Arsenic trioxide decreases AKT protein in a caspase-dependent manner

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Abstract

Arsenic trioxide (As_2O_3) is used clinically to treat acute promyelocytic leukemia but is less successful in other malignancies. To identify targets for potential combination therapies, we have begun to characterize signaling pathways leading to As_2O_3-induced cytotoxicity. Previously, we described the requirement for a reactive oxygen species–mediated, SEK1/c-Jun NH_2-terminal kinase (JNK) pathway to induce apoptosis. AKT inhibits several steps in this pathway; therefore, we postulated that As_2O_3 might decrease its activity. Indeed, As_2O_3 decreases not only AKT activity but also total AKT protein, and sensitivity to As_2O_3 correlates with the degree of AKT protein decrease. Decreased AKT expression further correlates with JNK activation and the release of AKT from the JNK-interacting protein 1 scaffold protein known to assemble the mitogen-activated protein kinase cascade. We found that As_2O_3 regulates AKT protein stability without significant effects on its transcription or translation. We show that As_2O_3 decreases AKT protein via caspase-mediated degradation, abrogated by caspase-6, caspase-8, caspase-9, and caspase-3 inhibitors but not proteosome inhibitors. Furthermore, As_2O_3 enhances the ability of a heat shock protein 90 inhibitor to decrease AKT expression and increase growth inhibition. This suggests that As_2O_3 may be useful in combination therapies that target AKT pathways or in tumors that have constitutively active AKT expression. [Mol Cancer Ther 2008;7(6):1680–7]

Introduction

Arsenic trioxide (As_2O_3) is an effective treatment for patients with acute promyelocytic leukemia (APL) but has been less successful in other malignancies at tolerable doses. Several groups have begun preclinical and early-stage clinical efforts to develop rational combination therapies that increase the efficiency of As_2O_3. Some of these combinations show promise in non-APL acute myelogenous leukemia and multiple myeloma (1–3). To further identify therapeutic targets for such combination therapies, we have begun to characterize signaling pathways leading to As_2O_3-induced cytotoxicity.

As_2O_3 initiates many signaling cascades within a cell, but identifying those pathways essential for the induction of apoptosis is critical. As_2O_3 treatment results in production of reactive oxygen species (ROS) in leukemic cells (4, 5). Several compounds that enhance ROS production can sensitize cells to As_2O_3-induced apoptosis. We have shown that either L-buthionine sulfoximine (BSO; ref. 4), which depletes glutathione stores, or Trolox (6), a vitamin E analogue that selectively acts as a pro-oxidant in malignant cells (6, 7), can enhance As_2O_3-induced cytotoxicity. In addition, antioxidant compounds such as *N*-acetylcysteine inhibit As_2O_3-induced apoptosis (8). ROS production by As_2O_3 is linked to the induction of stress pathways, including mitogen-activated protein kinases (MAPK). As_2O_3 treatment results in c-Jun NH_2-terminal kinase (JNK) activation, which is required for maximal induction of apoptosis in several cell types (9). Genetic deletion of the upstream activator kinase SEK1/MAPK kinase 4 in mouse embryo fibroblasts inhibits both As_2O_3-induced JNK activity and apoptosis (9).

In addition to activated pathways leading to death, we hypothesized that survival signals would be inhibited by As_2O_3. AKT, a serine/threonine protein kinase, mediates the cell survival signals coming through phosphoinositide 3-kinase by phosphorylation and inactivation of several proapoptotic proteins, including BAD (10), caspase-9 (11, 12), and members of the forkhead family of transcription factors (13, 14), rendering them inactive. AKT also negatively regulates the MAPK pathways required for As_2O_3-induced apoptosis. AKT phosphorylates SEK1 on Ser^{78} and inhibits its function (15). AKT also phosphorylates and inactivates ASK1, a MAPK kinase kinase upstream of SEK1 (15, 16). Oxidative stress initiates the dissociation of AKT from JNK-interacting protein 1 (JIP1), a scaffolding protein, which facilitates the activation of the ASK-SEK-JNK pathway (17). AKT binding to JIP1 negatively regulates this signal transduction pathway (17); thus, a decrease in AKT as a result of oxidative stress could enhance JNK signaling.

Here, we present data showing a correlation between sensitivity to As_2O_3-induced death and decreased AKT activity. We further show that As_2O_3 decreases total AKT protein levels without affecting transcription or translation of AKT. Instead, we found that As_2O_3 decreased AKT

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protein expression in a caspase-dependent manner. In addition, we show that As_2O_3 acts to enhance the growth inhibition seen with the heat shock protein 90 (hsp90) inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG), suggesting possible combination therapies to target AKT pathways.

Materials and Methods

Reagents

As_2O_3, BSO, anti-β-actin antibody and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent were purchased from Sigma. LY294002, MG132, and caspase inhibitors were purchased from Calbiochem (EMD Biosciences). AKT antibody and the nonradioactive AKT kinase assay kit were purchased from Cell Signaling Technology.

Cell Culture and Treatments

NB4 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin. The arsenic-resistant NB4-MR-AsR2 and AsR3 cell lines (AsR2 and AsR3) were originally derived from the arsenic-sensitive NB4 cells and are cultured in the presence of 2 μmol/L As_2O_3 (4). AsR2 and AsR3 cells were washed thoroughly before all experiments. MDA-MB-468 cells were cultured in α-MEM containing 10% fetal bovine serum. MDA-MB-231 cells were cultured in DMEM/F-12 containing 10% fetal bovine serum. Breast cancer cells were plated at 10^4 per well of a 24-well plate for 3-day growth curve analyses.

