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Dembitz, Vilma; Lalić, Hrvoje; Ostojić, Alen; Vrhovac, Radovan; Banfić, Hrvoje; Višnjić, Dora

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The mechanism of synergistic effects of arsenic trioxide and rapamycin in acute myeloid leukemia cell lines lacking typical t(15;17) translocation

Vilma Dembitz[1], Hrvoje Lalic[1], Alen Ostojic[2], Radovan Vrhovac[2, 3], Hrvoje Banfic[1], Dora Visnjic[1]*

Running head: Synergism of ATO and rapamycin in non-APL AML

Original article

[1]Department of Physiology and Croatian Institute for Brain Research, School of Medicine, University of Zagreb, Salata 3, 10 000 Zagreb, Croatia

[2]Department of Hematology, University Hospital Centre Zagreb, Kispaticeva 12, 10 000 Zagreb, Croatia

[3]Department of Internal Medicine, School of Medicine, University of Zagreb, Salata 3, 10 000 Zagreb, Croatia

* Corresponding author: prof dr Dora Visnjic, Department of Physiology and Croatian Institute for Brain Research, School of Medicine, University of Zagreb, Salata 3, POB 978, 10 001 Zagreb, Croatia.

Tel: 385-1-4596-831,

Fax: 385-1-4590-207.

E-mail: visnjic@mef.hr

Abstract

Arsenic trioxide (ATO) has potent clinical activity in the treatment of patients with acute promyelocytic leukemia (APL), but is much less efficient in acute myeloid leukemia (AML) lacking t(15;17) translocation. Recent studies indicated that the addition of mammalian target of rapamycin (mTOR) inhibitors might increase the sensitivity of malignant cells to ATO. The aim of this study was to test for the possible synergistic effects of ATO and rapamycin at therapeutically achievable doses in non-APL AML cells. In HL-60 and U937 cell lines, the inhibitory effects of low concentrations of ATO and rapamycin were synergistic and more pronounced in U937 cells. The combination of drugs increased apoptosis in HL-60 cells and increased the percentage of cells in G_0/G_1 phase in both cell lines. In U937 cells, rapamycin alone increased the activity of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and the addition of ATO decreased the level of phosphorylated ERK, Ser473 phosphorylated Akt and antiapoptotic Mcl-1 protein. Primary AML cells show high sensitivity to growth-inhibitory effects of rapamycin alone or in combination with ATO. The results of this study revealed the mechanism of the synergistic effects of two drugs at therapeutically achievable doses in non-APL AML cells.

Keywords

arsenic trioxide; rapamycin; U937; HL-60; CD64

Introduction

Arsenic trioxide (ATO) has potent clinical activity in the treatment of patients with acute promyelocytic leukemia (APL), a rare form of acute myeloid leukemia (AML) carrying typical t(15;17) translocation [1]. Differentiation therapy of APL with all-*trans* retinoic acid (ATRA) in combination with chemotherapy induces complete remission in more than 90% of patients, and the introduction of ATO into treatment of refractory or relapsed APL further improves the clinical outcome of the disease. The latest results of clinical studies instituting ATO in first-line treatment showed that ATRA plus arsenic trioxide might be superior to ATRA plus chemotherapy in the treatment of patients with low-to-intermediate-risk APL, suggesting the possibility of leukemia treatment without any DNA-damaging chemotherapy [2]. Mechanistically, both ATRA and ATO act on the promyelocytic leukemia (PML)/retinoic acid receptor α (RARα) fusion protein, which is encoded by the t(15;17) translocation typical for APL. Pharmacological doses of ATRA bind to RARα, relieve a transcription block and allow granulocytic differentiation, while ATO binds directly to the PML moiety, stimulates ubiquitination and degradation of PML-RARα, and induces apoptosis or partial differentiation of leukemia cells [3]. Importantly, the combination of ATRA and ATO promotes clearance of PML-RARα+ leukemia-initiating cells (LIC), resulting in APL eradication in murine models and patients [4]. In other types of AMLs, much higher concentrations of ATO are needed for antiproliferative effects, and the mechanisms of ATO-mediated responses are only partially understood [5-7]. However, many recent studies suggest a therapeutic potential of ATO in non-APL AMLs, especially in combination with drugs that target proliferative or survival pathways [8-10].

The phosphatydilinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is one of the principal signaling mechanisms regulating cell growth,

survival, proliferation and metabolism [11]. The most established inhibitors of the pathway are mTOR inhibitors, rapamycin and its analogs (rapalogs), which have been used as immunosuppressants for decades. Although rapalogs have been shown to exert antiproliferative and differentiative effects on AML cells in vitro [12, 13], clinical trials have revealed only a modest efficacy when they are used as a single agent [11]. However, several recent studies indicated that the addition of ATO may increase the sensitivity of malignant cells to mTOR inhibitors; ATO-mediated degradation of PML abolished resistance of glioblastoma cells to rapamycin [14], ATO synergized with everolimus to induce cytotoxicity of ovarian cancer cells [15], and anti-tumor effects of rapamycin in breast cancer were enhanced by the addition of ATO [16]. In studies using primary hematopoietic progenitors from patients with AML, Altman et al. [17] found that pharmacological inhibition of mTOR enhances the suppressive effects of ATO on leukemic progenitor colony formation.

Our previous studies suggested that rapamycin-mediated enhancement of the expression of differentiation markers in AML cell lines depends on the activity of the PI3K/Akt pathway and that the effect is not common to all differentiation inducers [18-20]. The aim of the present study was to investigate the possible synergistic effects of ATO and rapamycin on the proliferation, survival and differentiation of AML cell lines and primary AML cells that lack typical t(15;17) translocation.

