Rapamycin, the mTOR kinase inhibitor, sensitizes acute myeloid leukemia cells, HL-60 cells, to the cytotoxic effect of arabinozide cytarabine

Agnieszka Janus[a], Anna Linke[a], Barbara Cebula[b], Tadeusz Robak[a] and Piotr Smolewski[b]

The mammalian target of rapamycin (mTOR) kinase is a key regulator of cell growth and proliferation. Overexpression of the mTOR signaling pathway has been described in several tumor cells, including the majority of acute myeloid leukemia (AML) cases. The anti-tumor efficacy of mTOR inhibitors was shown in several preclinical and clinical studies. In AML, however, the potential antineoplastic effect of mTOR inhibitors has received little attention thus far. In this in-vitro study of the human AML cell line, HL-60, we aimed to assess the antileukemic activity of rapamycin (RAPA), an mTOR inhibitor, alone and in combination with cytarabine (Ara-C). The study showed that RAPA in concentrations of 1–10 nmol/l arrested the cell cycle progression of Hl-60 cells in the G_1 phase, without evident cytotoxic effect. This effect was associated with significant inhibition of cyclin E expression. At concentrations higher than 10 nmol/l, RAPA exerted a significant proapoptotic effect, with the collapse of mitochondrial potential and caspase-3 activation. The most prominent proapoptotic effect was observed for a combination of 1 nmol/l of RAPA and 50 nmol/l of Ara-C, especially when Ara-C was added at a 24-h interval after RAPA. In conclusion, these data indicate that RAPA might be effective in the treatment of acute leukemia patients, especially in combination with Ara-C, the drug routinely used in AML treatment. On the basis of these results, attempts to combine classical induction chemotherapy with an inhibitor of the mTOR kinase in AML treatment could be warranted. *Anti-Cancer Drugs* **20:693–701 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Departments of [a]Hematology and [b]Experimental Hematology, Medical University of Lodz, Poland

Correspondence to Professor Piotr Smolewski, MD, PhD, Department of Experimental Hematology, Medical University of Lodz, Ciolkowskiego 2, Lodz, Poland
Tel: +48 42 689 55 83; e-mail: piotr_smolewski@wp.pl

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease in which hematopoietic progenitor cells acquire genetic lesions that lead to a block in cell differentiation, increased self-renewal, and unregulated proliferation. These events are responsible for the accumulation of malignant myeloid cells in the bone marrow and other organs [1].

Recent studies have shown that two major types of genetic alterations are required to induce leukemic transformation. There are alterations in the activity of transcription factors that control hematopoietic differentiation and activation of components of receptor tyrosine kinase signaling pathways [2,3]. Receptor tyrosine kinase mutations have been found in 50–60% of all AML cases [4]. In 70% of the cases, the deregulated activity of proteins was described in a mammalian target of the rapamycin (mTOR) signaling pathway [5]. This serine/threonine kinase controls the translation of mRNA that encodes the proteins (cyclins) required for cell cycle progression from the G_1 to the S phase. Overproduction of cyclins causes uncontrolled proliferation and leads to tumorigenesis, including AML [6]. Inhibition of the mTOR resulting in prolonged G_1 phase or arrest in G_1 has consequently become a matter of interest as a relevant therapeutic target in AML [7,8].

The mTOR kinase activity is potently inhibited by rapamycin (RAPA), an agent known for its immunosuppressant and antiproliferative activities [9–11]. The anticancer activity of RAPA and its derivatives (RAD001-everolimus; CCI-779-temsirolimus, AP23573-deforolimus) has recently been shown in numerous preclinical and clinical studies [12–16]. In addition, in May 2007, the FDA approved Torisel for the treatment of advanced renal cell carcinoma [17]. Phase III clinical trials with Torisel in advanced breast cancer and mantle cell lymphoma and AP23573 in sarcomas, and phase II clinical studies with RAD001 in advanced renal cell cancer, endometrial carcinoma, and hematological malignancies (Hodgkin/non-Hodgkin lymphomas, Waldenstrom macroglobulinemia) are still ongoing [18–26].

The potential antineoplastic effect of mTOR inhibitors in AML has received little attention thus far. Taking

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into account the promising results obtained with mTOR inhibitors in clinical trials, the high similarity of all the compounds, and the proven antiproliferative and anti-angiogenic properties of RAPA, we decided to investigate its potential effect on the growth of the myeloid cell line, HL-60. To intensify the antileukemic activity, we used a combination of RAPA with S-phase-specific cytarabine (Ara-C), commonly used for the treatment of AML. The combined action of these agents should result in an effective blockade of the cell cycle at G_1–S transition and S phase.

