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Phosphoinositide 3-Kinase/Akt Involvement in Arsenic Trioxide Resistance of Human Leukemia Cells

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The purpose of this study was to evaluate the possible involvement of the phosphoinositide 3-kinase (PI3K)/Akt survival pathway in determining resistance to arsenic trioxide (As_2O_3)-induced apoptosis. We employed a HL60 cell clone (HL60AR) with a constitutively active PI3K/Akt survival pathway, as well as U937 and K562 cells. In addition, we used parental (PT) HL60 cells overexpressing a constitutively active Akt. Selective pharmacological inhibitors of the PI3K/Akt axis (LY294002, wortmannin) were employed to influence the sensitivity to As_2O_3. While HL60PT cells were sensitive to 2.5 μM As_2O_3 and died of apoptosis, HL60AR cells were resistant up to 5 μM As_2O_3. Treatment with either LY294002 or wortmannin lowered resistance of HL60AR cells to As_2O_3. Also in U937 and K562 cells, inhibitors of the PI3K/Akt axis caused a decrease in As_2O_3 resistance. Overexpression of constitutively active Akt in HL60PT cells caused the induction of resistance to 2.5 μM As_2O_3. Conversely, forced expression of a dominant negative Akt in HL60AR cells resulted in a decrease in As_2O_3 resistance. Moreover, HL60 cell resistance to 2.5 μM As_2O_3 could be significantly reduced by incubation with SN50, a peptide inhibitor selective for the NF-κB transcription factor. Taken together our findings suggest that a constitutive activation of the PI3K/Akt pathway, which is increasingly detected in some types of acute myeloid leukemia, may contribute to As_2O_3 resistance, most likely through NF-κB activation. Selective pharmacological inhibitors of this survival pathway, as well as of NF-κB, might be usefully employed in the future to reverse resistance to this treatment. J. Cell. Physiol. 202: 623–634, 2005. © 2004 Wiley-Liss, Inc.

As_2O_3 is an effective agent against acute promyelocytic leukemia (APL), both in patients with newly diagnosed disease and in those with refractory and relapsed forms (Bachleitner-Hofmann et al., 2002; Hu et al., 2003a). As far as leukemic cell lines are concerned, the therapeutic effects of As_2O_3 are not restricted to parental NB4 cells, but they are seen also in retinoic acid-resistant cells (Chen et al., 1997; Bachleitner-Hofmann et al., 2002). Moreover, it has been shown that As_2O_3 is effective also against other cell lines, both of myeloid and of lymphoid lineage (Wang et al., 1998a; Zhang et al., 1998), and against drug-resistant sublines (Perkins et al., 2000; Grad et al., 2001).

The mechanisms of action of As_2O_3 in APL and other malignancies are thought to involve growth inhibition and apoptosis induction (Chen et al., 1997; Zhang et al., 1998; Akao et al., 2000; Zhang and Nie, 2001; Choi et al., 2002).

Giovanna Tabellini and Alessandra Cappellini equally contributed to this work.

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Available evidence suggests that As_2O_3 causes apoptotic cell death through various mechanisms. As_2O_3 treatment of U937 leukemia cells induces mitochondrial transmembrane potential collapse and release of cytochrome c (Cai et al., 2000; Choi et al., 2002). Hence, As_2O_3 should upregulate the intrinsic apoptotic pathway which leads to activation of caspase-9 (Hengartner, 2000). Indeed, caspase-9 activation in response to As_2O_3 has been recently reported (Liu et al., 2003). Another important factor in As_2O_3-mediated apoptosis induction is the generation of reactive oxygen species (Choi et al., 2002; Yi et al., 2002).

Moreover, recent findings have highlighted that As_2O_3 also up-regulates the CD95/CD95 ligand expression in leukemic cell lines. This results in the activation of the receptor-mediated apoptotic pathway and cleavage of procaspase-8 (Zhu et al., 2003), one of the apical caspases of this signaling network (Chen and Wang, 2002).

As_2O_3 activates the p38 and JNK pathways. This activation may be responsible for the carcinogenic effects of As_2O_3 but it is not required for the induction of apoptosis (Cavigelli et al., 1996; Maeda et al., 2001; Mathas et al., 2003).

As_2O_3 resistance has been reported at diagnosis or as arising during treatment with As_2O_3 in some cases of APL. The resistance has been linked with induction of P-glycoprotein and multidrug resistance-associated protein 1, but this does not seem to be the only mechanism which underlies As_2O_3 resistance.

It has been shown that As_2O_3 down-regulates NF-κB target genes, including c-IAP2 (Mathas et al., 2003). Interestingly, NF-κB transcription factor is a critical down-stream target of the PI3K/Akt signaling network. This pathway conveys signals of utmost importance for cell survival and has a powerful anti-apoptotic effect (Nicholson and Anderson, 2002; Chang et al., 2003). In this connection, it is interesting to emphasize that As_2O_3 decreases Akt activity in HL60 (Porosnicu et al., 2001) or U937 (Choi et al., 2002) human leukemia cells.

In light of these findings, we decided to investigate whether or not the PI3K/Akt signaling pathway is involved in influencing sensitivity of human leukemia cells to As_2O_3. To this end, we took advantage of a HL60 cell clone, HL60AR cells, with an up-regulated PI3K/Akt signaling network. We have thoroughly characterized this clone in a series of recently published papers (Tazzari et al., 2002; Bortul et al., 2003; Cappellini et al., 2003; Neri et al., 2003). Stimulation of the PI3K/Akt axis of HL60AR cells is caused by an autocrine production of insulin-like growth factor-1. As a consequence, HL60 AR cells are resistant to a variety of apoptotic stimuli, including chemotherapeutic drugs, TRAIL, and ionizing radiation (Martelli et al., 2003).

Here, we show that HL60AR cells are resistant to clinically relevant (0.5–5 μM) concentrations of As_2O_3. In contrast, PT HL60 cells underwent apoptotic cell death when cultured with the same concentrations of As_2O_3. Down-regulation of the PI3K/Akt pathway by selective pharmacological inhibitors restored HL60AR cell sensitivity to As_2O_3. PT cells overexpressing a constitutively active Akt became resistant to As_2O_3, whereas AR cells with a forced expression of either a dominant negative Akt or a wild-type PTEN displayed a sensitivity to As_2O_3. Most likely, As_2O_3 resistance was partly linked with activation of the NF-κB transcription factor. Treatment with PI3K pharmacological inhibitors also increased As_2O_3 sensitivity of K562 and U937 leukemia cells.

Taken together, our findings point to the likelihood that an activation of the PI3K/Akt pathway may contribute to As_2O_3 resistance in human leukemia cells.

MATERIALS AND METHODS

Reagents

Bovine serum albumin (BSA), normal goat serum (NGS), mouse monoclonal antibody to β-tubulin, rabbit polyclonal to XIAP, and peroxidase-conjugated anti-mouse or anti-rabbit IgG were from Sigma (St. Louis, MO). The COMPLETE Protease Inhibitor Cocktail, and the Lumi-Light^{Plus} Enhanced chemiluminescence (ECL) detection kit were from Roche Applied Science (Milan, Italy). The Akt kinase nonradioactive assay kit was from Cell Signaling Technology (Beverly, MA).The Protein Assay kit (detergent compatible) was from Bio-Rad (Hercules, CA). LY294002, wortmannin, PD98059, Z-VAD-FMK, and mouse monoclonal antibody to lamin B were from Calbiochem (La Jolla, CA). The SN50 peptide was from Biomol Research Laboratories (Plymouth Meeting, PA). The following antibodies were from Cell Signaling Technology: rabbit polyclonals to total Akt (#9272), Ser 473 phosphorylated Akt (p-Akt), Thr-308 p-Akt, and mouse monoclonal to PTEN (# 9556). Rabbit polyclonal antibody to NF-κB p50 subunit was from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonals to cIAP-1, cIAP-2, TRAF-1, and TRAF-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). The ApoAlert Caspase-8 colorimetric assay kit, the ApoAlert Caspase-9/6, and the ApoAlert Caspase-3 fluorometric assay kits were from BD Biosciences Clontech (Palo Alto, CA). The Caspase-7 fluorometric assay kit was from Cell Signaling Technology. The Caspase-6 colorimetric assay kit was from Oncogene Research Products (La Jolla, CA). PTEN-G129E cDNA was a kind gift from Dr. W. Sellers, Dana-Farber Cancer Institute (Boston, MA) (Ramaswamy et al., 1999).

