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MOLECULAR TARGETS FOR THERAPY (MTT)

MTT
Leukemia

Inhibition of phosphatidylinositol 3-kinase dephosphorylates BAD and promotes apoptosis in myeloid leukemias

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The phosphatidylinositol 3-kinase (PI3K)/AKT protein kinase pathway is involved in cell growth, proliferation, and apoptosis. The functional activation of PI3K/AKT provides survival signals and blockade of this pathway may facilitate cell death. Downstream targets of PI3K-AKT include the proapoptotic protein BAD, caspase-9, NF-κB, and Forkhead. We have previously reported that BAD is constitutively phosphorylated in primary acute myeloid leukemia (AML) cells, a post-transcriptional modification, which inactivates its proapoptotic function. In this study, we tested the hypothesis that the inhibition of PI3K by LY294002 results in the dephosphorylation of AKT and BAD, and thus promote leukemia cell apoptosis. We investigated the effects of LY294002 in megakaryocytic leukemia-derived MO7E cells, primary AML and normal bone marrow progenitor cells. In MO7E cells, LY294002 reduced AKT kinase activity, induced dephosphorylation of AKT and BAD, and increased apoptosis. Concomitant inhibition of mitogen-activated protein kinase signaling or combination with all-*trans* retinoic acid further enhanced apoptosis of leukemic cells. In primary AML samples, clonogenic cell growth was significantly reduced. Normal hematopoietic progenitors were less affected, suggesting preferential targeting of leukemia cells. In conclusion, the data suggest that the inhibition of the PI3K/AKT signaling pathway restores apoptosis in AML and may be explored as a novel target for molecular therapeutics in AML.

Introduction

Phosphatidylinositol 3-kinase (PI3K) can transduce survival signals from growth factors and cytokines. It contains two subunits: a p85 regulatory subunit, which activates the enzyme by translocating it to the plasma membrane upon receiving signals from receptors, and a p110 subunit with catalytic activity.[1,2] One of the downstream targets of PI3K is the serine–threonine kinase AKT[3] that was initially identified as an oncogenic retrovirus (AKT 8) in murine lymphomas.[4] Binding of phosphatidylinositol-3,4,5-triphosphate to the PH domain of AKT induces translocation of AKT to the plasma membrane.[5,6]

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The PI3K-dependent kinases PDK1 and PDK2 (which may be PDKI, ILK, or AKT itself) phosphorylate T308 and S473, which are required for complete AKT activation.[5,7]

The PI3K/AKT pathway is believed to be one of the pivotal signaling pathways regulating cell growth, proliferation, and apoptosis.[3] Cytokines including GM-CSF and IL-3, upon binding their receptors, activate PI3K and AKT kinase, which leads to the induction of a downstream signaling cascade including phosphorylation of BAD, caspase-9, NF-κB, and Forkhead proteins.[8–18] Phosphorylation of these AKT substrates results in antiapoptotic effects. Phosphorylation by AKT of the Forkhead transcription factor leads to the retention of the protein in the cytoplasm where it is unable to induce transcription of proapoptotic FAS-L and to increase the protein levels of p27kip1, a cell cycle inhibitor.[19] Cytokines including IL-3, GM-CSF, SCF, and TNF have been reported to induce phosphorylation of the Bcl-2 family member BAD.[11,20,21] It is believed that the balance between antiapoptotic and proapoptotic proteins dictates whether a cell will survive or undergo apoptosis.[22] Nonphosphorylated BAD dimerizes with Bcl-2 or Bcl-X_L and abrogates their anti-apoptotic function at the mitochondrial membrane. In the presence of IL-3, the PI3K/AKT pathway is activated, and BAD is phosphorylated at S112. Phosphorylation of BAD disables its ability to bind to BCL-X_L; it instead binds to 14-3-3 in the cytosol resulting in the inactivation of its proapoptotic function, and the balance is shifted towards survival.[23,24] Furthermore, BAD protein can be phosphorylated as a consequence of the activation of the mitogen-activated protein kinase (MAPK) pathway.[25,26] This indicates that alternative pathways may affect the function of BAD, and that the AKT and MAPK pathways intersect and may share common targets. We have recently reported that BAD is constitutively phosphorylated on both residues in all primary acute myeloid leukemia (AML) samples studied.[27] Thus, BAD phosphorylation may reflect the state of AKT and MAPK/ERK signaling.

The PI3K pathway is negatively regulated by phosphatases. PTEN/MMAC1[28–31] and SHIP-2[32,33] removes the 3-phosphate and 5-phosphate from the PI3K lipid product PI(3,4,5)P_3 to yield PI(4,5)P_2 and PI(3,4)P_2, respectively, which prevents AKT activation. This results in increased signaling through the PI3K pathway contributing to cell proliferation and resistance to apoptosis. PTEN is considered a tumor suppressor gene. The inactivation of PTEN is commonly observed in solid and some hematopoietic tumors.[34–36]

Recent evidence suggests that the PI3K/AKT pathway is constitutively activated in a subset of leukemias. TEL/platelet-

derived growth factor (PDGF) beta R fusion protein resulting from the t(5;12) in CMML activates the kinase activity of PI3K and stimulates phosphorylation of its downstream substrates including AKT.[37] The BCR/ABL fusion protein, a hallmark of CML, also results in the activation of PI3K/AKT signaling.[38] A recent report demonstrated that factors present in plasma promote basal survival of B-CLL cells and resistance to cytotoxic drugs via stimulation of the AKT cytoprotective signaling pathway.[39] Therefore, pharmacological blockade of the PI3K/AKT pathway may have a potential as a novel strategy for leukemia therapy.