Materials and methods

Cell culture and reagents

The human myeloid leukemia cell line (HL-60) was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). The cell line was cultured at 37°C in a humidified atmosphere containing 5% CO_2, in RPMI 1640 (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum, 100 j/ml of penicillin, 100 mg/ml of streptomycin, and 2 mmol/l of L-glutamine (all reagents purchased from GIBCO, Life Technologies, Scotland, UK).

RAPA was purchased from A.G. Scientific Inc. (San Diego, California, USA). Ara-C was obtained from EBEWE Pharma Ges.m.b.h. Nfg.KG (Unterach, Austria).

In the first phase of experiments, the cell cultures were treated *in vitro* in increasing concentrations with RAPA (0.1–200 nmol/l) and Ara-C (10–250 nmol/l). On the basis of preliminary results, the optimal concentration of the agents provided for the next phase of the experiments was chosen (RAPA, 1 nmol/l; Ara-C, 50 nmol/l). Drug administration to cell lines was then performed as follows:

(1) Cells without drugs incubated for 24 and 48 h.
(2) Cells incubated with RAPA/Ara-C for 24 and 48 h.
(3) Cells incubated with RAPA and Ara-C for 24 and 48 h.
(4) Cells incubated with RAPA for 24 h, then Ara-C added for the 24 h incubation (RAPA 48 h and Ara-C 24 h).

The cells incubated without drugs were considered negative control.

In addition, the PI3-K inhibitor, LY294002 (Sigma Chemical Co., St. Louis, Missouri, USA) was used in optimal doses of 50–100 μmol/l.

The cells destined for cell cycle analysis and protein expression were fixed as follows: into 450 μl cells suspended in 0.5 ml of phosphate-buffered saline (PBS) (Sigma-Aldrich, Copenhagen, Denmark), and then 4.5 ml of 70% ethanol was added. To prevent the clustering of cells during fixation, the suspension was vortexed at half speed while adding ethanol. The suspension was incubated overnight at −20°C.

Assessment of apoptosis

The proapoptotic effect of the drugs was assessed by means of DNA fragmentation and confirmed by assays detecting active caspase-3 and the collapse of mitochondrial potential in the cell.

Sub-G_1 fraction

Before staining, the cells were fixed in 1% methanol-free formaldehyde (Polysciences Inc., Warrington, Pennsylvania, USA) for 15 min at 0°C, and then permeabilized in 70% ethanol (30 min at 0°C). After a 60 min incubation with the reagents, the cellular DNAs were counterstained with 5 μg/ml of propidium iodide (PI) in the presence of 100 μg/ml of RNase A (DNase-free) (Sigma-Aldrich) in PBS. Red (PI) fluorescence was measured during the next 15 min by flow cytometry. The apoptotic, hypodiploid sub-G_1 fraction was evaluated on the basis of the DNA histogram. In addition, the cell cycle phase specificity of the apoptosis triggered by the study drugs was assessed by using the same settings. The percentage of sub-G_1 cells was presented as an apoptotic index (AI).

Caspase-3 activation

Active caspase-3 was detected by using fluorescein isothiocyanate (FITC)-conjugated, monoclonal, rabbit anti-active caspase-3 antibody (BD PharMingen, San Diego, California, USA). Incubation cells were fixed and permeabilized using Cytofix/Cytoperm (BD PharMingen) solution (20 min, on ice), then washed twice and resuspended in the Perm/Wash buffer (BD PharMingen). The antibody was added at 60 μl per 300 μl of cell suspension [30 min of incubation at room temperature (RT)]. The fluorescence was measured directly after staining and washing with the Perm/Wash buffer by flow cytometry using an FL-1 filter to detect the green fluorescence of the anti-active caspase-3 antibody.

Collapse of mitochondrial potential

The collapse (dissipation) of the mitochondrial transmembrane potential ($\Delta\Psi_m$) occurs early during apoptosis and is often considered as a marker of apoptosis activated by the mitochondrial pathway. As a probe of $\Delta\Psi_m$, we used MitoTracker Red 580 dye (Molecular Probes, Eugene, Oregon, USA), which accumulates to the active mitochondria of living cells. The stock solution of MitoTracker Red (1 mmol/l) was diluted to a working concentration of 50 nmol/l by adding to the growth culture medium (20 min incubation, at RT). The drop of $\Delta\Psi_m$ has been visualized by the decrease in red fluorescence of the dye, and detected by flow cytometry using a FL-3 fluorescence filter.