Propidium Iodide Staining

Quantitation of apoptotic cells was done as previously described (18). Cells were washed in 4°C PBS/5% fetal bovine serum/0.01 mol/L NaN_3, pelleted, and resuspended in 0.5 mL of hypotonic fluorochrome solution containing 50 μg/mL propidium iodide (Sigma), 0.1% sodium citrate, and 0.1% Triton X-100. Cells undergoing DNA fragmentation and apoptosis were shown to be those in which propidium iodide fluorescence was weaker than the typical G_0-G_1 cell cycle peak.

AKT Kinase Assay

Cells were processed following the protocol from Cell Signaling Technology. Briefly, cells were lysed in 1× cell lysis buffer and 200 μL of protein extracts were incubated with AKT monoclonal antibody immobilized on agarose beads for 2 h at 4°C. Beads were then washed in 1× cell lysis buffer and in 1× kinase buffer. For the kinase assay, beads were resuspended in the 1× kinase buffer supplemented with 200 μmol/L ATP and glycogen synthase kinase 3α/β (GSK3α/β; $Ser^{21/9}$) fusion protein and incubated 30 min at 30°C. To stop the reaction, 2× SDS sample buffer was added to the samples, which were then boiled 5 min and loaded on a 12% SDS-PAGE gel. Samples were analyzed by immunoblotting with a phosphorylated GSK3α/β ($Ser^{21/9}$) antibody. Anti-AKT immunoblots were done as a control for the immunoprecipitation.

JNK Kinase Assay

Assays for JNK activity were done as previously described (9). Briefly, following treatment, cells were washed and lysed in the presence of phosphatase and

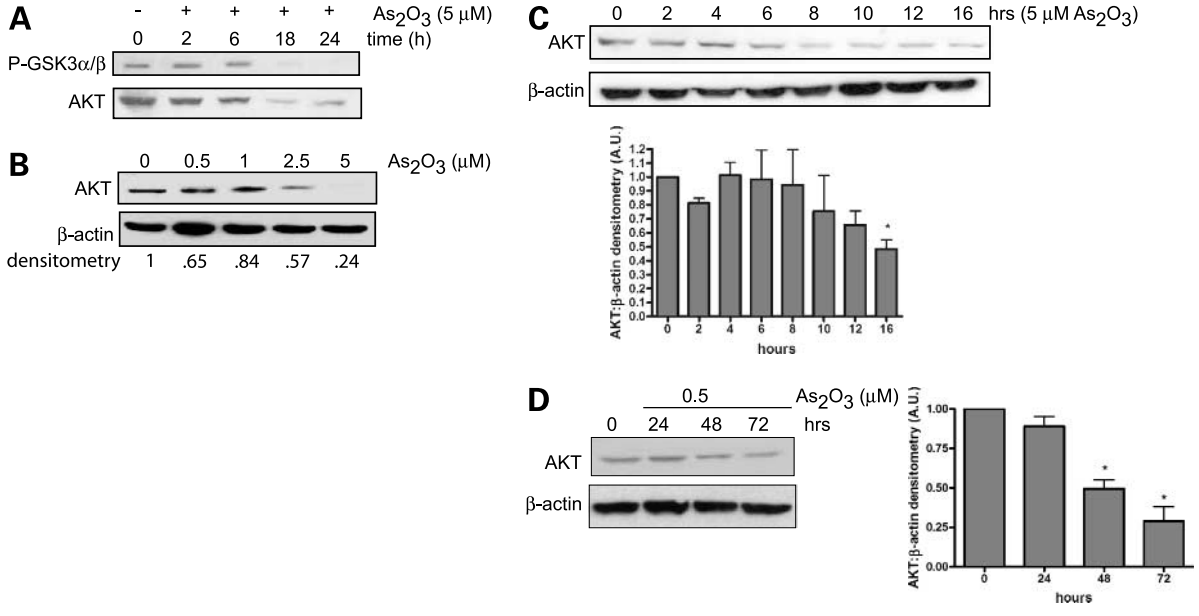

Figure 1. As_2O_3 decreases AKT activity and protein levels. **A,** NB4 cells were treated with 5 μmol/L As_2O_3 over 24 h. AKT was immunoprecipitated from extracts and used in a nonradioactive AKT kinase assay, where phosphorylated GSK3α/β (*P-GSK3α/β*) is detected by immunoblotting (*top*) and the amount of AKT protein immunoprecipitated was detected by immunoblotting of the same membrane. NB4 cells were treated with varying doses of As_2O_3 (0.5 – 5 μmol/L; **B**) for 24 h or varying times with 5 μmol/L As_2O_3 (**C**). **D,** low doses of As_2O_3 (0.5 μmol/L) were used to treat NB4 cells over 3 d. Total AKT and β-actin expression was assessed by immunoblotting. Densitometry of immunoblotting is expressed in arbitrary units (*A.U.*) compared with untreated (0 h) cells, where the ratio of AKT to β-actin was set at 1.

