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MOLECULAR TARGETS FOR THERAPY (MTT)

A new selective AKT pharmacological inhibitor reduces resistance to chemotherapeutic drugs, TRAIL, all-*trans*-retinoic acid, and ionizing radiation of human leukemia cells

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It is now well established that the reduced capacity of tumor cells of undergoing cell death through apoptosis plays a key role both in the pathogenesis of cancer and in therapeutic treatment failure. Indeed, tumor cells frequently display multiple alterations in signal transduction pathways leading to either cell survival or apoptosis. In mammals, the pathway based on phosphoinositide 3-kinase (PI3K)/Akt conveys survival signals of extreme importance and its downregulation, by means of pharmacological inhibitors of PI3K, considerably lowers resistance to various types of therapy in solid tumors. We recently described an HL60 leukemia cell clone (HL60AR cells) with a constitutively active PI3K/Akt pathway. These cells were resistant to multiple chemotherapeutic drugs, all-*trans*-retinoic acid (ATRA), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Treatment with two pharmacological inhibitors of PI3K, wortmannin and Ly294002, restored sensitivity of HL60AR cells to the aforementioned treatments. However, these inhibitors have some drawbacks that may severely limit or impede their clinical use. Here, we have tested whether or not a new selective Akt inhibitor, 1L-6-hydroxymethyl-chiro-inositol 2(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate (Akt inhibitor), was as effective as Ly294002 in lowering the sensitivity threshold of HL60 cells to chemotherapeutic drugs, TRAIL, ATRA, and ionizing radiation. Our findings demonstrate that, at a concentration which does not affect PI3K activity, the Akt inhibitor markedly reduced resistance of HL60AR cells to etoposide, cytarabine, TRAIL, ATRA, and ionizing radiation. This effect was likely achieved through downregulation of expression of antiapoptotic proteins such as c-IAP1, c-IAP2, cFLIP$_L$, and of Bad phosphorylation on Ser 136. The Akt inhibitor did not influence PTEN activity. At variance with Ly294002, the Akt inhibitor did not negatively affect phosphorylation of protein kinase C-ζ and it was less effective in downregulating p70S6 kinase (p70S6K) activity. The Akt inhibitor increased sensitivity to apoptotic inducers of K562 and U937, but not of MOLT-4, leukemia cells. Overall, our results indicate that selective Akt pharmacological inhibitors might be used in the future for enhancing the sensitivity of leukemia cells to therapeutic treatments that induce apoptosis or for overcoming resistance to these treatments.
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Introduction

Chemotherapy for the treatment of some types of neoplastic disease has been one of the success stories of medicine in the last 30 years. Survival figures for certain hematological malignancies have improved dramatically: 5-year survival from childhood acute lymphoblastic leukemia was less than 40% in the 1970s, while is now approaching 80%.[1,2] However, the chemotherapeutic treatment outcome for most adults with acute myeloid leukemia remains unacceptable.[3] Indeed, it seems that we have reached the limits of our ability to kill cancer cells that are intrinsically resistant to chemotherapy.

It is now clear that chemotherapeutic drugs kill cancer cells also through induction of apoptosis.[4] Among acute myeloid leukemias, acute promyelocytic leukemia can be successfully treated with all-*trans*-retinoic acid (ATRA).[5] Recent results showed that ATRA induces apoptosis in leukemia cells through the paracrine action of the tumor-selective death ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also called Apo-2L.[6] Since TRAIL displays minimal toxic effects on normal cells, its use in the treatment of acute promyelocytic leukemia has been proposed.[6]

The development of resistance to a wide spectrum of cytotoxic drugs frequently impedes the successful treatment of acute myeloid leukemia either at the initial presentation or following primary or subsequent relapses.[7] Moreover, ATRA resistance in acute promyelocytic leukemia is rare but markedly increases in frequency after relapses from chemotherapy-induced clinical remission.[8] In some cases, drug and ATRA resistance might be the consequence of failure of leukemic cells to undergo apoptosis.

It appears, therefore, increasingly necessary to elucidate the molecular mechanisms underlying the enhanced survival capability displayed by tumor cells in the presence of anticancer treatments. The future of chemotherapy and other therapies appears to be strictly linked with our capability of finding new tools to lower the sensitivity threshold of cancer cells to molecules that induce apoptosis.[9] In particular, it seems necessary to identify points in the apoptotic pathways at which disregulation occurs.

A wealth of data has indicated that in mammals the pathway based on phosphoinositide 3-kinase (PI3K)/Akt conveys signals of extreme importance for cell survival. Indeed, the serine/threonine kinase Akt (also known as PKB, protein kinase B) targets several proteins that either directly or indirectly influence

the capability of initiating cell death by apoptosis.[10–13] Akt is capable of interfering with both the intrinsic (mitochondrial) and extrinsic (receptor mediated) apoptotic pathway.[13] Some of the antiapoptotic effects of Akt are mediated through the activation of the transcription factor NF-κB.[13]

Recent findings from several laboratories have highlighted that Akt is constitutively activated and promotes cellular survival and resistance to chemotherapy, ionizing radiation, and TRAIL in solid tumors.[14–16] On the other hand, limited information is available regarding the role played by the PI3K/Akt axis in determining resistance to these treatments in cells of hematopoietic lineage.[17–19] In these studies, inhibition of the PI3K/Akt pathway was obtained by means of two pharmacological inhibitors of the PI3K catalytic subunit, wortmannin and Ly294002. However, these two inhibitors have some limitations. Wortmannin has a very short half-life, while Ly294002 also inhibits casein kinase-2, so that it cannot be considered entirely specific.[20] Moreover, use of PI3K inhibitors may lead to undesired side effects, such as blocking of PI3K-elicited pathways that do not entirely impinge on Akt, like the insulin-evoked glucose transport.[21,22]

Recently, a selective Akt pharmacological inhibitor, 1L-6-hydroxymethyl-chiro-inositol 2(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate (Akt inhibitor), has been synthesized.[23]

HL60 cells derived from a human acute promyelocytic leukemia and are usually sensitive to chemotherapeutic drugs and TRAIL. We have described an HL60 leukemia human cell clone (named HL60AR) with a constitutively activated PI3K/Akt axis.[24] We further demonstrated that this clone is resistant to TRAIL and that inhibitors of the PI3K/Akt pathway restored TRAIL sensitivity of this clone.[25] Subsequently, we have shown that HL60AR cells are resistant to multiple chemotherapeutic drugs and to ATRA.[26]

In this article, we show that the novel Akt inhibitor is capable of restoring sensitivity of HL60AR cells to chemotherapeutic drugs, TRAIL, ATRA, and ionizing radiation. Moreover, we demonstrate that this inhibitor increases sensitivity to apoptotic inducers of two other cell lines, that is, K562 and U937 human leukemia cells that display sizeable levels of phosphorylated (active) Akt (p-Akt). In contrast, the inhibitor did not enhance sensitivity of a cell line with very low levels of activated Akt, that is, MOLT-4 human T leukemia cells.

Therefore, our results strengthen the hypothesis that the PI3K/Akt pathway is an important target to lower the sensitivity threshold of hematopoietic cells that have become resistant to various types of conventional (chemotherapy, ATRA, ionizing radiation) or novel (TRAIL) therapeutic treatments. This might be achieved by selective pharmacological inhibitors of the serine/threonine protein kinase Akt.

Materials and methods

Materials

Etoposide, cytarabine, bovine serum albumin (BSA), normal rabbit IgG, protein A/G agarose, and peroxidase-conjugated secondary antibodies were from Sigma, St Louis, MO, USA. Histone H2B, the COMPLETE Protease Inhibitor Cocktail, and the Lumi-Light^{Plus} enhanced chemiluminescence (ECL) detection kit were from Roche Applied Science, Milan, Italy. Phosphatidylinositol (4,5) bisphosphate [PtdIns(4,5)P_2], Ly 294002, 1L-6-hydroxymethyl-chiro-inositol 2(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate (Akt inhibitor), and human recombinant TRAIL were from Calbiochem, La Jolla, CA, USA. PtdIns(3,4,5)P_3 was from Alexis Biochemical, Laufelfingen, Switzerland. The SN50 peptide was from Biomol Research Laboratories, Plymouth Meeting, PA, USA. The ApoAlert Caspase-8 colorimetric assay kit and the ApoAlert Caspase-9/6 fluorometric assay kit were from BD Biosciences Clontech (Palo Alto, CA, USA). cAMP-dependent protein kinase inhibitor peptide was from Bachem (Bubendorf, Switzerland). P-81 paper was from Whatman (Maidstone, UK). The PTEN and p70S6K assay kits were from Upstate Biotechnology Incorporated, Lake Placid, NY, USA.