Wortmannin and LY294002, effective inhibitors of PI3K, have been shown to decrease cell survival and enhance apoptosis in a variety of model systems.[40–45] This effect has been linked to changes induced in downstream effectors of AKT, including dephosphorylation of BAD and caspase-9, the activation of Forkhead protein, and the inactivation of NF-κB. In the present study, we report the effects of the inhibition of PI3K with LY294002 in cytokine-dependent leukemic MO7E cells, in primary AML cells and in normal bone marrow (BM) progenitors. We found that LY294002 dephosphorylates AKT and BAD, induces apoptosis in leukemic cell lines and in primary samples of AML, and reduces clonogenic growth of AML cells. However, less pronounced inhibition of colony-forming ability was observed in normal myeloid progenitor cells, suggesting that LY294002 may selectively target leukemic, while sparing normal hematopoietic cells. Combined inhibition of PI3K and MAPK pathways resulted in decreased cell growth and enhanced apoptosis. Furthermore, LY294002 exerts an additive effect with all-*trans* retinoic acid (ATRA) in inhibiting leukemic cell growth. These results demonstrate that targeting of the PI3K pathway, alone or in combination with MAPK inhibition or retinoids, may have therapeutic applications in the therapy of AML.

Materials and methods

Cells, cell culture, reagents, and preparation of patients sample

MO7E is a human megakaryocytic leukemia cell line requiring IL-3 or GM-CSF for proliferation.[46] MO7E cells were cultured in RPMI 1640 medium plus 10% FCS, 1% ampicillin, 1% streptomycin, with or without IL-3 (0.1 ng/ml), or GM-CSF (37 U/ml) as indicated. Cells were then incubated at 37°C in 5% CO$_2$. LY294002 (2-[4-morpholinyl]-8-phenyl-[4H]-1-benzopyran-4-one) and ATRA were purchased from Sigma (St Louis, MO, USA) and dissolved in DMSO. MEK inhibitor PD98059 (2′-amino-3′-methoxyflavone) was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA). DMSO (0.1%) was used as a control.

AML patient samples were obtained following informed consent according to institutional guidelines. All samples had >70% blasts after Ficoll–Hypaque separation. Normal BM cells were obtained from allogeneic BM transplant donors following informed consent. In some experiments, low-density BM cells were separated by Ficoll–Hypaque density-gradient centrifugation and then enriched by magnetic-activated cell sorting for CD34$^+$ cells, as described previously.[47]

Western blot, immunoprecipitation, and kinase assay

After cells were lysed in protein lysis buffer (0.2 million cells in 10 μl), an equal amount of protein lysate was placed on an 12% gel and subjected to SDS-PAGE for 2 h at 100 V, followed by transfer of the protein on Nytran membranes (S&S, Heween, NH, USA). Immunoblotting was performed by incubation at room temperature for 2 h with 5% milk, incubated with the primary antibody in a 1:1000 dilution for another 2 h, followed by three washes in phosphate-buffered saline (PBS). The procedure was repeated for the secondary antibody, after which the blot was soaked in ECL plus buffer for 1 min and then exposed to film. The Western blots were analyzed with Imagequant software in the STORM-860 system (Molecular Dynamics, Sunnyvale, CA, USA) for quantitation. For immunoprecipitation, 5 million cells were lysed in 1 ml RIPA buffer. After incubation at 4°C for 2 h, anti-human BAD antibody was added to the supernatant, followed by G-agarose beads for 1 h at 4°C. The immune complex was then collected for Western blotting, as described. The antibodies to human BAD, to S-112 and S-136 phosphorylated BAD, to AKT, and to T-308 and S-427 phosphorylated AKT were from New England BioLabs (Beverly, MA, USA); antibody to SHIP from Santa Cruz Biotech (Santa Cruz, CA, USA). The AKT kinase assay kit was obtained from New England BioLabs and used according to the manufacturer's instructions. Briefly, cell lysates (200 μg) were incubated for 2 h with immobilized AKT1 monoclonal antibody. After extensive washing, the kinase reaction was performed in the presence of ATP and GSK-3 substrate. Phosphorylation of GSK-3 was measured by Western blotting using phospho-GSK-3 antibody.