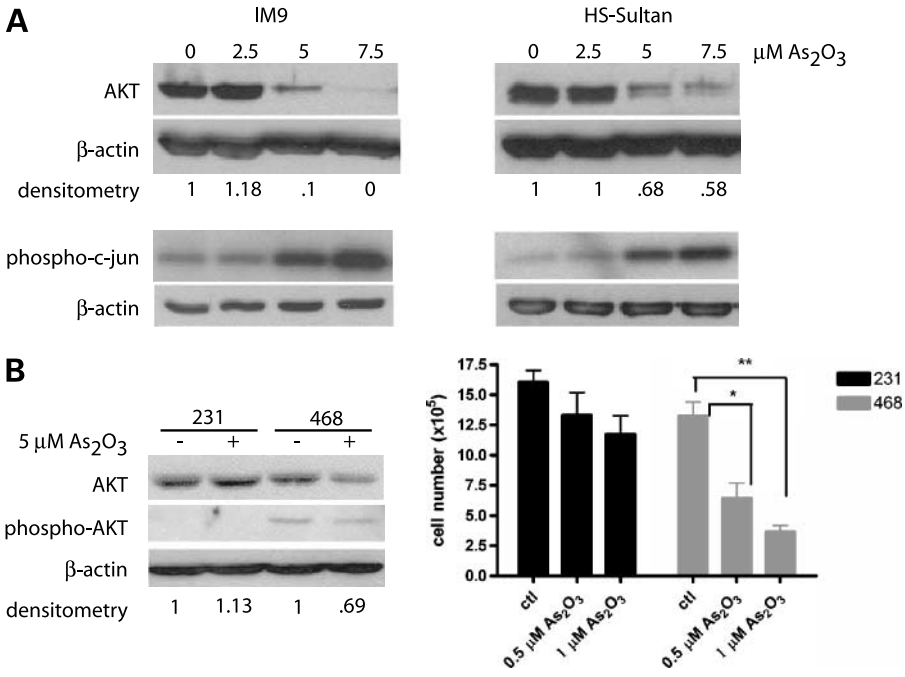

Figure 2. The As_2O_3-induced AKT decrease is not specific to APL cells. **A,** IM9 lymphoblastoid and HS-Sultan multiple myeloma cell lines were treated with 2.5 to 7.5 μmol/L of As_2O_3 for 24 h and whole-cell protein extracts were used in immunoblots for AKT or β-actin (*top*) or for *in vitro* JNK assays using c-Jun as a substrate and β-actin immunoblots as loading controls (*bottom*). **B,** *left,* MDA-MB-231 or MDA-MB-468 cells were treated with 5 μmol/L As_2O_3 for 24 h and the levels of total AKT, phosphorylated AKT (Ser^{473}), and β-actin were assessed by immunoblotting; *right,* cell number was determined by trypan blue exclusion assay after 3-d treatment with 0.5 or 1 μmol/L of As_2O_3. Densitometry of immunoblotting is expressed in arbitrary units compared with untreated (0 h) cells, where the ratio of AKT to β-actin was set at 1.

protease inhibitors. JNK1 was immunoprecipitated with an anti-JNK1 antibody (Santa Cruz Biotechnology, Inc.) and protein A-Sepharose beads by nutation at 4°C for 1 h. After washing, immune complexes were incubated with glutathione *S*-transferase-c-Jun (a gift from Sylvain Meloche, University of Montreal, Montreal, Quebec, Canada) and [γ-^{32}P]ATP for 30 min at 30°C. Glutathione *S*-transferase-c-Jun was visualized via SDS-PAGE and autoradiography. Immunoblotting was done before immunoprecipitation to ensure that treatment did not affect absolute JNK1 expression levels.

Protein Extraction and Immunoblotting

After treatment, cells were washed with cold PBS and lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 8), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS] containing protease/phosphatase inhibitors. Protein concentration was measured with 5× Bradford reagent (Bio-Rad). Proteins were separated by SDS-PAGE gel and then transferred onto a nitrocellulose membrane. Membranes were blocked (1 h, room temperature) in TBS containing 0.1% Tween 20 (TBST) and 5% nonfat milk and incubated with anti-AKT (1:500), anti-JIP1, anti-β-actin, or anti-JNK1 (overnight, 4°C). The next day, membranes were washed in TBST and incubated with horseradish peroxidase–conjugated secondary antibody (1 h, room temperature). Membranes were then washed and peroxidase activity was visualized with the enhanced chemiluminescence method (GE Healthcare Biosciences-Amersham).

Coimmunoprecipitation Assays

NB4 cells were lysed in buffer containing 50 mmol/L Tris (pH 8), 150 mmol/L NaCl, and 1% Triton X-100. After preclearing lysates, specific proteins were immunoprecipitated overnight at 4°C with anti-AKT antibody (sc-1618-R). Protein A-agarose beads were added for 4 h and then the beads were washed thrice in lysis buffer. Immunoprecipitates were run on SDS-PAGE gels and immunoblotted as described above.

Transfection Assays

Transient transfection of NB4 cells was done by electroporation using the Gene Pulser apparatus (Bio-Rad). For constitutively active AKT transfection, 1×10^7 NB4 cells were transfected by electroporation with 10 μg of either empty vector pCMV (mock) or a constitutively active AKT (Dr. Adriane Stoica, Georgetown University, Washington, District of Columbia). Cells were treated with 2 μmol/L As_2O_3. After 48 h, cell proliferation was assessed by MTT assay.

Proteosome Inhibition Assay

NB4 cells were seeded in the presence or absence of 5 μmol/L As_2O_3 for 18 h. For the last 6 h, MG132, a proteasome inhibitor, was added to some cultures. Cells were then harvested and lysed with radioimmunoprecipitation assay buffer. Immunoblotting analysis was done using an anti-AKT polyclonal antibody (Cell Signaling Technology) and β-actin antibody (Sigma) as a loading control.

Caspase Inhibition Assays

NB4 cells were treated with 5 μmol/L As_2O_3 in the presence or absence of Z-VAD (caspase inhibitor I), a pan-caspase inhibitor (Calbiochem), or with the Caspase Inhibitor Set II from Calbiochem: caspase-1 inhibitor VI (ZYVAD-fmk), caspase-2 inhibitor I (ZVDVAD-fmk), caspase-3 inhibitor II (Z-DEVD-fmk), caspase-5 inhibitor I (Z-WEHD-fmk), caspase-6 inhibitor I (Z-VEID-fmk), caspase-8 inhibitor (Z-IETD-fmk), caspase-9 inhibitor I

(Z-LEHD-fmk), and caspase inhibitor III (Boc-D-fmk). After 6 or 24 h, cells were counted and then harvested to prepare whole-cell extracts. Immunoblotting analysis was done to evaluate AKT protein levels.

