Regulatory Effects of Mammalian Target of Rapamycin-mediated Signals in the Generation of Arsenic Trioxide Responses*

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Arsenic trioxide (As$_2$O$_3$) is a potent inducer of apoptosis of leukemic cells in vitro and in vivo, but the mechanisms that mediate such effects are not well understood. We provide evidence that the Akt kinase is phosphorylated/activated during treatment of leukemia cells with As$_2$O$_3$, to regulate downstream engagement of mammalian target of rapamycin (mTOR) and its effectors. Using cells with targeted disruption of both the Akt1 and Akt2 genes, we found that induction of arsenic trioxide-dependent apoptosis is strongly enhanced in the absence of these kinases, suggesting that Akt1/Akt2 are activated in a negative feedback regulatory manner, to control generation of As$_2$O$_3$ responses. Consistent with this, As$_2$O$_3$-dependent pro-apoptotic effects are enhanced in double knock-out cells for both isoforms of the p70 S6 kinase (S6k1/S6k2), a downstream effector of Akt and mTOR. On the other hand, As$_2$O$_3$-dependent induction of apoptosis is diminished in cells with targeted disruption of TSC2, a negative upstream effector of mTOR. In studies using primary hematopoietic progenitors from patients with acute myeloid leukemia, we found that pharmacological inhibition of mTOR enhances the suppressive effects of arsenic trioxide on leukemic progenitor colony formation. Moreover, short interfering RNA-mediated inhibition of expression of the negative downstream effector translesional repressor 4E-BP1, partially reverses the effects of As$_2$O$_3$. Altogether, these data provide evidence for a key regulatory role of the Akt/mTOR pathway in the generation of the effects of As$_2$O$_3$, and suggest that targeting this signaling cascade may provide a novel therapeutic approach to enhance the anti-leukemic properties of As$_2$O$_3$.

Arsenic trioxide (As$_2$O$_3$) is a potent derivative that has potent antitumor effects in vitro and in vivo (1–3). This agent is approved by the Food and Drug Administration for the treatment of patients with acute promyelocytic leukemia (APL) (1–9), and there is considerable interest in its potential use for the treatment of other hematologic malignancies and solid tumors (10–16). Although the use of As$_2$O$_3$ in the treatment of patients with APL is well established, there are limitations in its application to other malignancies, because of a requirement for high concentrations for the induction of antineoplastic responses in non-APL cells. Therefore, studies to identify the mechanisms of action of As$_2$O$_3$ are of high interest, as they may ultimately allow the development of strategies to overcome the relative As$_2$O$_3$ resistance of malignant cells and allow induction of antitumor responses at lower concentrations of As$_2$O$_3$.

Extensive work over the years has attempted to define the mechanisms of action of As$_2$O$_3$. Previously described cellular events implicated in the generation of As$_2$O$_3$ responses include the following: degradation of the PML-RARα protein in APL cells (17); suppression of Bcl-2 levels and decreased mitochondrial transmembrane potential, resulting in cytochrome c release and activation of the caspase cascade (18–20); inhibition of nuclear receptor function via JNK-mediated RXRα phosphorylation (21); and induction of expression of the programmed cell death 4 (pDCD4) protein (22). There is also recent evidence indicating that the p38 MAP kinase (23) and its downstream effector kinase Msk1 (24), as well as the kinase Ask1 (25), are activated by As$_2$O$_3$. Interestingly, cells with targeted disruption of the genes for these kinases exhibit enhanced sensitivity to As$_2$O$_3$, suggesting that their function negatively regulates generation of As$_2$O$_3$ responses (23–25).

The Akt/mTOR cascade is an important signaling pathway that is activated by a variety of cellular signals and plays critical roles in cap-dependent mRNA translation and generation of cell proliferative responses (26–31). We have recently demonstrated that treatment of BCR-ABL-expressing cells with As$_2$O$_3$ results in paradoxical phosphorylation/activation of mTOR and the p70 S6 kinase (p70 S6K) (32), but the precise regulatory role of this pathway in the induction of As$_2$O$_3$ responses is, thus far, unknown.