Asessment of cytotoxicity using the MTT test

Cytotoxicity was evaluated using the MTT (thiazolyl blue) assay (Sigma, Steinheim, Germany). MTT stock

solution (5 mg/ml) was diluted to 0.1 mg/ml, adding a tenth volume to each culture, and incubated for 4 h. During incubation, a soluble yellow dye was converted into purple insoluble formazan by the active mitochondrial dehydrogenases of living cells. At the end of the incubation, the medium was removed and the converted dye was solubilized with 0.5 N HCl in absolute isopropranolol. The absorbance of converted dye was measured using Microplate Reader Model 550 (BioRad, Hercules, California, USA), at a wavelength of 570 nm, with background subtraction 690 nm.

Cell cycle analysis

The cell cycle was analyzed on the basis of DNA content distribution. Namely, the cells were stained with the mixture of 5 μg/ml of PI and 100 μg/ml of RNAse A (both Sigma-Aldrich) in PBS (see above). Cell green (FITC; Becton Dickinson, San Jose, California, USA) versus red (PI) fluorescence was measured over the next 15 min by dual-color flow cytometry. On the basis of the DNA histogram analysis, the percentage of cells in the G_1, S, G_2/M phases of the cell cycles was determined.

Cyclin expression

Cyclins were detected in the cells fixed with alcohol. For cell membrane permeabilization, the cells were washed twice with PBS, centrifuged at 1100*g* for 5 min and incubated with 0.25% Triton-100 for 1 min, at RT. Subsequently, specific antibodies directed against cyclins A, D3, and E (BD Pharmingen) were added to the sediment and the whole mixture was incubated for 2 h, at RT. The cells were then washed twice and resuspended in a 500 μl PI/RNase solution. All antibody dilutions were prepared in a 1% PBS–BSA solution. After 30-min incubation in the dark, at RT, fluorescence was measured using FL-1 fluorescence filter for FITC (anticyclin antibodies) and FL-3 fluorescence filter for PI fluorescence.

mTOR pathway protein expression

mTOR pathway proteins were assessed in the cells fixed with 70% alcohol and additionally permeabilized with 0.25% Triton-100 (see above). After washing with PBS, the cells were incubated with appropriate antibodies. The following proteins were assessed: phosphorylated Akt (Akt-P), p70 (S6K1), and 4EB1, using adequate primary antibodies (rabbit antibodies, all produced by Cell Signaling, Danvers, Massachusetts, USA). The cells were incubated with the antibodies at 1 : 25 dilution, at RT, in the dark, for 30 min. After washing twice and centrifugation, the secondary antibody at 1 : 20 dilution, specific to the primary antibody, was added, and the samples were incubated for 30 min, at RT, in the dark. After incubation and washing, the fluorescence was measured by flow cytometry.

Fluorescence measurements

All fluorescence measurements were performed by multicolor flow cytometry (FACScan; Becton-Dickinson), using standard emission filters.

Statistics

For the statistical analysis of the data obtained, the range of the measured variable, mean, median, and standard deviation were calculated, using statistical software (Statistica version 5.0, Tulsa, Oklahoma, USA). The comparison of more than two mean values was made to estimate the possible statistically significant differences between them and combined variance within the analyzed groups. Where significant differences existed, a comparison for all possible pairs was performed. The differences between values in more than two groups were evaluated by using the Kruskal–Wallis analysis of variance rank and median test. The Mann–Whitney test was then carried out for indirect comparisons. The correlation between the features was evaluated using the Spearman's rank coefficient. In all tests, P values of less than 0.05 were considered statistically significant.

Results

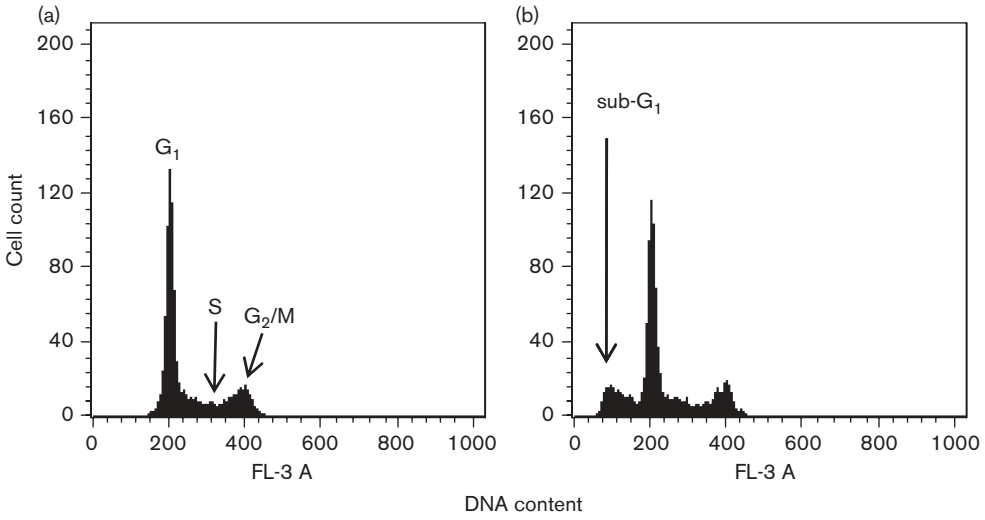
Antitumor effects of RAPA on the HL-60 cell line

