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Arsenic Trioxide-Dependent Activation of Thousand-and-One Amino Acid Kinase 2 and Transforming Growth Factor-β-Activated Kinase 1

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ABSTRACT

Arsenic trioxide (As_2O_3) has potent antileukemic properties in vitro and in vivo, but the mechanisms by which it generates its effects on target leukemic cells are not well understood. Understanding cellular mechanisms and pathways that are activated in leukemic cells to control the generation of As_2O_3 responses should have important implications in the development of novel approaches using As_2O_3 for the treatment of leukemias. In this study, we used immunoblotting and immune complex kinase assays to provide evidence that the kinases thousand-and-one amino acid kinase 2 (TAO2) and transforming growth factor-β-activated kinase 1 (TAK1) are rapidly activated in response to treatment of acute leukemia cells with As_2O_3. Such activation occurs after the generation of reactive oxygen species and regulates downstream engagement of the p38 mitogen-activated protein kinase. Our studies demonstrate that siRNA-mediated knockdown of TAO2 or TAK1 or pharmacological inhibition of TAK1 enhances the suppressive effects of As_2O_3 on KT-1-derived leukemic progenitor colony formation and on primary leukemic progenitors from patients with acute myelogenous leukemia. These results indicate key negative-feedback regulatory roles for these kinases in the generation of the antileukemic effects of As_2O_3. Thus, molecular or pharmacological targeting of these kinases may provide a novel approach to enhance the generation of arsenic-dependent antileukemic responses.

Arsenic trioxide (As_2O_3) has been used for medicinal purposes for thousands of years and has potent antitumor effects both in vitro and in vivo (Miller et al., 2002; Platanias, 2009). First used by investigators in China (Chen et al., 2002; Miller et al., 2002), it is now approved in the United States for the treatment of acute promyelocytic leukemia (APL), a rare subtype of acute myelogenous leukemia (AML). In addition to its promise in APL therapy, As_2O_3 has also been shown to inhibit the growth of various other types of malignant cells in vitro, including chronic myelogenous leukemia, multiple myeloma, prostate carcinoma, and neuroblastoma cells (Chen et al., 2002; Miller et al., 2002; O'Dwyer et al., 2002; Douer and Tallman, 2005; Platanias, 2009). The effects of As_2O_3 are known to be dose-dependent, with low doses ($\leq$0.5 μM) inducing differentiation in APL cells, whereas higher doses ($\geq$2 μM) are required for apoptosis (Chen et al., 2002; Miller et al., 2002; O'Dwyer et al., 2002; Douer and Tallman, 2005; Platanias, 2009). By elucidating the pathways through which the antineoplastic effects of As_2O_3 are regulated, it is possible that new strategies can be developed to enhance the effects of this agent on malignant cells, allowing for its broader use in the treatment of various cancers.

In previous work, we demonstrated that the p38 mitogen-activated protein kinase (MAPK) pathway is activated in leukemic cells in response to treatment with As_2O_3 (Verma et al., 2002; Giafis et al., 2006). The engagement of the p38 pathway seems to occur in a negative-feedback

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ABBREVIATIONS: APL, acute promyelocytic leukemia; TAO2, thousand-and-one amino acid kinase 2; TAK1, transforming growth factor-β-activated kinase 1; MAPK, mitogen-activated protein kinase; AML, acute myelogenous leukemia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ROS, reactive oxygen species; DCFDA, 2′7′-dichlorofluorescein diacetate; DTT, dithiothreitol; NAC, *N*-acetyl cysteine; siRNA, small interfering RNA; CFU-L, leukemic colony forming unit; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase-polymerase chain reaction; MAPKKK, mitogen-activated protein kinase kinase kinase.

regulatory manner, with enhanced proapoptotic and/or antiproliferative effects seen after pharmacological inhibition of p38 or in p38α knockout cells (Verma et al., 2002; Giafis et al., 2006). Furthermore, downstream effectors of this pathway activated by arsenic trioxide, including the mitogen- and stress-activated kinase 1 (Kannan-Thulasiraman et al., 2006) and the MAPK-interacting kinases 1 and 2 (Dolniak et al., 2008), have been identified, and their involvement in the negative control of generation of arsenic responses has been established (Kannan-Thulasiraman et al., 2006; Dolniak et al., 2008). Others have also shown recently that pharmacological targeting of the p38 MAPK pathway enhances arsenic trioxide-induced apoptosis in multiple myeloma cells (Wen et al., 2008), suggesting a similar negative feedback mechanism in these cells.

Because there is emerging evidence that the p38 MAPK pathway plays an important regulatory role in the generation of arsenic trioxide responses, we sought to identify the upstream effector signals that lead to its activation by arsenic in leukemic cells. In this study, we provide the first evidence demonstrating that thousand-and-one amino acid kinase 2 (TAO2) and TGF-β-activated kinase 1 (TAK1) are activated during treatment of leukemic cells with As_2O_3. Our data demonstrate that such phosphorylation occurs downstream of As_2O_3-induced reduction/oxidation reactions, and that phosphorylation of p38 by As_2O_3 is regulated by upstream engagement of these kinases. In addition, the suppressive effects of As_2O_3 on primitive leukemic progenitors are enhanced by knockdown of TAO2 and TAK1, suggesting that these kinases negatively regulate the generation of As_2O_3-mediated antileukemic responses.