Materials and methods

Chemicals and reagents

Arsenic trioxide (A1010) was obtained from Sigma (St. Louis, MO), dissolved in 1M NaOH to a concentration of 100 mM and further diluted in RPMI-1640 to a stock concentration of 1

mM. Rapamycin (#553210), all-*trans* retinoic acid (ATRA) (#554720) and PD 98059 (#513000) were purchased from Calbiochem (San Diego, CA) and dissolved in 100% DMSO to stock concentrations of 20 μM, 1mM and 50 mM, respectively. Thiazolyl blue tetrazolium bromide (for MTT assay, M2128), color markers, bovine serum albumin (BSA), Triton X-100, sodium dodecyl sulfate (SDS), leupeptin, phenylmethylsulfonyl fluoride (PMSF), propidium iodide (PI), RNase A, Igepal and monoclonal anti-β-actin antibody (#A5441) were purchased from Sigma; enhanced chemiluminescence (ECL) substrate for the detection of horseradish peroxidase (HRP) was from Pierce (Rockford, IL); and RPMI-1640, fetal bovine serum (FBS) and penicillin/streptomycin were from Gibco/Invitrogen (Grand Island, NY). Cell lysis buffer (#9803) and antibodies against Mcl-1 (#5453), total S6K (#9202), phosphorylated (Thr389) S6K (#9205), total p44/42 mitogen activated protein kinase MAPK (#9102), phosphorylated (Thr202/Tyr204) MAPK (#9101), total Akt (#4691), phosphorylated (Ser473) Akt (#4060), secondary HRP-conjugated anti-rabbit immunoglobulin G (IgG) (#7074) and anti-mouse IgG (#7076) were obtained from Cell Signaling Technology (Beverly, MA). The annexin V - fluorescein isothiocyanate (FITC) kit (IM3546), FITC-conjugated monoclonal antibodies against CD11b (IM0530), CD64 (IM1604) and FITC-conjugated mouse IgG1 (IM0639) were obtained from Immunotech Beckman Coulter (Marseille, France).

Cell culture and viability

HL-60 cells (ECACC no. 88112501) were purchased from the European Collection of Animal Cell Cultures (HPA, Porton Down, UK). NB4 and U937 were kindly provided by Dr. M. Golemovic (Clinical Hospital Zagreb, Croatia). All cells were passaged routinely in RPMI-1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin in a 5% CO_2 humidified atmosphere at 37 °C. For the experiments, exponentially growing cells were harvested by centrifugation and resuspended in

fresh medium containing 10% FBS. Unless stated otherwise, the cells were seeded at an initial cell density of $2x10^5$/mL in BD Falcon six-well plates (BD Biosciences, Franklin Lakes, NJ). ATO, rapamycin and other compounds were added to the cultures at the times and concentration indicated in the Figure legends. For longer incubations, 0.3 mL of fresh medium was added to each well at day 2. Viable cells were determined by trypan blue exclusion and quantified using a hemocytometer.

Cell cycle analysis

At the end of incubation, cells were collected, washed with ice-cold phosphate buffered saline (PBS) and stained directly with PI solution (50 μg/mL of PI, 10 mM Tris, pH 8.0, 10 mM NaCl, 10 μg/mL RNase A, 0.1% Igepal) for 30 min. Monoparametric DNA analyses were performed on at least 10 000 cells for each sample using the FACSCalibur system and CellQuest software (Becton Dickinson). The percentages of cells in different phases of the cell cycle and in the sub-G_1 region were determined using ModFit software (Becton Dickinson).

MTT assay

To determine the effects of ATO and rapamycin on the proliferation and viability of HL-60 and U937 cells, colorimetric assays were performed. Cells were seeded at an initial density of $1.2x10^4$/well in 96 well culture plates (BD Falcon) and incubated with six different concentrations of ATO, rapamycin, and their combinations. After 96 h, 20 μL of MTT solution (5 mg thiazolyl blue tetrazolium bromide per 1mL of PBS) were added to each well, and cells were incubated for 3 h at 37 °C. After incubation, plates were centrifuged at 250xg for 5 min, supernatant was discharged, and remaining crystals were dissolved in 180 μL of DMSO. The absorbance of the sample was read at 540 nm (Dynatech MR5000, Dynatech Technology, Inc., Horsham, PA, USA).

Synergism analysis

Synergistic effects for the combination of arsenic trioxide and rapamycin were assessed by applying the method of Chou and Talalay [21]. Briefly, results of the MTT assay were expressed as fractions of cells affected (Fa) in comparison to control and the Combination Index (CI) was calculated using CompuSyn software (ComboSyn Inc.). CI>1 indicates antagonism, CI=1 indicates additive effects and CI<1 indicates synergism.

Detection of apoptosis

After incubation, cells were collected, washed with ice-cold PBS and labeled with annexin V – FITC and PI, according to the manufacturer's instructions. Samples were analyzed using the FACSCalibur system and Cell Quest software (Becton Dickinson).

Expression of differentiation markers

After incubation, cells were collected, washed and incubated with FITC-conjugated monoclonal antibodies against CD11b, CD64, or with isotypic (FITC-IgG_1) control for 20 min. Samples were analyzed using a FACSCalibur system and Cell Quest software (Becton Dickinson), as previously described [18-20]. Briefly, live cells were gated based upon forward and side scatter patterns and a total of 15 000 events were collected for each marker from the gated area. Results were expressed as both mean fluorescence intensity (MFI) of the sample and percentage of positive cells, with isotypic control MFI levels and percentage deducted from all values.

Isolation of total cell lysates and Western blot analysis

Cells were seeded at a concentration of $0.3x10^6$/mL in T25 cm^2 flasks and incubated for 3 or 24 h. After incubation, cells were collected by centrifugation, washed in ice-cold PBS, resuspended in 1x cell lysis buffer containing freshly added 1 mM PMSF and 1 μg/ml leupeptin, and incubated on ice. After 10 min, cells were disrupted by seven passages through a 23-gauge needle. Lysates were incubated on ice for an additional 10 min, and then

centrifuged at 14 000 x *g* for 10 min at 4 °C. The supernatants were collected and stored at -80 °C. The protein concentration in each sample was determined by a Bio-Rad protein assay.

Western blot analysis was performed as previously described [20]. Briefly, equal amounts of proteins (50 μg) in each sample were loaded on two parallel 8% SDS-polyacrylamide gels. Electrophoresis and transfer were carried out using Bio-Rad mini-Protean and mini Trans-Blot systems. After transfer, nitrocellulose membranes were blocked 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, the membranes were washed three times in TBS-Tween and incubated overnight at 4 °C with primary antibodies diluted (1:20000 for actin; 1:1000 for other antibodies) in TBS-Tween containing 5% BSA. After incubation, membranes were washed and incubated in appropriate secondary antibodies for 120 min at room temperature. The antibody binding was detected using the ECL kit according to the manufacturer's suggestion.