Cell culture and treatment with As_2O_3

HL60PT (obtained from the American Type Culture Collection-CCL 240) and HL60AR cells were routinely maintained in RPMI 1640 supplemented with 10% fetal calf serum at an optimal cell density of 3 to 8×10^5 cells/ml. Medium was changed every 3 days. HL60 PT cell clones stably overexpressing constitutively active Akt and HL60AR cell clones stably overexpressing either dominant negative Akt or full length wild-type (normal) PTEN were as previously reported (Bortul et al., 2003; Neri et al., 2003). K562 and U937 human leukemia cells were cultured in the same medium as HL60 cells. In some experiments, cells were incubated with nontoxic concentrations of the PI3K pharmacological inhibitors LY294002 (10 μM), wortmannin (0.1 μM), or of the ERK-pathway inhibitor PD98059 (20 μM). As_2O_3 was dissolved in 1.65 mM NaOH to make a 1 mM stock solution which was serially diluted in RPMI 1640 (Perkins et al., 2000).

Detection of apoptosis

For sub-G1 (apoptotic cells) peak analysis, cells were harvested by centrifugation at 200*g* for 10 min, fixed with 70% cold (4°C) ethanol for 1 h, and subsequently stained with propidium iodide (PI, DNA-Prep kit, Beckman-Coulter Immunology, Miami, FL). The sub-diploid DNA content was analyzed and calculated using an Epics XL flow cytometer with the appropriate software (Beckman-Coulter). At least 10,000 events were acquired.

Cell transfection

Transfection of HL60AR cells was performed by electroporation with a Bio-Rad Gene Pulser apparatus. Twenty micrograms of plasmid DNA (PTEN-G129E cloned in pUSEamp) was mixed with 10^7 cells in 0.5 ml of phosphate-buffered sucrose (272 mM sucrose and 7 mM Na_2HPO_4, pH 7.4). Cells were electroporated with a pulse of 250 V for 18–20 msec. Control cells were mock-transfected in the same conditions with the empty vector. Following electroporation, cells were allowed to recover in 20 ml of culture medium for 48 h before selection with 600 μg/ml of G418. G418-resistant transfected clones were obtained by limited dilution.

Preparation of cytoplasmic fraction and isolated nuclei

Cells were washed once in phosphate buffered saline (PBS), pH 7.4 (without Ca^{2+} and Mg^{2+}) and resuspended to 1.5×10^7/ml in 10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of aprotinin and leupeptin, 1.0 mM Na_3VO_4, and 20 nM okadaic acid. After 5 min at 0°C, cells were sheared by six passages through a 22 gauge needle fitted to a 30 ml plastic syringe. The concentration of Mg^{2+} was brought to 5 mM and nuclei were sedimented at 400*g* for 6 min. The post-nuclear supernatant was saved for analysis of cytoplasmic fraction. Nuclei were washed once in 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and resuspended to 2 mg DNA/ml in 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 5 mM $MgCl_2$. This procedure allowed the preparation of nuclei free of cytoplasmic contamination as demonstrated by transmission electron microscopy analysis (data not shown).

Protein assay

This was performed according to the instruction of the manufacturer using the detergent compatible Bio-Rad Protein Assay.

Preparations of cell homogenates for 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, boiled for 5 min to solubilize protein. Protein separated on sodium dodecylsulphate (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 re-probed.

Preparation of cell homogenates, immunoprecipitation, and Akt kinase assay

Cells were resuspended in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, supplemented with the protease and phosphatase inhibitor cocktail as above and homogenized by 30 passages through a 25 gauge needle. 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, samples were incubated for 2 h at 4°C under constant agitation with 5 μg of the antibody to total Akt. Ten micrograms of 50% Protein A/G Agarose was then added and incubation proceeded for 1 h at 4°C under constant agitation.

The resulting immunoprecipitates were incubated with GSK-3 fusion protein in the presence of ATP, following the Manufacturer's instructions. This allowed Akt to phosphorylate GSK-3. Phosphorylation of GSK-3 was measured by Western blotting using a polyclonal antibody to Ser 21/9 p-GSK-3α/β.

Caspase activity assay

These activities were measured using fluorometric or colorimetric assays according to the Manufacturer's instructions. Briefly, cells (2×10^6) were first lysed in 50 mM HEPES/KOH, pH 7.5, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol (DTT), 20 μg/ml leupeptin, 10 μg/ml pepstatin and aprotinin. For caspase-3 activity samples were incubated with the caspase-3 fluorometric substrate DEVD-AFC (0.5 mM) for 2 h at 37°C. Samples were read in a fluorometer with a 400-nm excitation filter and a 505-nm emission filter. For caspase-7 assay, the substrate was Ac-DEVD-AFC. Antibody to caspase-7 was added to the cell lysate and incubation proceeded for 2 h. Protein A/Sepharose beads were added and further incubated for 2 h at 4°C. The immunoprecipitates were washed twice in 50 mM HEPES/KOH, pH 7.5, 75 mM NaCl, 5 mM DTT, 0.01% CHAPS. Ac-DEVD-AFC was added to a final concentration of 0.4 mM and incubation was at 37°C overnight. AFC was excited at 400 nm and the emission recorded at 505 nm. For caspase-9, the fluorometric substrate was LEHD-7-amino-4-trifluoremethyl coumarin, and 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, while for caspase-6 it was VEID-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. Data of caspase activities were expressed as arbitrary units: one arbitrary unit corresponds to a fluorescence intensity of 0.02 or an absorbance of 0.01 on the reading scale.

RESULTS

As_2O_3 induces apoptosis and activate caspases in HL60PT but not AR cells

In a first series of experiments, we performed an analysis of the sensitivity to As_2O_3-induced apoptosis of