Caspase-3 Activity Assay

Caspase-3 activation was detected using the Caspase-Glo 3/7 Assay (Promega). NB4 and AsR2 cells were treated for 18 h. Subsequently, cells were incubated for 30 min at room temperature with an equal volume of Ac-DEVD-pNA substrate in the buffer provided. The amount of light was measured using a GloMax 20/20 luminometer (Promega) and normalized to cell number.

Results

AKT Activity and Protein Levels Decrease in NB4 Cells following As_2O_3 Treatment

Based on our previous findings that an intact JNK signaling pathway is required for As_2O_3-induced apoptosis, we hypothesized that As_2O_3 may concomitantly decrease negative regulators of this pathway. We chose to assess AKT expression and activity in our model system of the arsenic-sensitive NB4 APL cell line after treatment for increasing amounts of time with 5 μmol/L As_2O_3. AKT activity was measured using a nonradioactive kinase assay with GSK3α/β ($Ser^{21/9}$) fusion protein as a substrate. Indeed, As_2O_3 decreases AKT activity within 18 h (Fig. 1A, *top*). In addition, we observed a time-dependent decrease in total AKT protein immunoprecipitated (Fig. 1A, *bottom*). To verify that this was not an artifact of the kinase assay, we then assessed total AKT levels by immunoblot and found a dose-dependent (Fig. 1B) and time-dependent (Fig. 1C) decrease in AKT protein levels, confirming our immunoprecipitation results that 5 μmol/L As_2O_3 decreases AKT by 6 to 8 h. In addition, longer exposures with low concentrations of As_2O_3 (0.5 μmol/L), well within the clinically achievable range, also decreased AKT protein expression (Fig. 1D).

The As_2O_3-induced decrease in AKT protein levels was not specific to APL cells. In both the IM9 B lymphoblastoid cell line and the HS-Sultan multiple myeloma cell line, As_2O_3 decreased AKT protein expression in a dose-dependent manner after 24 h, which correlated with the ability of As_2O_3 to activate JNK as assessed by *in vitro* kinase assay where JNK was immunoprecipitated from cell lysates and used to phosphorylate a glutathione *S*-transferase-c-Jun construct *in vitro* (Fig. 2A). Notably, higher doses of As_2O_3 were required to initiate the signaling events in these cell lines, which correlates with their decreased sensitivity to As_2O_3. A decrease in AKT levels correlated with sensitivity to As_2O_3 in breast cancer cell lines as well. AKT expression as well as AKT phosphorylation at Ser^{473} was decreased in MDA-MB-468 cells (Fig. 2B), which have a high level of constitutively active AKT. However, MDA-MB-231 cells, which are significantly less sensitive to As_2O_3-induced growth inhibition at day 3, did not have a decreased level of AKT after exposure to As_2O_3.

As_2O_3 Decreases AKT Protein Levels in NB4 (Arsenic-Sensitive APL Cell Line) but not in AsR2 and AsR3 (Arsenic-Resistant APL Cell Lines)

If the arsenic-induced decrease of AKT protein facilitates cell death, we hypothesized that cells resistant to