In the first set of experiments, we analyzed the sensitivity of the HL-60 cells to increasing concentrations of RAPA (1–200 nmol/l). No significant apoptosis induction was observed when RAPA was used in concentrations of 1 and 5 nmol/l. However, for low concentrations of RAPA, especially of 5 nmol/l, we observed cell growth inhibition that was mainly the result of arrest in the G_1 phase of the cell cycle. The significant proapoptotic effect, as measured by the rate of sub-G_1 fraction cells (Fig. 1), was apparent for RAPA in concentrations of 10 nmol/l and higher. The mean AI for 10 nmol/l of RAPA was $9.5 \pm 1.0\%$ after 24 h and $19.3 \pm 1.9\%$ after 48 h of incubation (vs. control, $P = 0.041$ and $P = 0.027$, respectively; Fig. 2). At this dose, the cytotoxic effect of RAPA was also significant, as assessed by the MTT assay (1.5-fold and 1.7-fold increase in cytotoxicity after RAPA 10 nmol/l added for 24 and 48 h, respectively; vs. control, $P = 0.031$ and 0.021, respectively).

In samples treated with RAPA at the highest concentration used in our experiments (200 nmol/l), the mean AI was $45.3 \pm 5.3\%$ after 24 h and $61.3 \pm 4.3\%$ after 48 h of incubation (vs. controls, $P < 0.001$ for both time points; Fig. 2).

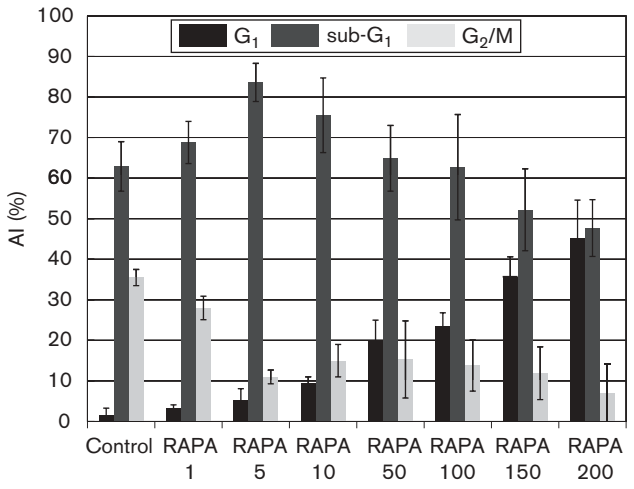
Incubation of HL-60 cells with 10 nmol/l of RAPA induced significant increase in caspase-3 activation after 24 h ($8.5 \pm 3.1\%$) and 48 h ($14.9 \pm 2.7\%$) of culture (vs. $1.8 \pm 0.7\%$ and $4.8 \pm 2.1\%$ in the respective controls; $P = 0.037$ and $P = 0.009$, respectively; Fig. 3). Moreover, we found a significant drop in mitochondrial potential in response to RAPA ($9.9 \pm 2.5\%$ cells after 24 h and

Fig. 1



Proapoptotic effect on cell cycle of rapamycin (RAPA) at concentration of 1 nmol/l in combination with cytarabine (Ara-C) at concentration of 50 nmol/l (b) in comparison with control cells (a), following 24 h incubation. The cell cycle was assessed by the DNA content analysis (staining with propidium iodide/RNase). The apoptotic cells (apoptotic index) are identified as the sub-G_1 fraction. The figure shows result of one of measurements as an example.

Fig. 2



Proapoptotic effect of rapamycin (RAPA) on HL-60 cell line. RAPA was used at concentrations 1–200 nmol/l, following 24 h incubation. Bars represent the mean apoptotic index, AI (± SD) estimated by the rate of cells in the appropriate cell cycle fractions: sub-G_1, S, G_2/M.

20.1 ± 5.9% after 48 h), compared with untreated controls ($P = 0.033$ and $P = 0.005$, respectively; Fig. 4).

Cyclins and mTOR downstream protein expression in response to RAPA treatment

RAPA in a concentration of 10 nmol/l decreased the levels of cyclin A, D3, and E. However, statistical significance has been achieved only for differences in the cyclin E level in RAPA-treated samples in comparison with the control ($P = 0.039$) (Fig. 5).