Flow cytometry detection of apoptotic cells

Annexin V staining: Cells were washed in PBS and resuspended in 100 μl of binding buffer containing Annexin V (Roche Diagnostic Corporation, Indianapolis, IN, USA). Cells were analyzed by flow cytometry after the addition of propidium iodide (PI).[48] Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of those cells with a compromised cell membrane. This allows for live cells (unstained with either fluorochrome) to be discriminated from apoptotic cells (stained only with Annexin V) and necrotic cells (stained with both Annexin and PI).[49]

DNA fragmentation: Aliquots (80 μl) of cells were mixed with 100 μl of solution containing 0.1% (v/v) Triton X-100, 0.05 mol/l HCl, 0.15 mol/l NaCl, and 8 μg/ml acridine orange (Polysciences, Warrington, PA, USA). Cell fluorescence was measured within 5 min of staining using the logarithmic scale of the FACScan flow cytometer with 488-nm excitation of a 15-mW argon laser and filters set for green (530 nm) (DNA) and red (585 nm) (RNA) fluorescence.[50] In all experiments, 10 000 events were stored in list mode for analysis. The percentage of cells in the 'sub-G$_1$ region' defined the proportion of apoptotic cells in the tested populations. Cell debris was defined as events in the lowest 10% range of fluorescence and was eliminated from analysis.

Statistical analysis

Statistical analysis was performed using two-tailed Student's *t*-tests and paired *t*-tests. Statistical significance was considered when $P < 0.05$. Unless otherwise indicated, average values were expressed as mean$\pm$s.e. of the mean. All experiments were repeated at least three times.

AML blast and normal BM colony assay

BM mononuclear cells (1 or 2 × 10^5) containing more than 80% blasts from the patients with AML were plated in methylcellu-

lose medium (Methocult, Stem Cell Technologies, Vancouver, Canada) containing human recombinant growth factors, as follows: erythropoietin (3 U/ml), IL-6 (20 ng/ml) IL-3 (20 ng/ml), GM-CSF (20 ng/ml), G-CSF (20 ng/ml), and stem cell factor (SCF) (50 ng/ml). LY294002 was added at the initiation of cultures at concentrations of 1, 5, 10, 15, and 20 μM, and control cultures had DMSO only. Duplicate cultures were incubated in 35-mm Petri dishes at 37°C in a humidified atmosphere of 5% CO_2 in air. AML blast colonies were microscopically evaluated on days 8–10. A blast colony was defined as a cluster of 40 or more cells.

In three experiments, mononuclear cells from random allogeneic BM transplant donors were plated in methylcellulose as described above. Cultures were evaluated for the number of CFU-GM colonies, defined as a cluster of 40 or more granulocyte, monocyte—macrophages, or both.

Results

### BAD protein is phosphorylated in all primary AML samples on S-112 and S-136

AML samples with high blast count were analyzed for BAD expression by Western blot ($n = 44$). Immunoblot showed a small shift in BAD protein mobility, suggesting phosphorylation (data not shown). Using phosphorylation site-specific antibodies, this finding was subsequently confirmed. Figure 1 shows the results from 11 patient samples. Analysis of leukemic samples demonstrated that the majority of samples analyzed contained phosphorylated BAD. The expression of total BAD was variable; however, in most cases Ser136 and Ser112 phosphorylation levels correlated with the levels of BAD expression. The constitutive BAD phosphorylation indicates the activation of the upstream PI3K/AKT or MAPK pathways, which may promote cell proliferation and survival. It is likely that the functional protein kinases or phosphatases differ between patient samples, and that variations in phosphorylation may be of clinical importance.

Expression and regulation of AKT kinase in AML

Since BAD is phosphorylated in all AML samples, the related upstream kinase must be functional. Our recent report demonstrated constitutive activation of MAPK in the majority of primary AML blasts.[51] To assess the activation status of AKT, cell lysates from primary AML samples with high blast count were blotted with antibodies recognizing phosphorylated T-308 and S-473 in AKT (Figure 2a). Total AKT was expressed in all 8/8 AML samples studied and in normal CD34$^+$ cells. AKT was found to be phosphorylated at S473 and T308 in the majority of
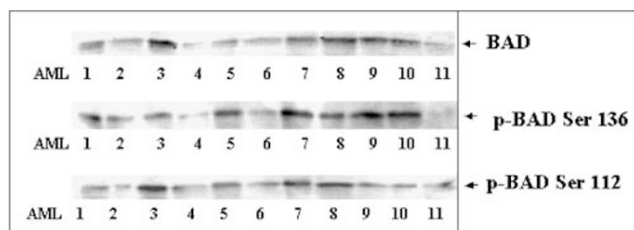
Figure 1 Detection of BAD, p-S-112 BAD, and p-S-136 BAD protein by Western blot using phosphorylation site specific antibodies in 11 AML patient samples containing >60% blasts.
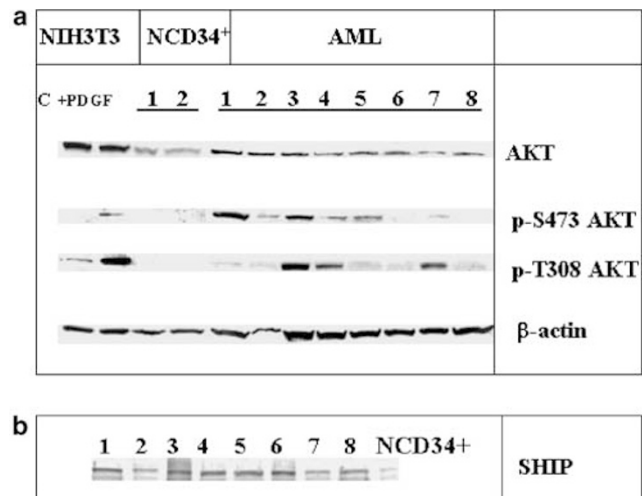
Figure 2 (a) Detection of AKT, p-S-473 AKT, and p-T-308 AKT proteins by Western blot using phosphorylation site-specific antibodies in eight AML patient samples. Normal BM CD34$^+$ (NCD34$^+$) cells was used as a normal control, NIH 3T3 cells treated with PDGF was blotted as a positive control for the antibodies. β-actin as a loading control. (b) Expression of p145 SHIP in the same AML samples and in normal BM CD34$^+$ (NCD34$^+$) cells.