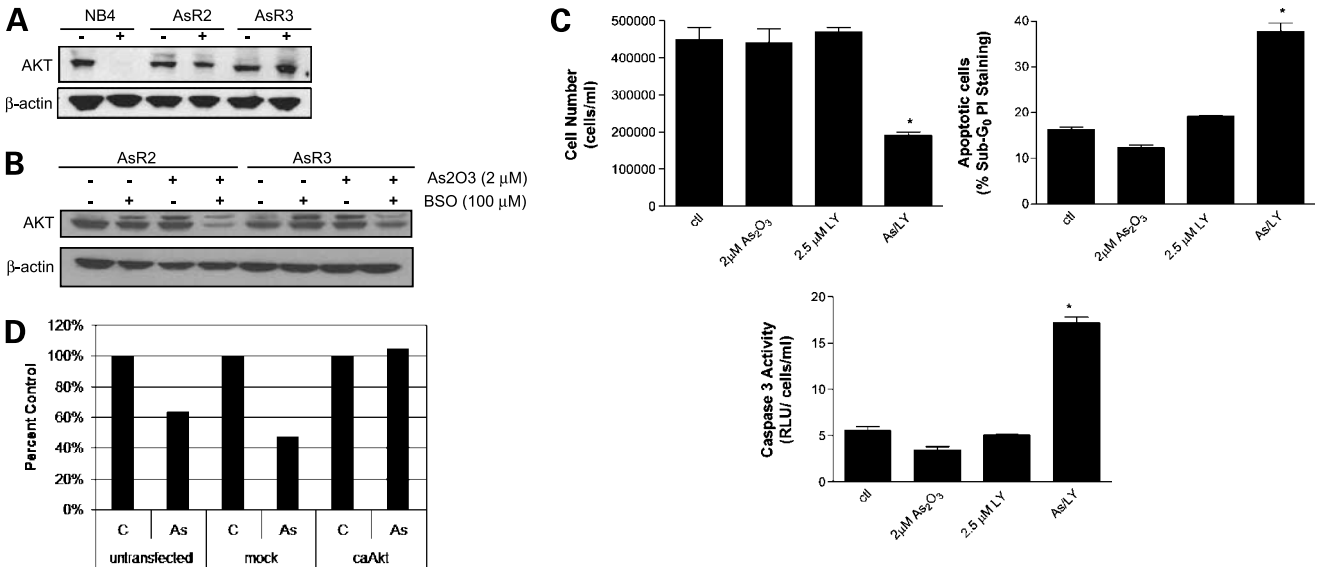

Figure 3. The decrease in AKT expression correlates with sensitivity to As_2O_3-induced apoptosis. **A,** NB4, AsR2, and AsR3 cell lines were treated with 5 μmol/L As_2O_3 for 24 h and whole-cell protein extracts were used in immunoblots for AKT (*top*) or β-actin (*bottom*). **B,** AsR2 and AsR3 cells were pretreated with 100 μmol/L BSO for 18 h and then treated with 2 μmol/L As_2O_3 for 24 h. Protein whole-cell extracts were used in immunoblots for AKT (*top*) or β-actin (*bottom*). **C,** AsR2 cells were treated with 2 μmol/L As_2O_3 with or without 2.5 μmol/L LY294002 (*LY*) for 48 h. Viable cells were enumerated using trypan blue exclusion. Apoptosis was defined as the sub-G_0 population in propidium iodide (*PI*) assays. Caspase-3 activation was assessed by Caspase-Glo assay and is represented as relative luciferase unit (*RLU*) per cells per mL. For all assays, $n = 3$ and is representative of at least two independent experiments. **D,** NB4 cells were transiently transfected by electroporation with empty vector pCMV (mock) or a constitutively active AKT. Cells were treated with 2 μmol/L As_2O_3. After 48 h, cell proliferation was assessed by MTT assay.

As_2O_3-induced apoptosis would not decrease AKT expression in response to arsenic. To test this, we used arsenic-resistant NB4 subclones developed in our laboratory, which are grown under the selective pressure of 2 μmol/L As_2O_3. These cells have increased glutathione levels, which results in their resistance to As_2O_3 (4). When NB4 cells as well as the resistant clones AsR2 and AsR3 were treated with 5 μmol/L As_2O_3 for 24 h, AKT protein expression was decreased in the parental line but not in the resistant clones (Fig. 3A). Furthermore, when glutathione levels were decreased by a pretreatment with BSO for 18 h followed by 2 μmol/L As_2O_3 treatment for 24 h, the AKT protein decreased and apoptosis was induced (Fig. 3B; data not shown). AKT levels were not affected by treatment with BSO or As_2O_3 alone in these cell lines. We have previously shown that BSO, which decreases intracellular reduced glutathione levels, can sensitize these arsenic-resistant cells to As_2O_3-induced ROS production and apoptosis (4). Therefore, these data suggest that AKT is downstream of ROS generation by As_2O_3.

We then asked if pharmacologic inhibition of AKT in the arsenic-resistant cell lines could sensitize them to As_2O_3-induced death. AsR2 cells were treated with 2 μmol/L As_2O_3 over 2 days in the presence or absence of 2.5 μmol/L LY294002. LY294002 is an inhibitor of phosphoinositide 3-kinase, an activating kinase upstream of AKT. Interestingly, this inhibitor can overcome resistance to As_2O_3 in the AsR2 cells as assessed in growth curves, propidium iodide staining, and caspase-3 activation (Fig. 3C). To confirm that AKT down-regulation was necessary for As_2O_3-induced death, we asked whether overexpression of AKT in the NB4 cells results in resistance to As_2O_3-induced growth inhibition. Indeed, NB4 cells transiently transfected with a constitutively active AKT construct were completely resistant to a dose of As_2O_3 that substantially inhibited growth in untransfected or vector-transfected cells (Fig. 3D). Together, these data show that decreased AKT is an essential part of the mechanism of As_2O_3-induced apoptosis in APL cells, and resistance to As_2O_3 is associated with persistent AKT expression.

As_2O_3 Treatment Decreases AKT-JIP1 Association

We then asked how AKT could inhibit As_2O_3-induced cell death. We have previously shown that JNK activation is critical for As_2O_3-induced apoptosis in NB4 cells. AKT is a negative regulator of this pathway, by binding to JIP1, the scaffold protein that integrates both positive and negative regulators of JNK signaling. When AKT is bound to JIP1, the MAPK cascade cannot assemble and JNK is not activated. AKT and JNK are coordinately and inversely regulated in our model system; therefore, we postulated that inhibition of AKT would increase JNK activation induced by As_2O_3. NB4 cells were serum starved for 24 h and subsequently treated for 24 h with 0.5 μmol/L As_2O_3 in the presence or absence of 2.5 μmol/L LY294002. As we have previously shown, 0.5 μmol/L As_2O_3 induced JNK activity at this time point (Fig. 4A). Addition of LY294002 enhanced the JNK activation alone and the combination of these drugs further enhanced JNK activation (Fig. 4A). These data further correlate decreased AKT activity with JNK activation. Next, we investigated whether As_2O_3-induced JNK activity was correlated with a decreased association of AKT with JIP1. Endogenous AKT and JIP1 were coimmunoprecipitated from NB4 cells treated for 24 h with control or 1 to 5 μmol/L of As_2O_3. AKT and JIP1 were strongly associated in untreated cells, but this association was significantly decreased even at the lowest dose of As_2O_3, which did not decrease AKT protein at this time point (Fig. 4B), indicating that As_2O_3-induced JNK activation correlates with decreased AKT-JIP1 binding. Interestingly, as AKT protein levels are decreasing with As_2O_3 treatment, JIP1 levels increase.