RNA: MEF, mouse embryonic fibroblast; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; MAP, mitogen-activated protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; APL, acute promyelocytic leukemia; JNK, c-Jun NH$_2$-terminal kinase; S6K, S6 kinase; CFU, colony-forming unit; CFU-L, CFU-blast.
In this study we sought to identify As$_2$O$_3$-dependent upstream regulatory effectors of the mTOR pathway and to directly address the functional relevance of mTOR-mediated signals in the induction of As$_2$O$_3$ responses. For this purpose, cells with targeted disruption of genes encoding for various effectors of the mTOR pathway were used. Our data demonstrate that Akt is phosphorylated/activated in an As$_2$O$_3$-inducible manner to regulate downstream activation of mTOR. Moreover, induction of As$_2$O$_3$-dependent apoptosis and growth suppression is enhanced in cells with targeted disruption of both the Akt1 and Akt2 genes (Akt$_1^{-/-}$Akt$_2^{-/-}$), as well as in double knock-out cells for both isoforms of the p70 S6 kinase (S6k$_1^{-/-}$S6k$_2^{-/-}$). On the other hand, As$_2$O$_3$-inducible pro-apoptotic responses are diminished in cells with targeted disruption of TSC2, a negative upstream effector of mTOR. We also demonstrate that pharmacological or molecular targeting of mTOR effectors in primary progenitors from AML patients regulates As$_2$O$_3$-dependent growth inhibition, consistent with a critical role for this pathway in the generation of As$_2$O$_3$-induced antileukemic effects.

**MATERIALS AND METHODS**

**Cells and Reagents**—The KG-1, MM6, K562, and U937 leukemia cell lines were grown in RPMI 160 medium supplemented with 10% fetal bovine serum and antibiotics. Arsenic trioxide was purchased from Sigma. Antibodies against the phosphorylated forms of Akt, p70 S6 kinase, eIF4B, and 4E-BP1 were obtained from Cell Signaling Technology, Inc. The FRAP/mTOR inhibitor, rapamycin, was obtained from Calbiochem. Immortalized mouse embryonic fibroblasts (MEFs) from Akt$_1^{-/-}$Akt$_2^{-/-}$ mice (34) and from S6k$_1^{-/-}$S6k$_2^{-/-}$ mice (35) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotics. Immortalized TSC2$_{1,2}^{-/-}$ and TSC2$_{1,2}^{-/-}$ MEFs (36, 37) were from Dr. Kwiatkowski and were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotics. siRNA against 4E-BP1 was obtained from Dharmacon Inc.

**Cell Lysis and Immunoblotting**—Cells were treated with the indicated doses of arsenic trioxide for the indicated times and subsequently lysed in phosphorylation lysis buffer as described previously (38, 39). In some experiments the cells were serum-starved for 24 h before treatment with As$_2$O$_3$. Immunoprecipitation and immunoblotting using an enhanced chemiluminescence (ECL) method were performed as described previously (38, 39).

**S6 Kinase Assays**—Assays to detect the arsenic-dependent activation of the p70 S6 kinase were performed as described previously (32, 39, 40). Briefly, U937 cells were lysed in phosphorylation lysis buffer, and cell lysates were immunoprecipitated with an antibody against p70 S6 kinase or control nonimmune rabbit immunoglobulin (RlgG). *In vitro* kinase assays were performed using a synthetic peptide substrate (AKRRRLSSLRA), and p70 S6 kinase activity was measured using an S6 kinase assay kit (Upstate Biotechnology, Inc.) according to the manufacturer’s instructions. Values were calculated by subtracting nonspecific activity, detected in RlgG immunoprecipitates, from kinase activity detected in anti-p70 S6K immunoprecipitates (32, 39, 40).

**S6 Kinase Assays**—Cell proliferation assays using the MTT methodology were subsequently performed as described previously (42).

**Evaluation of Apoptosis**—Cells were exposed to arsenic trioxide for the indicated times. Flow cytometric assays to evaluate apoptosis by annexin and propidium iodide staining were performed as described previously (24).