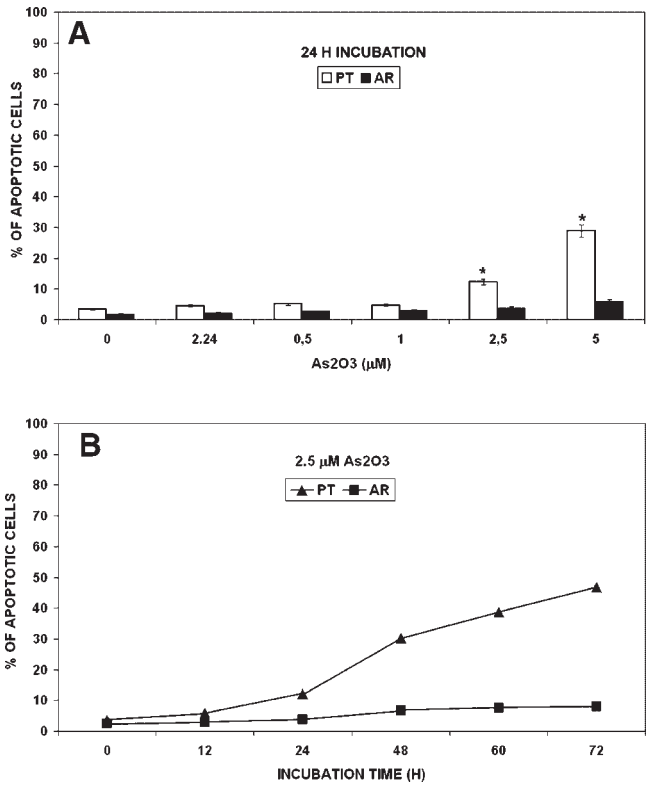


Fig. 1. HL60AR cells are resistant to As_2O_3-induced apoptosis. **A**: HL60PT and AR cells were incubated for 24 h in the presence of the indicated As_2O_3 concentration. **B**: HL60PT and AR cells were incubated for indicated times in the presence of 2.5 μM As_2O_3. Samples were fixed with 70% ethanol, stained with PI, and analyzed by flow cytometry. Results are the mean of three different experiments ±SD. Asterisks indicate significant differences ($P < 0.01$).

HL60 cells. As shown in Figure 1A, an incubation of 24 h resulted in a significant increase in the amount of apoptosis in HL60PT cells treated with 2.5 μM As_2O_3. The percentage of apoptotic cells more than doubled if the concentration was raised to 5 μM. In contrast, HL60AR did not show any significant increase in the amount of apoptosis even at the highest As_2O_3 concentration (Fig. 1A).

A time course study (Fig. 1B) showed that, at a concentration of 2.5 μM As_2O_3, the percentage of apoptotic HL60PT cells steadily increased up to nearly 45% after a 72 h incubation.

On the other hand, even after a 72 h incubation no more than 6% of HL60AR cells were apoptotic. Overall, our findings regarding HL60PT cells were in agreement with the data reported recently by Rojewski et al. (2002) who have investigated the differential sensitivity to As_2O_3 displayed by several human acute leukemia cell lines, including HL60.

In the following experiments, caspase activity assays indicated a marked activation of caspase-3, -6, -8, and -9 (but not of caspase-7) in PT cells at 24 and 48 h since exposure to As_2O_3. In striking contrast, caspases were not activated in AR cells (Fig. 2). The importance of caspase activation in determining apoptotic cell death of HL60PT cells was demonstrated by the finding that a broad range caspase inhibitor, Z-VAD-FMK, completely

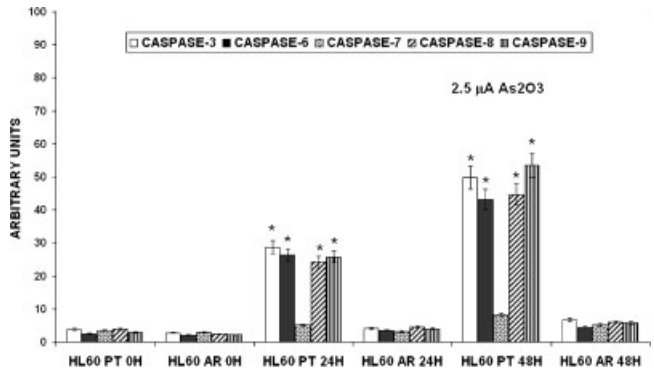


Fig. 2. Caspase activities in response to 2.5 μM As_2O_3. HL60PT and AR cells were treated with 2.5 μM As_2O_3 for 24 or 48 h. In vitro caspase-3, -6, -7, -8, and -9 activities were then measured in cell homogenates using fluorometric (caspase-3, -7, and -9) or colorimetric (caspase-6 and -8) assays. Results are the mean of three different experiments ±SD. Asterisks indicate significant differences ($P < 0.01$).

inhibited apoptosis of As_2O_3-treated HL60PT cells (data not shown).

Pharmacological inhibitors of the PI3K/Akt pathway increases sensitivity to As_2O_3 of HL60AR cells and activate caspases

We next investigated whether or not two unrelated pharmacological inhibitors of the PI3K/Akt pathway, LY294002 and wortmannin, could restore the sensitivity to As_2O_3 of HL60AR cells. HL60 cells were exposed to pharmacological inhibitors and to 2.5 μM As_2O_3 for 48 h.

As shown in Figure 3, the amount of total Akt did not decrease in PT or AR cells under all the conditions tested. Ser 473 p-Akt, one of the active forms of the enzyme, did not markedly decrease in untreated cells (either PT or AR) from the beginning of experiment to 48 h. In contrast, LY294002 and wortmannin were effective in reducing the levels of Ser 473 p-Akt especially in AR cells after a 48 h incubation. Similar data were obtained also for Thr 308 p-Akt, the other active form of the kinase. These experiments demonstrated that the two inhibitors markedly reduced the amount of active Akt after a 48 h incubation.

Flow cytometric analysis of PI-stained samples showed that, after a 48 h incubation, LY294002 and wortmannin did not increase per se the percentage of apoptotic HL60 cells and the same was true of PD98059, an inhibitor of the ERK pathway, which is also very important for cell survival (Kohno and Pouyssegur, 2003).

However, both LY294002 and wortmannin dramatically increased the sensitivity of HL60AR cells to 2.5 μM As_2O_3 over a 48 h incubation (Fig. 3). The two inhibitors only slightly increased the percentage of apoptotic cells in As_2O_3-treated PT cells. In contrast, incubation of PT or AR cells with PD98059 did not result in an increased sensitivity to As_2O_3, suggesting the ERK pathway is not involved in cell resistance to As_2O_3.

As shown in Figure 4, while the PI3K inhibitors per se did not increase caspases activities, the combination of a PI3K inhibitor (either LY294002 or wortmannin) plus 2.5 μM As_2O_3 led to a dramatic increase in the activity of capases-3, -6,-8, and -9, but not of caspase-7, in HL60AR cells.