Source of antibodies

The following primary antibodies were used in this study. From Upstate Biotechnology, Lake Placid, NY, USA: rabbit polyclonals to total Akt, cytochrome *c*, and p85 regulatory subunit of PI3K. From Santa Cruz Biotechnology (Santa Cruz, CA, USA): mouse monoclonals to FLICE-inhibitory protein (cFLIP$_{S/L}$), Mcl-1, rabbit polyclonals to A1/Bfl-1, cIAP-1, and cIAP-2. From Cell Signaling Technology (Beverly, MA, USA): mouse monoclonal to PTEN, rabbit polyclonals to Thr 308 p-Akt, Ser 473 p-Akt, total Bad, Ser 136 phosphorylated Bad (p-Bad), Ser 112 p-Bad, Thr 410 phosphorylated protein kinase C (p-PKC)-ζ, and p70S6K. From Sigma: mouse monoclonal to β-tubulin, rabbit polyclonal to total PKC-ζ.

Cell culture and differentiation

HL60PT (for parental, obtained from the American Type Culture Collection-CCL 240), HL60AR (for Apoptosis Resistant), K562, U937, and MOLT-4 human leukemia cells were routinely maintained in RPMI 1640 supplemented with 10% fetal calf serum at an optimal cell density of $3–8 \times 10^5$ cells/ml.

For differentiation, exponentially growing HL60 cells were seeded in fresh media at a concentration of 5×10^5 cells/ml in the presence of ATRA (1×10^{-6} M). The culture media was changed every 24 h and contained, when required, the following inhibitors: Ly294002 (10 μM), Akt inhibitor (20 μM), or SN50 (30 μg/ml). After 96 h, differentiation was evaluated by flow cytometric analysis of CD11b expression. Antibody to CD11b was purchased from Beckman-Coulter Immunology (Miami, FL, USA), as fluorescein isothiocyanate conjugate.

Induction and detection of apoptosis

At 16 h after medium change, cells in exponential growing phase were treated with etoposide (5 μg/ml), cytarabine (40 μg/ml), or TRAIL (500 ng/ml) for 6 h at 37°C. In some experiments, prior to apoptotic induction, cells had been preincubated for 24 h with Ly294002 (10 μM) or the Akt inhibitor at the indicated concentration, or SN50 at 30 μg/ml. For detection of apoptosis, samples containing $2–5 \times 10^5$ cells were harvested by centrifugation at 200 *g* for 10 min, fixed with 70% cold ethanol for 1 h, and subsequently stained with propidium iodide (DNA-Prep kit, Beckman-Coulter Immunology). The subdiploid DNA content was calculated using an Epics XL flow cytometer with the appropriate software (Beckman-Coulter Immunology).[25]

Irradiation

Cells were irradiated with 50 Gy of 250 kVp X-rays (2.74 Gy/min) using a γ-cell irradiator. The dose was administered in two

equal fractions with 3 min between fractions to prevent the anode of the X-ray unit from overheating during irradiation. Samples to be irradiated were cooled on ice for at least 5 min prior to irradiation and were held on ice during the irradiation. All samples, including unirradiated control cells, were held on ice for an equivalent time (total time approximately 25 min). After irradiation, flasks were placed in a 37°C incubator, with aliquots of cells removed at 24 h after irradiation for analysis. The ice treatment did not cause a decrease in viability or a significant lag in the growth rate once the cells were returned to the 37°C incubator and allowed to re-equilibrate.

Immunoprecipitation

Cell homogenates (1 ml, containing 500 μg of protein) were precleared by adding 5 μg of normal rabbit IgG and 10 μg of 50% protein A/G agarose, followed by incubation for 1 h at 4°C and centrifugation at 12 000 g for 10 min at 4°C. Then, the samples were incubated for 2 h at 4°C under constant agitation with 5 μg of the primary antibody. In total, 10 μg of 50% protein A/G agarose was then added and incubation proceeded for 1 h at 4°C under constant agitation.

PI3K activity assay

The immunoprecipitates were washed twice with each of the following buffers: (i) phosphate-buffered saline (PBS) pH 7.4, containing 1% Nonidet P-40; (ii) 100 mM Tris-HCl (pH 7.4), 0.5 M LiCl; and (iii) 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA. All washing solutions contained 1 mM Na_3VO_4. The PI3K activity assay was then performed by adding sonicated PtdIns(4,5)P_2 (0.5 mg/ml in 10 mm HEPES-NaOH, pH 7.5, 1 mM EDTA), 10 mM $MgCl_2$, and 50 μM [γ-^{32}P]ATP (10 Ci/mmol). Incubation was for 15 min at room temperature. In some cases, the Akt inhibitor was also present during the assay at the indicated concentrations.[27] The reaction was stopped by the addition of chloroform/methanol/HCl (200:100:0.75, v:v), followed by two washes with chloroform/methanol/HCl 0.6 N (3:48:47). The lipid containing organic phase was resolved on thin-layer chromatography plates developed in isopropanol: acetic acid:H_2O (65:1:34). The radiolabeled PtdIns(3,4,5)P_3 was identified by comparison with standard PtdIns(3,4,5)P_3. After autoradiography, the spots were excised and quantified by scintillation counting.

Akt activity assay

The immunoprecipitates were washed twice in lysis buffer, once in distilled water and twice in the Akt kinase buffer (20 mM HEPES-NaOH, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT). Assays (100 μl) contained 20 mM HEPES-NaOH, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 1 μM cAMP-dependent protein kinase inhibitor peptide, 5 μg histone H2B as exogenous substrate, 2 μM ATP, 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol). Samples were incubated for 30 min at 30°C and the reaction was then stopped by spotting 80 μl on to P-81 filter papers and immersing in 1% (v/v) orthophosphoric acid. The papers were washed several times, rinsed in ethanol, air-dried, and the radioactivity was determined by scintillation counting. Background values, obtained by samples in which the anti-Akt antibody was replaced by normal rabbit IgG, were subtracted from all values.

PTEN activity

The immunoprecipitates were washed in a buffer containing 20 mM HEPES-NaOH, pH 7.7, 50 mM NaCl, 0.1 mM EDTA, and 2.5 mM $MgCl_2$, followed by a wash in phosphatase assay buffer lacking PtdIns(3,4,5)P_3 (100 mM Tris-HCl, pH 8.0, 10 mM DTT). Samples were incubated in a 50 μl volume containing mixed phospholipid vesicles (final concentrations: 415 μg/ml dioleyl phosphatidylcholine, 415 μg/ml dioleyl phosphatidylserine, 200 μg/ml PtdIns(3,4,5)P_3 in 10 mM HEPES-NaOH, pH 7.4, 1 mM EGTA, 1 mg/ml BSA). Reactions proceeded for 15 min at 37°C and were stopped by the addition of 200 μl of Malachite Green solution. Absorbance was measured at 640 nm. Phosphate standard curves were obtained employing a recombinant PTEN-GST fusion human protein.

P70S6K assay

For p70S6K activity, immunoprecipitation products were washed twice in lysis buffer followed by a wash in kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 0.4 mM DTT). The kinase reaction was performed for 15 min at 30°C with provided S6 peptide pseudosubstrate (125 μM) in the presence of 100 μM ATP and 0.06 μM [γ-^{32}P]ATP. The reaction was stopped by spotting samples onto Whatman P-81 filter papers and immersing in 1% (v/v) orthophosphoric acid. Then, the procedure was continued as for PI3K activity assays.

Preparation of cell homogenates and Western blot analysis

Cells were washed twice in PBS containing the COMPLETE Protease Inhibitor Cocktail supplemented with 1.0 mM Na_3VO_4 and 20 nM okadaic acid. Cells were then lysed at $\sim 10^7$/ml in boiling electrophoresis sample buffer containing the protease and phosphatase inhibitor cocktail. Lysates were briefly sonicated to shear DNA and reduce viscosity, and boiled for 5 min to solubilize protein. Protein separated on SDS-polyacrylamide gels was transferred to nitrocellulose sheets using a semi-dry blotting apparatus. Bands were visualized by the ECL method. To ensure equal loading, blots were always first probed with an antibody to β-tubulin, then stripped and reprobed.