**Human Hematopoietic Progenitor Cell Assays**—Bone marrow or peripheral blood was obtained from patients with acute myeloid leukemia (AML) or acute lymphoid leukemia after obtaining consent, approved by the Institutional Review Board of Northwestern University. Bone marrow or peripheral blood mononuclear cells were cultured in the presence or absence of arsenic trioxide (0.5 μM), with or without the indicated concentrations of rapamycin (10 nM) or LY294002 (10 μM), and used for clonogenic assays in methylcellulose as described previously (40, 44). CD34$^+$ cells or total mononuclear cells in CD34-neg-ative or unknown cases were transfected with either control siRNA or siRNAs specifically targeting 4E-BP1 and subsequently incubated in a methylcellulose, in the presence or absence of arsenic trioxide.

**RESULTS**

We initially examined whether the upstream regulator of mTOR, kinase Akt, is activated in an As$_2$O$_3$-dependent manner in leukemic cell lines. Cells were incubated for different times with As$_2$O$_3$, and the phosphorylation/activation of Akt was assessed by immunoblotting using a specific antibody against the phosphorylated form of Akt on Ser-473. As shown in Fig. 1, treatment of cells with As$_2$O$_3$ resulted in strong phosphorylation/activation of Akt that was detectable as early as 10 min after treatment of cells with As$_2$O$_3$ (Fig. 1, A and B). Similarly, As$_2$O$_3$ treatment resulted in phosphorylation of Akt on the PKD1 phosphorylation site, Thr-308 (Fig. 1C). In addition, As$_2$O$_3$ treatment of the cells resulted in phosphorylation of PRAS40 (Fig. 1D), a substrate for phosphorylation by mTORC1, which was recently shown to be upstream of the mTOR effectors S6k1 and 4E-BP1 (45). These data suggested that Akt may be the upstream effector that regulates downstream engagement of mTOR/p70 S6K (32) in response to arsenic trioxide. To address the functional role of engagement of Akt during As$_2$O$_3$-dependent treatment of cells, studies were subsequently performed using MEFs with targeted disruption of both the Akt1 and Akt2 genes (34). In experiments in which the induction of phosphorylation of p70 S6K was compared in parental MEFs and Akt1/2 double knock-out MEFs, we found that such phosphorylation is significantly decreased in the absence of Akt1/2 (Fig. 2A), consistent with regulatory effects of Akt kinases on As$_2$O$_3$-dependent mTOR/p70 S6K activation. Likewise, we found that As$_2$O$_3$-induced phosphorylation of 4E-BP1 was blocked in the absence of Akt1/2 (Fig. 2B). To directly address the functional relevance of Akt1/Akt2 kinases in the generation of the effects of arsenic trioxide, the induction of apoptosis in Akt1/2 negative MEFs was subsequently determined. Akt$_1^{+/+}$/Akt$_2^{+/+}$ and Akt$_1^{-/-}$/Akt$_2^{-/-}$ MEFs were treated with As$_2$O$_3$ for 72 h, and the percentage of apoptotic cells was determined by flow cytometry for annexin V staining. Treatment of parental Akt$_1^{+/+}$/Akt$_2^{+/+}$ MEFs with arsenic tri-
FIGURE 1. Arsenic trioxide-dependent phosphorylation/activation of the Akt kinase. A, MM6 acute myeloid leukemia cells were incubated with  \( \text{As}_2\text{O}_3 \) (1 \( \mu \text{M} \)) for the indicated times. Total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of Akt on serine 473. The same blot was subsequently re-probed with an antibody against GAPDH to control for protein loading. The signals for the different bands were quantitated by densitometry. Data are expressed as ratios of phosphorylated Akt to GAPDH levels and represent means ± S.E. of two independent experiments. Paired t test analysis for the phosphorylation of Akt from lysates of cells treated for 10 min versus control untreated cells showed a \( p \) value = 0.036. B, U937 cells were incubated with  \( \text{As}_2\text{O}_3 \) (1 \( \mu \text{M} \)) for the indicated times. Total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of Akt on serine 473. The same blot was subsequently re-probed with an antibody against GAPDH to control for protein loading. The signals for the different bands were quantitated by densitometry. Data are expressed as ratios of phosphorylated Akt to GAPDH levels and represent means ± S.E. of two independent experiments. Paired t test analysis for the phosphorylation of Akt from lysates of cells treated for 10 min versus control untreated cells showed a \( p \) value = 0.016. C, serum-starved K562 cells were incubated in the presence or absence of  \( \text{As}_2\text{O}_3 \) for 5 min, as indicated. Total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of Akt on threonine 308. The same blot was subsequently re-probed with an antibody against GAPDH to control for protein loading. The signals for the different bands were quantitated by densitometry. Data are expressed as ratios of phosphorylated Akt to GAPDH levels and represent means ± S.E. of four independent experiments in which the cells were treated with  \( \text{As}_2\text{O}_3 \) for 5 or 10 min. Paired t test analysis for the phosphorylation of Akt from  \( \text{As}_2\text{O}_3 \)-treated cell lysates versus control untreated cells showed a \( p \) value = 0.037. D, serum-starved K562 cells were incubated with  \( \text{As}_2\text{O}_3 \) for the indicated times. Total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of PRAS40 on threonine 246. The same blot was subsequently re-probed with an antibody against GAPDH to control for protein loading. The signals for the different bands were quantitated by densitometry. Data are expressed as ratios of phosphorylated PRAS40 to GAPDH levels and represent means ± S.E. of two independent experiments. Paired t test analysis for the phosphorylation of PRAS40 from lysates of cells treated for 60 min versus control untreated cells showed a \( p \) value = 0.021.
oxide resulted in minimal induction of apoptosis (Fig. 3A).