Primary patient samples

Peripheral blood was obtained from patients with newly diagnosed AML (with exclusion of APL) at the time of diagnosis. All patients provided written informed consent in accordance with the Declaration of Helsinki and the study was approved by the Institutional Review Board of the University of Zagreb School of Medicine. Mononuclear cells were isolated by density gradient centrifugation with NycoPrep^{TM} 1.077 (Fresenius Kabi Norge AS for Axis-Shield PoC AS, Oslo, Norway) and further purified by overnight adherence to plastic. The next day, cells were collected, washed, resuspended in fresh RPMI-1640 medium supplemented with 20% FBS and seeded at an initial density of $1.0x10^6$/well in 96-well plates (BD Falcon). Cell viability was tested by MTT assay after 96-hours incubation with ATO (0.5 or 1 μM), rapamycin (20 nM), ATRA (1 μM) or their combination. Each experiment was done in triplicate and results are expressed as percentage of control.

Statistical analysis

Data are shown as means ± SEM. The Student's *t* -test was used to assess the difference between each group and control, and one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test, was used to assess the difference between dual-compound treated cells versus cells treated with the same concentration of each agent alone. Statistical significance was considered to be achieved when $p<0.05$.

Results

Sensitivities of a typical APL, and two AML cell lines lacking t(15;17) translocation, were tested first by measuring the sub-G_1 fraction of propidium labeled cells 24–48 h after the addition of increasing doses of ATO. As shown in Figure 1a, the addition of ATO significantly increased the sub-G_1 fraction of NB4 cells carrying typical t(15;17) translocation. The HL-60 cell line was derived from a patient that was initially considered to have APL or AML-M3, but had atypical clinical and cytogenetic features and was more appropriately classified as an acute myeloblastic leukemia with maturation (AML-M2) [22]. As previously reported [5, 6], the sensitivity of myeloblastic HL-60 and promonocytic U937 cell lines to ATO was low, and the sub-G_1 fraction of HL-60 cells was significantly increased only after addition of the highest concentration of ATO (5 μM). Therefore, we next tested whether the addition of rapamycin might potentiate the effects of ATO in non-APL AMLs.

Colorimetric MTT assays were performed on HL-60 and U937 cells that were incubated in the presence of ATO (0.25–16 μM), rapamycin (5-320 nM), and the combination of agents for 96 h. As shown in Figure 1b, the presence of rapamycin alone, when applied at doses that are therapeutically achievable, had no marked effects on the proliferation of

leukemia cells. However, adding increasing doses of rapamycin to ATO resulted in increased cytotoxicity when lower concentrations of ATO were used. In U937 cells, combination indices that were calculated using CompuSyn software were below 0.3 for the combination of low doses of rapamycin (10-20 nM) and low doses of ATO (0.5-1 μM), indicating that the interaction between rapamycin and ATO was highly synergistic. The inhibitory effects of these concentrations of rapamycin and ATO on the number of viable cells were further confirmed using a hemocytometer and trypan blue staining (Figure 1c).

To further characterize the mechanism of synergistic effects in AML cell lines, the effects of 20 nM rapamycin on the percentage of sub-G_1 in control and ATO-treated HL-60 and U937 cells were tested. As shown in Figure 2a, no significant increase in the sub-G_1 fraction was measured 48 h after addition of drugs. Annexin V/PI staining confirmed that no significant increase in the percentage of annexin V positive cells could be observed 48 h after exposure (Figure 2b). However, longer (96 h) incubation of cells with the combination of 20 nM rapamycin and 1 μM ATO significantly increased the percentage of apoptotic cells. Previous studies demonstrated that some growth-arresting properties of rapamycin are due to arrest in G_0/G_1 phase [19, 20]. To test the effects of the combination of agents, cell cycle analyses were performed on HL-60 and U937 cells that were incubated in the presence of the drugs for 24 h. As shown in Figure 2c, rapamycin significantly reduced the percentage of HL-60 cells in S phase and U937 cells in G_2/M phase, while the percentage of cells arrested in G_0/G_1 phase was significantly higher following treatment with the combination of agents.

Rapamycin-mediated effects may be limited by activation of feedback mechanisms resulting in survival signals, including engagement of both ERK and Akt signaling pathways [16, 23, 24]. To study the effects of the combination of the agents on the activity of ERK, Western blot analyses were performed on total cell lysates isolated from HL-60 and U937 cells treated with ATO (0.5 and 1 μM), 20 nM rapamycin, and the combination of agents for

30 min. As shown in Figure 3a, the basal level of Thr389 phosphorylated p70 S6K, a downstream target of an activated mTOR, was high in U937 cells, as previously described [20], but the presence of 20 nM rapamycin completely abolished the phosphorylation in control and ATO-treated HL-60 and U937 cells. In U937 cells, rapamycin alone induced an increase in the level of Thr202/Tyr204 phosphorylated MAPK that was inhibited by the addition of ATO (Figure 3b). In contrast, no increase in the level of phosphorylated MAPK was detected in rapamycin-treated HL-60 cells, as previously described [19]. The presence of ATO (1 μM) alone had modest stimulatory effects on the phosphorylation of both S6K and MAPK in HL-60 cells, but no similar effects in U937 cells. To further test for the effects of ATO alone on the activity of both signaling pathways in U937 cells, cells were incubated with increasing doses of ATO for 3 and 24 h. As shown in Figure 4, longer incubation of U937 cells with higher doses of ATO inhibits the phosphorylation of both p70 S6K and MAPK.

In NB4 cells, ATO-induced inhibition of ERK signaling is correlated with reduced levels of an anti-apoptotic protein Mcl-1 [10]. The expression of Mcl-1 has previously been reported to decrease as a downstream effect of mTOR inhibition [25]. We studied next the levels of Mcl-1 in U937 cells treated with ATO, rapamycin and their combination for 24 h. As shown in Figure 5a, the level of Mcl-1 was lower in cells treated with combination of rapamycin and higher doses of ATO (1 μM). To further test for the activity of other enzymes known to mediate proliferative signals, the level of activated Akt was measured in U937 cells. In some cancer cell lines, treatment with mTOR complex 1 inhibitors has been reported to induce phosphorylation of Akt on Ser473 by negative feedback involving mTOR complex 2 molecules [16]. As shown in Figure 5b, short duration (3 h) treatment with rapamycin had some modest stimulatory effects on the level of phosphorylated Akt, but the level was low after 24 h and further decreased in cells treated with the combination of rapamycin and ATO.