Measurement of caspase-9 and -8 activity

This was performed using a fluorometric (for caspase-9) or colorimetric (for caspase-8) assay according to the manufacturer's instructions. Briefly, samples (from 10^6 cells) were incubated with the caspase-9 fluorometric substrate (LEHD-7-amino-4-trifluoremethyl coumarin) for 2 h at 37°C. Samples were read in a fluorometer with a 380-nm excitation filter and a 460-nm emission filter. For caspase-8 assay, the colorimetric substrate was IETD-pNA. Incubation of the samples (from 10^6 cells) was at 37°C for 2 h. The optical density of the reaction mixture was quantified spectrophotometrically at a wavelength of 405 nm.

Preparations of mitochondria and cytosol

Cell pellets were resuspended with 5 vol ice-cold buffer A (20 mM HEPES-KOH, pH 7.5, 0.1% BSA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml

leupeptin, 10 μg/ml of both aprotinin and pepstatin A) containing 250 mM sucrose. After swelling on ice for 5 min, the cells were homogenized with 20 strokes of a number 22 Kontes Dounce homogenizer with the B pestle (Kontes Glass, Vineland, NJ, USA,) and the homogenates were centrifuged at 750 g for 15 min at 4°C. Supernatants were then pelleted at 10 000 g for 15 min at 4°C. Resultant pellets containing mitochondria were resuspended in cold buffer A. Supernatants were further cleared at 20 000 g for 30 min at 4°C and used as cytosol.

Results

PI3K and Akt activity in HL60 cells

We recently described an HL60 cell clone (named HL60AR) with a constitutively active PI3K/Akt pathway.[24] This clone is resistant to TRAIL[25] and to several chemotherapeutic drugs and ATRA.[26] We first made a comparison between the effects of a PI3K (Ly294002) and of an Akt inhibitor on the *in vitro* enzymatic activity of either PI3K or Akt. Since no information was available if the Akt inhibitor effect was irreversible, when we measured PI3K activity the inhibitor was also present during the assays.

As shown in Figure 1a and b, the immunoprecipitable enzymatic activities of PI3K and Akt were higher in AR than in PT cells, in agreement with our own previous data.[26]

Ly294002 (10 μM) effectively reduced both activities. The Akt inhibitor did not significantly affect PI3K activity up to a concentration of 20 μM. At 80 μM, 54% of PI3K activity was inhibited. In contrast, a 20 μM concentration inhibited approximately 73% of Akt activity. In light of these results, we employed a 20 μM concentration of the Akt inhibitor in the following studies. A cell permeable inhibitor of the nuclear translocation of the p50 subunit of the NF-κB transcription factor (SN50) did not affect either PI3K or Akt activity (Figure 1).

Results obtained by enzymatic assays were corroborated by Western blot analysis for both Thr 308 p-Akt and Ser 473 p-Akt, the active forms of the enzyme.[10] As shown in Figure 1c, the levels these p-Akt forms were markedly higher in AR than in PT cells and were dramatically reduced by either Ly294002 or the

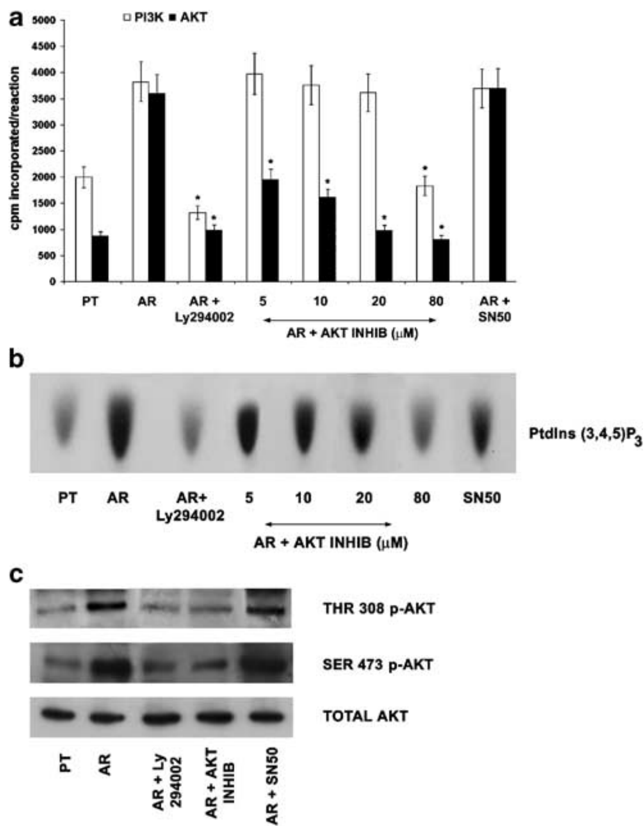

Figure 1 PI3K and Akt activities in HL60PT and AR cells treated with Ly294002, Akt Inhibitor (Akt inhib), or SN50. (a) PI3K or Akt were immunoprecipitated from cell homogenates and their activities were tested *in vitro*. The inhibitors were present for 24 h prior to immunoprecipitation. Ly294002 was employed at 10 μM, SN50 at 30 μg/ml. For PI3K activity assays only, the Akt inhibitor was also present during the assay at the same concentrations. Results are the mean of three different experiments ± s.d. The asterisks indicate significant differences ($P<0.01$) in comparison with untreated AR cells. (b) A representative thin-layer chromatography showing *in vitro* synthesized PtdIns(3,4,5)P_3. (c) Western blot analysis for expression of Thr 308 p-Akt, Ser 473 p-Akt, and total Akt. The inhibitors were used for 24 h. A total of 80 μg of protein from cell homogenates was blotted to each lane.

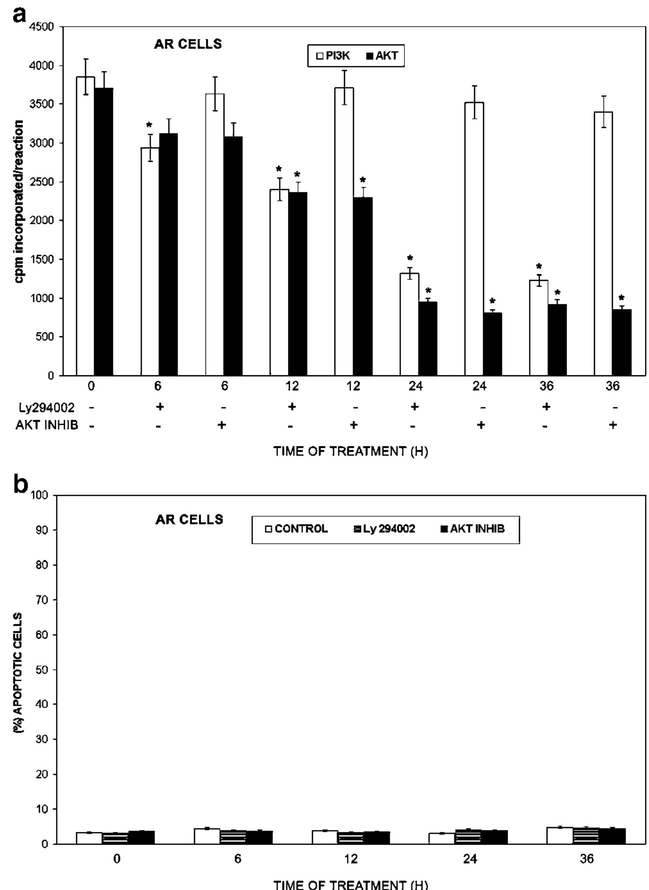

Figure 2 Time-course study of PI3K and Akt inhibition in AR cells. (a) Cells were treated for increasing periods of time with Ly294002 (10 μM) or Akt inhibitor (Akt inhibt, 20 μM). For the 36 h time point, the inhibitors were readded after 24 h of incubation. Enzyme activity assays were then performed on the immunoprecipitates. Results are the mean of three different experiments ± s.d. The asterisks indicate significant differences ($P<0.01$) in comparison with untreated AR cells (time 0). (b) Parallel samples were analyzed for the percentage of apoptotic cells by flow cytometry. Results are the mean of three different experiments ± s.d.