However, in double knockout MEFs for both Akt1 and Akt2 (Akt1<sup>-/-</sup>Akt2<sup>-/-</sup>), there was a dramatic enhancement of As<sub>2</sub>O<sub>3</sub>-dependent apoptosis (paired p value = 0.045) (Fig. 3A), strongly suggesting negative regulatory roles for Akt kinases in the control of As<sub>2</sub>O<sub>3</sub>-inducible apoptosis. Similarly, when the antiproliferative effects of As<sub>2</sub>O<sub>3</sub> were compared in double Akt1<sup>-/-</sup>Akt2<sup>-/-</sup> knock-out and parental MEFs, there was a substantial augmentation of the inhibitory effects of different concentrations of As<sub>2</sub>O<sub>3</sub> in the absence of Akt1 and Akt2 (Fig. 3B). Taken together, these findings strongly suggested that Akt kinases are activated in a negative feedback regulatory manner during As<sub>2</sub>O<sub>3</sub> treatment of cells to regulate downstream activation of p70 S6K and to negatively control induction of apoptosis and growth inhibition.

The Tsc2 gene product (tuberin) along with the Tsc1 gene product (hamartin) are present in a protein complex that acts as a negative upstream regulator of the mTOR pathway (26, 29). To determine the effects of this negative regulator of the mTOR pathway in the induction of As<sub>2</sub>O<sub>3</sub> responses, experiments were performed using mouse embryonic fibroblasts with targeted disruption of the Tsc2 gene. TSC2<sup>+/+</sup> and TSC2<sup>-/-</sup>-MEFs were incubated with As<sub>2</sub>O<sub>3</sub> for 48 h, and the percentage of apoptotic cells was subsequently assessed. As<sub>2</sub>O<sub>3</sub>-dependent induction of apoptosis was significantly decreased in TSC2<sup>−/−</sup>-cells, as compared with TSC2<sup>+/+</sup>-cells (paired p value = 0.013) (Fig. 3C). Likewise, the generation of As<sub>2</sub>O<sub>3</sub>-dependent growth inhibitory effects was impaired in TSC2<sup>−/−</sup>-MEFs, as compared with TSC2<sup>+/+</sup>-cells MEFs (Fig. 3D). Thus, induction of arsenic trioxide pro-apoptotic responses is impaired in the absence of the negative upstream effector of mTOR, TSC2, further supporting the existence of a negative feedback, mTOR-regulated, mechanism to control induction of arsenic trioxide responses.

