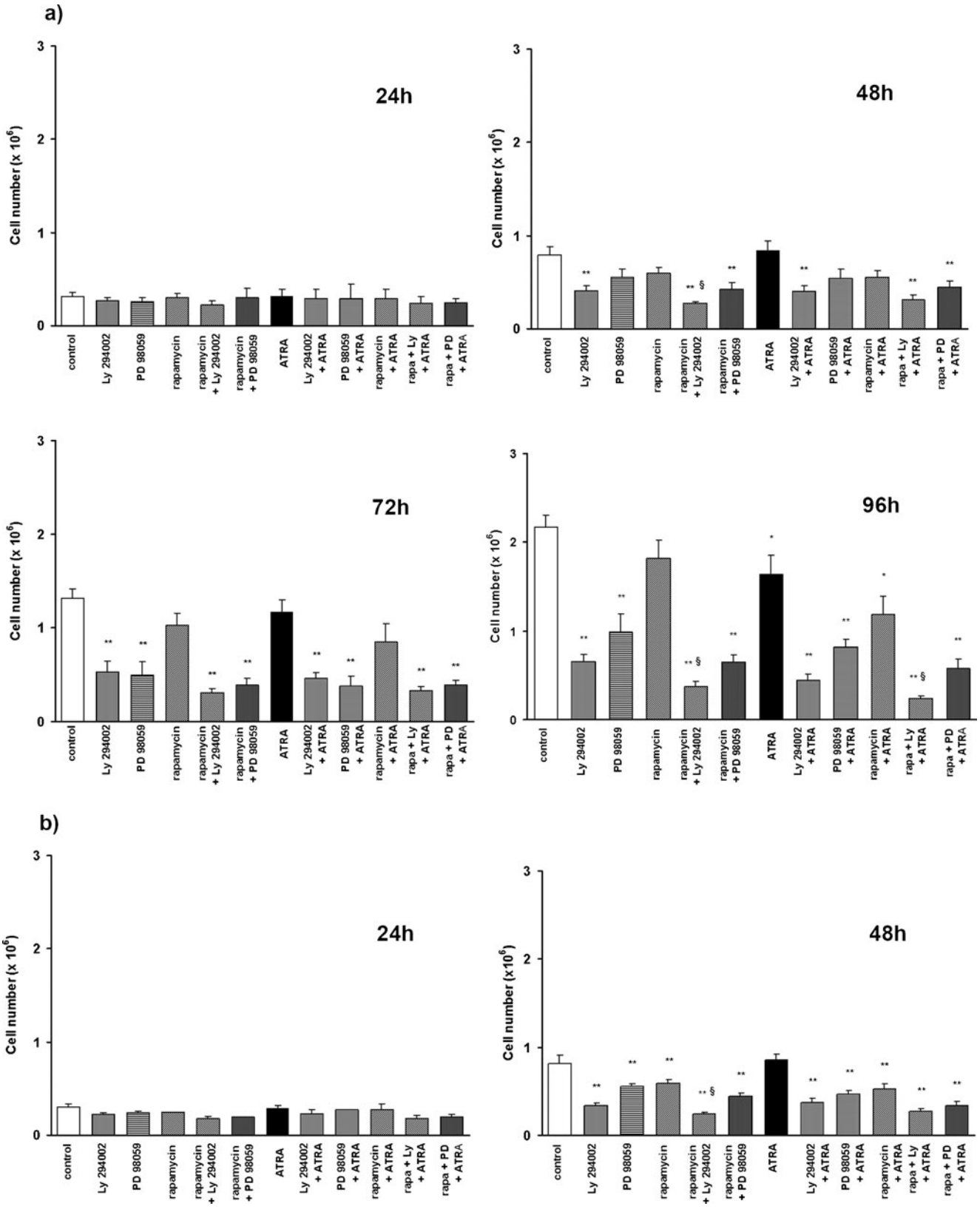
a)
24h
48h
72h
96h
b)
24h
48h
Cell number (x 10^6)
Cell number (x10^6)
control
Ly 294002
PD 98059
rapamycin
rapamycin + Ly 294002
rapamycin + PD 98059
ATRA
Ly 294002 + ATRA
PD 98059 + ATRA
rapamycin + ATRA
rapa + Ly + ATRA
rapa + PD + ATRA