The levels of two mTOR substrates, 4E-BP1 and p70S6K1, were diminished under the influence of RAPA, when compared with the control without the drug. However, statistical significance has been reached only for 4E-BP1 ($P = 0.007$). Interestingly, treatment with RAPA seemed to increase the expression of the Akt-P protein (Fig. 6).

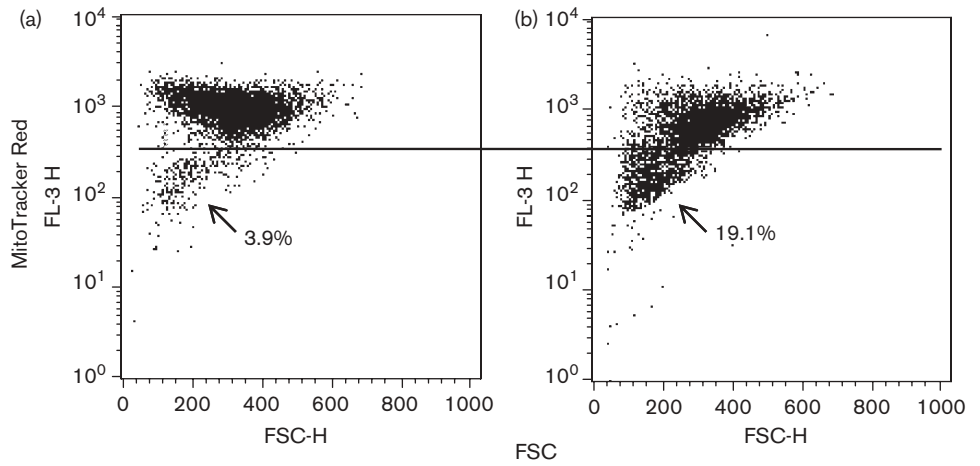
Effect of the combination of RAPA and Ara-C on the HL-60 cell line

On the basis of the initial results, we have chosen the most optimal drug concentrations for the next phase of experiments: RAPA at a concentration of 1 nmol/l (no apoptosis induction and no significant impact on cell cycle at this concentration) and Ara-C-50 nmol/l (at this concentration, Ara-C caused moderate apoptosis of HL-60 cells, with AI 15–20% after 24 h of incubation). The mean AI induced by the combination of RAPA and Ara-C after 24 h incubation was 30.1 ± 4.9% and was statistically significantly higher when compared with apoptosis induced by the drugs used alone (the mean AI for 1 nmol/l of RAPA, 3.2 ± 1.1%; for 50 nmol/l of Ara-C, 18.5 ± 5.4%; $P < 0.001$ and $P = 0.03$, respectively; Fig. 7). The combination of RAPA and Ara-C significantly increased the rate of cells, with a drop in mitochondrial potential and with activated caspase-3 (Table 1).

Pretreatment with RAPA sensitizes HL-60 cells to the proapototic effect of Ara-C

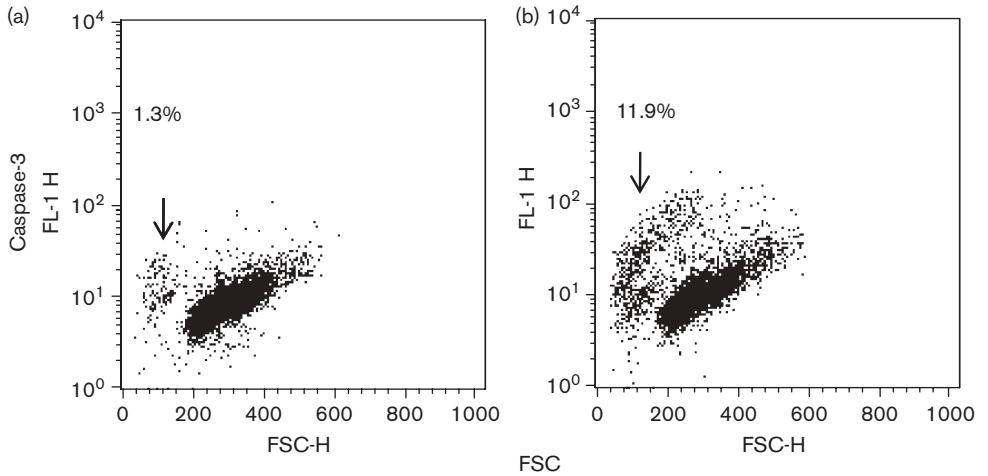
One of the most interesting observations was that preincubation with RAPA most effectively increased apoptosis. The mean AI for this combination was

Fig. 3



Effect of rapamycin (RAPA) at a concentration of 10 nmol/l on active caspase-3 expression in HL-60 cells (population of cells below the line) (b) in comparison with control cells (a). The figure shows an example of measurement. Arrows show cells with activation of caspase-3. Percentages of cells with caspase-3 activation in control and RAPA-treated sample are also shown. FSC, forward scatter.