One of the two major downstream effectors of mTOR is the p70 S6 kinase, which in turn regulates downstream phosphorylation/activation of the S6 ribosomal protein and the eukaryotic initiation factor 4B (eIF4B) (26–31, 46, 47). Similar to what we previously observed in BCR-ABL-transformed cells (32), treatment of cells of acute myelomonocytic leukemia origin with As<sub>2</sub>O<sub>3</sub> resulted in strong activation of p70 S6 kinase, as shown by immune-complex kinase assays in anti-p70 S6K immuno-

FIGURE 2. Arsenic trioxide-inducible activation of p70 S6K and phosphorylation of 4E-BP1 are Akt-dependent. A, serum-starved Akt1/2<sup>+/+</sup> and Akt1/2<sup>-/-</sup> mouse embryonic fibroblasts (MEFs) were incubated in the absence or presence of As<sub>2</sub>O<sub>3</sub> (5 μM) for 30 min. Total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of p70 S6 kinase on threonine 389 (upper panel). The same blot was re-probed with an antibody against Akt (middle panel). The same blot was re-probed with an antibody against GAPDH to control for protein loading (lower panel). The signals for the different bands in the upper and lower panels were quantified by densitometry. Data are expressed as ratios of phosphorylated p70 S6K to GAPDH levels and represent means ± S.E. of three independent experiments (right panel). Paired t test analysis for the phosphorylation of p70 S6K in Akt1/2<sup>-/-</sup> versus Akt1/2<sup>+/+</sup>-MEFs showed a p value = 0.035. ATO, arsenic trioxide. B, serum-starved Akt1<sup>+/+</sup>Akt2<sup>−/−</sup> and Akt1<sup>-/-</sup>Akt2<sup>−/−</sup>-MEFs were incubated in the absence or presence of As<sub>2</sub>O<sub>3</sub> (5 μM) for 30 min. Total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of 4E-BP1 on threonine 70 (upper panel). The same blot was re-probed with an antibody against Akt (middle panel). The same blot was re-probed with an antibody against GAPDH to control for protein loading (lower panel). The signals for the different bands in the upper and lower panels were quantified by densitometry. Data are expressed as ratios of phosphorylated 4E-BP1 to GAPDH levels and represent means ± S.E. of three independent experiments (right panel). Paired t test analysis for the phosphorylation of p70 S6K in Akt1/2<sup>-/-</sup> versus Akt1/2<sup>+/+</sup>-MEFs showed a p value = 0.028.
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precipitates from As$_2$O$_3$-treated cells (paired $p$ value $= 0.039$) (Fig. 4A), establishing that this kinase is activated by As$_2$O$_3$ in cells of diverse hematopoietic origin. To elucidate the role of the p70 S6 kinase pathway in the generation of arsenic trioxide responses, we evaluated the generation of arsenic trioxide responses in immortalized MEFs from mice with targeted disruption of both the S6k1 and S6k2 genes (35). In initial experiments, we found that As$_2$O$_3$ treatment of S6k$^{1-/-}$/S6k$^{2+/+}$ MEFs resulted in phosphorylation of eIF4B (Fig. 4B), but such phosphorylation was completely blocked in the double knock-out, S6k$^{1-/-}$/S6k$^{2-/-}$ MEFs (Fig. 4B). Importantly, the induction of As$_2$O$_3$-dependent apoptosis was dramatically enhanced in the double S6k1/S6k2 knock-out cells (Fig. 4C), establishing that S6 kinases are key mediators of the negative regulatory effects of the Akt/mTOR pathway on the induction of As$_2$O$_3$ responses.

The other major target for the kinase activity of mTOR is the translational repressor 4E-BP1 (26–31). mTOR-mediated phosphorylation of this protein in multiple sites (49) results in its inactivation and dissociation from eIF4E, an event that leads to initiation of cap-dependent translation (26–31). In experiments performed using several different acute leukemia cell lines, we found that As$_2$O$_3$ treatment induces phosphorylation of 4E-BP1 on Thr-70 and Thr-37/46 (Fig. 5, A–C), sites whose phosphorylation is required for deactivation of 4E-BP1 and initiation of mRNA translation. Taken together, these findings suggested that beyond downstream engagement of the p70 S6k/eIF4B pathway in acute leukemia cells, another mechanism by which the As$_2$O$_3$-activated form of mTOR may mediate induction of As$_2$O$_3$ responses in acute leukemia cells may involve regulation of 4E-BP1 activity.