Fig. 4 The effects of inhibitors on the growth of ATRA-differentiated HL-60 and NB4 leukemia cells. HL-60 (**a**) and NB4 cells (**b**) were seeded at an initial concentration of 0.2×10^6/mL. LY 294002 (10 μM), PD 98059 (20 μM), rapamycin (20 nM) or the combination of inhibitors were added 30 min before the addition of ATRA (1 μM) or 0.1% DMSO (vehicle). The number of viable cells was determined after 24–96 h (HL-60 cells) and 24–48 h (for NB4 cells). The results are the mean±SEM of at least three independent experiments. Student's *t*-test was used to analyze data. *$P<0.05$ with respect to the control. **$P<0.05$ in comparison to ATRA. ***$P<0.05$ in comparison to both control and ATRA. §$P<0.05$ with respect to the cells treated with the same concentration of LY 294002 or rapamycin alone

was added to rapamycin 30 min before the addition of ATRA, ATRA-mediated increase in the expression of CD11b was more inhibited than in the presence of PI3K inhibitor alone.

The presence of MEK inhibitor PD 98059 significantly decreased the expression of CD11b in ATRA-differentiated cells and abolished rapamycin-mediated potentiation of ATRA-effects. Several studies have previously documented a role of an increase in the activity of ERK during differentiation of leukemia cells in response to various agents, including ATRA and PMA [26–28]. To test for the possibility that rapamycin-mediated effects on differentiation are mediated by an increase in MAPK activity, total cell lysates were isolated from HL-60 and NB4 cells treated with inhibitors for 60 min or 24 h and analyzed for the activity of MAPK/ERK. As shown in representative blots (Fig. 6), no differences in the level of either phospho-Thr^{202}/Tyr^{204} p44/42 MAP kinase or total MAPK/ERK can be observed in cells treated with LY 294002, rapamycin or their combination at the time-points tested.

Discussion

A novel strategy has been suggested to enhance rapamycin-based cancer therapy through combining mTOR inhibitors with an inhibitor of the PI3K/Akt or MAPK/ERK pathway [14]. In bone marrow samples obtained from patients with AML, concomitant inhibition of mTORC1 and PI3K/Akt by RAD001 and IC87114 induced additive antiproliferative effects [15]. Results of the present data show that combination of mTOR inhibitor, rapamycin, and PI3K inhibitor, LY 294002 co-operates to give effective inhibition of proliferation of various leukemia cell lines. Using inhibitors at doses that were previously reported to effectively inhibit proliferation of T-cell leukemia cells [23, 24], synergistic effects were observed in typical APL cell line carrying t(15;17) translocation (NB4), AML-M2 (HL-60) and AML-M5

Table 3 The effects of inhibitors on the cell cycle and annexin-positivity of HL-60 and NB4 cells differentiated in the presence of ATRA. HL-60 and NB4 cells were seeded at an initial concentration of 0.2×10^6/mL. LY 294002 (10 μM), PD 98059 (20 μM), rapamycin (20 nM) or the combination of inhibitors were added 30 min before the addition of ATRA (1 μM) or 0.1% DMSO (vehicle). At the time indicated, cells were collected and either stained with propidium iodide or labeled with annexin V-FITC. The results are the mean±SEM of at least three independent experiments. *$P<0.05$ (Student's *t*-test) with respect to the control. **$P<0.05$ in comparison to ATRA. ***$P<0.05$ in comparison to both control and ATRA