Fig. 4



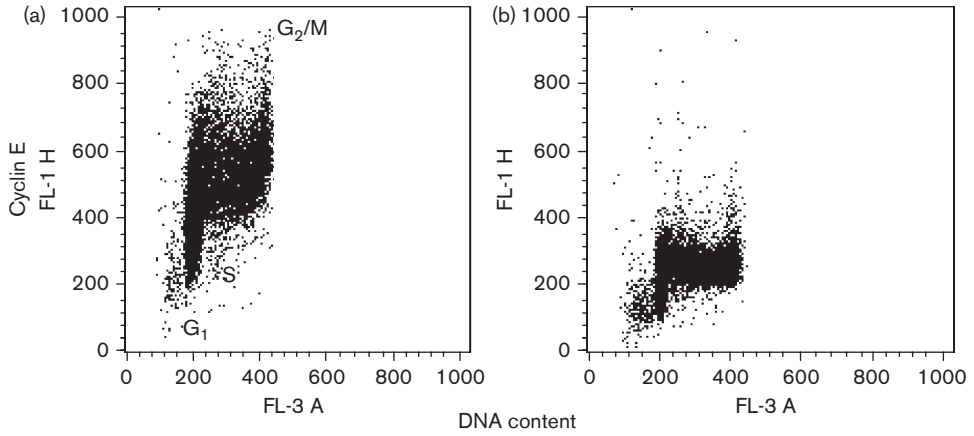
Effect of rapamycin (RAPA) at a concentration 10 nmol/l on mitochondrial membrane potential (a) of HL-60 cells in comparison with control cells (b), following 24-h incubation. Horizontal lines show the gate for discrimination of healthy cells and cells with drop of mitochondrial potential (additionally marked by arrows). The figure shows an example of measurement, with percentages of cells with drop of mitochondrial potential in control and RAPA-treated sample. FSC, forward scatter.

$50.9 \pm 6.1\%$, approximately 10-fold higher in comparison with RAPA (AI after 48 h incubation with 1 nmol/l of RAPA alone: $5.5 \pm 1.9\%$; the difference between the effect of combination and drugs used alone, $P < 0.001$) (Fig. 7).

Addition of PI3-K pathway inhibitor further increases cytotoxicity of the RAPA and Ara-C combination

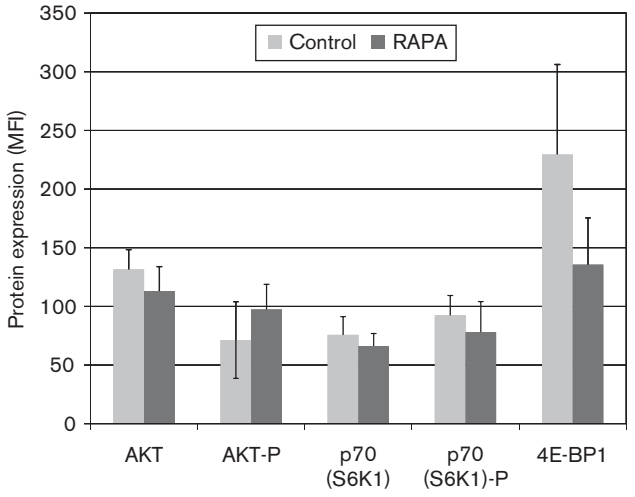
As RAPA was found to induce a slight increase in Akt protein expression, we decided to investigate the effect of an additional inhibitor of the upstream PI3-K pathway, LY294002. In combination with RAPA, LY294002 strongly enhanced its apoptotic effect. Namely, for the combination of 10 nmol/l RAPA with 25 µmol/l of LY294002, the mean AI ($36.2 \pm 5.4\%$) was significantly higher after 24 h of culture than AI for RAPA and LY294002 as single drugs (mean AIs 7.5 ± 1.1 and $28.1 \pm 4.9\%$, respectively; $P < 0.05$). The addition of Ara-C to this combination induced a further increase in cell apoptosis (mean AI $58.1 \pm 8.7\%$; vs. single agent, $P < 0.01$).