When applied in low doses, ATO promotes differentiation of NB4, a typical APL cell line [26]. To test for the possible differentiative effects of the combination of ATO and rapamycin in U937 cells, the expression of cell surface markers was determined by flow cytometry, and the presence of ATRA (1 μM) alone or in combination with 20 nM rapamycin was used as a positive control [27]. A slight increase in the mean fluorescence intensity (MFI) of U937 cells stained with either CD64 (Figure 6a) or CD11b (Figure 6b) was observed after 96 h incubation in the presence of 1μM ATO with no significant increase in the percentage of positive cells. Rapamycin alone had no significant effects on the expression of CD11b (Figure 6b) but significantly decreased both MFI and the percentage of cells expressing a monocytic CD64 marker (Figure 6a). When two different concentrations of ATO were added to rapamycin pretreated cells, some increase in the expression of CD64 was observed suggesting the potential of ATO to overcome a rapamycin-induced decrease in the expression of markers of differentiated monocytes. Although the expression of CD11b was significantly higher in cells treated with a combination of agents in comparison to control or cells treated with rapamycin alone, no significant differences were observed in the level of CD11b expression in comparison to cells treated with ATO alone. The possible role of ERK activation in rapamycin and ATO-mediated effects on the expression of differentiation markers was further tested by the addition of PD 98059, a pharmacological inhibitor of MAPK that acts by preventing the activation of an upstream kinase, mitogen-activated protein kinase kinase (MAPKK or MEK). As shown in Figure 5c, the pretreatment of cells with PD 98059, at a dose (20 μM) that was previously shown to inhibit ERK in U937 cells [28], reduced the basal level of CD11b and abolished an increase in the expression induced by ATO alone or in combination with rapamycin. In addition, PD 98059 significantly reduced the level of CD64 in control cells as well as the cells treated with rapamycin alone or in the combination with ATO.

Finally, combination effects of ATO and rapamycin were examined in primary AML cells isolated from the peripheral blood of three AML patients (Table 1). As shown in Figure 7, AML cells show different sensitivities to increasing doses of ATO, but the lowest number of viable cells was detected in cells treated with rapamycin alone or in the combination with 1 μM ATO.

Discussion

ATO has been shown to exert cytotoxic effects in various solid tumors and non-APL leukemias but at concentrations that may not be clinically achievable because of toxicities. Therefore, a possible approach to promote generation of its effects at lower concentrations is to combine this agent with other drugs that may inhibit growth, promote apoptosis or induce differentiation of leukemia cells. The results of the present study show that a combination of ATO and rapamycin at therapeutically achievable doses exerts synergistic antiproliferative effects in two AML cell lines lacking typical t(15;17) translocation. Similar to results obtained by Altman et al. [17], our data confirmed that rapamycin and ATO inhibit the growth of cells isolated from peripheral blood of newly diagnosed AML patients.

The beneficial effects of the combination of ATO and rapamycin, as measured by MTT assay, were more pronounced in promonocytic U937 cells that have been previously shown to have a high basal level of phosphorylated p70 S6K [20], a principal downstream target of an activated mTOR. Pharmacological inhibitors of mTOR are known to exert multiple effects on cell metabolism [29], and the effects of drugs that target metabolism are known to depend on the intrinsic metabolic characteristics of the used leukemia cell line [30]. It is important to note that tetrazolium dye reduction is dependent on NAD(P)H-dependent oxidoreductase enzymes and that formazan deposits increase in metabolically active and

decrease in quiescent viable cells. Therefore, results of MTT assays may be misleading as they depend more on the metabolic activity of the cell than on the viability itself [31]. However, results obtained by trypan blue exclusion confirmed that the addition of rapamycin significantly potentiated the inhibitory effects of ATO on the number of viable HL-60 and U937 cells.

The results of this study indicate that the synergistic effects of the combination of drugs at low concentrations may be partially explained by potentiation of apoptosis. In U937 cells, ATO (1 μM) alone increases the percentage of annexin V-positive cells and decreases the phosphorylation of ERK, while rapamycin alone decreases the percentage of annexin V-positive cells and increases the ERK activity. As rapamycin-mediated activation of ERK is reduced by co-treatment with ATO, these results suggest that co-treatment with ATO efficaciously reduces survival via ERK inhibition. Regarding the effects on the PI3K/Akt/mTOR signaling pathway, both ATO and rapamycin reduce the phosphorylation of p70 S6K, a downstream target of activated mTOR. In various cancer cell lines, treatment with mTORC1 inhibitors such as rapamycin induces upregulation of Akt phosphorylation by a negative feedback effect [16]. However, previous studies have suggested that some cell lines (including U937 cells) and primary AML cells showed dose-dependent alterations in p-Akt levels after prolonged treatment with an mTOR inhibitor [32, 33]. Results of our study that revealed a modest increase in the level of Ser473-phosphorylated Akt in rapamycin-treated U937 cells after short-term treatment (3 h) and a decrease after longer treatment (24 h) are consistent with previous reports in U937 cells [32, 34]. Again, the lowest levels of Ser473 phosphorylated Akt protein were detected in cells treated with combination of rapamycin and ATO suggesting that a part of the antiproliferative effects of ATO and rapamycin may be due to inhibition of Akt signaling pathway.

Similar to results reported previously, the presence of low doses of ATO alone for 24 h had no effects on cell cycle distribution of both HL-60 and U937 cells [35]. In contrast, the addition of rapamycin affected cell cycle transition and significantly increased the percentage of ATO-treated U937 cells arrested in G_1 phase. The mechanism responsible for rapamycin-mediated growth arrest in G_1 phase is well described in many cell types, including leukemia cells [20, 27], but recent studies indicated that activated mTOR may have a role in other phases, including G_2/M cell transition [36]. As the G_2/M phase has been described to be the most sensitive to arsenite-induced apoptosis in U937 cells [6], the significant decrease in the percentage of U937 cells in G_2/M phase observed in our study may be partially due to the synergistic effects of rapamycin-induced arrest in cell cycle and ATO-mediated toxicity.