	HL-60							NB4						
	24 h			48 h			96 h	24 h			48 h			
	Annexin	G_0/G_1	sub-G_1	Annexin	G_0/G_1	sub-G_1	Annexin	G_0/G_1	sub-G_1	Annexin	G_0/G_1	sub-G_1	Annexin	G_0/G_1	Sub-G_1
Control	7±2	45±2	4±1	5±1	41±1	2±1	4±1	49±2	2±1	8±2	46±5	10±3	10±1	38±1	8±3
Control + LY 294002	6±2	63±2*	3±1	5±1	58±2*	3±1**	4±1	54±2	5±1*	13±4	57±5*	11±4	21±4*	45±1*	13±3
Control + PD 98059	7±2	62±4*	3±1	5±1	43±1	2±1	6±2	50±1	3±1	8±2	56±3*	10±4	10±1	49±1*	7±2
Control + rapamycin	8±2	54±3*	3±1	6±1	45±1*	2±1	5±2	47±1	3±1	10±2	53±4*	8±2	11±1	43±1*	6±2
Control + rapamycin + LY	7±2	76±3*	6±1*	7±1	67±3*	4±1**	5±1	56±3	13±7	14±3	61±7*	11±4	22±2*	52±1*	30±8
Control + rapamycin + PD	6±2	69±5*	4±1	5±1	54±5*	3±1	6±2	50±4	5±2	9±2	60±3*	9±3	11±1	44±3*	10±2
ATRA	6±2	46±2	4±1	5±1	64±1*	2±1	6±1	75±2*	3±2	8±2	52±4	9±3	13±3	50±1*	10±1
ATRA + LY 294002	6±2	63±2***	4±1	5±1	64±2*	2±1	7±2	82±5*	27±9***	8±2	50±5	9±3	12±2	54±2*	13±3
ATRA + PD 98059	6±2	52±2***	4±1	4±1	54±1**	3±1	9±1*	55±3**	7±3*	7±2	68±1*	10±3	13±3	58±2*	8±2
ATRA + rapamycin	7±2	60±2***	4±1	7±1	69±1**	2±1	6±1	84±2***	7±5	7±2	53±4*	10±4	14±3	59±1***	7±2
ATRA + rapamycin + LY	8±3	73±2***	5±1	6±1	73±2**	4±1***	5±1	80±6*	16±9*	8±1	50±6	12±3	14±3	51±4*	19±5
ATRA + rapamycin + PD	7±2	63±2***	4±1	4±1	61±1*	3±1	6±1	62±8	7±5	7±2	66±2*	11±4	12±2	66±3***	12±2

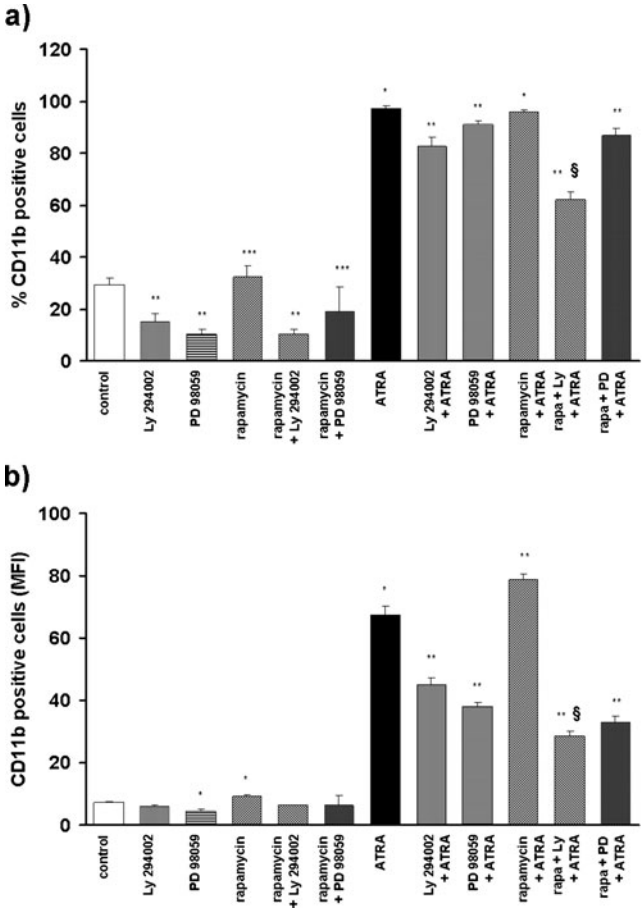

Fig. 5 The effects of inhibitors alone or in the combination on CD11b expression in NB4 cells differentiated in the presence of ATRA. NB4 cells were seeded at an initial concentration of 0.2×10^6/mL and treated with vehicle alone (control), inhibitors, ATRA or the combination of inhibitors and ATRA. LY 294002 (10 μM), PD 98059 (20 μM), rapamycin (20 nM) or the combination of inhibitors were added 30 min before the addition of ATRA (1 μM). After 48 h, cells were collected and analyzed for the percentage (**a**) and MFI (**b**) of CD11b-positive cells. The results are the mean±SEM of three independent experiments. Student's *t*-test was used to analyze data. $*P<0.05$ with respect to the control. $**P<0.05$ in comparison to ATRA. $***P<0.05$ in comparison to both control and ATRA. §$P<0.05$ with respect to the cells treated with the same concentration of LY 294002 or rapamycin alone