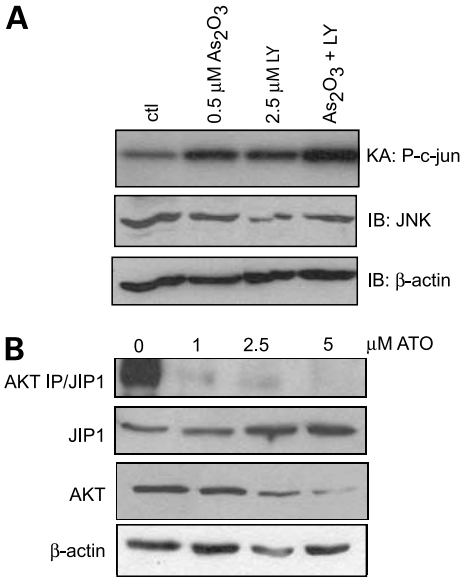

Figure 4. Decreased AKT correlates with JNK activation. **A,** NB4 cells were serum starved and then treated for 24 h with 0.5 μmol/L As_2O_3 in the presence or absence of 2.5 μmol/L LY294002. *In vitro* kinase assays (*top*) and JNK (*middle*) and β-actin (*bottom*) immunoblotting were done. **B,** AKT and JIP1 were coimmunoprecipitated from NB4 cell extracts after 24-h treatment with 1 to 5 μmol/L of As_2O_3. Immunoblotting of JIP1, AKT, and β-actin was done as controls for expression and loading.

As_2O_3 Regulates AKT Protein Levels through a Proteasome-Independent and Caspase-3–Dependent Pathway

We next investigated the mechanism by which As_2O_3 treatment decreases AKT expression. We found that As_2O_3 affects neither transcriptional activity of *AKT1* as assessed by transient transfection and chromosomal immunoprecipitation assays nor the mRNA stability (Supplementary Fig. S1).[1] Therefore, we hypothesized that As_2O_3 induced degradation of AKT protein. AKT degradation has been shown to occur in a proteasome-dependent manner (19, 20). We treated NB4 cells with 5 μmol/L As_2O_3 for 18 h. Six hours before harvesting the cells, MG132, a potent reversible cell-permeable proteasome inhibitor, was added. Immunoblotting for total AKT protein levels showed that

[1] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

inhibiting the proteasome pathway could not rescue AKT protein from As_2O_3-induced degradation (Fig. 5A). AKT has also been shown to be a target of caspase-mediated degradation, particularly caspase-3, caspase-6, and caspase-7 (21–23). To test whether caspases mediate the As_2O_3-induced decrease in AKT protein levels, we treated NB4 cells for 6 or 24 h with Z-VAD, a pan-caspase inhibitor, alone or in combination with 5 μmol/L As_2O_3. Following Z-VAD treatment, AKT protein levels were protected from As_2O_3-induced decrease (Fig. 5B), suggesting that one or more caspases are involved in As_2O_3 effect on AKT protein. We then treated NB4 cells with a wide range of caspase inhibitors in combination with As_2O_3. Inhibitors of caspase-1, caspase-2, and caspase-5 had no effect on arsenic-induced AKT protein degradation, whereas caspase-3, caspase-6, caspase-8, and caspase-9 could each slightly inhibit As_2O_3 effect on total AKT protein levels but not as strongly as pan-caspase inhibitors (Z-VAD and Boc-D-fmk; Fig. 5C). Of the caspases implicated in this inhibitor experiment, caspase-3 is the furthest downstream and likely to integrate all the initiator caspase signals. To correlate caspase-3 activity with sensitivity to As_2O_3, we assessed caspase-3 activity in NB4 and AsR2 cells after 18 h of treatment with As_2O_3 alone or combined with Z-DEVD-fmk (Fig. 5D). As_2O_3 alone could increase caspase-3 activity in NB4 cells, which was decreased when As_2O_3 was used in combination with the caspase-3–specific inhibitor. However, in the arsenic-resistant AsR2 cell line, no increase in caspase-3 activity was detected in the presence or absence of As_2O_3 (Fig. 5D). Increased caspase-3 activity in the arsenic-sensitive NB4 cells correlates with the ability of As_2O_3 to decrease AKT protein levels.

As_2O_3 Enhances the Cytotoxic Effects of 17-AAG

17-AAG is a geldanamycin derivative currently undergoing clinical development, which binds to hsp90 and enhances degradation of hsp90 client proteins (24, 25). AKT is a hsp90 client protein, whose activity and, subsequently, expression are decreased after treatment with 17-AAG *in vitro* (26). Interestingly, As_2O_3 and 17-AAG act synergistically to induce apoptosis of HL-60 and Jurkat cells (26). We investigated whether As_2O_3 enhanced 17-AAG–induced cytotoxicity in NB4 cells. As_2O_3 (0.5 μmol/L), 17-AAG (0.125 or 0.25 μmol/L), and the combinations were used in 3-day growth curves to determine the effects of combination therapy. Indeed, the combination of As_2O_3 with 17-AAG was significantly more inhibitory than either drug alone (Fig. 6A). Higher concentrations of As_2O_3 (1 μmol/L) also enhanced the efficacy of 17-AAG even at 24 h (data not shown). Total AKT levels also were assessed at 24 h using immunoblotting. As_2O_3 and 17-AAG alone decreased total AKT levels, but the combination further decreased AKT expression (Fig. 6B and C). These data suggest that the enhanced cytotoxic effects of the combination treatment may be due to a decrease in prosurvival signals from AKT.