Materials and Methods

Cells and Reagents. The NB4 human acute promyelocytic leukemia, the U937 acute myelomonocytic leukemia, and the KT-1 chronic myelogenous leukemia-blast crisis cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. The NB4.306 retinoic-acid-resistant variant cell line (Dermime et al., 1993; Sassano et al., 2007) was provided by Dr. Saverio Minucci (European Institute of Oncology, Milan, Italy) and was also grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. As_2O_3, dithiothreitol (DTT), and *N*-acetyl cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO). An antibody against the phosphorylated form of TAO2 (Ser181) was purchased from Abcam (Cambridge, MA). Antibodies against p38 MAPK and the phosphorylated forms of both TAK1 (Ser412) and p38 MAPK (Thr180/Tyr182) were purchased from Cell Signaling Technology (Danvers, MA). An antibody against TAO2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the p38 substrate activating transcription factor 2 were obtained from Millipore (Billerica, MA). The TAK1 inhibitor 5*Z*-7-oxozeaenol was purchased from Calbiochem (La Jolla, CA).

Cell Lysis and Immunoblotting. Cells were incubated with the indicated doses of As_2O_3 for the indicated times and subsequently lysed in phosphorylation lysis buffer as described previously (Uddin et al., 1995). Immunoblotting using an enhanced chemiluminescence method was done as described previously (Uddin et al., 1995).

Kinase Assays. Cells were incubated with As_2O_3 for the indicated times. Total cell lysates were immunoprecipitated with an antibody against TAO2 or nonimmune rabbit IgG. In vitro kinase assays were performed as described previously (Verma et al., 2002; Kannan-Thulasiraman et al., 2006).

Generation of Reactive Oxygen Species. The generation of reactive oxygen species (ROS) was measured by monitoring the oxidation of 2′7′-dichlorofluorescein diacetate (DCFDA; Invitrogen, Carlsbad, CA) to 2′7′-dichlorofluorescein as described previously (Evens et al., 2005). DCFDA is a nonfluorescent compound that permeates cells and interacts with intracellular oxidants to form the fluorescent compound 2′7′-dichlorofluorescein. In brief, after treatment with As_2O_3 in the absence or presence of the reducing agent DTT, cells were incubated in 5 μM DCFDA for 30 min at 37°C. Cells were then analyzed for fluorescent intensity by flow cytometry.

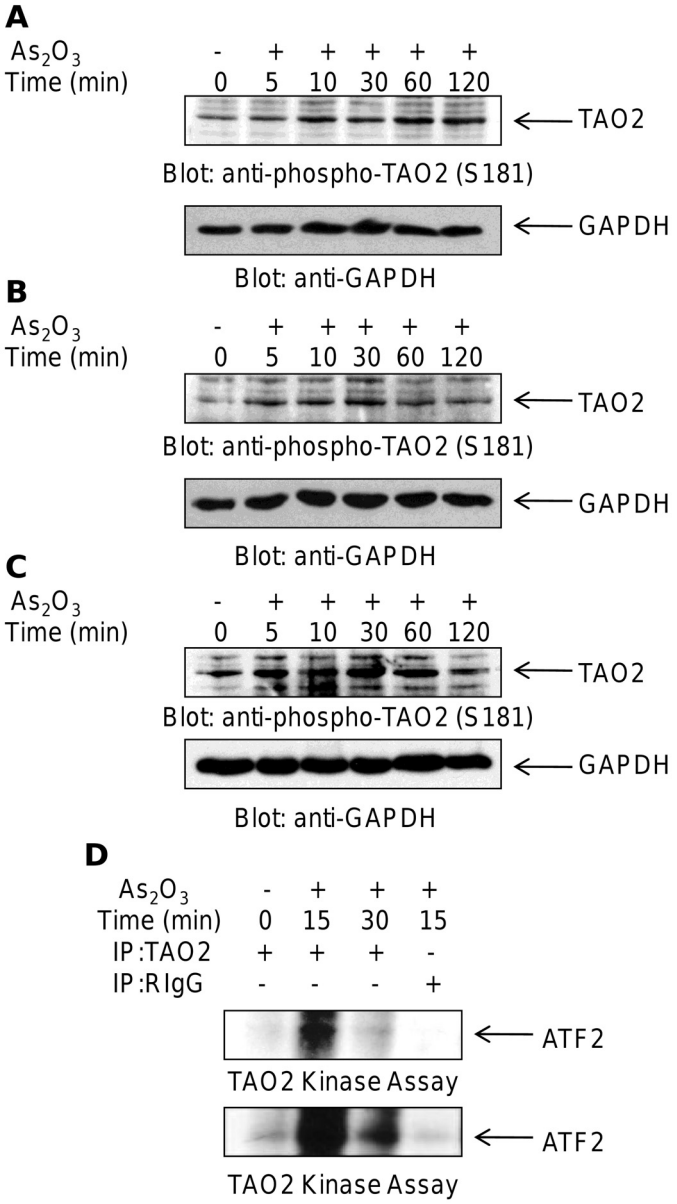

Fig. 1. As_2O_3-dependent phosphorylation of TAO2 in leukemic cell lines. A, U937 cells were incubated in the absence or presence of As_2O_3 (2 μM) for the indicated times. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-TAO2 (Ser181) antibody (top). The same blot was reprobed with an anti-GAPDH antibody to control for protein loading (bottom). B, as in A, but using NB4 cells. C, as in A, but using NB4.306 cells. D, U937 cells were incubated with As_2O_3 (2 μM) as indicated. Cell lysates were subjected to in vitro kinase assays using activating transcription factor 2 as an exogenous substrate. Proteins were resolved by SDS-PAGE, and phosphorylated proteins were detected by autoradiography (top). Longer exposure of the same membrane is also shown (bottom).