(U937) cell lines that lack the typical translocation, but nevertheless respond to differentiation agents, like ATRA and PMA, and finally in K562 cells, a BCR/ABL-positive line developed from CML in blast crisis. In all of the cell lines tested, rapamycin alone exerted a mild antiproliferative effect, and effects of LY 294002 were more pronounced, which is in accordance with previous data suggesting that rapamycin mostly inhibits cell cycle progression with minimal induction of apoptosis [3, 8–10]. The effects of LY 294002 cannot be ascribed solely to specific PI3K inhibition, since the inhibitor affects broad spectrum of different classes of PI3K and closely related kinases, which are known to have distinct biological roles [29]. However, the results obtained with the use of LY 294002 are similar to those obtained with highly isoform-specific compounds, IC87114 [15], or dual inhibitor PI-103, which inhibits both PI3Kα and δ and mTOR, and exerted antiproliferative effects in T-cell lines [30] and MOLM14 and OCI-AML3 leukemia lines [31]. In conclusion, all of these data confirm that blocking two components of PI3K/Akt/mTOR is more effective in inhibiting proliferation of leukemia cells than the inhibition of a single component of the pathway.

However, acute myeloid leukemia is characterized not only by an uncontrolled proliferation, but also by a differentiation block at the early stage of myelopoiesis. The success of ATRA-based therapy of APL provides a proof that an approach aimed to induce cellular differentiation may be less toxic and more effective than killing in the treatment of leukemia [18]. Although pharmacological inhibitors of PI3K/Akt/mTOR have been proposed in the treatment of leukemia based on their antiproliferative effects, several studies suggested that components of the pathway activate during ATRA-mediated differentiation; an increase in the level of PI3K [32], or phosphorylated Akt [20] has been observed in nuclei of ATRA-treated leukemia cells, and phosphorylation/activation of mTOR, which regulates downstream activation of the p70 S6 kinase has been observed in ATRA-differentiated NB4 cells [33]. Both nonspecific PI3K inhibitors and specific down-regulation of Akt [20] or p85α-subunit of PI3K [32] reduced CD11b expression in ATRA-stimulated leukemia cells. Recent studies demonstrated that the inhibition of the more distal components of the pathway by a rapamycin derivative actually potentiates ATRA-mediated differentiation in human acute myelogenous cells in conjunction with upregulation of C/EBPε, RTP800 and $p27^{kip}$ and downregulation of cyclin D1 and c-Myc [21]. No effects of specific reduction of mTOR level by siRNA has been examined leaving the possibility that the effects of mTOR inhibitors may be simply ascribed to non-specific effects of rapamycin derivative on other cellular targets; similar to some differences that have been previously observed in the effects of commercially available Akt-inhibitors on ATRA-mediated differentiation in comparison to the effects of siRNA-mediated specific reduction in the level of Akt in HL-60 cells [20]. Another possible explanation for the differentiative effects of rapamycin may be attributed to the modest effects of rapamycin, in comparison to LY 294002 or MEK-inhibitor, on leukemia cell proliferation, as some authors suggest that cell division is pre-requisite for leukemia cell differentiation [26]. However, the most likely explanation is that rapamycin-mediated inhibition of more distal component of the pathway interferes with complicated Akt-mTOR interrelationship. Rapamycin binds to FKBP12 to