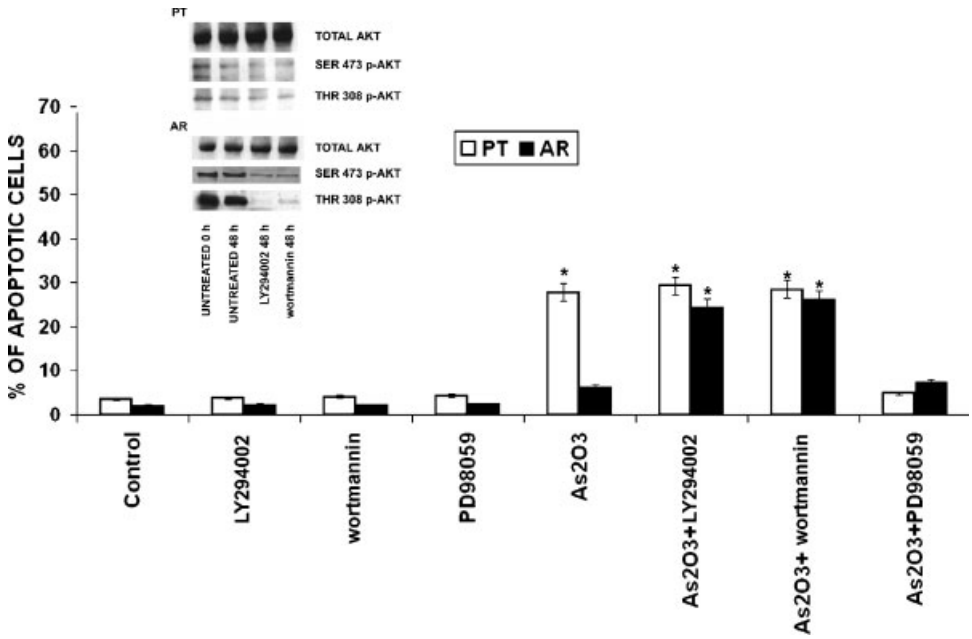


Fig. 3. Inhibitors of the PI3K/Akt pathway increase sensitivity of HL60AR cells to As_2O_3. Cells were incubated for 48 h in the presence of 2.5 μM As_2O_3. LY294002 (10 μM), wortmannin (0.1 μM), or PD98059 (20 μM) were added at time 0, 12, 24, and 36 h. Samples were fixed with ethanol, stained with PI, and analyzed by flow cytometry. Results are the mean of three different experiments ±SD. Asterisks indicate significant differences ($P < 0.01$). Western blot analysis shows the amount of total Akt and of its phosphorylated forms in HL60PT and AR cells. Cells were incubated with 2.5 μM As_2O_3 for 48 h. The PI3K inhibitors were present as specified above and samples were collected after a 48 h incubation. Protein (80 μg/lane), separated by SDS-polyacrylamide gels, was blotted to nitrocellulose sheets that were then probed with polyclonal antibodies specific for total Akt or its phosphorylated forms. Bands were detected by ECL. A representative blots from three different experiments is shown.

Inhibition of the PI3K/Akt pathway increases As_2O_3 sensitivity of K562 and U937 leukemia cells

It remained important to investigate whether the involvement of the PI3K/Akt signaling network in conferring resistance to As_2O_3 was restricted to HL60 cells or could be a more general phenomenon.

To address this issue, we analyzed the effects of the PI3K/Akt pharmacological inhibitors on two other cell lines which have high levels of Akt activity, K562 and

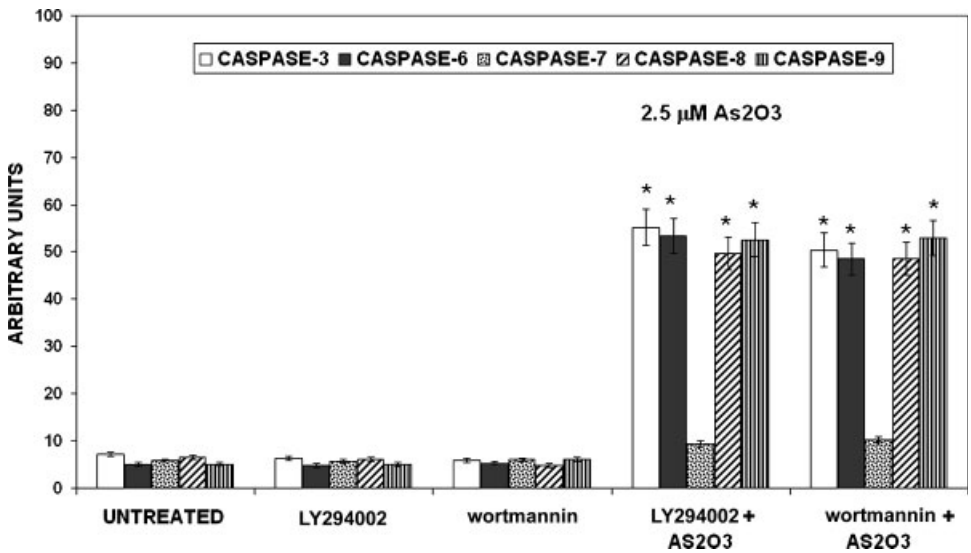


Fig. 4. Inhibitors of the PI3K/Akt pathway increase caspase activities in HL60AR cells treated with As_2O_3. HL60AR cells were incubated for 48 h in the presence of 2.5 μM As_2O_3. LY294002 (10 μM), or wortmannin (0.1 μM) were added at time 0, 12, 24, and 36 h. Cells were then lysed and in vitro caspase-3, -6, -7, -8, and -9 activities were measured in cell homogenates using fluorometric (caspase-3, -7, and -9) or colorimetric (caspase-6 and -8) assays. Results are the mean of three different experiments ±SD. Asterisks indicate significant differences ($P < 0.01$).

U937 cells (Skorski et al., 1995; Plo et al., 1999; Klejman et al., 2002; Martelli et al., 2003). According to previous results (Rojewski et al., 2002), K562 and U937 are resistant to 2.5 μM As_2O_3. Therefore, we employed a 5.0 μM concentration for 48 h.

As shown in Figure 5, such a concentration of As_2O_3 induced apoptosis in approximately 20% of the cells. Treatment of K562 cells with either LY294002 or wortmannin resulted in a marked decrease of Akt phosphorylation (not shown, but see Martelli et al., 2003).

If K562 or U937 cells were incubated with two PI3K inhibitors alone, the percentage of apoptotic cells did not change in comparison with control cells. However, the combination of As_2O_3 + LY294002 or As_2O_3 + wortmannin, markedly increased the number of cells that underwent apoptotic cell death.

Transfection experiments

Because our findings demonstrated that PI3K inhibitors sensitize HL60AR cells to As_2O_3, we used a genetic approach to up-regulate Akt activity in PT cells or to down-regulate it in AR cells. In our previous papers, we have described PT cell clones overexpressing constitutively active Akt, and AR cell clones overexpressing either dominant negative Akt or wild-type PTEN. Briefly, PT cell clones overexpressing constitutively active Akt (12FC, 17GC, 81M) have an Akt activity higher than untransfected PT cell, whereas AR cell clones overexpressing either dominant negative Akt (2W, 3G, 3Y) or wild-type PTEN (GH1, GH9, 7E5) have an Akt activity lower than untransfected AR cells (24, 25).

In the present study, we also stably transfected HL60AR cells with a PTEN mutant (PTEN-G129E) to assess the role of the catalytic motif of PTEN in rendering lipid phosphatase activity (Ramaswamy et al., 1999). PTEN is a fundamental negative regulator of the PI3K/Akt axis because it dephosphorylates phoshatidylinositol 3,4,5-trisphophate and this results in Akt down-regulation (Sulis and Parsons, 2003).

Therefore, we first characterized these new clones. We selected three stably transfected clones (40G, 71F, 71M) with higher amounts of mutated PTEN, as demonstrated by western blot analysis (Fig. 6). In these clones, the expression of total Akt and the levels of either Ser 473 p-Akt or Thr-308 p-Akt were not different from those detected in untransfected HL60AR cells, as expected.

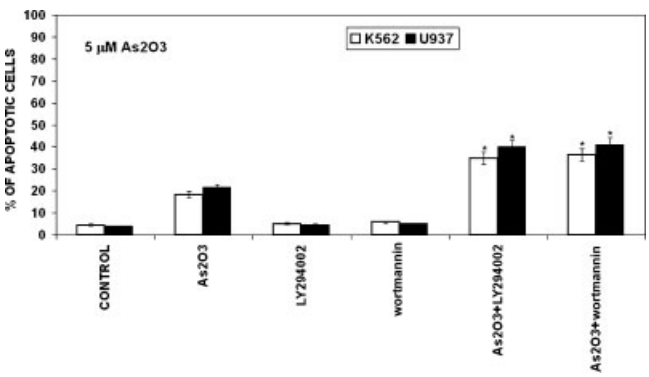


Fig. 5. Inhibitors of the PI3K/Akt pathway increase sensitivity of K562 and U937 cells to As_2O_3. Cells were incubated for 48 h in the presence of 5.0 μM As_2O_3. LY294002 (10 μM) and wortmannin (0.1 μM) were added at time 0, 12, 24, and 36 h. Samples were fixed with ethanol, stained with PI, and analyzed by flow cytometry. Results are the mean of three different experiments ±SD. Asterisks indicate significant differences ($P < 0.01$).