AML samples, albeit to different degree. In contrast, AKT phosphorylation was not detected in normal CD34$^+$ cells. Reprobing of the blot with actin showed equal loading in all lanes. These results are suggestive of constitutive phosphorylation of AKT in the majority of primary AML samples.

Constitutive phosphorylation of AKT may result from either the activation of upstream PI3K or from loss of activity of negative regulators – the phosphatases PTEN or SHIP. In CML, BAD is phosphorylated in a PI3K-dependent fashion, partially mediating resistance to apoptosis,[52] and the expression of SHIP was recently reported to be absent or substantially reduced.[53] We therefore investigated SHIP expression in primary AML. As shown in Figure 2b, SHIP was found to be expressed in all AML samples tested and in normal CD34$^+$ cells.

Since cytokines activate the protein kinase pathway, we studied the regulation of AKT phosphorylation in primary AML blasts in response to cytokines. AML blasts were cultured with GM-CSF, G-CSF, and SCF, alone and in combination. After 48 h, phosphorylation levels of AKT were induced by each of the growth factors in comparison with control culture (media only), as shown in Figure 3. In contrast to cells assayed directly from the patient where AKT was phosphorylated (Figure 2, patient #1), incubation in serum-free media with no cytokines led to a decrease of AKT phosphorylation likely due to a loss of the microenvironmental stimuli that exert kinase stimulation through cellular interactions and cytokines. Further, this indicates that AKT phosphorylation is not cell autonomous. Hence, the presence of cytokines in cultures may restore part of the microenvironment's supportive functions. Therefore, cytokine-supplemented culture was used to examine the effects of PI3K inhibition on AKT.

PI3K inhibitors block downstream AKT phosphorylation and kinase activity, resulting in BAD dephosphorylation in cytokine-dependent leukemic cell lines

To demonstrate that the constitutive phosphorylation of AKT and BAD in AML is PI3K dependent, we used PI3K inhibitor
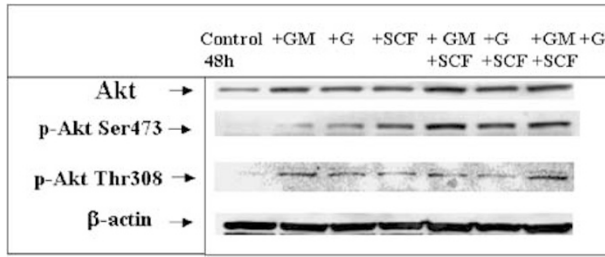
Figure 3 Expression of AKT and phosphorylated AKT on S-473 and T-308 following 48 h stimulation with GM-CSF (GM), G-CSF (G), SCF, and their combinations in AML patient sample. β-actin was used as a loading control.
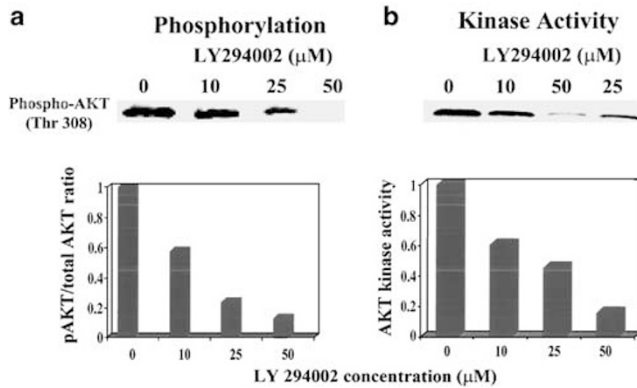

Figure 4 Inhibition of the AKT phosphorylation (left) and kinase activity (right) by LY294002 at 10, 25, and 50 μM concentration in MO7E cells. MO7E cells were starved for 8 h and LY294002 was added for 1–4 h, followed by stimulation with either IL-3 or GM-CSF for 4 h. (a) Cell lysates were analyzed by Western blotting using phospho-Akt antibody (left panel); blots were stripped and reprobed with total Akt antibody. The bar graph represents the ratio of pAkt to total Akt; the experimental values are normalized to control untreated sample designated as '1'. (b) Cell lysates were analyzed for activated Akt kinase using the *in vitro* kinase assay, as described. The bar graph represents Akt kinase activity normalized to 'no treatment control' (designated as '1').