Akt inhibitor, but not by SN50. The expression of total Akt did not vary between PT and AR cells and was not affected by treatment with pharmacological inhibitors.

A time-course study (Figure 2a) revealed that maximal inhibition of either PI3K or Akt activity was achieved after a 24 h incubation in the presence of Ly294002 or Akt inhibitor. Increasing the exposure to the inhibitors to 36 h did not result in lower enzymatic activity, even if, after 24 h, the inhibitors were readded to the culture medium. It should be emphasized that, having established that the Akt inhibitor (20 μM) did not influence PI3K activity also if present during the *in vitro* assay, for the experiments of the time-course study the inhibitor was present only in the culture medium during pretreatment of cells.

Since the decrease in PI3K or Akt inhibitor could be a consequence of cell death, parallel samples were analyzed by flow cytometry for detection of apoptotic cells. However, as shown in Figure 2b, Ly294002 or Akt inhibitor did not increase *per se* the percentage of apoptotic cells.

The Akt inhibitor restores sensitivity of HL60AR cells to chemotherapeutic drugs, TRAIL, and ionizing radiation

In previous investigations,[25,26] we showed that HL60AR were markedly resistant to several chemotherapeutic drugs and TRAIL. As presented in Figure 3a, we confirmed these data by showing that HL60AR were resistant to etoposide, cytarabine, and TRAIL. In contrast, HL60PT cells were sensitive to these treatments. Furthermore PT, but not AR cells, were sensitive to ionizing irradiation (Figure 3a).

We next verified whether treatment with Ly294002, Akt inhibitor or SN50 could restore sensitivity of HL60AR cells to the apoptosis inducers. As shown in Figure 3b, Ly294002, Akt inhibitor, or SN50 alone did not significantly increase the percentage of apoptotic HL60AR cells. However, either Ly294002 or Akt inhibitor were able to increase markedly the sensitivity of AR cells to chemotherapeutic agents or ionizing radiation. In contrast, SN50 failed to increase significantly the percentage of apoptotic cells in response to these conventional therapies. On the other hand, the sensitivity of HL60AR cells to TRAIL was dramatically increased by all the three inhibitors.

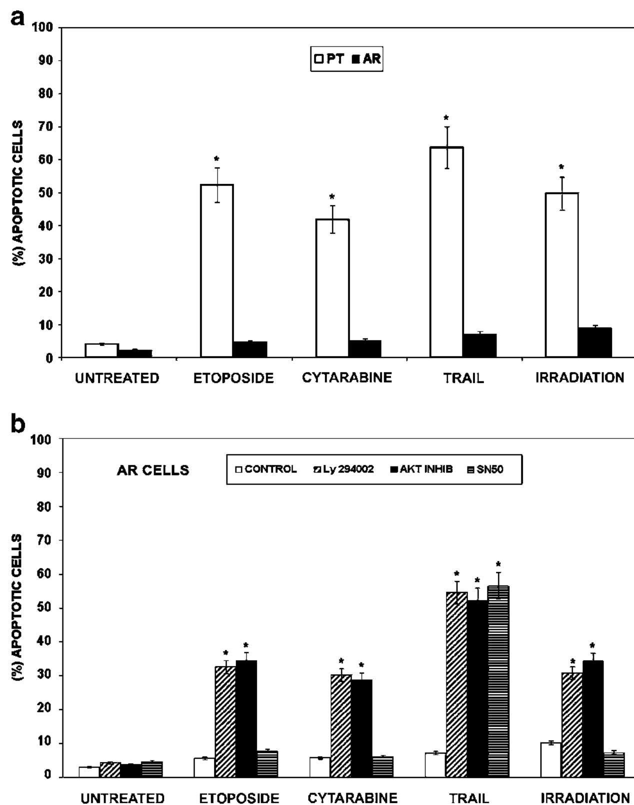

Figure 3 HL60AR cells are resistant to several apoptosis inducers (a) and resistance is diminished by Ly294002 (10 μM), Akt inhibitor (Akt inhib, 20 μM), or SN50 (30 μg/ml) (b). The inhibitors had been present for 24 h prior to treatment with the apoptosis inducers. Cells were treated with etoposide (5 μg/ml), cytarabine (40 μg/ml), TRAIL (500 ng/ml) for 6 h or irradiated with 50 Gy. In case of ionizing radiation, cells were analyzed 24 h later. Apoptotic cells were evaluated by flow cytometry analysis as a subdiploid peak in propidium iodide-stained samples. Results are the mean of three different experiments $\pm$ s.d. The asterisks indicate significant differences ($P<0.01$) in comparison with untreated AR cells.

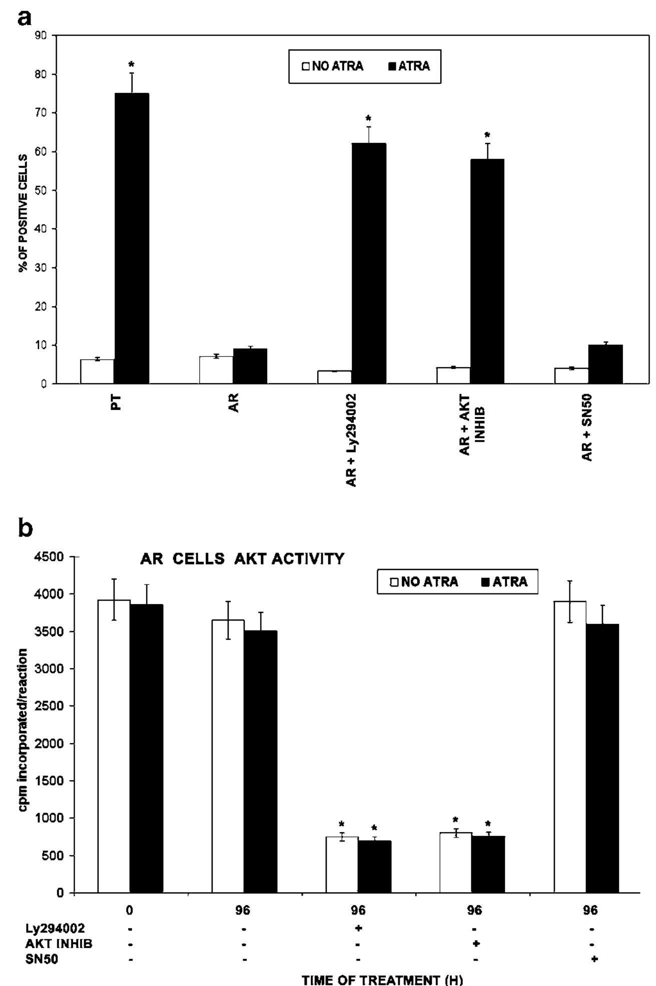

Figure 4 ATRA-dependent differentiation of HL60 cells. (a) HL60PT and AR cells were exposed to 1×10^{-6} M ATRA for 4 days. The medium was changed every day and, when required, contained the inhibitors. Results show the percentage of CD11b-positive cells, as analyzed by flow cytometry. Data are the mean of three different experiments $\pm$ s.d. The asterisks indicate significant differences ($P<0.01$) in comparison with untreated (no inhibitors) AR cells. (b) *In vitro* Akt kinase activity in AR cells treated for 96 h with ATRA. The inhibitor concentrations were: 10 μM Ly294002, 20 μM Akt inhibitor (Akt inhib), 30 μg/ml SN50. Results are the mean of three different experiments $\pm$ s.d. The asterisks indicate significant differences ($P<0.01$) in comparison with control (time 0) AR cells.

The Akt inhibitor restores HL60AR cell sensitivity to ATRA

Since our previous results showed that AR cells were resistant to ATRA,[26] we wanted to determine whether or not sensitivity to ATRA could be restored by the three inhibitors employed in this study. As presented in Figure 4a, after a 96 h exposure to ATRA approximately 75% of PT cells were differentiated. In contrast, only 8% of AR cells showed CD11b expression. However, if AR cells had been exposed to either Ly294002 or the Akt inhibitor they became much more sensitive to ATRA, with about 60% of cells showing CD11b expression. SN50 was ineffective in restoring ATRA sensitivity of HL60AR cells.

Measurement of *in vitro* Akt kinase activity demonstrated that at the end of ATRA exposure it was low in cells exposed to either Ly294002 or Akt inhibitor, whereas it was not affected by SN50 (Figure 4b).