Fig. 5



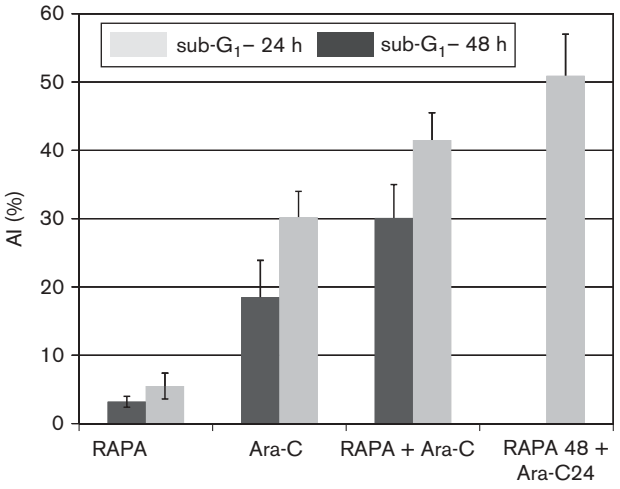
Effect of rapamycin (RAPA) at concentration of 10 nmol/l on cyclin E expression in HL-60 cells (a) in comparison with control cells (b). The figure shows the result of one measurement as an example. Expression of cyclin E was measured by means of mean fluorescence intensity.

Fig. 6



Expression of mTOR pathway proteins in the HL-60 cells after the use of rapamycin (RAPA) at concentration of 10 nmol/l in comparison with control cells. The expression of proteins was measured by mean fluorescence intensity (MFI).

Fig. 7



Incidence of apoptosis in HL-60 cells after the use of rapamycin (RAPA) at concentration of 1 nmol/l and cytarabine (Ara-C) at concentration of 50 nmol/l, alone or in combination. The cells were incubated 24–48 h or pretreated with RAPA for 24 h and subsequently exposed to Ara-C. The bars present the mean apoptotic index, AI (±SD).

Discussion

Emerging data indicate that mTOR inhibitors induce growth inhibition and apoptosis in proliferating cells [27–29]. mTOR blockade inhibits growth signal transduction and uncontrolled proliferation [30,31] Hence, cellular stress caused by mTOR inhibition activates the MAPK signaling pathway [32]. The phosphorylation of JNK (c-JUN *N*-terminal kinase) proteins activates caspases and leads to programmed cell death [33]. The identification of the cytometric DNA fragments (sub-G_1 fraction the DNA content histogram) undoubtedly confirms the proapoptotic effect of RAPA. The results, including the decrease in the mitochondrial potential and increased activity of caspase-3, show that RAPA invokes apoptosis through the mitochondrial pathway.

The effectiveness of RAPA use exists only when there is proof of constitutive mTOR activation. We checked the state of phosphorylation of Akt, S6K1, and 4E-BP1 in AML cells. We found increased activation of all the mentioned mTOR proteins and their inhibition after the use of RAPA. The antiproliferative effect of RAPA is

Table 1 Effects of 1 nmol/l RAPA alone and in combination with 50 nmol/l of Ara-C on caspase-3 activation and collapse of mitochondrial potential ($\Delta\Psi_m$), after 24-h of incubation and 48-h pretreatment with RAPA

Drugs/time points (h)	Caspase-3 activation, mean ± SD (%)	$\Delta\Psi_m$, mean ± SD (%)	Statistics
RAPA/24	3.5 ± 0.9	9.9 ± 2.5	Vs. control 24 h, $P > 0.05$
RAPA/48	6.0 ± 1.5	20.1 ± 5.9	Vs. control 48 h, $P > 0.05$
Ara-C/24	17.0 ± 4.1	14.5 ± 4.8	Vs. control 24 h, $P = 0.015$
Ara-C/48	27.1 ± 9.7	24.5 ± 8.7	Vs. control 48 h, $P = 0.003$
RAPA/24 + Ara-C/24	32.0 ± 5.6	35.1 ± 6.5	Vs. single drugs, $P < 0.01$
RAPA/48 + Ara-C/24	54.7 ± 10.3	60.3 ± 11.5	Vs. single drugs, $P < 0.001$

Ara-C, cytarabine; RAPA, rapamycin.

attributed mainly to the inhibition of cyclins activation, which is crucial for cell cycle progression from the G_1 to the S phase. In our experiments, we confirmed the diminished activation of cyclins A, D, E under the influence of RAPA. As shown in the analysis by Decker *et al.* [34,35] this cell cycle block was also associated with an inhibition of pRb (retinoblastoma protein, pRb) phosphorylation. Having obtained confirmation of sensitivity to RAPA, we moved to the subsequent phase of experiments assessing the utility of mTOR inhibitors in acute leukemia.