LY294002 in cytokine-dependent MO7E cells. MO7E cells were starved for 8 h and LY294002 was added for 1–4 h, followed by stimulation with either IL-3 or GM-CSF for 4 h. As demonstrated in Figure 4, the inhibition of PI3K signaling with LY294002 induces a dose-dependent inhibition of AKT phosphorylation and a similar reduction of AKT kinase activity. Figure 5 shows the inhibition of BAD phosphorylation by LY294002 in GM-CSF- or IL-3-stimulated MO7E cells. These data demonstrate that PI3K is involved in the constitutive phosphorylation of AKT and BAD in AML cells.

PI3K inhibition decreases viability and induces apoptosis in MO7E and in primary AML cells

To investigate if the inhibition of AKT and BAD phosphorylation by LY294002 will affect leukemic cell growth, we determined MO7E cell viability by Trypan blue staining following 48 h of treatment with 10 μM with LY294002. The treated samples showed more than 50% reduction in the number of viable cells compared to DMSO controls (Figure 6, left panel). In parallel, cells were analyzed by DNA/RNA flow cytometry, and the number of 'sub-G_1' cells was determined as a measure of DNA fragmentation. The apoptosis rate detected by flow cytometry for
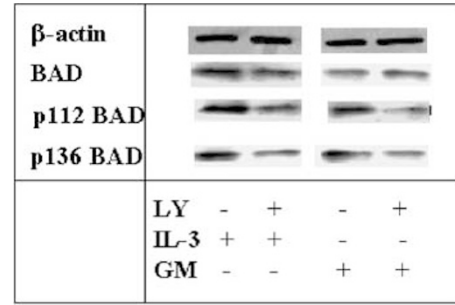

Figure 5 Detection of BAD and phosphorylated BAD protein on S-112 and S-136 in MO7E cells. The cells were starved for 8 h in cytokine-free medium, and GM-CSF (GM, 4 h culture) or IL-3 was replenished after PI3K blockade with LY294002 (LY) for 4 h. β-actin was used as a loading control.
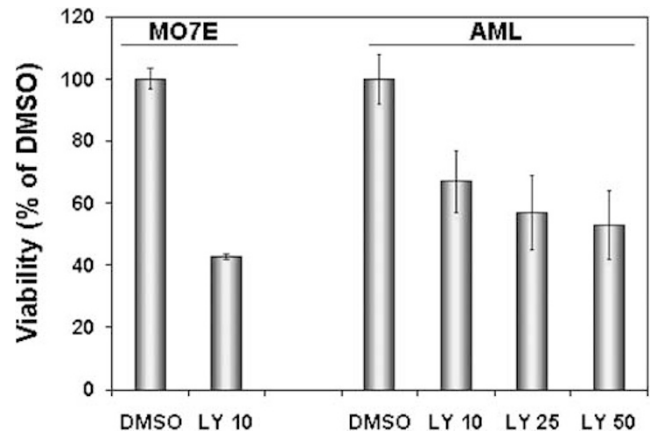
Figure 6 MO7E cells (in triplicate) and cells from primary AML samples ($n = 10$) were treated with PI3K inhibitor LY294002 (LY) at the indicated concentrations. At 48 h, the live cells were stained with Trypan blue and counted; the control cells were treated with same amount of DMSO. Data are presented as the percentage of viability compared to DMSO-treated cultures.

the 'sub-G_1' population was 15–20%. In addition, a decrease in the number of S cells was also observed, indicating blockade of cell proliferation at G_1/S by PI3K inhibition (data not shown).

To examine the role of PI3K signaling in primary AML cells, we tested the effects of LY294002 in a short-term culture assay of AML samples. A total of 10 separate AML patient samples were cultured in media containing IL-3 and SCF, conditions known to support survival of AML blasts. Cells were treated with LY 294002 at 10, 25, and 50 μM; controls were incubated with equivalent amounts of DMSO. AML cell survival was analyzed at 48 h by cell count with Trypan blue exclusion. Two of the 10 samples showed decreased survival only at the highest concentration (50 μM) of LY294002. However, in 8/10 cases a consistent dose response to LY294002 was observed, with decreased viability starting at 10 μM ($P<0.01$). On average, cell survival was reduced by 33, 43, and 47% at 10, 25, and 50 μM of LY294002, respectively (Figure 6, right panel). In order to understand the mechanism of decreased AML growth following the inhibition of PI3K signaling, we analyzed the induction of apoptosis by sub-G_1 flow cytometry (using acridine orange). The average percentage of apoptotic cells increased by 10–20% (data not shown, $P<0.01$). In the same assay, no significant changes in cell cycle distribution were observed. These data suggest that the inhibition of PI3K signaling abrogates AML cell growth primarily through the induction of apoptosis.
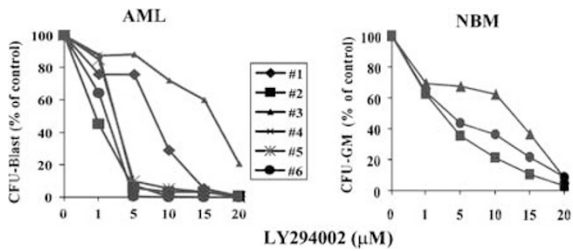
Figure 7 LY294002 inhibition of AML clonogenic progenitor growth. AML samples ($n=6$, left panel) were cultured for colony formation of leukemic blast with indicated concentrations of LY294002. Right panel, effect of LY294002 on the growth of myeloid progenitors (CFU-GM) from normal BM samples (NBM, $n=3$). Results are expressed as the mean±s.e.m. of the percentage of colonies in the presence of increasing concentrations of LY294002 (1, 5, 10, 15, and 20 μM) compared with the number in DMSO-treated cells.