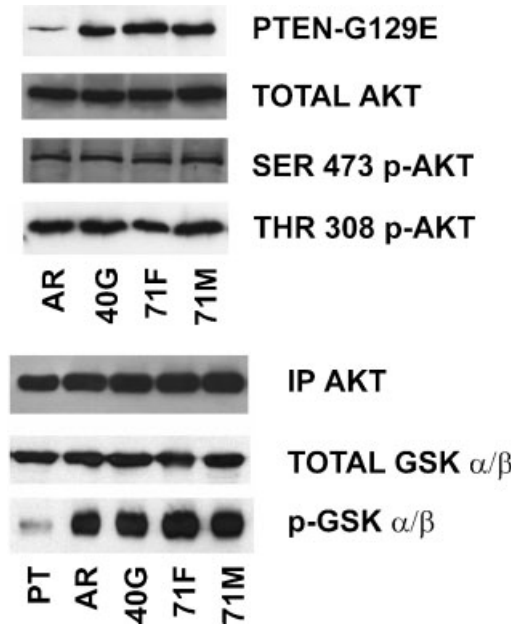
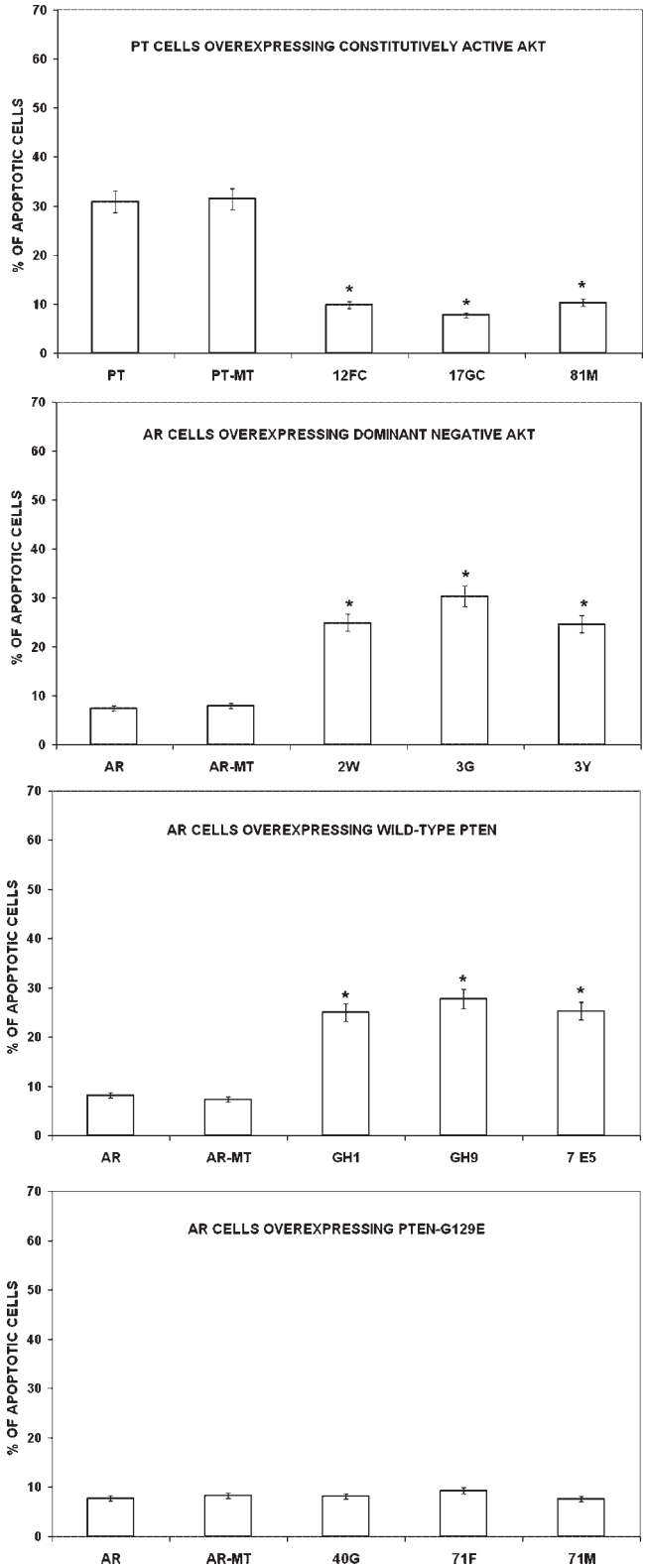


Fig. 6. Characterization of HL60AR cell clones (40G, 71F, 71M) stably transfected with a PTEN mutant (PTEN-G129E). Western blot analysis showing the expression levels of PTEN, total Akt, and phosphorylated Akt forms. Eighty μg of protein, separated by SDS–PAGE, was blotted to each lane. The blot is representative of three different experiments. Akt activity assay on GSKα/β performed on HL60PT and AR cells, and in the clones stably transfected with PTEN-G129E cDNA. Immunoprecipitation (IP) was performed with an antibody to total Akt. The immunoprecipitates were subjected to an in vitro kinase assay using GSK α/β as a substrate (1 μg). Reaction products were resolved on a 12% SDS-polyacrylamide gel and subjected to western blot analysis using anti-p-GSK β/β antibody. One representative experiment of three is shown.

We also analyzed the in vitro Akt kinase activity of these clones using GSK-3 as substrate, to detect Akt-induced phosphorylation of GSK-3. As presented in Figure 6, the immunoprecipitable Akt activity of the three PTEN-G129E expressing clones was similar to the activity of AR cells. PT cells had much lower Akt activity, in agreement with our own previous data (24, 25).

We next assessed the As_2O_3 (2.5 μM for 48 h) sensitivity of all the stably transfected HL60 cell clones, described in our previous papers (24, 25) and in this article. The results are presented in Figure 7.

In PT cells, the overexpression of a constitutively active Akt led to a significant decrease in the sensitivity to As_2O_3. In contrast, AR cell clones overexpressing either dominant negative Akt or wild-type PTEN were nearly as sensitive to As_2O_3 as HL60PT cells. Finally, overexpression of PTEN-G129E, did not increase to sensitivity of AR cells to 2.5 μM As_2O_3, thus demonstrating the importance of the lipid phosphatase activity. In mock transfected clones, there were no differences as far as As_2O_3 sensitivity was concerned (Fig. 7).



An inhibitor of NF-κB lowers As_2O_3 resistance of HL60 cells with an enhanced Akt activity

One of the downstream targets of the PI3K/Akt axis is represented by the NF-κB transcription factor which controls the expression of several anti-apoptotic factors (Nicholson and Anderson, 2002). Our own findings have clearly established a link between activation of the PI3K/Akt signaling pathway and up-regulation of NF-κB in HL60 cells (Bortul et al., 2003).

As demonstrated in Figure 8, cell fractionation experiments coupled with Western blotting showed the p50 subunit of NF-κB to be predominantly located in the cytoplasm of HL60PT cells, whereas it was mostly nuclear in HL60AR cells. Treatment of HL60AR cells with either LY294002 or wortmannin resulted in a mostly cytoplasmic localization of p50. These results further confirmed that in HL60AR cells NF-κB localization, and hence its activity, are under the control of the PI3K/Akt network.