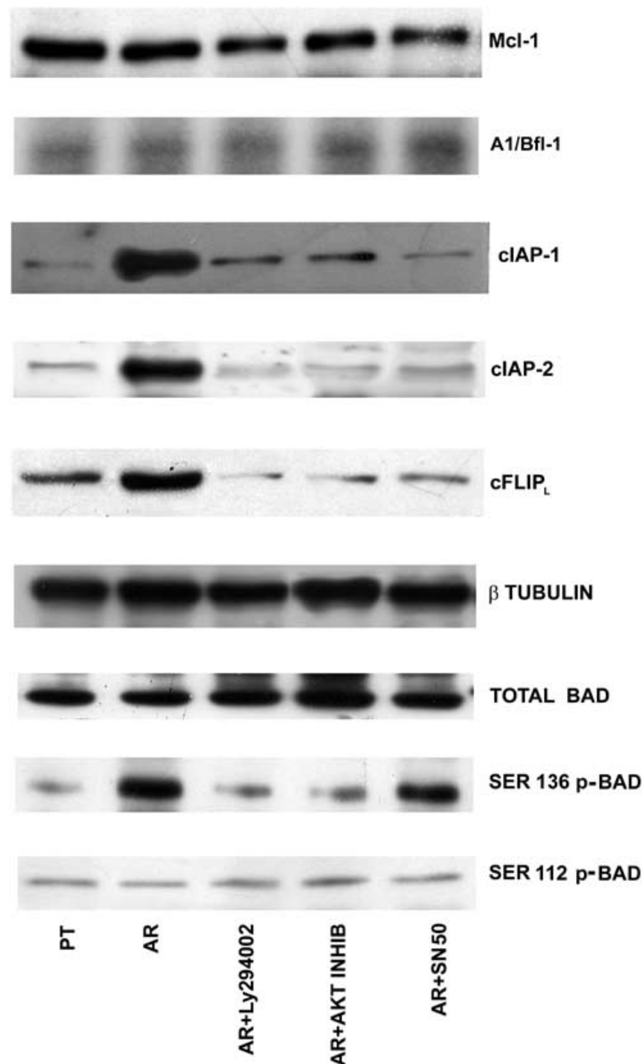

Figure 5 Expression of antiapoptotic proteins and p-Bad in HL60 cells. The inhibitors were present for 24 h and their concentrations were as in Figure 4b legend. Protein from cell homogenates was separated by SDS-PAGE and blotted to nitrocellulose paper. A total of 80 μg of protein was blotted to each lane. Bands were visualized by the ECL technique. For Mcl-1, A1/Bfl-1, cIAP-1, cIAP2, and cFLIP$_L$, immunostaining for β-tubulin confirmed equal loading.

The Akt inhibitor affects expression or phosphorylation state of antiapoptotic proteins

We next investigated the expression and/or the phosphorylation state of some antiapoptotic proteins that are known to be regulated through the PI3K/Akt pathway.[13] The expression level of two antiapoptotic members of the Bcl-2 family, Mcl-1, and A1/Bfl-1 was the same in HL60 PT and AR cells and was not sensitive to Ly294002, Akt inhibitor or SN50 (Figure 5). HL60AR cells expressed levels of the antiapoptotic proteins cIAP-1, cIAP-2, and cFLIP$_L$ higher than PT cells, in agreement with our own previous results.[25,26] The amount of these three proteins was sensitive to Ly294002, Akt inhibitor, or SN50, suggesting that their expression is mediated by the transcription factor NF-κB under the control of Akt.

Furthermore, when compared with PT cells, HL60AR cells had higher levels of Ser 136 p-Bad, a direct substrate of Akt. The levels of total Bad were similar in both the cell types and were not affected by the inhibitors. Bad phosphorylation on Ser 136 was inhibited by either Ly294002 or Akt inhibitor but not by SN50. As a further control, we investigated Ser 112 p-Bad, a target of the MAP kinase pathway.[28] However, in this case there was no difference between PT and AR cells, and the levels of phosphorylation were totally unaffected by the inhibitors tested.

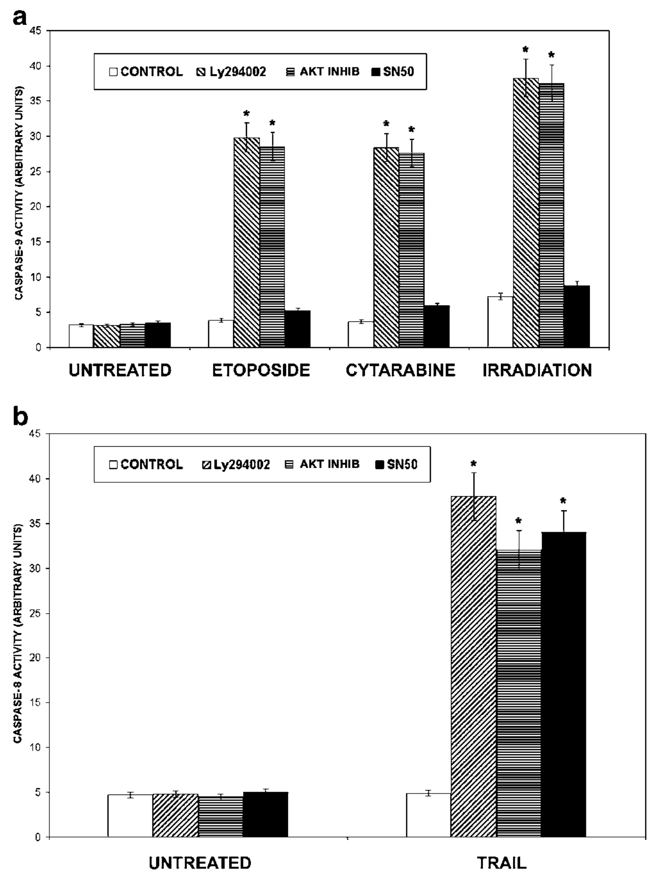

Figure 6 Caspase-9 (panel a) and caspase-8 (panel b) activation in HL60AR cells. Cells were treated with the apoptosis inducers as indicated in Materials and methods. The inhibitors had been present for 24 h prior to treatment. Concentrations of the inhibitors were as in Figure 4b legend. Caspase-9 was assayed with a fluorometric assay, while caspase-8 activity was analyzed by means of a colorimetric assay. Results are the mean of three different experiments$\pm$s.d. The asterisks indicate significant differences ($P<0.01$) in comparison with untreated AR cells.

Caspase activation

We next investigated caspase activation in response to apoptotic treatments in HL60AR cells. Since chemotherapeutic drugs and ionizing radiation are known to activate the mitochondrial pathway of apoptosis,[29] we first analyzed caspase-9 activation. As shown in Figure 6a, etoposide, cytarabine, or ionizing radiation failed to activate caspase-9 in AR cells. However, pretreatment with either Ly294002 or Akt inhibitor led to a dramatic increase in caspase-9 activity. In contrast, pretreatment with SN50 did not result in activation of caspase-9 in response to drugs or ionizing radiation.

According to our own previous studies, TRAIL induces apoptosis in HL60 cells through the receptor pathway and in this case the apical caspase was shown to be caspase-8.[25] As presented in Figure 6b, there was no activation of caspase-8 following exposure of HL60AR cells to TRAIL. Nevertheless, all three inhibitors markedly activated caspase-8.