To assess the effects of LY294002 on clonogenic AML progenitors, blast colony assays were performed. LY294002 induced complete inhibition of clonogenic cell growth in 4/6 AML samples at 5 μM (Figure 7). This suggests that LY294002 reduces the viability of the majority of leukemic cells in culture and that the approximately 50% decrease in viability of suspension cultures at 48 h underestimates the growth-inhibitory activity of LY294002 in clonogenic AML cells. In contrast, normal myeloid progenitors ($n=3$, Figure 7) were inhibited to a lesser degree (52% inhibition at 5 μM and 60% at 10 μM, $P<0.01$).

Combined blockade of PI3K and MAPK pathways increase apoptosis in leukemic cells

Since the MAPK pathway may contribute to BAD phosphorylation[25,26] and is frequently activated in AML,[51,54] we tested the effects of simultaneous blockade of the PI3K and MAPK pathways. When PI3K inhibition was combined with MAPK inhibitor PD98059 in MO7E cells, additional induction of apoptosis was observed (Figure 8b). This effect was associated with markedly decreased viability (Figure 8a). Similar results were obtained in OCI-AML3 leukemic cells (Figure 9). OCI-AML3 cells are cytokine independent with constitutively activated MAPK pathway,[51] and express phospho-AKT (not shown).

LY294002 and ATRA increase apoptosis in MO7E cells

LY294002 inhibits PI3K activity and downstream AKT and BAD phosphorylation, which facilitate the activation of the apoptotic cascade in MO7E cells. We then postulated that the reduction of levels of proteins agonistic to BAD, such as Bcl-2 and Bcl-X$_L$, would further increase the susceptibility to apoptosis. Since ATRA was found by us to downregulate Bcl-2 and Bcl-X$_L$ mRNA and protein,[27,55] the efficacy of a combination of LY294002 with ATRA in inducing apoptosis was assessed (Figure 10). Cell survival decreased by 71±7% with the combined treatment, while ATRA alone reduced survival only by approximately 30%. This result was confirmed by the determination of apoptotic cells by Annexin V binding assay (Figure 10). Similar effects were observed in primary AML cases (Figure 11).

Discussion

In this study, we report on the phosphorylation of the Bcl-2 family member BAD and the activation of the upstream PI3K/
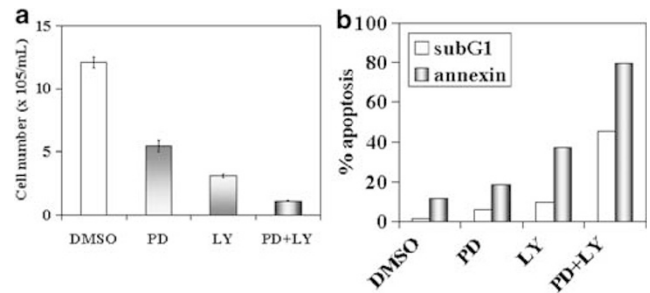
Figure 8 Effects of combined use of PI3K and MAPK inhibitors. To block the MEK/ERK and the PI3K pathways, MO7E were washed twice with serum-free RPMI 1640 medium, resuspended at 0.2×10^6 cells/ml, and cultured overnight in the absence of GM-CSF. MEK inhibitor PD98059 (20 μM), LY294002 (15 μM), or the appropriate concentration of DMSO was added for 4 h at 37°C before the addition of GM-CSF (100 U/ml). Effects on cell growth were analyzed at 72 h by cell count with Trypan blue exclusion (a), induction of apoptosis (b) by Annexin V staining and sub-G$_1$ DNA flow cytometry (acridine orange). Experiment was repeated twice with identical results.
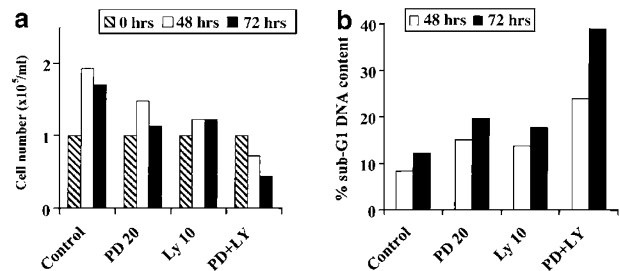