Similar to the study by Recher *et al.* [36], we showed a rather modest cytotoxic effect of RAPA on the HL-60 cell line. After 24-h incubation with increasing concentrations, the highest rate of apoptotic cells observed was 61.3%, and this was achieved with the highest concentration used (200 nmol/l). Although the low concentrations of RAPA (1–5 nmol/l) did not show any antiapoptotic effect, 5 nmol/l of RAPA caused an increase in rate of G_1-phase cells, which confirms the antiproliferative features of the drug. The proliferative status of the cell seems to be crucial for the effectiveness of the mTOR inhibitors. According to French data, 80% effectiveness could be observed in the most immature AML cell line – KG1 – exposed on drug action in a concentration of 10 nmol/l [37]. In addition, the analysis of the cell cycle showed that inhibition of growth was entirely the result of arrest in the G_1 phase. In contrast, the cell lines, UT-7GM and UT-EPO, which are supposed to be equivalents of the most differentiated acute leukemias, were completely resistant to the cytotoxic action of RAPA.

The increased state of phosphorylation was confirmed in the experiments carried out *ex vivo* by Recher *et al.* [37], and became a reason for the French group to carry out a pilot study with RAPA alone in nine patients with refractory/recurrent AML. However, partial remission (defined as > 50% decrease of blasts in bone marrow or peripheral blood) was observed in just four patients, and normalization of neutrophil count and red blood cell/platelets transfusion independence was observed in two other patients, and the mean response time was 38 days [37].

Emerging data indicate that the antiapoptotic effect of mTOR pathway inhibitors might be strengthened by adding cytostatics [38–40]. Plo *et al.* [41] found a more potent antiapoptotic effect on the U937 cell line by evaluating the combination of daunorubicin with LY294002. Sensitization of the HL-60 human cell line under the influence of RAPA was also shown by Shi *et al.* [42]. The apoptotic effect of cisplatin on leukemic cells was detected at concentrations of 5–10 μmol/l of the drug. The use of just 1 nmol/l of RAPA allowed a decrease in the dose of cisplatin to 2.5 μmol/l to obtain the comparable proapoptotic activity. The studies by O'Gorman *et al.* [43], in turn, back up the crucial role of the PI3-K/Akt pathway in the development of drug resistance. As a result of the use of LY294002, the authors observed a 2.5-fold increase in sensitivity to cytostatics (mitoxantrone, etoposide, and doxorubicin) on the HL-60 cell line obtained from the patient with refractory AML. The determination of mTOR pathway protein activity in these cells showed upregulation of Akt, which was effectively diminished by LY294002 at a concentration of 30 μmol/l [43]. In addition, RAPA was shown to increase the activity of Akt kinase [44]. This may be a mechanism diminishing the antitumor effect of RAPA. We showed a similar tendency with regard to leukemic cells *in vitro*. Therefore, in one phase of experiments, we investigated the activity of RAPA in combination with Ara-C and the PI3-K inhibitor, LY294002. The addition of LY294002 significantly increased the proapoptotic action of the RAPA + Ara-C combination.

Given the data on the combined therapy, we addressed the question of whether the combination with Ara-C, commonly used for the treatment of AML, would strengthen the apoptotic effect on leukemic cells. As expected, AI for cells incubated with a combination of the both agents was significantly higher than the apoptotic effect observed after adding single drugs. However, the most potent proapoptotic effect that we observed was for the combination of RAPA and Ara-C added after a 24 h interval. This extremely interesting observation enables us to conclude that pretreatment with RAPA sensitizes the cells to chemotherapy through unknown mechanisms. Similarly, it allows the best possible therapeutic effect to be obtained.

The mechanism of enhanced chemotherapy toxicity after the addition of the mTOR kinase inhibitor remains inexplicable. The studies carried out by Beuvink *et al.* on nonmicrocellular lung cancer cell lines showed that

everolimus, RAPA derivative inhibited expression of p21 protein and, in this way, enhanced sensitivity to cisplatin [45,46].

Cisplatin acts by cross-linking DNA, which interferes with the replication process and induces p53-dependent repair mechanisms in the cell [47,48]. Low-dose cisplatin activates p53 and p53-dependent activation of p21, and then binds with cyclin D1 and inhibits the cell cycle in the G_1 phase. High-dose cisplatin inhibits p21 activation, acts in a proapoptotic manner, but is simultaneously more toxic [49,50]. By adding everolimus and mTOR blockade, the translation of cell cycle proteins, including p21, is inhibited, without increasing toxicity and impairment of anticancer activity.

Our study, and the results of pilot clinical trials, indicate that RAPA – used alone or in combination – might be effective in the treatment of acute leukemia patients. We showed the antitumor activity of the mTOR kinase inhibitor, RAPA, against AML cells *in vitro*. The activity was significantly increased in combination with a drug routinely used in AML treatment, Ara-C. Additional inhibition of upstream PI3-K further enhanced this cytotoxicity. As RAPA and Ara-C exert a significantly increased antileukemic effect, attempts to combine the standard induction chemotherapy ('3 + 7' regimen) with the mTOR kinase inhibitor in AML treatment could be warranted.

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