siRNA-Mediated Knockdown of TAO2 or TAK1 in Human Leukemic Cells. Cells were transfected with SMARTpool predesigned TAO2- or TAK1-specific siRNAs from Dharmacon RNA Technologies (Lafayette, CO), using Nucleofector kits from Amaxa Biosystems (Gaithersburg, MD) according to the manufacturer's instructions. Expression of mRNA was evaluated by real-time RT-PCR using TAO2- or TAK1-specific primers purchased from Applied Biosystems (Foster City, CA).

Human Hematopoietic Progenitor Cell Assays. Peripheral blood from patients with AML was collected after obtaining informed consent approved by the Institutional Review Board of Northwestern University (Chicago, IL). The effects of As_2O_3 on leukemic progenitor colony formation (CFU-L) were assessed by clonogenic assays in methylcellulose as described previously (Kannan-Thulasiraman et al., 2006; Altman et al., 2008). The suppressive effects of arsenic trioxide on leukemic progenitor colony formation from KT-1 leukemic cells were assessed by clonogenic assays in methylcellulose as in previous studies (Kroczynska et al., 2009).

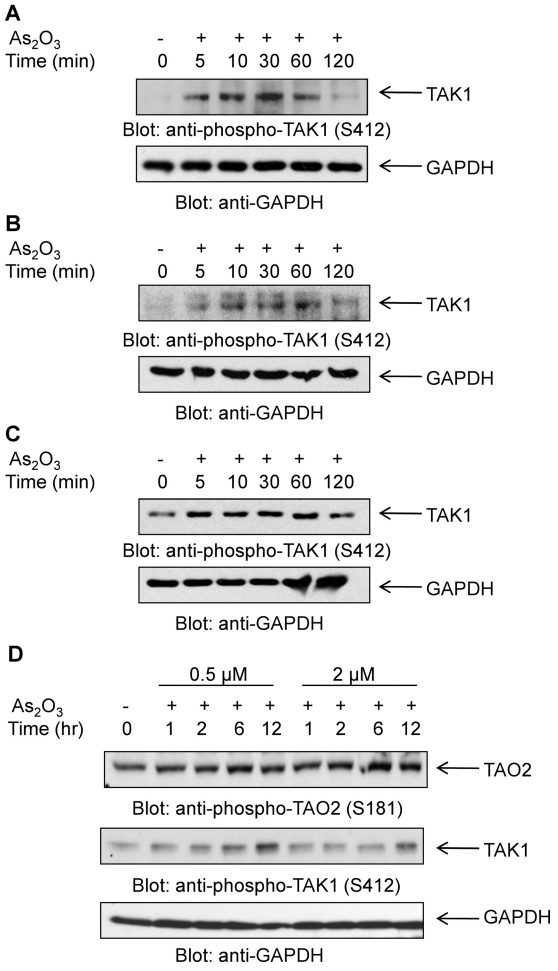

Fig. 2. As_2O_3-dependent phosphorylation of TAK1 in leukemic cell lines. A, NB4 cells were incubated in the absence or presence of As_2O_3 (2 μM) for the indicated times. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-TAK1 (Ser412) antibody (top). The same blot was reprobed with an anti-GAPDH antibody to control for protein loading (bottom). B, as in A, but using KT-1 cells. C, as in A, but using NB4.306 cells. D, KT-1 cells were incubated in the absence or presence of As_2O_3 at varying times and concentrations as indicated. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-TAO2 (Ser181) antibody (top) or an anti-phospho-TAK1 (Ser412) antibody (middle). The same blot was reprobed with an anti-GAPDH antibody to control for protein loading (bottom).

Results

We first determined whether As_2O_3 treatment of leukemic cells leads to phosphorylation of TAO2 or TAK1. Different acute leukemia cell lines were incubated in the absence or presence of As_2O_3 for various times, and cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of TAO2 on serine 181. As_2O_3 treatment resulted in phosphorylation of TAO2 in both U937 (Fig. 1A) and NB4 cells (Fig. 1B). Likewise, As_2O_3 treatment induced phosphorylation of TAO2 in the NB4.306 variant cell line (Fig. 1C) that is resistant to the differentiating and growth inhibitory effects of all-*trans*-retinoic acid (ATRA) (Dermime et al., 1993; Sassano et al., 2007). In addition, such phosphorylation led to activation of the TAO2 kinase domain, as evidenced in immune complex kinase assay experiments (Fig. 1D). In other parallel studies in which we examined the effects of As_2O_3 on the phosphorylation/activation of TAK1, a similar pattern of phosphorylation of TAK1 on serine 412 was seen in response to the treatment of various acute leukemia cell lines with As_2O_3 (Fig. 2, A–C). It is interesting that more baseline TAK1 phos-