Therefore, we sought to determine whether or not selective inhibition of NF-κB activity would result in lowered resistance to As_2O_3 of HL60 cells displaying an up-regulated Akt. To this end, we used SN50, a synthetic peptide which inhibits intranuclear translocation and transcriptional activity of NF-κB (Wen et al., 2000; Bortul et al., 2003).

By Western blot analysis, we checked first whether or not SN50 affected the intracellular distribution of the p50 subunit of NF-κB. As expected, treatment with SN50 resulted in p50 being mostly cytoplasmic in AR cells (Fig. 8). p50 subunit was enriched in the cytoplasmic fraction also in clones 12FC, 17GC, and 81M treated with SN50. These clones derived from PT cells transfected with a constitutively active Akt (Neri et al., 2003), and we have previously shown that they have a predominantly nuclear p50 subunit of NF-κB under basal conditions (Bortul et al., 2003). As shown in Figure 8, flow cytometric analysis of PI-stained samples revealed that SN50 (30 μg/ml) was capable of significantly increasing the percentage of As_2O_3-evoked apoptosis in AR cells and in the three PT cell clones overexpressing constitutively active Akt. It should be pointed out, however, that treatment with SN50 was not as effective as incubation with LY29402 or wortmannin in restoring sensitivity of AR cells to As_2O_3 (see Fig. 3).

Inhibition of NF-κB results in caspase activation and down-regulation of anti-apoptotic proteins in HL60AR cells

To gain insight into the mechanisms of action of NF-κB inhibition, we measured caspase activities after treatment with SN50 in AR cells. Figure 9 shows that the inhibitor per se did not increase caspase activities in these cells. However, SN50, when employed together with As_2O_3, caused a significant increase in the activities of caspase-3, -6, -8, and -9.

Fig. 7. Modulation of the PI3K/Akt pathway influences sensitivity of HL60PT and AR cells to As_2O_3. Stably transfected cells clones (or clones mock-transfected with the empty vector only, MT) were incubated for 48 h in the presence of 2.5 μM As_2O_3. Samples were fixed with 70% ethanol, stained with PI, and analyzed by flow cytometry. Results are the mean of three different experiments ±SD. Asterisks indicate significant differences ($P < 0.01$).

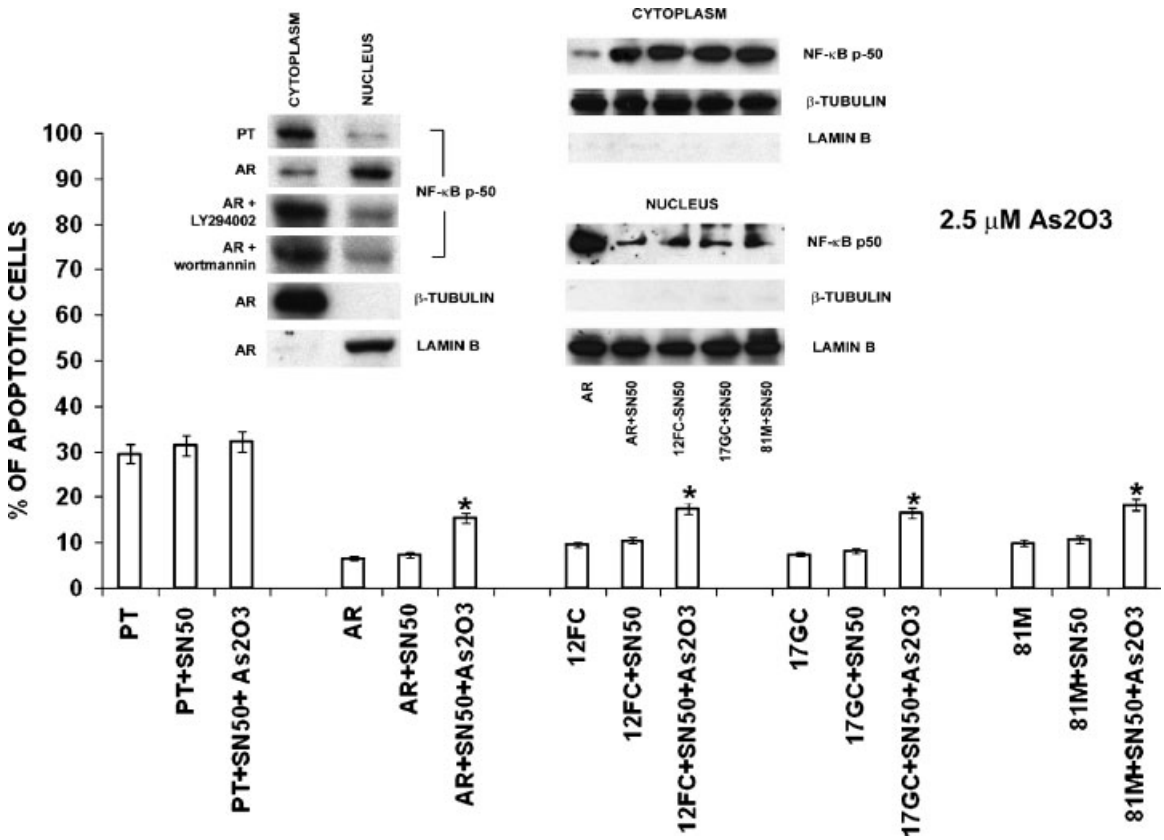


Fig. 8. NF-κB transcription factor influences resistance of HL60 cells to As_2O_3.Western blot analysis shows the subcellular distribution of NF-κB p50 subunit in HL60AR cells, and in clones 12FC, 17GC, and 81M. The SN50 peptide inhibitor (30 μg/ml) was added at time 0, 12, 24, and 36 h. Samples were collected after a 48 h incubation. Eighty micrograms of protein, from either cytoplasmic or nuclear fraction, was blotted to each lane. Blots were then probed with antibodies to NF-κB p50 subunit, or to β-tubulin or lamin B to demonstrate purity of the fractions. Bands were visualized by ECL. The blot is representative of three different experiments. For flow cytometric analysis, cells were incubated for 48 h in the presence of 2.5 μM As_2O_3. SN50 was added as described above. Samples were ethanol fixed, stained with PI, and then analyzed. Results are the mean of three different experiments ±SD. Asterisks indicate significant differences ($P < 0.01$). Note that all the samples employed for flow cytometric analysis have been treated with 2.5 μM As_2O_3.

It needs to be emphasized that in this case the activities of caspase-3 and -6 did not reach the levels seen after treatment with As_2O_3 plus either LY294002 or wortmannin (see Fig. 4), whereas that of caspase-8 did. Indeed, the increase in caspase-9 activity induced by incubation with SN50 plus As_2O_3 was modest. There was no activation of caspase-7 in response to SN50 plus As_2O_3. It is important to emphasize here that HL60 cells express caspase-7 (e.g., Germain et al., 1999).

Subsequently, we investigated by Western blot analysis the expression of a number of anti-apoptotic proteins whose synthesis is known to be regulated through NF-κB transcription factor.

As shown in Figure 9A, the expression of cIAP-1, cIAP-2, XIAP, TRAF-1, and TRAF-2 in HL60AR cells was sensitive to LY294002, wortmannin, or SN50. All the inhibitors markedly decreased the amount of these proteins. Similar results, regarding caspase activation, were seen with a clone (12FC) representative of HL60 PT cells overexpressing constitutively active Akt (Fig. 9B).

DISCUSSION

In the present study we have demonstrated, for the first time to our knowledge, that the PI3K/Akt signaling pathway may contribute to resistance to As_2O_3-induced apoptosis of human leukemia cell lines.