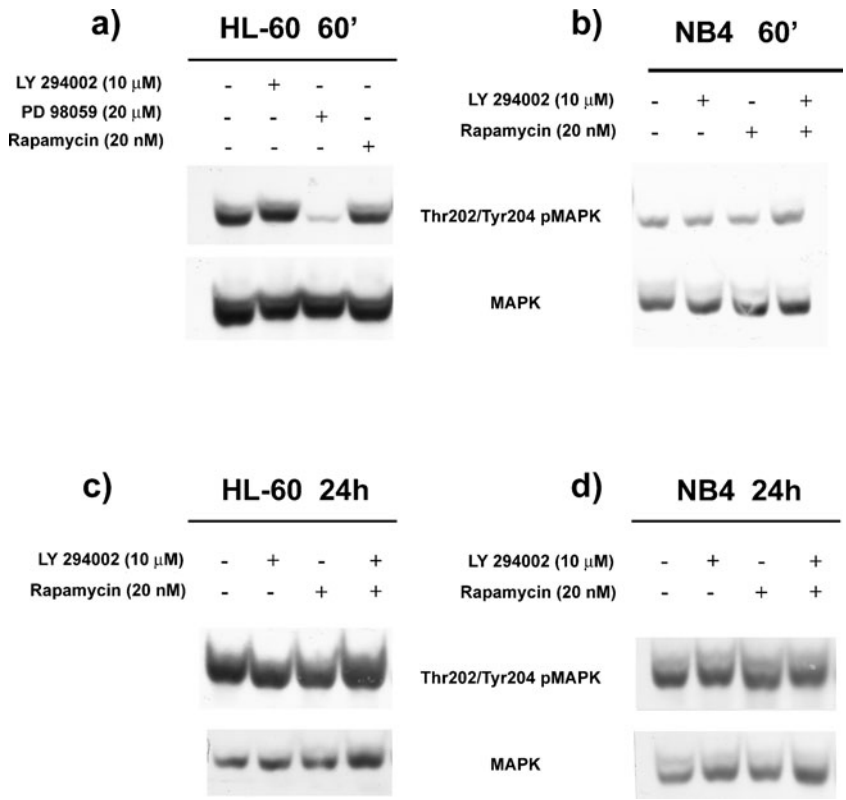

Fig. 6 MAPK activity in HL-60 and NB4 cells treated with PI3K and mTOR inhibitors. HL-60 cells (**a** and **c**) and NB4 cells (**b** and **d**) were incubated in the presence of 10 μM LY 294002, 20 nM rapamycin or the combination of inhibitors. HL-60 cells (**a**) were treated with 20 μM PD 98059 for 60 min. Cells were harvested after 60 min (**a** and **b**) or 24 h (**c** and **d**) and Western blot analysis for expression of phosphorylated and total MAPK was performed using equal amount (75 μg) of total cell lysates

inhibit mTOR complex 1 (mTOR/raptor/mLST8), mTORC1 activity down-regulates IGFR-signaling, and rapamycin-mediated inhibition of mTOR complex 1 causes an increase in IGF/PI3K/Akt-activity which may contribute to differentiation and/or increased survival of differentiated cells [8, 20, 34].

Independently of the precise mechanism involved, our data show that longitudinal inhibition of two components of the PI3K/Akt/mTOR pathway by combination of LY 294002 and rapamycin significantly inhibited ATRA-induced expression of the differentiation marker. As previously described, rapamycin alone potentiated the differentiative and antiproliferative effects of ATRA in a typical APL-cell line [21], and LY 294002 alone exerted a strong antiproliferative effect and significantly inhibited ATRA-induced expression of CD11b in NB4 cells [20]. However, the combination of rapamycin and LY 294002 inhibited ATRA-mediated increase in the expression of the differentiation marker more than the presence of PI3K alone. As previously described, MEK-inhibitor, the inhibitor of a parallel pro-survival signaling pathway, inhibited both proliferation and the expression of CD11b in leukemia cells [20, 26], but the combination of rapamycin and MEK inhibitor showed no synergistic effects.

The present study further demonstrates that the effects of the concomitant inhibition of PI3K and mTOR are not restricted only to ATRA-induced differentiation of leukemia cells, as the synergistic effects of LY 294002 and rapamycin are confirmed in a model of leukemia cells differentiated along monocytic pathway. PMA-mediated adherence and an increase in the expression of differentiation marker occurred as early as 24 h after the addition of agonist, LY 294002 inhibited the expression of CD11b, as previously described [35], rapamycin had no significant effects on PMA-induced increase, but the combination of rapamycin and LY 294002 inhibited the expression of CD11b more than PI3K inhibitor alone. The effects of MEK-inhibitors in PMA-mediated differentiation are interesting since MEK-inhibitor completely abolished PMA-mediated growth arrest, which is probably due to the role of the pathway in PMA-mediated adherence [27], but nevertheless inhibited the expression of CD11b in HL-60 cells. Again, the effects of combination of rapamycin and MEK-inhibitors were not significantly different from the effects of MEK-inhibitor alone.

In conclusion, data from the present study show that longitudinal inhibition of two components of PI3K/Akt/mTOR signaling pathway exert synergistic antiproliferative effect on several human myelogenous leukemia cell lines. However, the concomitant inhibition of PI3K and mTOR by LY 294002 and rapamycin has more inhibitory effects on ATRA-mediated differentiation than the presence of PI3K alone, and diminishes positive effects of rapamycin on leukemia cell differentiation.

Acknowledgements We thank Ms Dunja Tankovic for valuable technical help and assistance. This work was supported by the Ministry of Science, Education and Sport of the Republic of Croatia, grants No. 108-1081347-1448 (to D. V.) and 108-1081347-0173 (to H. B.).

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