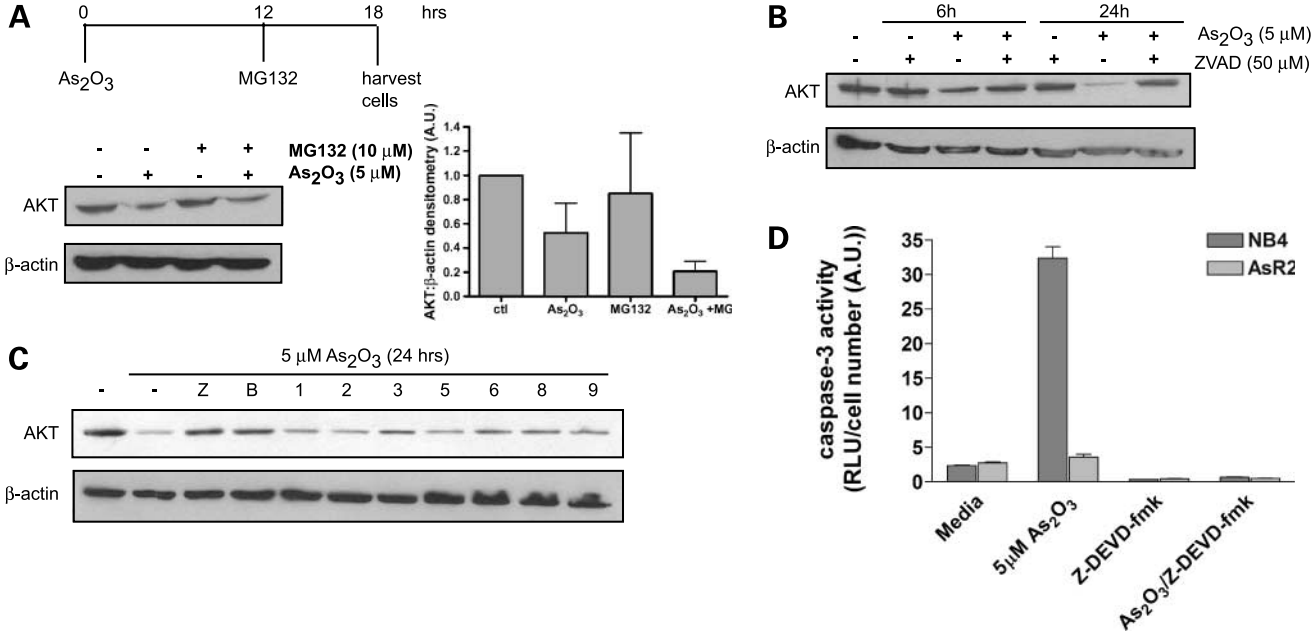

Figure 5. As_2O_3-induced degradation of AKT protein is caspase dependent. **A,** NB4 cells were treated with 5 μmol/L As_2O_3 for 18 h and 10 μmol/L of the proteasome inhibitor MG132 (*MG*) were added to the samples 6 h before harvesting the cells. Whole-cell extracts were used to assess total AKT levels by immunoblotting. Densitometry is expressed as the ratio of AKT to β-actin compared with control-treated cells; the value of which was set to 1. **B,** NB4 cells treated with 5 μmol/L As_2O_3 alone or in combination with 50 μmol/L of a general caspase inhibitor, Z-VAD, for 6 and 24 h. Whole-cell extracts were used to evaluate total AKT protein level by Western immunoblotting. **C,** NB4 cells treated for 24 h with 5 μmol/L As_2O_3 alone or in combination with generic caspase inhibitors Z-VAD and Boc-D-fmk or specific caspase inhibitors indicated as in Materials and Methods. Total AKT protein level was assessed by immunoblotting ($n = 2$). **D,** NB4 and AsR2 cells treated as described in **B** for 18 h, stained with a fluorescent caspase-3 inhibitor, Z-DEVD-fmk, for 1 h at 37°C, and analyzed for caspase-3 activity.

Discussion

As_2O_3 is used effectively to treat patients with APL and shows promise as a treatment for multiple myeloma and myelodysplastic syndrome. Understanding the mechanism by which As_2O_3 induces apoptosis may identify potential targets for combination therapies and broaden the malignancies for which arsenic can provide effective treatment. Previous data from our laboratory and others show that generation of ROS and activation of the JNK signaling pathway mediate the arsenic-induced apoptotic signal in APL cells (4, 5, 8, 9). Based on these data, we hypothesized that As_2O_3 would inhibit negative regulators of this pathway.

Several laboratories have investigated a role for AKT in As_2O_3-induced death, although the data are confusing. In one report, pretreatment with inhibitors of phosphoinositide 3-kinase had no effect on As_2O_3-induced death in NB4 cells but enhanced the death in MOLT-4 T-cell leukemia line (27). However, another report showed that a cotreatment with the same inhibitors enhances As_2O_3-induced apoptosis in NB4 cells but not the cytotoxicity associated with ionidamine, camptothecin, or cisplatin (28). The cytotoxic effect is correlated with a decrease in phosphorylated AKT but not total AKT (28). Our data show that cotreatment with phosphoinositide 3-kinase inhibitors can sensitize arsenic-resistant NB4 subclones (Fig. 3C) and confirm that overexpression of a constitutively active AKT construct rescues cells from As_2O_3-induced apoptosis (Fig. 3D). Of note, we also observed the As_2O_3-induced decrease of AKT protein in other cell lines, including several multiple myeloma lines and MDA-MB-468 breast cancer cells, which overexpress phosphorylated AKT (Fig. 2). In addition to confirming an association between dominant-negative AKT and As_2O_3-induced JNK activation and apoptosis, we focused on the observed decrease in AKT total protein and show that As_2O_3 does not decrease AKT transcription or translation (Supplementary Fig. S1) but causes decreased AKT protein expression that can be blocked by selective caspase inhibitors.