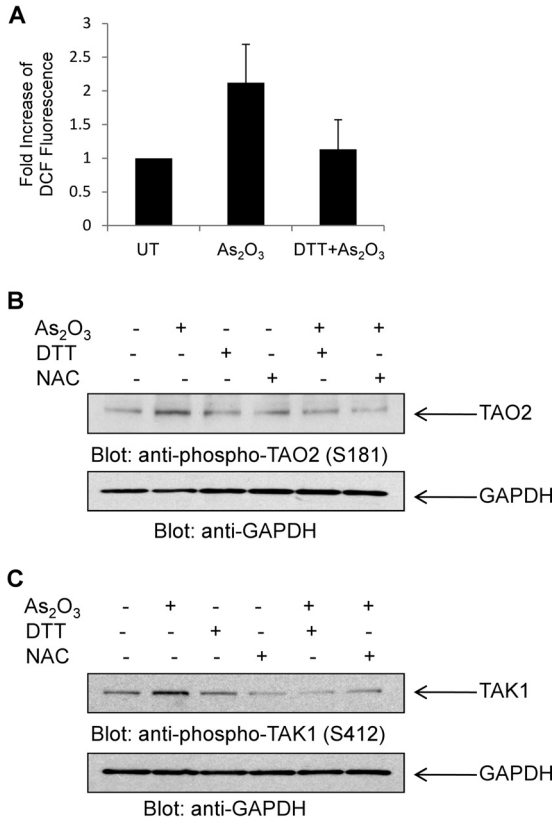

Fig. 3. As_2O_3-induced phosphorylation of TAO2 and TAK1 is diminished by the reducing agents DTT and NAC. A, NB4 cells were preincubated for 1 h with DTT (1 mM) and subsequently incubated with As_2O_3 (2 μM) for 30 min. Cells were then analyzed by flow cytometry for the presence of ROS as described under *Materials and Methods*. Data are expressed as the fold increase in mean fluorescence over untreated samples and represent the means ± S.E. of two independent experiments. B, NB4 cells were incubated with or without combinations of DTT (1 mM), NAC (10 mM), and As_2O_3 (2 μM) as indicated. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-TAO2 (Ser181) antibody (top). The same blot was reprobed with an anti-GAPDH antibody to control for protein loading (bottom). C, similar experiment as in B, demonstrating immunoblotting with an anti-phospho-TAK1 (Ser412) antibody (top).

phorylation was detected in NB4.306 cells compared with NB4 cells (Fig. 2, A and C). Phosphorylation of both kinases was rapid, occurring within 5 min of treatment of cells, with signal intensity peaking at 30 to 60 min (Figs. 1, A–C, and 2, A–C). In time- and dose-response experiments, we found that low concentrations of As_2O_3 also resulted in phosphorylation/activation of these kinases, and such activity was detectable after prolonged treatment of the cells (Fig. 2D). Thus, treatment of acute leukemia cells with As_2O_3 results in phosphorylation/activation of the kinases TAO2 and TAK1, suggesting their involvement in the generation of the antileukemic properties of arsenic trioxide.

There is extensive previous evidence in the literature implicating increases in cellular H_2O_2 stores and production of ROS in the generation of various arsenic responses (Dai et al., 1999; Jing et al., 1999; Miller et al., 2002; Platanias, 2009). As expected, treatment of cells with As_2O_3 also resulted in the generation of ROS in our system (Fig. 3A). Such ROS induction seems to be necessary for As_2O_3-dependent phosphorylation/activation of TAO2 or TAK1, because pretreatment of cells with the reducing agents DTT or NAC resulted in the inhibition of arsenic-inducible phosphorylation of both TAO2 (Fig. 3B) and TAK1 (Fig. 3C). Thus, similar to what was observed previously in the case of the p38 MAPK pathway (Verma et al., 2002), phosphorylation of TAO2 and TAK1 occurs downstream of As_2O_3-induced redox reactions.

Previous studies have demonstrated that TAO2 and TAK1 are upstream effectors in the p38 MAPK pathway in response to stress stimuli (Chen and Cobb, 2001; Huangfu et al., 2006). Because the p38 MAPK pathway is activated in an arsenic-dependent manner in leukemia cell lines (Verma et al., 2002; Giafis et al., 2006; Kannan-Thulasiraman et al., 2006; Dolniak et al., 2008) and plays a key role in the control of generation of antileukemic responses, we examined whether the inhibition of expression of TAO2 or TAK1 results in defective activation of p38 in response to treatment of acute leukemia cell lines with As_2O_3. TAO2- or TAK1-specific siRNAs were used to knock down the corresponding kinases (Fig. 4A), and the effects of such knockdown on p38 phosphorylation/activation were determined. U937 cells were nucleofected with either TAO2- or TAK1-specific siRNAs, and the phosphorylation of p38 in response to As_2O_3 was examined. Knockdown of TAO2 or TAK1 blocked As_2O_3-induced p38 phosphorylation (Fig. 4, B and C), establishing that these kinases act as upstream effectors of the As_2O_3-induced p38 MAPK pathway. We also performed experiments using the TAK1 inhibitor, 5*Z*-7-oxozeaenol. This compound is a resorcylic acid lactone of fungal origin, which has been shown to be a highly effective and specific inhibitor of TAK1 (Ninomiya-Tsuji et al., 2003; Choo et al., 2006). Pretreatment of cells with 5*Z*-7-oxozeaenol inhibited the phosphorylation of p38 (Fig. 4D), establishing that TAK1 is necessary for As_2O_3-induced p38 phosphorylation.