This conclusion was based on several lines of evidence. An HL60 human promyelocytic leukemia cell clone (HL60AR) with a constitutively active PI3K/Akt axis (Bortul et al., 2003; Neri et al., 2003) did not undergo apoptotic cell death following exposure to clinically achievable (0.5–5 μM) concentrations of As_2O_3, while parental HL60PT cells did. Treatment of HL60AR cells with pharmacological inhibitors of the PI3K/Akt signaling network restored the sensitivity of HL60AR cells to 2.5 μM As_2O_3.

Moreover, resistance to As_2O_3 was detected in PT cell clones overexpressing a constitutively active Akt. In contrast, transfection of AR cells with either a dominant negative Akt or a wild-type PTEN resulted in a higher sensitivity to As_2O_3. It is important to recall here that our previous results have shown that there are no differences between PT and AR cells as far as expression and activity of PTEN are concerned (Neri et al., 2003).

Since PTEN is a dual-specificity protein phosphatase which can also dephosphorylate 3-phosphorylated inositol lipids, we stably transfected AR cells with a mutant PTEN (G129E) which lacks the capability of dephosphorylating inositol lipids. In this case, the cells did not

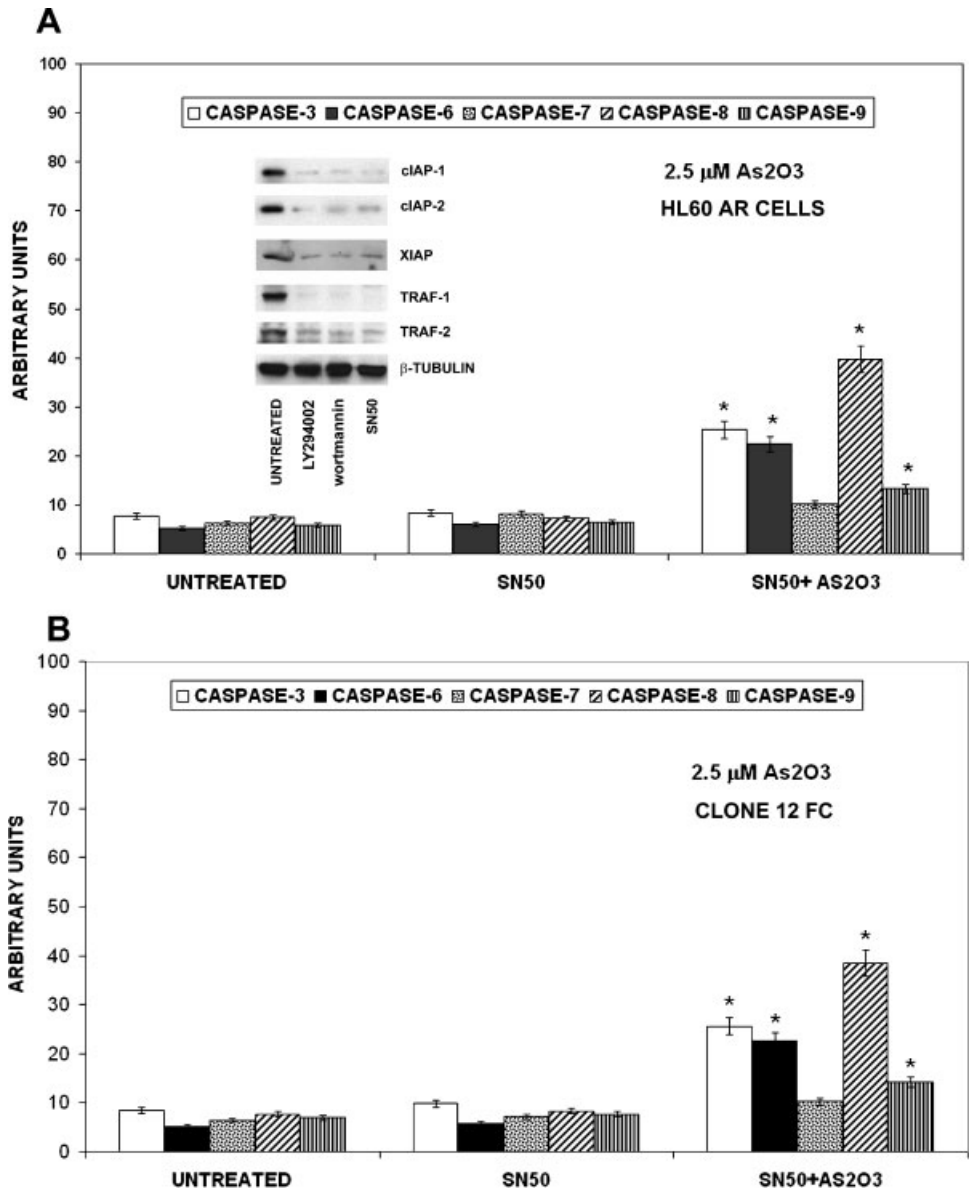


Fig. 9. Modulation of NF-κB transcription factor influences caspase activities in HL60 cells treated with As_2O_3 and affects expression of anti-apoptotic proteins. **A**: HL60 AR cells were incubated for 48 h in the presence of 2.5 μM As_2O_3. SN50 was added as detailed above. In vitro caspase-3, -6, -7, -8, and -9 activities were then measured in cell homogenates using fluorometric (caspase-3, -7, and -9) or colorimetric (caspase-6 and -8) assays. Results are the mean of three different experiments ±SD. Asterisks indicate significant differences ($P < 0.01$). Western blot analysis shows expression of anti-apoptotic proteins. The inhibitors were added at time 0, 12, 24, and 36 h. Samples were collected after a 48 h incubation. Eighty micrograms of protein, separated by SDS–PAGE, was blotted to each lane. Immunoreactive bands were visualized by the ECL technique. The blot is representative of three different experiments. **B**: caspase activities in clone 12FC (HL60 PT cells overexpressing constitutively active Akt). For the details see part A.

acquire sensitivity to As_2O_3. This finding clearly demonstrated that it is the lipid phosphatase activity of PTEN which is important for regulating the sensitivity to treatment with As_2O_3.

Taken together, the above results highlighted an important role played by the PI3K/Akt axis in determining resistance to As_2O_3 in HL60 cells. It is very interesting, however, that down-regulation of the PI3K/Akt signaling pathway by either LY294002 or wortmannin, resulted in enhanced apoptosis induced by As_2O_3 also in K562 and U937 leukemia cells, that are two cell lines of hematopoietic lineage with sizable levels of Akt activity.

The mechanism that blocks As_2O_3-dependent apoptosis in HL60AR cells seems to be at the caspase activation level. Indeed, while in HL60PT cells treated with As_2O_3 we measured an up-regulation of the activities of caspase-3, -6, -8, and -9, we were unable to do so in HL60AR cells. Nevertheless, pre-treatment of HL60AR cells with either LY294002 or wortmannin resulted in activation of caspases in response to As_2O_3.

The issue of resistance to As_2O_3 treatment has not been thoroughly investigated yet. Even though As_2O_3 was found to induce expression of P-glycoprotein and multidrug resistance-associated protein 1 (Takeshita et al., 2003), this cannot be the main mechanism of resistance because multidrug-resistant cells both of myeloid and lymphoid lineage undergo apoptosis in response to As_2O_3 (Perkins et al., 2000; Hu et al., 2003a,b).