In addition to protective and antiapoptotic effects, the activation of ERK is commonly attributed to have a role in differentiation of myeloid leukemia cell lines [37], including PMA-mediated differentiation of U937 cells into macrophages [38] or SET-induced differentiation into dendritic-like cells [39]. Both ATO and rapamycin have been previously described to induce some differentiation; ATO induced the expression of differentiation markers at doses that are too low to induce apoptosis [26, 40] and rapamycin only at concentrations that are much higher than the range of therapeutically achievable doses [41]. However, at the concentration used in our study, rapamycin enhanced expression of CD11b in response to ATRA [19, 28] and vitamin D_3 [42], and had no effects in cells treated with PMA [19] or DMSO [20]. The results of the present study show some modest increase in the level of CD11b in ATO-treated cells but these levels were not significantly enhanced by the addition of rapamycin. However, when the effects of both agents were tested by measuring the levels of monocytic marker CD64, ATO and rapamycin exerted different effects and rapamycin alone significantly reduced the level of CD64. Although the effects of rapamycin or its derivatives on the expression of CD64 in U937 cells have not been reported, a decrease in

CD64 surface marker expression has been recently reported in rapamycin-treated human macrophages *in vitro* [43] and similar inhibitory effects on endocytosis were reported in bone marrow-derived dendritic cells [44]. In both studies, the inhibitory effects of rapamycin on the expression of receptors involved in phagocytosis and antigen uptake occurred independently of either M1 and M2 polarization of macrophages [43] or dendritic cell maturation [44] suggesting that these effects are not linked to differentiation. In our study, the results obtained with MEK-inhibitors suggest that ERK activity may be important for the expression of CD11b as a differentiation marker, but the presence of MEK-inhibitor did not prevent rapamycin - mediated decrease in CD64. In addition, our recent study showed that the presence of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) increased the activity of ERK, decreased the level of phosphorylated p70 S6K and induced the expression of both CD11b and CD64 in U937 cells [28]. Since both AICAR and rapamycin have similar effects on the activity of signaling pathways, yet different effects on CD64 expression, these results further suggest that neither ERK nor p70 S6K activity are responsible for the rapamycin-mediated CD64 decrease. To conclude, results of this study indicate that the synergistic antiproliferative effects of ATO and rapamycin cannot be ascribed to synergistic effects on differentiation.

In summary, results of the present study show that ATO and rapamycin at concentrations that are therapeutically achievable have synergistic antiproliferative effects in two AML cell lines lacking typical t(15;17) translocation. As both drugs are already in clinical use, these results further support the role that combination therapies may have in non-APL AML patients.

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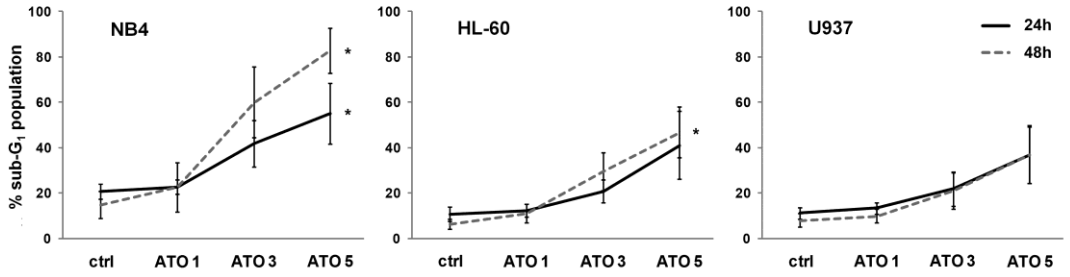
Figure Legends:

Fig. 1 Arsenic trioxide (ATO) decreases AML cell viability in a dose-dependent manner and acts in synergy with rapamycin

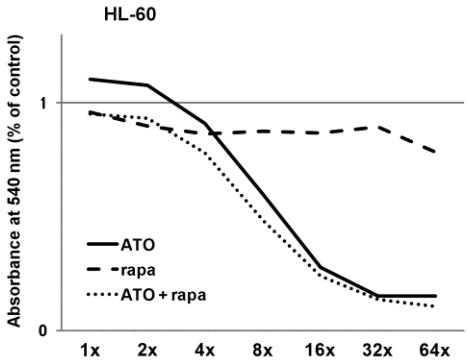
a) NB4, HL-60 and U937 cells were seeded at an initial concentration of $2x10^5$/mL and incubated for 24 or 48 h with increasing doses of ATO (1-5 μM) or vehicle (control). After incubation, cells were stained with propidium iodide and the proportion of sub-G_1 fraction was determined using flow cytometry. b) HL-60 and U937 cells were treated with increasing concentrations of ATO (0.25-16 μM), rapamycin (5-320 nM) and their combination (0.25 μM+5 nM - 16 μM+320 nM). After 96 h, cell viability was determined using MTT assay. The Combination Index (CI) was calculated using CompuSyn software. CI < 1 indicates synergism, CI = 1 indicates additive effects and CI > 1 indicates antagonism. Fa = fraction of cells affected. x = lowest concentration of the agent used. c) Cells were preincubated with rapamycin (20 nM) or vehicle (control) for 20 minutes before addition of ATO (0.5 and 1 μM). After 96 h, the number of viable cells was determined by trypan blue exclusion. Results are means ± SEM of at least three independent experiments. * $p<0.05$ (Student t-test) with respect to control. § $p<0.05$ (ANOVA and Tukey test) with respect to either agent alone.