To assess the functional relevance of TAO2 and TAK1 in the

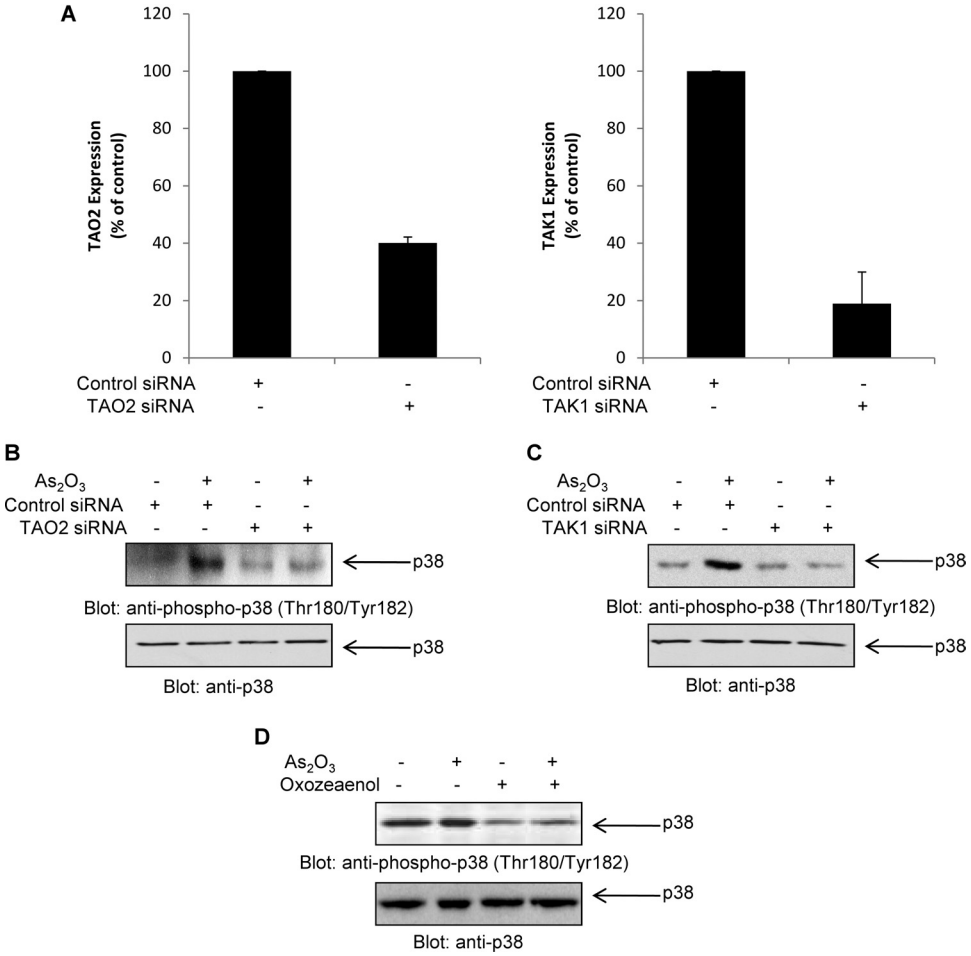

Fig. 4. Knockdown of TAO2 or TAK1 and pharmacological inhibition of TAK1 block As_2O_3-induced phosphorylation of p38 MAPK. A, left, U937 cells were transfected with control siRNA or TAO2-specific siRNA. Expression of mRNA for *TAO2* gene was evaluated by quantitative real-time RT-PCR using *GAPDH* gene for normalization. Data represent means ± S.E. of two experiments. Right, as on the left, but using TAK1-specific siRNA. B, U937 cells were transfected with control siRNA or TAO2-specific siRNA, and cells were incubated in the absence or presence of As_2O_3 (2 μM) for 30 min. Total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-p38 (Thr180/Tyr182) antibody (top). Equal amounts of cell lysates from the same experiment were resolved separately by SDS-PAGE and immunoblotted with an anti-p38 antibody (bottom). C, similar experiment as in B, but using TAK1 siRNA instead of TAO2 siRNA. D, KT-1 cells were pretreated for 60 min with 5*Z*-7-oxozeaenol (500 nM) and were subsequently incubated with As_2O_3 (2 μM) for 30 min in the continuous absence or presence of 5*Z*-7-oxozeaenol as indicated. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-p38 (Thr180/Tyr182) antibody (top). The same blot was reprobed with an anti-p38 antibody to control for protein loading (bottom).

generation of As_2O_3 antileukemic properties, we determined whether siRNA-mediated knockdown of TAO2 or TAK1 or pharmacological inhibition of TAK1 enhances the suppressive effects of As_2O_3 on leukemic progenitors. TAO2- or TAK1-specific siRNAs were used to knock down the corresponding kinases (Fig. 5A), and KT-1-derived CFU-L colony formation was examined in clonogenic assays in methylcellulose. As_2O_3-dependent suppression of CFU-L colony formation was clearly enhanced in cells transfected with either TAO2 or TAK1 siRNA compared with controls (Fig. 5, B and C). Concomitant treatment of KT-1 cells with the TAK1 inhibitor 5*Z*-7-oxozeaenol also led to enhanced growth-suppressive effects of As_2O_3 on CFU-L colony formation (Fig. 5D).