To directly address the physiological relevance of the Akt/mTOR pathway in the induction of the antileukemic properties of arsenic trioxide in acute leukemia, we performed studies using primary leukemia progenitors, collected from bone marrows or peripheral blood from a large number of patients with acute leukemia (AML). Treatment of bone marrow or peripheral blood-derived leukemia progenitors with As$_2$O$_3$ consistently inhibited leukemic properties of arsenic trioxide in acute leukemia, we performed studies using primary leukemia progenitors, collected from bone marrows or peripheral blood from a large number of patients with acute leukemia (AML). Treatment of bone marrow or peripheral blood-derived leukemia progenitors with As$_2$O$_3$ consistently inhibited leukemia CFU-blunt (CFU-L) colony formation (Fig. 6, A and B). However, concomitant addition to the cultures of the mTOR inhibitor rapamycin strongly enhanced the growth inhibitory effects of As$_2$O$_3$ (paired $p$ value $= 0.00015$, $n = 11$) (Fig. 6A). Similarly, LY294002, an inhibitor of the phosphatidylinositol 3-kinase pathway that acts as an upstream effector of Akt and mTOR (29, 30), also strongly enhanced As$_2$O$_3$-dependent suppression of CFU-L colony formation (paired $p$ value $= 0.0091$, $n = 5$) (Fig. 6B). Thus, pharmacological inhibition of phosphatidylinositol 3-kinase and the Akt/mTOR cascade appears to enhance the suppressive effects of arsenic trioxide on primitive leukemic progenitors from patients with acute leukemia, suggesting an important negative regulatory effect of Akt and mTOR effectors in the induction of the antileukemic properties of arsenic trioxide.

To further establish the relevance of mTOR-regulated signals in the suppression of leukemic hematopoietic progenitors, we sought to selectively target the negative downstream effector of this pathway, translational repressor 4E-BP1, and to determine the effects of such inhibition on As$_2$O$_3$-inducible antileukemic responses. Prior to this, the phosphorylation of the protein in primary AML leukemia blasts was examined. As
shown in Fig. 6, similarly to what we previously observed in acute leukemia cell lines, phosphorylation of 4E-BP1 was inducible in an As$_2$O$_3$-dependent manner in primary leukemia cells (Fig. 7A). To directly determine the effects of 4E-BP1 on the generation of arsenic trioxide-dependent responses in leukemic hematopoiesis, siRNA specifically targeting 4E-BP1 (Fig. 7B) was utilized. Such inhibition of 4E-BP1 expression with siRNA targeting did not result in a detectable compensatory increase in the activation of the other major cascade regulated by mTOR, as reflected by the lack of an increase in the phosphorylation of rpS6 (Fig. 7C). We examined the effects of siRNA-mediated inhibition of 4E-BP1 on the growth of primary leukemic progenitors from patients with acute leukemia (three patients with AML, one with acute lymphoid leukemia, and one with myelodysplastic syndrome in transformation to AML). As$_2$O$_3$ treatment suppressed the growth of primary CFU-L leukemic progenitors transfected with control siRNA. However, 4E-BP1 knockdown significantly reversed the suppressive effects of arsenic trioxide (paired p value = 0.0085) (Fig. 7D). Thus, pharmacological inhibition of the mTOR pathway potentiates, whereas siRNA-mediated knockdown of its negative downstream effector 4E-BP1 diminishes, the generation of the antileukemic effects of arsenic trioxide, indicating a key regulatory role for this pathway in the generation of the effects of arsenic trioxide.

**DISCUSSION**

The importance of arsenic trioxide as an effective agent for the treatment of one form of acute leukemia (APL) and its promise as a potential therapeutic agent for the treatment of a variety of other malignancies has resulted in extensive efforts to understand the mechanisms by which this agent mediates its effects on cells and tissues. Several key mechanisms that participate in the induction of arsenic tri-
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FIGURE 5. As2O3-dependent phosphorylation of the translational repressor 4E-BP1 in AML cell lines. KG-1 (A), MM6 (B), or U937 (C) cells were incubated with As2O3 (1 μM) for the indicated times. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with antibodies against the phosphorylated forms of 4E-BP1 on Thr-70 (top left panels) or Thr-37/46 (top right panels). The same blots were then re-probed with an anti-GAPDH antibody to control for protein loading (lower panels).