Figure 9 Combination of PI3K and MAPK inhibitors decreases growth and induces apoptosis in OCI-AML-3 cells. OCI-AML-3 cells were washed twice with serum-free medium and incubated with 10 μM of PD98059, 20 μM of LY294002, their combinations or DMSO. Effects on cell growth and apoptosis were analyzed at 48 and 72 h by cell counts with Trypan blue exclusion and DNA flow cytometry (sub-G$_1$, acridine orange), respectively.
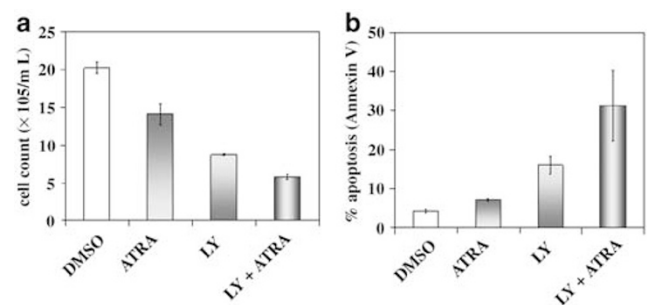
Figure 10 MO7E cells were cultured in the presence of LY294002 (10 μM), all-trans retinoic acid (ATRA, 1 μM) or the combination of PI3K inhibitor and ATRA. The control cells were treated with same amount of DMSO. At 48 h, the live cells were stained with Trypan blue and counted (a). The apoptotic cells were stained by Annexin V and measured by flow cytometry (b). These were the average of two experiments.

AKT signaling pathway in AML. Phosphorylation of BAD on serine 112 and 136 inactivates its proapoptotic function and is associated with survival. Published results have connected BAD phosphorylation to two major protein kinase pathways, PI3K-AKT and MAPK.[10,11,25,26] In primary AML cells, we were first to

Induction of DNA Fragmentation

Effect on Cell Proliferation
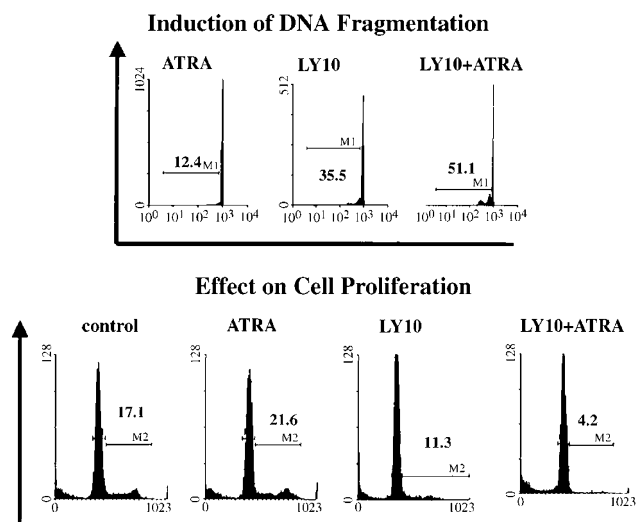
Figure 11 An example of primary AML blasts cultured with PI3K inhibitor LY294002 (LY, 10 μM), ATRA (1 μM) and the combination of LY294002 and ATRA for 48 h. The effects on proliferation (% S-phase cells) and apoptosis (sub-G_1 DNA content) was analyzed by DNA flow cytometry (acridine orange).

observe BAD phosphorylation in all samples studied, a finding that indicates constitutive activation of the PI3K/AKT and/or MAPK signaling pathways. Next, we examined AKT phosphorylation and activation by Western blot in AML samples and examined normal CD34$^+$ cells for comparison. We noted that Western blot analysis using phosphorylation-specific antibodies and kinase assay are both very sensitive in detecting AKT activation. We found that both AKT sites were phosphorylated in most AML samples tested, while no phosphorylation was detected in normal CD34$^+$ cells. Another group recently reported the constitutive phosphorylation of AKT in a majority of AML samples,[56] confirming our initial observation.[57] These reports establish AKT activation in the vast majority of AML. This may result from the constitutive activation of the upstream PI3K pathway, that is, as a result of cytokine stimulation. Indeed, we demonstrated that cytokines induce AKT phosphorylation *in vitro*. However, our studies of AKT phosphorylation were carried out in fresh samples from AML samples in the absence of exogenous cytokines, suggesting the existence of genetic alterations resulting in PI3K/AKT activation. As such, the amplification of multiple components of the PI3K pathway is a hallmark of ovarian cancers,[58] and a mutated form of the p85 subunit of PI3K has recently been isolated in a Hodgkin's lymphoma-derived cell line (CO).[59] In addition, deregulated activation of PI3K may be caused by some Ras mutations[60] or result from the aberrant cytokine signaling, that is, FLT3[61] or c-kit mutations.[62] Alternatively, the loss of expression or function of the phosphatases PTEN and SHIP that act as negative regulators of PI3K can induce constitutive activation of AKT. In our study, SHIP was expressed in all AML samples studied. However, a dominant-negative mutation of SHIP with the loss of the catalytic activity was recently described in 1/30 primary AML samples,[63] suggesting a possible tumor suppressor role of SHIP in selected AML cases. Furthermore, mutations in PTEN were described in a limited number of AML cases studied.[35]