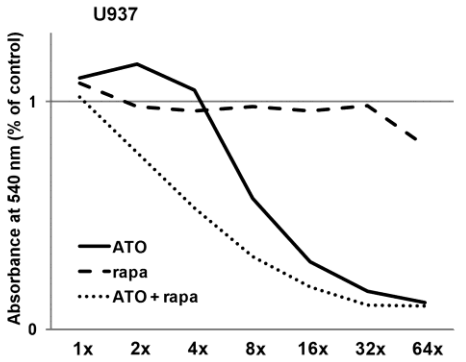
Figure 1

a)



b)

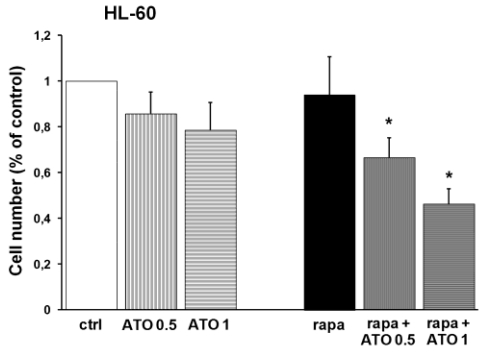




Arsenic trioxide (μM)	Rapamycin (nM)	Fa (combination)	Combination Index
0,25	5	0,050	3,064
0,5	10	0,067	2,481
1	20	0,220	0,591
2	40	0,519	0,529
4	80	0,760	0,581
8	160	0,865	0,784
16	320	0,896	1,333

Arsenic trioxide (μM)	Rapamycin (nM)	Fa (combination)	Combination Index
0,25	5	≈ 0,0	NA
0,5	10	0,223	0,215
1	20	0,468	0,256
2	40	0,681	0,341
4	80	0,813	0,491
8	160	0,893	0,728
16	320	0,897	1,426

c)



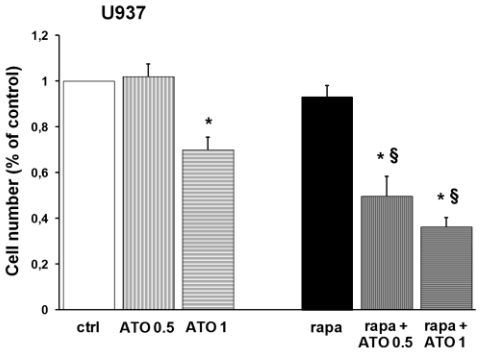


Fig. 2 Effects of arsenic trioxide (ATO) and rapamycin on apoptosis and cell cycle progression

HL-60 and U937 cells were incubated with vehicle (control), ATO (0.5 and 1 μM), rapamycin (20 nM) or their combination. Rapamycin was added 20 minutes prior to addition of ATO. a) The percentage of sub-G_1 fraction after 48 h incubation. b) The percentage of annexin V-FITC positive (upper bars, annexin+/PI-; lower bars, annexin+/PI+) cells after 48 and 96 h. *$p < 0.05$ (Student *t*-test) with respect to control for annexin+/PI- cells. #$p < 0.05$ (Student *t*-test) with respect to control for annexin+/PI+ cells. c) The percentage of cells in G_0/G_1, S and G_2/M phases of cell cycle after 24 h. * $p<0.05$ (Student t-test) with respect to control. Results are means ± SEM of at least three independent experiments.

Figure 2

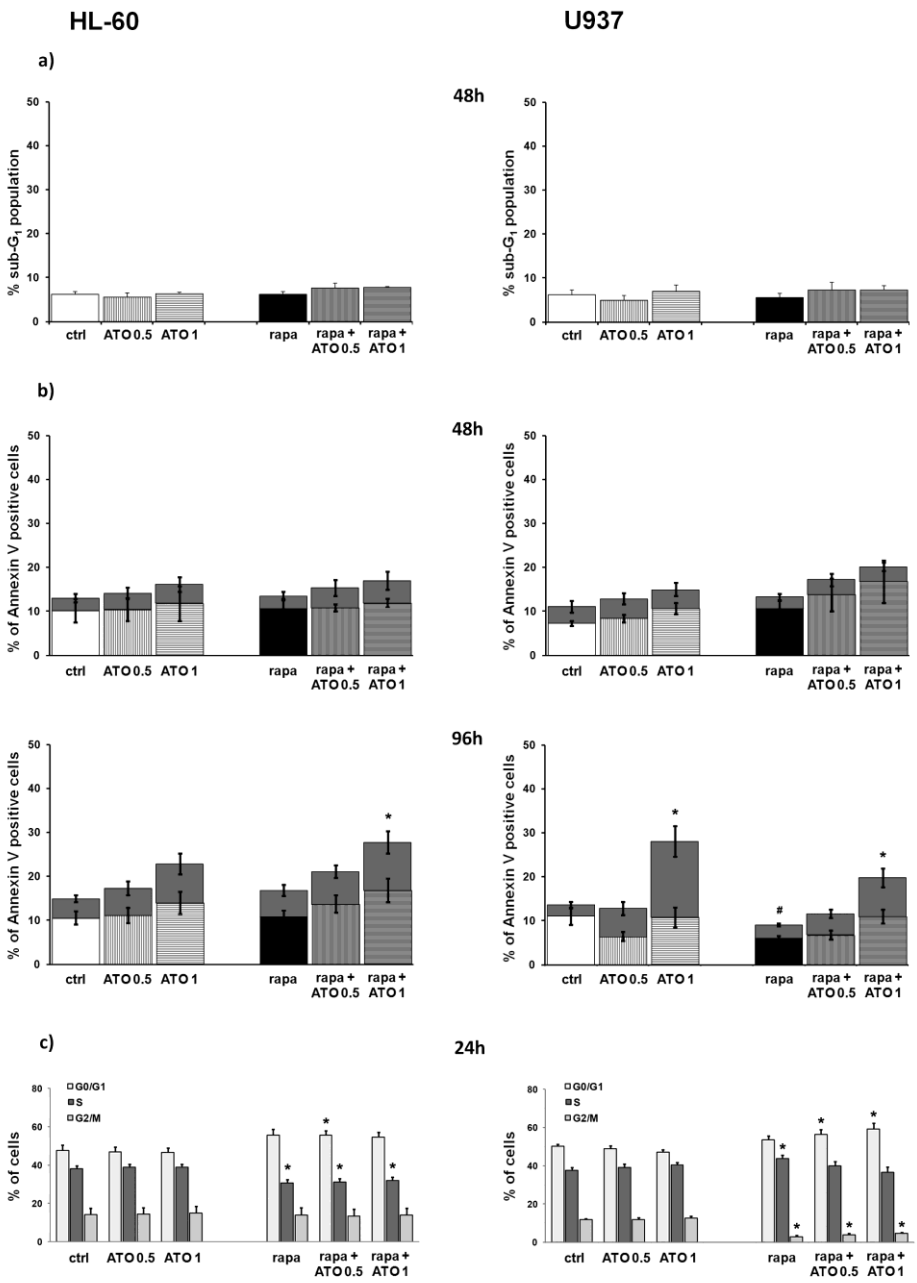

HL-60
U937
a)
48h
% sub-G1 population
b)
48h
% of Annexin V positive cells
96h
% of Annexin V positive cells
c)
24h
% of cells
G0/G1
S
G2/M
ctrl
ATO 0.5
ATO 1
rapa
rapa + ATO 0.5
rapa + ATO 1

Fig. 3 Effects of arsenic trioxide (ATO), rapamycin and their combination on the activity of mTOR and MAPK in HL-60 and U937 cell lines

Rapamycin (20 nM) or vehicle (control) were added to HL-60 and U937 cells 20 min before ATO (0.5 or 1 μM), and incubated for 30 min. After incubation, total cell lysates were isolated, loaded on two parallel gels and analyzed for the expression of phosphorylated and total proteins. a) The level of Thr389 phosphorylated and total p70 S6K. b) The level of Thr202/Tyr204 phosphorylated and total MAPK. All blots were reprobed with anti-β-actin antibodies to verify equal loadings.