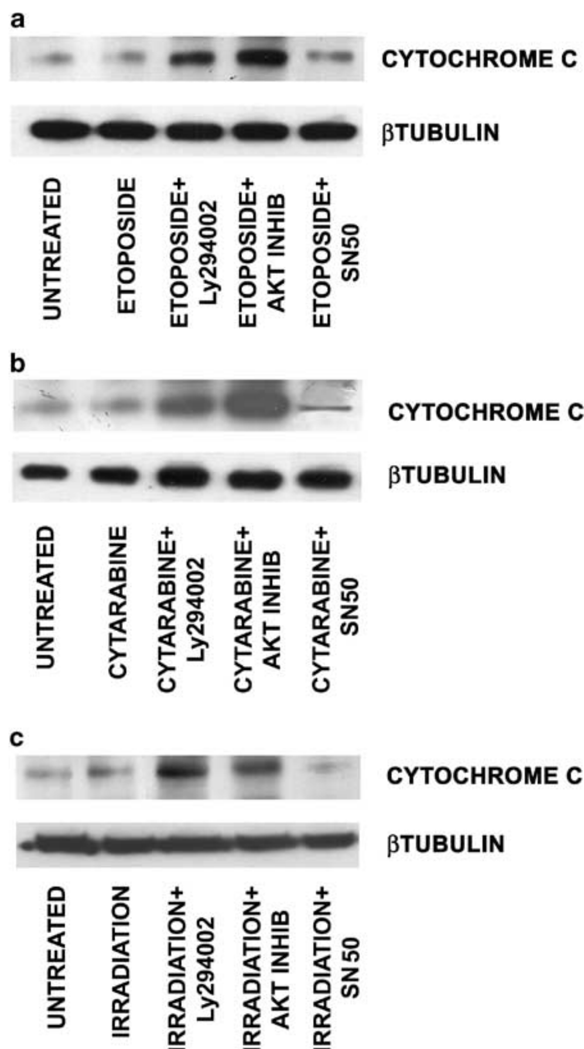

Figure 7 Cytochrome *c* release in the cytosolic fraction of HL60AR cells. Western blot analysis showing the release of cytochrome *c* from mitochondria following treatment with etoposide, cytarabine or ionizing radiation. Cells were treated with etoposide (5 μg/ml), cytarabine (40 μg/ml) for 6 h, or irradiated with 50 Gy. In case of ionizing radiation, cells were analyzed 24 h later. The inhibitors were present for 24 h prior to treatment with the apoptosis inducers and their concentrations were the same as reported in the legend of Figure 4b. A total of 80 μg of protein was blotted to each lane. Immunostaining with an antibody to β-tubulin confirmed equal loading.

Cytochrome c *release*

The activation of caspase-9 in HL60AR cells treated with chemotherapeutic drugs or ionizing agents in the presence of inhibitors of the PI3K/Akt pathway suggested the release of cytochrome *c* from mitochondria. We, therefore, investigated, by Western blot, the presence of cytochrome *c* released in the cytosol of AR cells. As shown in Figure 7, both etoposide and cytarabine, when employed alone, failed to increase the levels of cytosolic cytochrome *c*. In contrast, in cells pretreated for 24 h with either Ly294002 or Akt inhibitor, there was a marked increase in the amount of cytosolic cytochrome *c*. On the other hand, SN50 failed to induce the release of cytochrome *c* from mitochondria. The same pattern of cytochrome *c* release was also detected in samples exposed to ionizing radiation.

Akt inhibitor effect on PTEN activity, PKC-ζ phosphorylation, and p70S6K activity

PTEN is a phosphatase which dephosphorylates PtdIns(3,4,5)P_3. By doing so, PTEN is a fundamental negative regulator of Akt activity.[30] Therefore, we investigated whether PTEN activity was affected by the Akt inhibitor. As presented in Figure 8a, neither Ly294002 nor the Akt inhibitor caused a decrease in the expression levels of PTEN in AR cells, as evidenced by immunoblotting performed on cell homogenates. Moreover, the activity levels of PTEN were unchanged in AR cells exposed to either of the inhibitors.

The atypical PKC-ζ isoform is phosphorylated on Thr 410 of its activation loop in a PI3K-dependent manner by phosphoinositide-dependent protein kinase 1 (PDK-1, see Le Good *et al*,[31] Chen *et al*,[32] and Katso *et al*[33]). Also in HL60 cells PKC-ζ has been shown to be a downstream target of PI3K.[34] Thus, we sought to determine whether or not the Akt inhibitor affected the phosphorylation of PKC-ζ in HL60AR cells. Since the antibody to Thr 410 p-PKC-ζ we employed for these studies also recognizes PKC-λ phosphorylated on threonine 403, we first immunoprecipitated total PKC-ζ from cell homogenates with a specific polyclonal antibody. Then, the blots were probed with either the antibody to total PKC-ζ or to Thr 410 p-PKC-ζ. As shown in Figure 8b, it was evident that the expression of total PKC-ζ was the same in HL60PT and AR cells and was not affected by pretreatment with either of the inhibitors. On the other hand, the use of the antibody to Thr 410 p-PKC-ζ, demonstrated that in HL60AR cells, the kinase was more phosphorylated when compared with PT cells, consistently with the activation of the PI3K/PDK-1 pathway. PKC-ζ phosphorylation in AR cells was strongly inhibited by the PI3K inhibitor Ly294002, but not by the Akt inhibitor.

Another target of PDK-1 is represented by p70S6K, a protein kinase that is required for cell growth and cell cycle progression.[35] The activity of p70S6K is controlled by multiple phosphorylation events located within the catalytic, linker, and pseudosubstrate domains.[36] PDK-1 phosphorylates p70S6K on Thr 229. However, p70S6K is also phosphorylated on Thr 389 by FRAP/mTOR, a downstream target of Akt.[37,38] The amount of p70S6K protein was the same in HL60 PT and AR cells, and was not influenced by treatment with Ly294002 or Akt inhibitor (Figure 8c). When the activity of p70S6K was assayed, we found

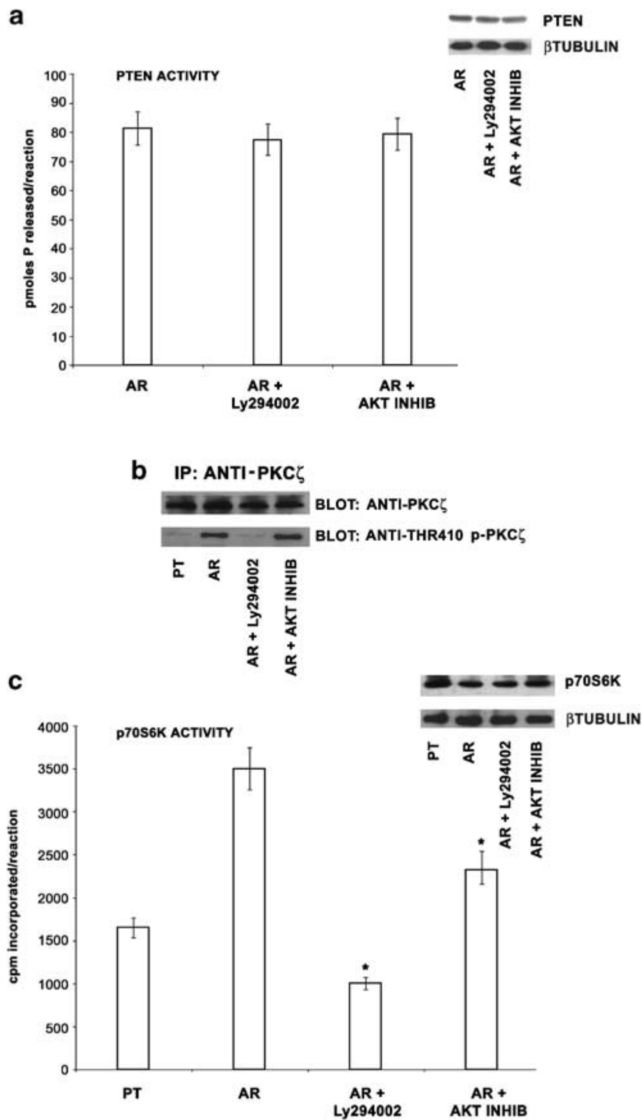

Figure 8 Effect of Akt inhibitor on PTEN activity, Thr 410 p-PKC-ζ levels, and p70S6K. (a) PTEN activity and Western blot analysis for PTEN expression. Cells had been incubated with the inhibitors (Ly294002, 10 μM; Akt inhibitor, 20 μM) for 24 h. Results of the activity assays are the mean of three different experiments ± s.d. For Western blot analysis, 80 μg of protein from cell homogenates was blotted to each lane. Immunoblotting analysis with antibody to β-tubulin confirmed equal loading. (b) Western blot analysis showing the levels of either total PKC-ζ or Thr 410 p-PKC-ζ in immunoprecipitates (IP) from HL60 PT, HL60 AR, and inhibitor-treated HL60AR cells. The inhibitors were present for 24 h at the same concentrations as in (a). Total PKC-ζ was immunoprecipitated with a polyclonal antibody. The blots were probed with either anti-PKC-ζ or anti-Thr 410 p-PKC-ζ. (c) p70S6K activity and Western blot analysis for p70S6K expression levels. Results of the activity assays are the mean of three different experiments ± s.d. The inhibitors were present for 24 h at the same concentrations as in (a). The asterisks indicate significant differences (P<0.01) in comparison with untreated AR cells. For Western blot analysis, 80 μg of protein was blotted to each lane. Immunoblotting analysis with antibody to β-tubulin confirmed equal loading.

it was higher in AR when compared with PT cells, in agreement with our own previous data.[26] In AR cells incubated with Ly294002 more than 70% of p70S6K activity was inhibited. In contrast, treatment of AR cells with the Akt inhibitor resulted in about 35% inhibition of p70S6K activity (Figure 8c).