To assess the functional role of PI3K in the activation of AKT, BAD phosphorylation, and survival of leukemic cells, we utilized the specific PI3K inhibitor LY294002. First, we tested the effects of LY294002 in the cytokine-dependent cell line

MO7E. Following treatment with LY294002, AKT phosphorylation and activity were blocked, and downstream BAD phosphorylation was inhibited, resulting in the decrease of cell survival and induction of apoptosis. Furthermore, LY294002 inhibited cell growth and promoted apoptosis in 8/10 primary AML samples in suspension culture. Of importance, LY294002 abrogated clonogenic leukemic cell growth in 4/6 samples tested at 5 μM and in 5/6 cases at 10 μM. It is conceivable that nonresponders lack expression of the target protein. Although the AKT phosphorylation status was not specifically tested in these samples, the response rate would correlate with the estimated percentage of PI3K/AKT activation observed in this and other studies.[56] Collectively, these results demonstrate that the activation of PI3K/AKT signaling supports growth and survival of primary AML cells.

The differential phosphorylation of AKT suggests that the activity of the PI3K pathway is elevated in AML, therefore PI3K inhibition could be a viable therapeutic approach to suppress leukemic cell growth. In addition, LY294002 has been found to sensitize leukemic HL-60 cells to chemotherapy.[64] In tumor-bearing mice, LY294002 was reported to reduce the tumor burden and ascitis. The only side effect observed was reversible dermatitis; of importance, no hematological toxicity was observed.[43,65] In our study, normal myeloid progenitors were less affected in clonogenic assays compared to AML blasts; however, toxicity was observed at high concentration of LY294002. This may suggest at least partial selectivity for leukemia cells. Results are also consistent with the lack of phosphorylated AKT protein in normal CD34$^+$ cells. However, LY294002 appears to affect all PI3K family proteins including Class I, II, and III PI3K. Recent results of gene expression analysis in purified hematopoietic stem cells demonstrated the presence of the catalytic subunit α in the early hematopoietic progenitors;[66] this observation likely explains the observed toxicity in normal BM progenitors. For clinical use, more specific PI3K and/or AKT inhibitors need to be identified. So far, there have been few candidate agents likely to be specific PI3K inhibitors, including the triterpine glycoside F035 and its derivatives,[67] and LY294002 analogs. Recently, the pyridinyl imidazole inhibitor SB203580[68] was described as blocking PI3K, and farnesyltransferase inhibition was linked to this pathway as well.[69] Of interest, recent clinical trials with farnesyltransferase inhibitors demonstrated activity in patients with poor-prognosis leukemias.[70,71] Novel AKT inhibitors are being developed, and our preliminary results demonstrate marked proapoptotic activity in leukemic cells.[72] Alternatively, specific inhibitors of the downstream mediators of AKT signaling, such as mTOR, may be of clinical utility.[73]

AKT has been associated with drug resistance in daunorubicin-treated leukemic cells,[74] and more recently also in ATRA resistance.[75] Clinical trials have documented retinoid resistance of AML, with the notable exception of APL.[76] When LY294002 was combined with ATRA, enhanced inhibition of cell growth and induction of apoptosis were noted. The induction of apoptosis following inhibition of the PI3K/AKT signaling may be associated with caspase-3 activation,[77] Bax conformational changes and its translocation to mitochondria,[78] Bcl-2[79] and Mcl-1 downregulation,[80] and cytochrome *c* release from mitochondria.[81] While the mechanism of enhanced apoptosis remains to be investigated, this may provide additional rationale for using LY294002 in overcoming drug resistance. Future studies should explore the ability of LY294002 to enhance retinoid sensitivity or overcome retinoid resistance in AML.

It is known that the MAPK pathway may contribute to phosphorylation of BAD, and we have recently demonstrated

that both, the PI3K and MAPK pathways, are also involved in the regulation of other downstream antiapoptotic members of the IAP family (survivin and XIAP).[82,83] Furthermore, Raf/MEK/ERK and PI3K/AKT pathways can synergize to induce cell survival and cellular transformation,[84] and the activation of PI3K contributes for MEK1-responsive growth and survival.[85] We therefore proposed that the apoptotic effects of PI3K/AKT inhibitor could be enhanced by cotreatment with MEK inhibitor. Indeed, the combination of the MAPK inhibitor PD98059 with LY294002 dramatically decreased cell growth and increased apoptosis in two different cell lines studied. These results suggest that multiple signal transduction pathways interact to regulate apoptosis in AML and that strategies aimed at targeting multiple signal transduction pathways could be effective in chemotherapy-resistant leukemias.

Acknowledgements

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