Our results point to the likelihood that part of the resistance to As_2O_3 effected by the PI3K/Akt axis in HL60 cells is due to an up-regulation of the NF-κB transcription factor. Indeed, treatment with SN50 of HL60AR cells and of PT cell clones overexpressing a constitutively active Akt partially restored sensitivity to As_2O_3.

These findings are in full agreement with the recent data by Mathas et al. (2003). These authors employed Hodgkin/Reed-Sternberg (HRS) cell lines to elucidate the role of NF-κB in As_2O_3-induced apoptosis. They found that a transient overexpression of NF-κB-p65 in 540Cy HRS cells protected cells from As_2O_3-induced apoptosis. These authors, however, did not investigate whether or not NF-κB was a target of the PI3K/Akt axis.

However, SN50 was not as effective as either LY294002 or wortmannin in restoring sensitivity of HL60AR cells to As_2O_3. Indeed, while both LY294002 and wortmannin increased caspase activation in HL60AR cells to levels similar to those measured in PT cells, SN50 was less effective, especially as far as caspase-9 activation was concerned.

NF-κB targets a number of anti-apoptotic genes, including cIAP-1 and cIAP-2, XIAP, TRAF-1, and TRAF-2, A1 (Bfl-1), and cFLIP (reviewed in Gupta, 2003). IAP proteins directly block activation of effector caspase-3 and -7, and of procaspase-9 (e.g., Deveraux et al., 1998). XIAP is considered to be the most potent member of the IAP gene family in terms of its ability to inhibit caspases and suppress apoptosis (Hu et al., 2003b; Kashkar et al., 2003). The anti-apoptotic effect of TRAF-1 and TRAF-2 may be due to an increased activation of NF-κB, hence providing an autocrine loop for activation signal (Gupta, 2003). A1 (Bfl 1) is an anti-apoptotic member of the Bcl-2 family (Choi et al., 1995), while cFLIP is capable of binding to the adapter protein FADD which is a key component of the death-inducing signaling complex. However, cFLIP cannot be cleaved to an active caspase thus preventing the initiation of the death pathway (Kreuz et al., 2001).

We have previously reported that an up-regulation of the PI3K/Akt pathway determines in HL60 cells an enhanced expression of cIAP-1, cIAP-2, and cFLIP (Bortul et al., 2003; Neri et al., 2003), but not of A1 (Bfl-1) (Martelli et al., 2003). We also reported that cFLIP expression was controlled through NF-κB (Bortul et al., 2003).

Here, we have demonstrated that also the expression of cIAP-1, c-IAP-2, XIAP, TRAF-1, and TRAF-2 is under NF-κB control in HL60AR cells, since it was down-regulated by treatment with SN50. This finding may explain why treatment with SN50 almost completely restored caspase-8 activity of As_2O_3-treated AR cells. Indeed, it is well known that the combined action of cIAPs and TRAFs inhibits activation of caspase-8, the apical caspase activated by death receptor signaling (Wang et al., 1998b). Moreover, a contribution from cFLIP, which is also up-regulated in AR cells (Bortul et al., 2003), could not be excluded.

However, Akt targets procaspase-9 by phosphorylating it (Nicholson and Anderson, 2002), therefore preventing its activation. Since SN50 does not directly affect the levels of Akt activity (Martelli et al., 2003) it appears logical that it does not markedly up-regulate caspase-9 activity. These observations are also consistent with the fact that SN50 treatment does not result in an effector caspase activation as high as after exposure to either of the PI3K inhibitors. Indeed, both caspase-3 and -6 are targeted by caspase-8 and -9 (Chen and Wang, 2002). A summary schematic drawing of these conclusions is shown in Figure 10.

One major function of the PI3K/Akt signaling network is mediating cell survival in a broad spectrum of cancer cells. Initially, investigations of this role of PI3K/Akt focused on solid tumors, but recently it being increasingly appreciated that also in a number of malignancies of hematopoietic lineage also this survival pathway may be up-regulated (Barragán et al., 2002; Klejman et al., 2002; Mitsiades et al., 2002; Ringshausen et al., 2002).

So far, the PI3K/Akt pathway has been scarcely investigated in patients suffering from acute leukemia. It should be considered, however, that there are some cases of acute myeloid leukemia in which the PTEN tumor suppressor is absent or non-functional (e.g. Dahia et al., 1999). Since PTEN is a fundamental negative regulator of Akt activity it might be that in these leukemias the Akt pathway is up-regulated with a consequent resistance to multiple apoptotic stimuli and high proliferation rates.

Moreover, very recent evidence has shown the occurrence, in acute myeloid leukemias, of a possible

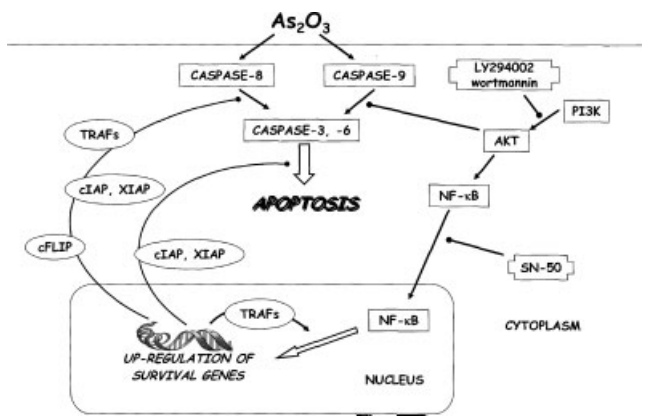


Fig. 10. Schematic drawing showing some of the possible interactions between the PI3K/Akt axis and the death pathways elicited by As_2O_3 in HL60 cells. As_2O_3 exposure activates both caspase-8 and -9, which, in turn, up-regulate effector caspase-3 and -6, resulting in apoptosis. Following activation through PI3K, Akt may prevent caspase-9 activation by directly phosphorylating it. Moreover, Akt, by promoting nuclear translocation of NF-κB, increases the transcription of several survival genes (cIAPs, TRAFs, cFLIP, XIAP) whose products block at various levels caspase activation. Note, however, that TRAFs may also function by further up-regulating NF-κB activity. SN50 stops NF-κB nuclear translocation, while LY294002 and wortmannin are pharmacological inhibitors of PI3K p110 catalytic subunit.

dominant-negative mutation of SHIP, a hematopoietic-specific inhibitory phosphatase which dephosphorylates phosphatidylinositol (3,4,5)P_3 to phosphatidylinositol (3,4)P_2, thus negatively influencing Akt activity (Luo et al., 2003).

Finally, it is noteworthy that Akt seems to be activated in acute myeloid leukemia blasts (Xu et al., 2003) and that the phosphorylation of the Akt-targeted FKHR transcription factor have been proposed as a new tool for identifying acute myeloid leukemias with an unfavourable outcome (Cheong et al., 2003; Min et al., 2003). Taken together, these findings point to the likelihood that also in acute myeloid leukemias the PI3K/Akt pathway might be an important molecular target for various types of therapy.

In conclusion, our results demonstrate that the PI3K/Akt pathway may be one of the factors linked with resistance to As_2O_3-induced apoptosis in human acute leukemia cell lines. However, a word of caution is necessary because we do not know if our findings bear any relationship with As_2O_3 resistance in NB4 cells or in primary leukemia cells. Further work will be necessary to elucidate this fundamental issue. In this connection, it is important to emphasize here that very recently it has been shown that in primary acute myeloid leukemia cells NF-κB is frequently activated through a PI3K/Akt-dependent pathway (Birkenkamp et al., 2004).

Therefore, it might be that future innovative therapeutic strategies for acute myeloid leukemia will consist of inhibitors of the PI3K/Akt pathway in addition to As_2O_3.

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