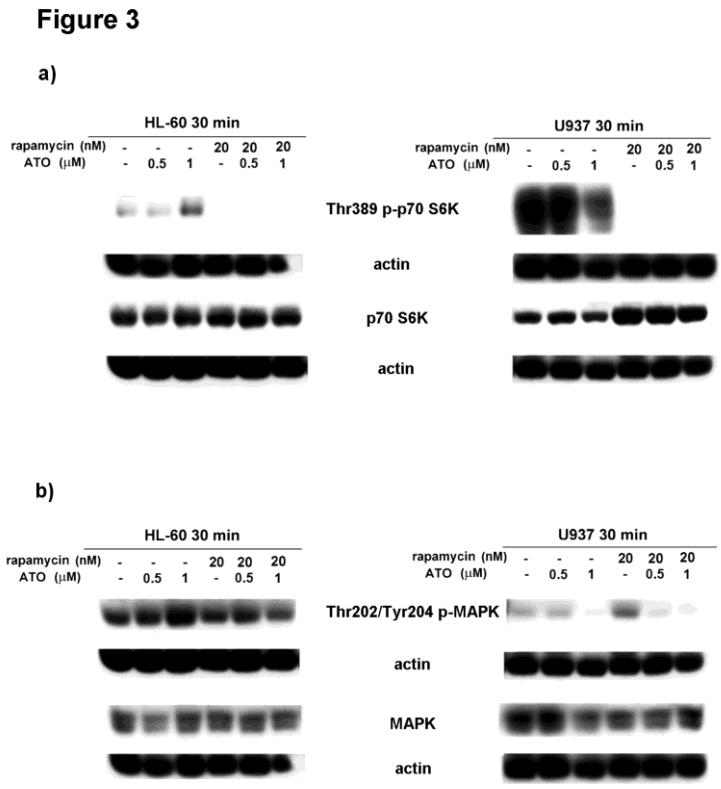


Fig. 4 Effects of increasing doses of arsenic trioxide (ATO) on the activity of mTOR and MAPK in U937 cells after 3 and 24 h

U937 cells were incubated with increasing doses of ATO (0.5 – 2 μM) for 3 and 24 h. After incubation, total cell lysates were isolated, loaded on two parallel gels and analyzed for the expression of phosphorylated and total proteins. a) The level of Thr389 phosphorylated and total p70 S6K. b) The level of Thr202/Tyr204 phosphorylated and total MAPK. All blots were reprobed with anti-β-actin antibodies to verify equal loadings.

Figure 4

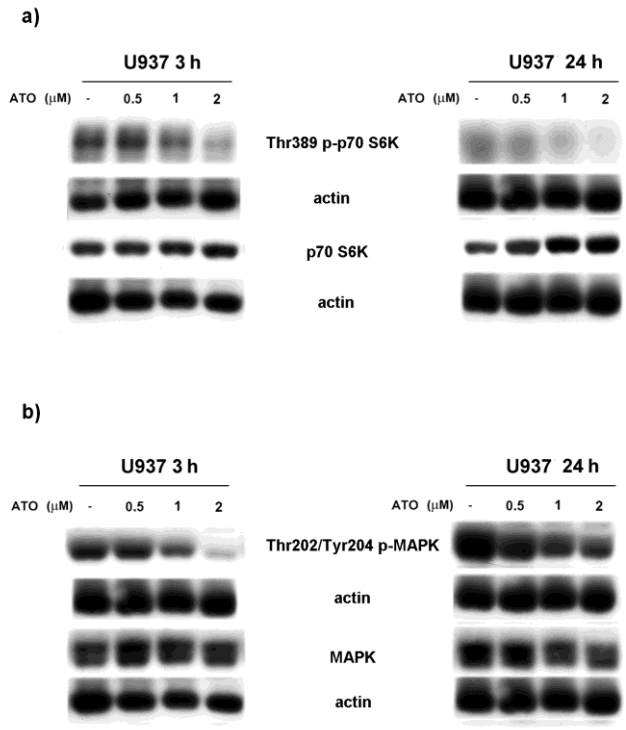


Fig. 5 Effects of arsenic trioxide (ATO), rapamycin and their combination on the levels of Mcl-1 and Ser473 phosphorylated Akt in U937 cells

Rapamycin (20 nM) or vehicle (control) were added to U937 cells 20 min before ATO (0.5 or 1 μM), and incubated for 3 or 24 h. After incubation, total cell lysates were isolated, loaded on two parallel gels and analyzed for the expression of phosphorylated and total proteins. a) The level of Mcl-1. b) The level of Ser473 phosphorylated and total Akt. All blots were reprobed with anti-β-actin antibodies to verify equal loadings.