FIGURE 6. Pharmacological inhibition of mTOR and its upstream effector components enhances the suppressive effects of arsenic trioxide on leukemic CFU-GM progenitors from patients with AML. A. Bone marrow or peripheral blood mononuclear cells from patients with AML were plated in a methylcellulose culture assay system with As2O3 (ATO) (0.5 μM) and rapamycin (Rapa) (10 nM), as indicated. Data are expressed as percent control of CFU-granulocyte colony numbers for untreated cells. Means ± S.E. of the values from 11 experiments using different patient samples are shown. Paired t test analysis of the combinations of As2O3 plus rapamycin, as compared with As2O3 alone, showed p value = 0.00015. B. Bone marrow or peripheral blood mononuclear cells from patients with AML were cultured in methylcellulose culture system with As2O3 (0.5 μM) and LY294002 (Ly) (10 μM) as indicated. The data are expressed as percent of CFU-granulocyte macrophage colony numbers for untreated cells. Means ± S.E. from five experiments are shown. Paired t test analysis of the combination of As2O3 plus LY294002 as compared with As2O3 alone showed p value = 0.0091.

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Methylcellulose culture system with As2O3 (0.5 μM) was used to culture peripheral blood mononuclear cells from patients with AML. These cells were plated in a methylcellulose culture system with As2O3 and rapamycin. The treatment was compared with As2O3 alone, and the results showed a decrease in cell colony numbers. The data are expressed as percent of CFU-granulocyte colony numbers for untreated cells. The means ± S.E. of the values from 11 experiments using different patient samples are shown. Paired t test analysis of the combinations of As2O3 plus rapamycin, as compared with As2O3 alone, showed a p value of 0.00015.

Bone marrow or peripheral blood mononuclear cells from patients with AML were also cultured in a methylcellulose culture system with As2O3 (0.5 μM) and LY294002 (Ly) (10 μM). The data are expressed as percent of CFU-granulocyte macrophage colony numbers for untreated cells. Paired t test analysis of the combination of As2O3 plus LY294002 as compared with As2O3 alone showed a p value of 0.0091.

Arsenic trioxide-dependent apoptosis have been identified and extensively described in the literature. Such mechanisms include an As2O3-dependent increase in reactive oxygen species (ROS), loss of mitochondrial membrane potential, release of cytochrome c, and activation of caspases (19, 20, 50). Generation of As2O3-inducible ROS appears to depend on cellular glutathione stores (21), and there is some evidence that reduced cellular GSH is an inhibitor of As2O3-dependent apoptosis. Because of its ability to conjugate arsenic as As(GS)3 complexes and to sequester ROS (53), consistent with this, GSH depletion with buthionine sulfoximine restores sensitivity to As2O3-induced apoptosis (54, 55), which may be related to enhanced activation of the JNK kinase pathway (56). There is accumulating evidence that the coordinated functions of different signaling cascades, some with common and some with opposing biological functions, account for the balanced generation of arsenic trioxide responses. For instance, MAP kinase pathways appear to play critical roles in the regulation of arsenic trioxide-dependent apoptosis, either by mediating pro-apoptotic signals or by generating anti-apoptotic effects that impede induction of arsenic responses (57). In that regard, there is definitive evidence that engagement of the JNK kinase pathway is a critical and necessary event for induction of apoptosis by As2O3 (58). On the other hand, it has been shown previously that the p38 MAP kinase pathway suppresses the generation of arsenic trioxide responses and that pharmacological inhibition of its activation enhances As2O3-mediated apoptosis and antiproliferative effects (23, 24, 44).

Interestingly, although elements of the p38 pathway, such as the upstream effectors Mkk3 and Mkk6, the p38 MAP kinases p38α and p38β, and the downstream p38 effector, Msk1, are all activated during treatment of cells with As2O3, they all mediate signals that negatively control arsenic trioxide-induced cell death (23, 24, 44). Similarly, the kinase Ask1 has been shown previously to be activated by arsenic in leukemic cell lines via accumulation of reactive oxygen species, but paradoxically, such activation plays a negative role in the induction of apoptosis (25). Such findings have raised the possibility that during As2O3 treatment of normal and malignant cells, there is ROS-mediated activation of signaling cascades in a negative feedback regulatory manner to protect the cells from free radical-induced damage. It is therefore possible that the generation of such anti-apoptotic signals may constitute a physiological defense mechanism of normal cells and tissues that has also been preserved in malignant cells.