Several other apoptotic stimuli result in cleavage of AKT protein. Etoposide and Fas stimulation results in AKT cleavage in U937 and Jurkat cells (21). Tumor necrosis factor-α treatment of adipocytes causes AKT cleavage, which can be inhibited by caspase inhibitors but not proteosome inhibitors (29). Glucose deprivation–enhanced, tumor necrosis factor–related apoptosis-inducing ligand–induced cytotoxicity is associated with caspase-3–mediated AKT cleavage (30). Oxidative stress triggered by treatment with H_2O_2 also resulted in proteolysis of AKT (23). Cytokine withdrawal in 32D cells causes caspase-3–mediated cleavage at the Asp^{462} residue (31), but mutation of this residue did not alter the ability of As_2O_3 to degrade AKT (data not shown). In addition, individual mutation of two other amino acids, previously shown to be important in AKT cleavage by caspases following death receptor activation (22), had no effect on As_2O_3-mediated AKT protein decrease (data not shown). Interestingly, hsp90 inhibitors target AKT for degradation via the ubiquitin-proteosome pathway (20).

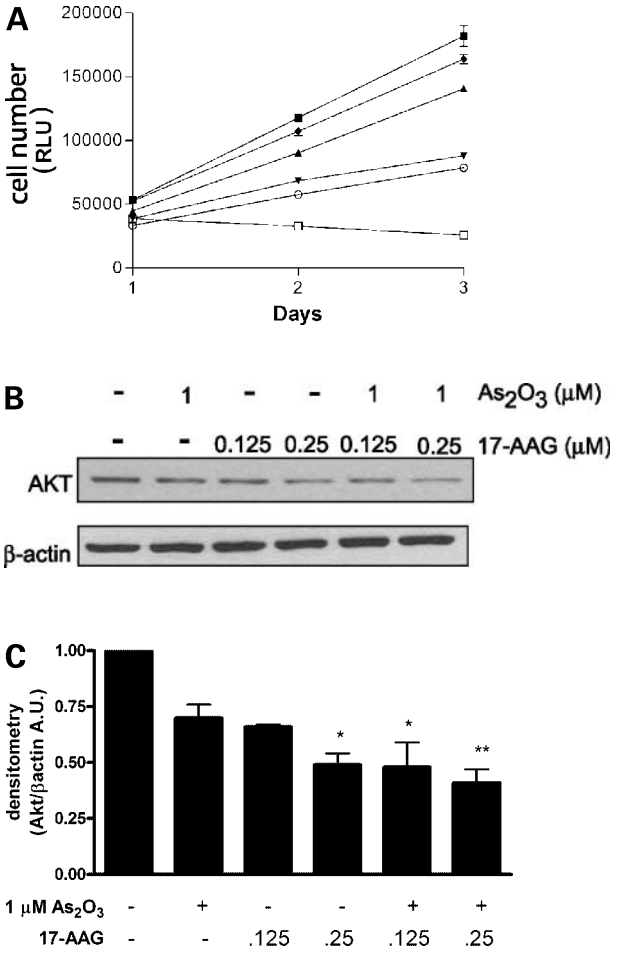

Figure 6. As_2O_3 enhances 17-AAG–induced AKT degradation and cytotoxicity. **A,** NB4 cells were treated for 3 d with 0.5 μmol/L As_2O_3 in the presence or absence of 0.125 or 0.25 μmol/L of 17-AAG. Cells were enumerated using trypan blue exclusion. Cell treatments were as follows; ■, control; ♦, 0.5 μmol/L As_2O_3; ▲, 0.125 μmol/L 17-AAG; ▼, 0.25 μmol/L 17-AAG; ○, 0.5 μmol/L As_2O_3 + 0.125 μmol/L 17-AAG; □, 0.5 μmol/L As_2O_3 + 0.25 μmol/L 17-AAG. **B,** AKT protein levels were assessed after 24-h treatment with 1 μmol/L As_2O_3 in the presence or absence of 0.125 or 0.25 μmol/L of 17-AAG. β-Actin levels were assessed as loading controls. **C,** average densitometry of AKT/β-actin protein levels as seen in **B** and expressed as arbitrary units (n = 2).

We show that AKT and JNK are inversely activated in response to As_2O_3. In addition, As_2O_3 treatment decreases the association of AKT with JIP1, the scaffolding protein on which the MAPK cascade assembles. Despite several reports that AKT inhibits association of JNK with JIP1, it is unclear what triggers release of AKT from JIP1. Clearly, release occurs after As_2O_3 treatment at a time before total AKT protein is degraded (Fig. 4B). This suggests that release of AKT from the JIP1/JNK complex may be the initiating event that activates the JNK cascade leading to loss of AKT and induction of apoptosis.

Our data suggest that As_2O_3 could be used in combination with new targeted agents to increase the cytotoxicity and therapeutic spectrum. This includes agents that decrease AKT activity or expression, such as 17-AAG

(Fig. 6). Alternatively, it may be used to enhance other promising drugs that unexpectedly increase AKT. CCI-779 (temsirolimus) is a rapamycin derivative that targets mammalian target of rapamycin, which lies downstream of AKT signaling. CCI-779 effectively inhibits growth of tumor cells *in vitro* and *in vivo* but also results in an increase in activated AKT via an ill-defined feedback mechanism (32, 33). Targeting the activated AKT by combining As_2O_3 with CCI-779 may result in enhanced tumor killing. Malignancies where the tumor suppressor PTEN is often silenced and AKT signaling is constitutively activated, such as glioblastoma multiforme, prostate cancer, endometrial cancer (34), and Herceptin-resistant breast cancer (35), are being targeted in clinical trials with mammalian target of rapamycin inhibitors. The combination of As_2O_3 and mammalian target of rapamycin inhibitors in these tumor settings is particularly intriguing and is currently under investigation in our laboratory.

Disclosure of Potential Conflicts of Interest

All authors claim no conflict of interest.

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