To further evaluate the role of TAO2 and TAK1, we explored the effects of siRNA-mediated knockdown or pharmacological inhibition of these kinases on the suppressive effects of As_2O_3 on primary leukemic progenitors from different patients with AML. Peripheral blood mononuclear cells from such patients were isolated, and CFU-L colony formation was assessed in clonogenic assays in methylcellulose. Similar to the results obtained in leukemic cell lines, As_2O_3 suppressed the growth of primary leukemic CFU-L progenitors, and such growth inhibition was further enhanced by knockdown of either TAO2 or TAK1 (Fig. 6, A and B) or by concomitant treatment of cells with 5*Z*-7-oxozeaenol (Fig. 6C), underscoring the importance of these kinases in the regulation of As_2O_3 responses.

Discussion

The ability of As_2O_3 to induce apoptosis and inhibit the growth of malignant cells both in vitro and in vivo has been well documented over the years (Chen et al., 2002; Miller et al., 2002; O'Dwyer et al., 2002; Douer and Tallman, 2005; Platanias, 2009). It is now known that a major mechanism by which As_2O_3 exhibits its effects on target neoplastic cells involves the generation of ROS, loss of mitochondrial membrane potential, and release of cytochrome *c*, resulting in programmed cell death (Wang et al., 1998; Jing et al., 1999; Park et al., 2000; Mahieux et al., 2001). Generation of intracellular ROS is de-