In this study we provide evidence that the Akt/mTOR signaling pathway plays a critical regulatory role in the generation of arsenic trioxide-induced apoptosis and growth-suppressive effects. Using knock-out cells for various components of this signaling cascade, we definitively establish the functional roles of various elements of the pathway in the control of arsenic trioxide-mediated apoptosis. Our data demonstrate that Akt is phosphorylated/activated in an arsenic trioxide-dependent manner and regulates downstream engagement of the p70 S6 kinase. Moreover, the induction of As2O3-dependent apoptosis is strongly enhanced in Akt1/−/− Akt2/−/− cells, as compared with parental cells. Similarly, induction of arsenic trioxide-mediated apoptosis is enhanced in cells with targeted disruption of both isoforms of the p70 S6K (S6k1/−/− S6k2/−/−), suggesting that sequential Akt → mTOR → p70 S6K activation ultimately results in the generation of signals that suppress apoptosis. The identity of the downstream effectors of the Akt/mTOR/
in previous studies we demonstrated that the S6 ribosomal protein is phosphorylated by As$_2$O$_3$ (32). It is therefore possible that these two downstream effectors of the p70 S6 kinase participate in the negative regulatory effects of the pathway in the generation of arsenic trioxide responses. In addition, S6k1 is known to inhibit the function of elongation factor 2 (EF2) kinase (51, 52), a kinase that phosphorylates and inhibits the activity of EF2 (43), and to phosphorylate the tumor suppressor PDCD4 (programmed cell death protein 4), resulting in its degradation via the ubiquitin ligase SCF (βTRCP) (53). Thus, multiple distinct mechanisms may participate in the control of arsenic-dependent apoptosis, but the precise contribution of distinct signaling events downstream of the p70 S6K remains to be determined in future studies.

Our data also show that rapamycin enhances the effects of arsenic trioxide on primary hematopoietic progenitors from acute leukemia patients. Such findings are consistent with arsenic trioxide and rapamycin acting directly on separate pathways to promote apoptosis of primitive leukemic progenitors. Previous studies have demonstrated a requirement for JNK (58) and Mkk4 (44) in the induction of As$_2$O$_3$-dependent apoptosis, establishing that activation of the JNK pathway is the key signaling event for the induction of apoptotic responses. Our findings indicate that arsenic trioxide activates the mTOR pathway, apparently in a negative feedback regulatory manner, as arsenic trioxide responses are modified in knock-out cells for various components of the mTOR pathway. It is therefore possible that mTOR-generated signals counteract the action of JNK-generated signals in leukemic hematopoietic progenitors, and the enhancing effects of rapamycin result by the suppression of such negative feedback pathway in leukemic precursors.

Our data also demonstrate a key role for the translational repressor 4E-BP1 in the regulation of the antileukemic effects of arsenic trioxide. Consistent with our previous observations in BCR-ABL-expressing cells (32), we found that arsenic trioxide treatment of various acute leukemia cell lines results in

S6K pathway that mediates such responses remains to be identified. It is possible that one of the mechanisms by which engagement of Akt during arsenic treatment of the cells negatively controls apoptosis involves phosphorylation of BAD on serine 136, resulting in its inactivation (59, 60). On the other hand, our data demonstrated that the eukaryotic translation initiation factor 4B, a protein that stimulates RNA helicase activity of eIF4A and binds to 18 S RNA (41, 43, 51), is phosphorylated in an As$_2$O$_3$-inducible manner in S6k$^{+/+}$S6k2$^{+/+}$, but not S6k$^{-/-}$S6k2$^{-/-}$ MEFs, whereas...
phosphorylation of 4E-BP1 in sites required for its inactivation and dissociation from eIF4E (26, 29). Importantly, in studies in which the expression of 4E-BP1 was knocked down in primitive hematopoietic progenitors from patients with acute leukemia, there was partial reversal of the inhibitory effects of As$_2$O$_3$. Altogether, our data support a model in which two distinct pathways downstream of mTOR, one involving p70 S6K and one phosphorylation/de-activation of 4E-BP1, are engaged in a negative feedback regulatory manner to regulate arsenic trioxide responses. Further under-standing of the precise elements involved downstream of these pathways to mediate negative feedback regulation of arsenic trioxide responses will be of importance, and it may lead to the identification of new specific targets for future therapeutic-translational efforts to overcome arsenic trioxide resistance.

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