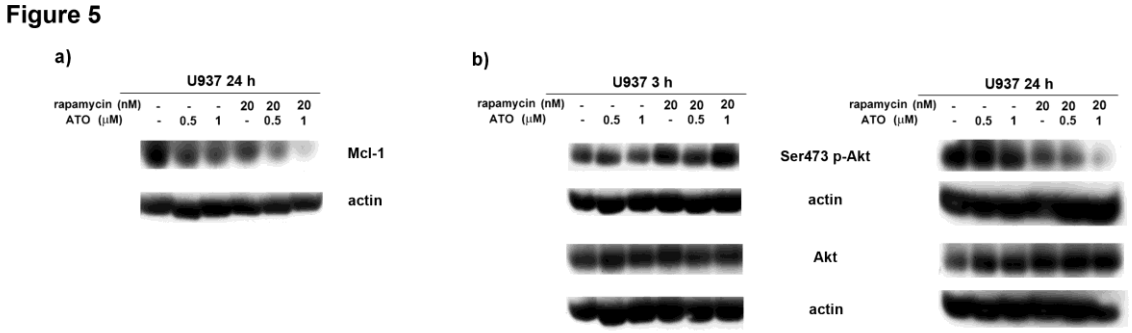


Fig. 6 Effects of rapamycin and arsenic trioxide (ATO) on the expression of differentiation markers in U937 cells

U937 cells were incubated for 96 h with vehicle (control), rapamycin (20 nM), MEK inhibitor PD 98059 (20 μM), ATO (0.5 or 1 μM), ATRA (1 μM) or their combination. Results are presented as mean fluorescence intensity (MFI) or percentage of CD64- and CD11b-positive cells. a) Effects of ATO, ATRA and rapamycin on the expression of CD64. b) Effects of ATO, ATRA and rapamycin on the expression of CD11b. c) Effects of PD 98059 on rapamycin- and ATO-mediated effects. Results are means ± SEM of at least three independent experiments. * $p<0.05$ (Student t-test) with respect to control. § $p<0.05$ (ANOVA and Tukey test) with respect to either agent alone.

Figure 6

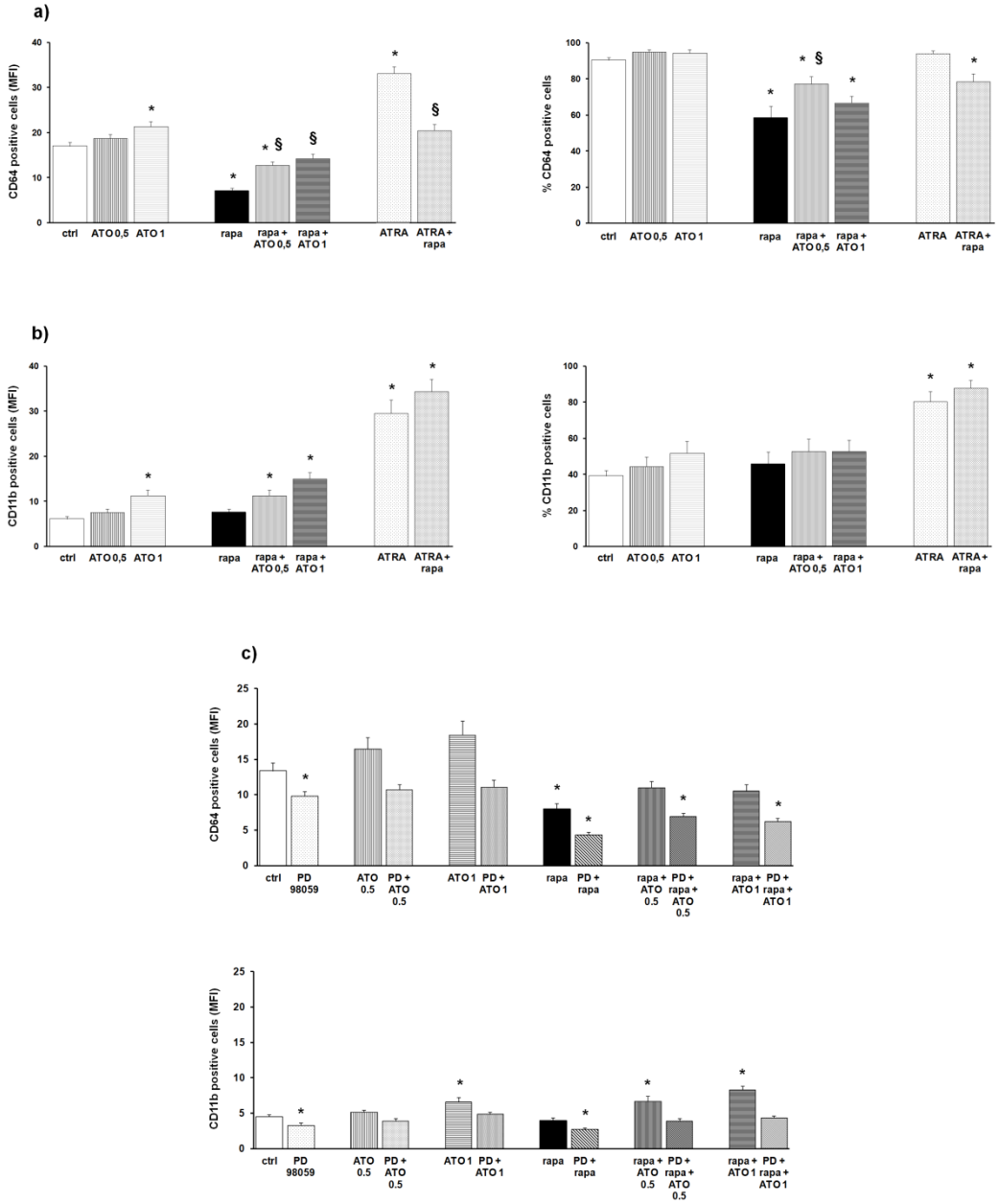

a)
CD64 positive cells (MFI)
% CD64 positive cells
ctrl
ATO 0,5
ATO 1
rapa
rapa + ATO 0,5
rapa + ATO 1
ATRA
ATRA + rapa
b)
CD11b positive cells (MFI)
% CD11b positive cells
c)
CD64 positive cells (MFI)
CD11b positive cells (MFI)
PD 98059
ATO 0.5
PD + ATO 0.5
ATO 1
PD + ATO 1
PD + rapa
rapa + ATO 0.5
PD + rapa + ATO 0.5
rapa + ATO 1
PD + rapa + ATO 1

Fig. 7 Effects of rapamycin, arsenic trioxide (ATO) and their combination on cells isolated from peripheral blood of newly diagnosed AML patients.

AML cells were incubated for 96 hours with vehicle (control), ATO (0.5 or 1 μM), rapamycin (20 nM), ATRA (1 μM) or their combination and cell viability was determined using MTT assay. Results are means of experiments done in triplicate and are presented as percentage of control.

Figure 7