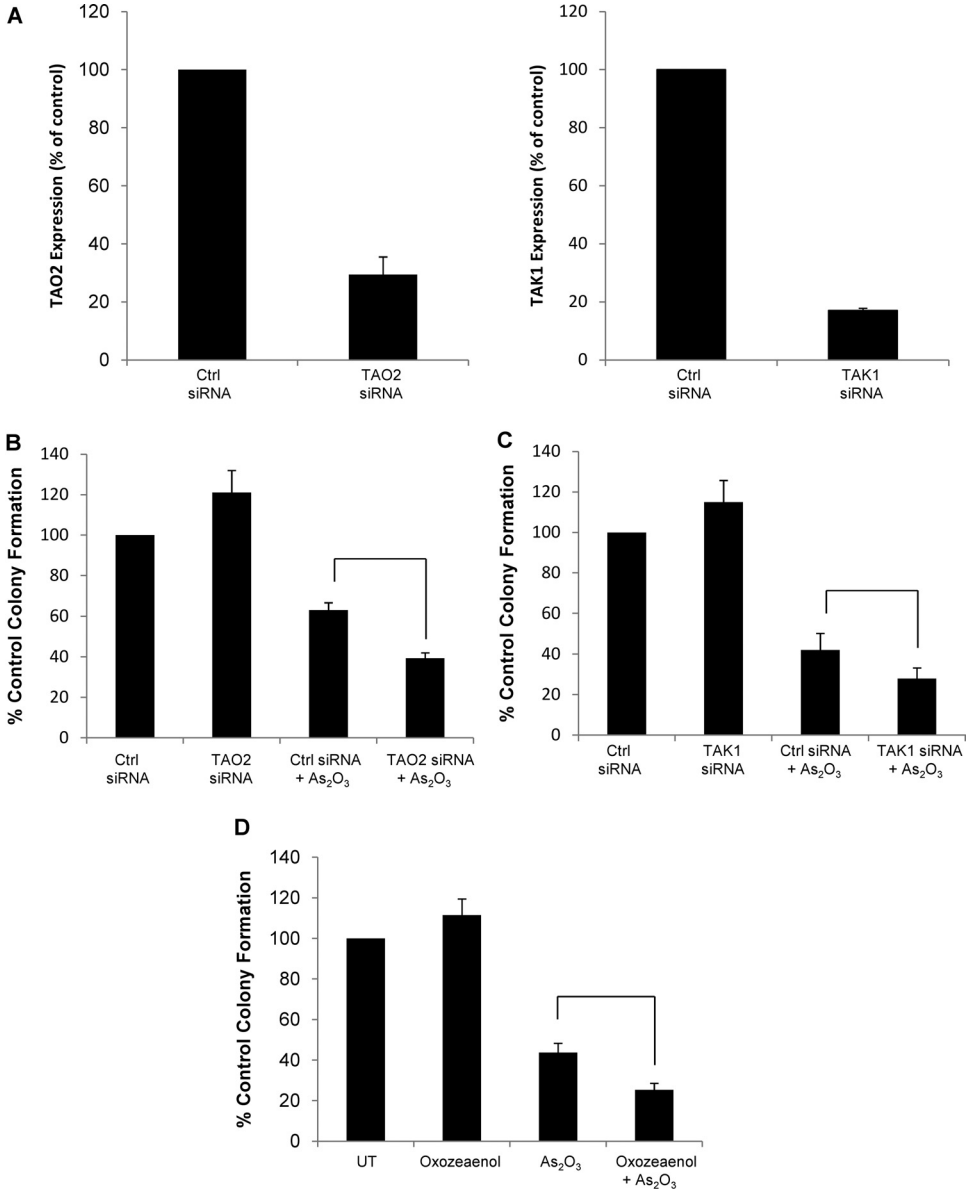

Fig. 5. siRNA-mediated knockdown of TAO2 and TAK1 and pharmacological inhibition of TAK1 enhance the suppressive effects of As_2O_3 on leukemic progenitor (CFU-L) growth. A, left, KT-1 cells were transfected with control siRNA or TAO2-specific siRNA. Expression of mRNA for *TAO2* gene was evaluated by quantitative real-time RT-PCR using *GAPDH* gene for normalization. Data are expressed as the percentage of control samples and represent means ± S.E. of three experiments. Right, as on the left, but using TAK1-specific siRNA. B, KT-1 cells transfected with control siRNA or TAO2-specific siRNA were subsequently incubated in methylcellulose in the absence or presence of As_2O_3 (0.5 μM), and leukemic CFU-L colony formation was assessed. Data are expressed as the percentage of control colony formation of samples treated with control siRNA and represent means ± S.E. of four independent experiments as shown. Paired *t* test analysis comparing the effects of As_2O_3 in the absence or presence of TAO2 siRNA showed a paired *p* value of 0.0006. C, as in B, but using TAK1-specific siRNA. Paired *t* test analysis comparing the effects of As_2O_3 in the absence or presence of TAK1 siRNA showed a paired *p* value of 0.0087. D, KT-1 cells were incubated in methylcellulose with As_2O_3 (0.5 μM), in the absence or presence of 5*Z*-7-oxozeaenol (100 nM) as indicated, and leukemic CFU-L colony formation was assessed. Data are expressed as the percentage of control colony formation of untreated samples and represent means ± S.E. of five independent experiments as shown. Paired *t* test analysis comparing the effects of As_2O_3 in the absence or presence of 5*Z*-7-oxozeaenol showed a paired *p* value of 0.005935. UT, untreated.

pendent on glutathione stores within cells, and lower levels of intracellular glutathione peroxidase and catalase have been demonstrated in malignant cell lines that are particularly sensitive to arsenic (Jing et al., 1999; Miller et al., 2002; Platanias, 2009). Consistent with this, there has been previous evidence that pretreatment of malignant cells with the reducing agent DTT prevents the loss of mitochondrial inner transmembrane potential and limits arsenic-induced apoptosis, whereas the effects of arsenic are augmented by pretreatment with buthionine sulfoximine, a glutathione synthesis inhibitor (Zhu et al., 1999; Miller et al., 2002). Other studies have shown that arsenic regulates cellular signaling pathways, with activation of the c-Jun NH_2-terminal kinase pathway (Davison et al., 2004; Mann et al., 2005) and inhibition of the nuclear factor-κB pathway (Mathas et al., 2003; Kerbauy et al., 2005; Wei et al., 2005) playing roles in As_2O_3-induced cell death.

In previous work, we have demonstrated that the p38 MAPK pathway is activated in a variety of leukemic cell lines after treatment with As_2O_3 (Verma et al., 2002; Giafis et al., 2006). Moreover, the upstream regulators Mkk3 and Mkk6 (Verma et al., 2002; Giafis et al., 2006) and the downstream regulators mitogen- and stress-activated kinase 1 (Kannan-Thulasiraman et al., 2006) and mitogen-activated protein kinase-interacting kinases 1 and 2 (Dolniak et al., 2008) were identified as arsenic-regulated kinases. Our previous work also suggested that the p38 MAPK pathway regulates arsenic responses in a negative-feedback regulatory manner, because we have found that the proapoptotic and antiproliferative effects of As_2O_3 are enhanced by pharmacological or siRNA-mediated inhibition of these kinases, or in corresponding knockout cells (Verma et al., 2002; Giafis et al., 2006; Kannan-Thulasiraman et al., 2006; Dolniak et al., 2008). These studies have raised the possibility that pharmacological targeting of p38 and/or its downstream effectors in leukemia cells could provide a novel approach to enhance the induction of antileukemic responses by As_2O_3. Identifying upstream effectors at the MAPK kinase kinase (MAPKKK) or MAPK kinase kinase kinase levels of the cascade is also of considerable interest, because the ability to enhance the antineoplastic effects of As_2O_3 could lead to broader uses of this agent at physiologically achievable concentrations.

TAO2 (Chen et al., 1999, 2003; Chen and Cobb, 2001; Dhillon et al., 2007) and TAK1 (Yamaguchi et al., 1995; Moriguchi et al., 1996; Hanafusa et al., 1999; Dhillon et al., 2007) have been identified as kinases that function as MAPKKKs. TAO2 has been shown to activate downstream Mkk3 and Mkk6 but not Mkk1, Mkk4, or Mkk7 (Chen et al., 1999; Chen and Cobb, 2001). Thus, this MAPK kinase kinase has specificity for the stress-activated p38 MAPK (Chen et al., 1999; Chen and Cobb, 2001). TAO2 is also known to be activated by a number of stress stimuli, including sorbitol, sodium chloride, ionizing radiation, ultraviolet radiation, and chemotherapy-induced stress by hydroxyurea (Chen and Cobb, 2001; Raman et al., 2007). TAK1 was initially identified as a mediator of TGF-β signal transduction (Yamaguchi et al., 1995) and was subsequently shown to activate both Mkk3 and Mkk6 (Moriguchi et al., 1996). Further studies demonstrated a signaling cascade linking TAK1 to p38 through Mkk6 in response to TGF-β stimulation (Hanafusa et al., 1999). Besides TGF-β, other cytokines such as tumor necrosis factor and interleukin-1 have been shown to induce TAK1 activation (Ninomiya-Tsuji et al., 1999; Takaesu et al., 2003; Shim et al., 2005; Inagaki et al., 2008). It has also been demonstrated that TAK1 is activated by chemical and physical stresses (Cheung et al., 2003; Huangfu et al., 2006) and plays a role in both c-Jun NH_2-terminal kinase (Huangfu et al., 2006; Frazier et al., 2007) and nuclear factor-κB signaling (Sakurai et al., 1998; Huangfu et al., 2006).