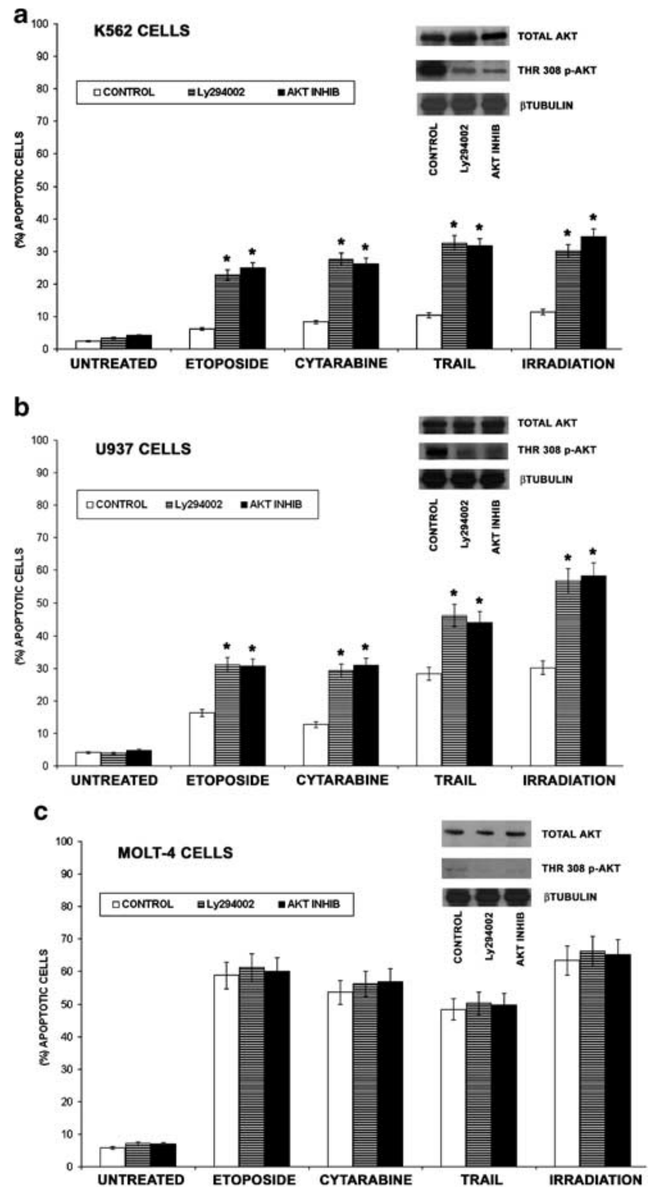

Figure 9 The Akt inhibitor enhances sensitivity to apoptosis inducers of K562 and U937, but not of MOLT-4, leukemia cells. (a) K562 cells. (b) U937 cells. (c) MOLT-4 cells. Concentrations of the inhibitors were: 10 μM for Ly294002 and Akt 20 μM for Akt inhibitor (Akt inhib). The inhibitors had been present for 24 h prior to treatment with the apoptosis inducers. Cells were treated with etoposide (5 μg/ml), cytarabine (40 μg/ml), TRAIL (500 ng/ml) for 6 h or irradiated with 50 Gy. In case of ionizing radiation, cells were analyzed 24 h later. Apoptotic cells were evaluated by flow cytometry analysis as a subdiploid peak in propidium iodide-stained samples. Results are the mean of three different experiments ± s.d. The asterisks indicate significant differences (P<0.01) in comparison with control cells. In each panel the levels of total Akt and Thr 308 p-Akt are shown. Immunoblotting analysis with antibody to β-tubulin confirmed equal loading of cell homogenates (80 μg protein/lane).

Akt inhibitor sensitizes to apoptosis inducers K562 and U937, but not MOLT-4, cells

We next sought to determine whether the Akt inhibitor was effective also in other cell lines with an upregulated PI3K/Akt pathway. K562 human erythroleukemia cells express the bcr/abl oncogene which, among other pathways, strongly stimulates

PI3K/Akt signaling.[39,40] Also because of the activation of the PI3K/Akt pathway, K562 cells are resistant to multiple apoptosis inducers.[41–43] As expected, K562 were found by immunoblotting to express high levels of Thr 308 p-Akt, which were effectively downregulated by treatment with either Ly294002 or Akt inhibitor (Figure 9a). K562 cells were resistant to etoposide, cytarabine, TRAIL, and irradiation. Nevertheless, pretreatment with Ly294002 or Akt inhibitor lowered resistance to these treatments (Figure 9a).

Another myeloid cell line which reportedly expresses sizeable levels of p-Akt is represented by U937 cells.[44] We confirmed this finding by Western blot analysis with an antibody to Thr 308 p-Akt (Figure 9b). Both Ly294002 and the Akt inhibitor downregulated the expression of Thr 308 p-Akt. U937 cells were more sensitive to apoptosis inducers than K562 cells. In any case, both the pharmacological inhibitors significantly enhanced the sensitivity to the treatments.

The concept of targeting the PI3K/Akt pathway in drug-resistant cells would have more validity if the observed increases in apoptosis were greater in drug-resistant, but not drug-sensitive, cells. To address this issue, we employed, as an experimental system, MOLT-4 cells which reportedly have very low levels of p-Akt.[45] Western blot analysis with the antibody to Thr 308 p-Akt confirmed these results (Figure 9c). The very low levels of p-Akt were completely downregulated by the pharmacological inhibitors. MOLT-4 cells were extremely sensitive to all the treatments and, more importantly, neither Ly294002 nor the Akt inhibitor did significantly raise their sensitivity (Figure 9c).

Discussion

Genetic instability contributes to the origin of cancer as well as to the ability of cancer cells to become resistant to various therapies. Many oncogenes and tumor suppressors mediate their effects by interfering with the signal transduction pathways that regulate cell survival or apoptosis. Thus, these pathways might be significantly altered in cancer cells relative to untransformed cells and the differences might present a therapeutic window that can be exploited for the development of cancer drugs. Historically, enzyme inhibitors have been the most straightforward molecules to identify and engineer into useful compounds.

The recent and remarkable success of the bcr/abl tyrosine kinase inhibitor STI-571 in patients suffering from chronic myeloid leukemia is, to date, the best example of a small molecule antagonist designed to inhibit the activity of a rationale target that is present in cancer cells only.[46]

The PI3K/Akt pathway plays a fundamental role in conveying signals essential for cell survival and appears to represent an attractive target for the development of new drugs for the treatment of acute myeloid leukemia also because previous results have indicated that this pathway is not essential for the growth of normal hematopoietic cells.[39]

In this study, we have investigated whether or not a new selective Akt pharmacological inhibitor could restore sensitivity to conventional (chemotherapeutic drugs, ATRA, ionizing radiation) and novel (TRAIL) therapeutic agents in an HL60 cell clone, known to be resistant to these treatments, which exhibits a constitutively active PI3K/Akt pathway. In HL60AR cells, the upregulation of the PI3K/Akt pathway is due to an autocrine production of insulin-like growth factor-1.[26] We reasoned that these cells could represent a good model for our studies because they would allow to investigate in greater depth the selectivity of the Akt inhibitor. Indeed, these cells also display an activation of PI3K-dependent signaling pathways that do not completely impinge on Akt, such as p70S6K.[26]

Our results showed that when used at 20 μM, the Akt inhibitor did not affect the activity of PI3K, whereas it was very effective in blocking Akt activity in HL60AR cells, as demonstrated by *in vitro* kinase assays and Western blotting analysis with an antibody to Thr 308 p-Akt or Ser 473 p-Akt. Inhibition of PI3K activity occurred at much higher concentrations. Overall, the Akt inhibitor was as effective as Ly294002 in blocking Akt activity and its phosphorylation. A time study showed that a 24 h incubation treatment was required to reach the maximal inhibition of Akt. These findings are consistent with the results of other groups that employed PI3K inhibitors (Ly294002 and wortmannin) for a similar length of time to downregulate its activity and that of Akt.[40,47]

The Akt inhibitor was able to sensitize AR cells to two chemotherapeutic drugs used for treatment of acute myeloid leukemia (ie etoposide and cytarabine), to TRAIL, ATRA, and ionizing radiation. Since it is thought that several of the antiapoptotic effects of Akt are mediated by the activation of the transcription factor NF-κB,[13] we investigated whether or not a cell permeable inhibitor of NF-κB nuclear translocation and activation (ie SN50) was also capable of sensitizing HL60AR cells to these treatments. However, SN50 was only capable of restoring sensitivity to TRAIL, but not to chemotherapeutic agents, ATRA or ionizing radiation. Therefore, we sought to investigate the possible molecular mechanisms underlying resistance of HL60AR cells.

As demonstrated by Western blot analysis, HL60AR cells, when compared with PT cells, expressed higher levels of several antiapoptotic proteins such as cIAP-1, cIAP-2, and cFLIP$_L$. Since the synthesis of these proteins is under the control of NF-κB, their expression in AR cells was lowered by Ly294002, Akt inhibitor or SN50. Nevertheless, HL60AR also displayed much higher levels of Ser 136 p-Bad, a direct target of Akt. Bad phosphorylation was markedly diminished by either Ly294002 or Akt inhibitor, but not by SN50. cIAP-1 antagonizes activation of effector caspase-3[29] while cIAP-2 and cFLIP$_L$ have been shown to inhibit caspase-8.[48]

p-Bad blocks activation of the mitochondrial apoptotic pathway, because it is sequestered in the cytosol by 14-3-3 proteins and cannot, therefore, antagonize antiapoptotic Bcl-X$_L$.[9] Therefore, Bad hyperphosphorylation could explain why both Ly294002 and Akt inhibitor sensitized AR cells to chemotherapeutic drugs and ionizing radiation (which activate the apoptotic mitochondrial pathway), while SN50 did not, even though it lowered c-IAP1 levels.

Indeed, both Ly294002 and Akt inhibitor enhanced caspase-9 activity and cytochrome *c* release from mitochondria in AR cells exposed to drugs or ionizing radiation, while SN50 was ineffective. In contrast, Ly294002 and Akt inhibitor were as effective as SN50 in increasing caspase-8 activation in response to TRAIL. Therefore, inhibitors of NF-κB nuclear translocation and activation may be useful in restoring sensitivity to TRAIL, but they appear to be useless in lowering resistance to chemotherapeutic drugs, ATRA, and irradiation in cells in which there is an increase of p-Bad.

We feel that these results underscore the importance of performing a comprehensive investigation of the apoptotic and survival signaling pathways if, in the future, therapies based on molecules interfering with these pathways will be employed for the treatment of hematological malignancies.

It is totally unclear how the PI3K/Akt pathway negatively influences sensitivity of HL60AR cells to ATRA. However,

similar results have recently been reported for human breast cancer cells.[49]

Since there was a lack of data regarding the selectivity of the Akt inhibitor we employed in this study, we investigated whether it might affect other signaling proteins. However, we have found that the inhibitor neither upregulated PTEN activity nor downregulated PKC-ζ phosphorylation, which is important, for example, in insulin-mediated glucose transport. In contrast, Ly294002 markedly diminished the phosphorylative state of this PKC isozyme. The Akt inhibitor inhibited p70S6K activity but not as much as Ly294002.

The Akt inhibitor enhanced sensitivity to apoptotic inducers of two other cell lines with sizeable levels of p-Akt, that is, K562 and U937 human leukemia cells. In contrast, the Akt inhibitor did not increase sensitivity of a cell line (MOLT-4 cells) in which the levels of p-Akt are very low. This finding is in agreement with our own previous data demonstrating that in HL60PT cells (which have low levels of p-Akt) PI3K inhibitors (Ly294002, wortmannin) did not increase sensitivity to chemotherapeutic drugs.[26]

At present, it is becoming evident that activation of the PI3K/Akt axis may be important for resistance to conventional (chemotherapeutic drugs) and novel (TRAIL) therapies in patients suffering from multiple myeloma.[19,50]

It should be considered, however, that there are some cases of acute myeloid leukemias in which the PTEN tumor suppressor is absent or non-functional.[51–53] Since PTEN is a fundamental negative regulator of Akt activity,[54] it might be that in these leukemias the Akt pathway is upregulated with a consequent resistance to multiple apoptotic stimuli, as it happens in PTEN-negative prostatic cancer cell lines.[17]

Furthermore, it should be emphasized that the mouse gene coding for the p110α catalytic subunit of PI3K has been mapped to band B of chromosome 3, a region syntenic with human chromosome 3q26.3.[55] It is of great interest that acute myeloblastic leukemias highly resistant to chemotherapy since initial presentation often display cytogenetic abnormalities (inversion, translocation) involving such a region of chromosome 3.[56] These anomalies could lead to overexpression of the PI3K catalytic subunit with an ensuing upregulation of Akt.

Lastly, very recent evidence has shown the occurrence of a possible dominant-negative mutation of the SHIP gene in acute myeloid leukaemia.[57] SHIP is a hematopoietic-specific inhibitory phosphatase,[58] which dephosphorylates PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 (59). It is of great interest that in SHIP−/− mast cells Akt was more active.[60]

Taken together, all the aforementioned findings point to the likelihood that an activation of the PI3K/Akt axis is possible in certain types of acute myeloid leukemias and this could have deep consequences on the outcome of therapy.

Our results also showed that the PI3K/Akt signaling pathway is important in determining resistance to ionizing radiation and pharmacological inhibitors of this pathway restored sensitivity of HL60AR cells to X-rays. This observation may have important implications for the treatment of acute myeloid leukemia by means of bone marrow transplantation. Indeed, the standard conditioning regimen for both allogeneic and autologous bone marrow transplantation consists of chemotherapy and total-body irradiation. However, the risk of relapse is high[61] and this might depend, at least in part, on leukemic cell resistance to chemotherapeutic drugs and ionizing radiation. Therefore, in the future, pharmacological inhibitors of PI3K/Akt axis might also be employed to sensitize leukemic cells to chemotherapeutic drugs and total-body irradiation prior to bone marrow transplantation.

Pharmacological inhibitors of PI3K such as wortmannin and Ly294002 have been shown to enhance the effect of STI571 in the inhibition of clonogenic growth of chronic myeloid leukemia cells, while sparing normal bone marrow cells.[40] The combination of STI571 plus wortmannin caused a more pronounced activation of caspase-3 and induced massive apoptosis, in comparison to STI571 and wortmannin alone.

Moreover, it should be emphasized that Ly294002 exerted a selective protection of mitogenically stimulated human lymphocytes but not of leukemic cell lines (HL60, MOLT-4, Jurkat) from cytosine arabinoside-induced apoptosis. This effect was likely due to the prevention of the exit of lymphocytes from the G_0 phase of the cell cycle.[62] Although these experiments were performed *in vitro*, taken together they suggest that inhibitors of the PI3K/Akt pathway might not exert a toxic effect on normal hematopoietic cells.

The problem of other systemic effects of these inhibitors has not been investigated so far, but may be substantial, since the PI3K/Akt pathway regulates many cell functions. However, our results have shown that the Akt inhibitor did not affect PKC-ζ phosphorylation, and inhibited p70S6K activity less than Ly294002. Additional selective Akt inhibitors have very recently been synthesized and they also produce only a moderate downregulation of p70S6K activity.[63]

Three distinct Akt isoforms have been identified and cloned.[10–12] Since they display a different tissue expression, it may be envisaged that the use of inhibitors that are selective for a given Akt isoform would diminish the risk of systemic toxicity. In this connection, it should be recalled that our unpublished data show that HL60 cells only express the Akt1 isoform.

Finally, our results also show that a thorough evaluation of the apoptotic and survival signaling pathways operating in patients suffering from acute leukemia may be of fundamental importance to set up a therapeutic treatment aimed at specific targets.

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