In this study, we provide the first evidence that TAO2 and TAK1 are activated by arsenic trioxide in leukemic cells in a rapid and transient manner. Our data demonstrate that both kinases are engaged downstream of As_2O_3-generated redox reactions and that the function of both of them is required for engagement of p38. This finding is of substantial interest be-

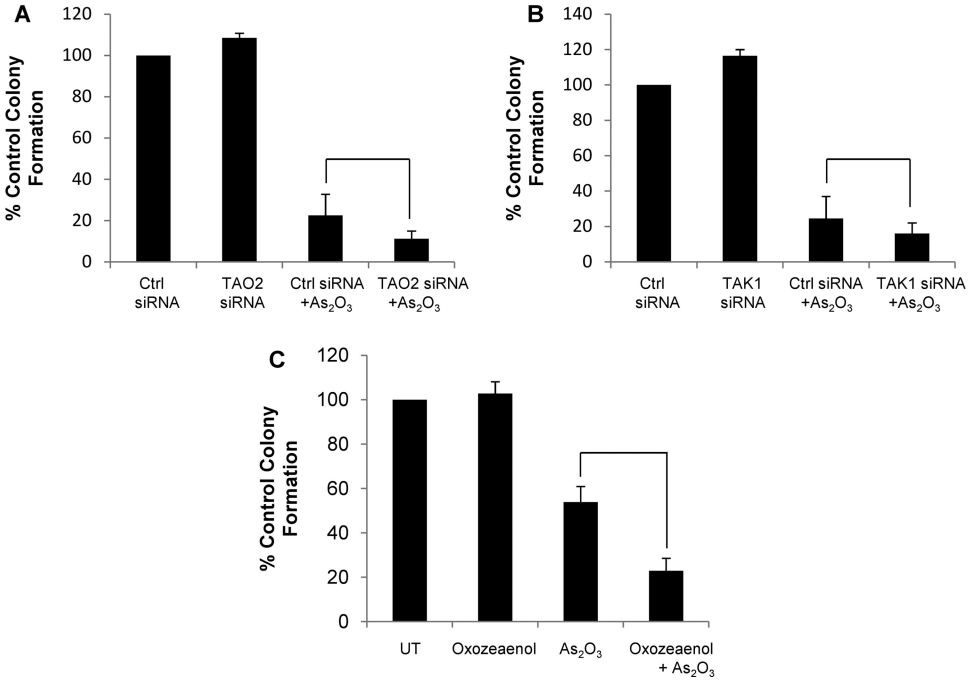

Fig. 6. siRNA-Mediated knockdown of TAO2 and TAK1 and pharmacological inhibition of TAK1 enhance As_2O_3-induced growth suppression of CFU-L colony formation from AML patients. A, peripheral blood mononuclear cells from two AML patients were transfected with control siRNA or TAO2 siRNA and were subsequently incubated in methylcellulose in the absence or presence of As_2O_3 (0.5 μM). CFU-L colony formation was assessed, and data are expressed as means ± S.E. of the percentage of colony formation of samples treated with control siRNA only. B, as in A, but using TAK1-specific siRNA. C, peripheral blood mononuclear cells from three AML patients were plated in a methylcellulose assay system with As_2O_3 (0.5 μM), in the absence or presence of 5*Z*-7-oxozeaenol (100 nM), as indicated. CFU-L colony formation was assessed and data are expressed as means ± S.E. of the percentage of colony formation of untreated samples. Paired *t* test analysis comparing the effects of As_2O_3 in the absence or presence of 5*Z*-7-oxozeaenol showed a paired *p* value of 0.01009. UT, untreated.

cause it suggests either sequential linear or parallel function of these kinases in the regulation of As_2O_3-dependent responses. These findings, taken together with a previous study from our group that demonstrated key roles for Mkk3 and Mkk6 in the generation of arsenic responses (Giafis et al., 2006), indicate that pairs of MAPKKKs (TAO2 and TAK1) and MAPKKs (Mkk3 and Mkk6) control arsenic-inducible p38 MAPK activation and generation of downstream effector signals. It is important to note that selective targeting of either TAO2 or TAK1 results in enhanced arsenic-dependent antileukemic responses. Such effects were seen using primary progenitors from patients with AML, indicating pharmacologically important and relevant roles for these kinases in the control of arsenic-induced antileukemic responses.

Altogether, our findings raise the potential of TAO2 and TAK1 as therapeutic targets for the treatment of leukemias. Although inhibiting the expression of TAO2 or TAK1 alone does not result in antileukemic effects, such inhibition results in potent enhancement of the antileukemic properties of arsenic trioxide. This suggests that pharmacological or molecular means to selectively target the kinase activities and/or expression levels of these kinases may provide a novel approach to promote the antileukemic effects of arsenic. In fact, because these kinases function at an early level of the p38 MAPK cascade, their targeting may provide a more complete blockade of the pathway and more effectively promote antileukemic responses than agents targeting downstream effectors, and clinical-translational efforts to target these kinases in vivo are warranted.

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