Arsenic Trioxide-Dependent Activation of TAO2 and TAK1

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Text Pages: 13
Figures: 6
References: 45
Words in Abstract: 192
Words in Introduction: 500
Words in Discussion: 1002

Abbreviations: As$_2$O$_3$, arsenic trioxide; TAO2, thousand-and-one amino acid kinase 2; TAK1, TGF-β-activated kinase 1; MAPK, mitogen-activated protein kinase; APL, acute promyelocytic leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; MSK1, mitogen- and stress-activated kinase 1; Mnk1/2, MAPK-interacting kinases 1 and 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ROS, reactive oxygen species; DCFDA, 2’7’-dichlorofluorescein diacetate; DTT, dithiothreitol; NAC, N-acetylcysteine; siRNA, small interfering RNA; CFU-L, leukemic colony forming unit; ATRA, all-trans retinoic acid
Abstract

Arsenic trioxide (As$_2$O$_3$) 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 generation of As$_2$O$_3$ responses should have important implications in the development of novel approaches using As$_2$O$_3$ for the treatment of leukemias. In this study, we used immunoblotting and immune complex kinase assays to provide evidence that the kinases TAO2 and TAK1 are rapidly activated in response to treatment of acute leukemia cells with As$_2$O$_3$. Such activation occurs after generation of reactive oxygen species and regulates downstream engagement of the p38 Map kinase. Our studies demonstrate that siRNA-mediated knockdown of TAO2 or TAK1 or pharmacological inhibition of TAK1 enhances the suppressive effects of As$_2$O$_3$ 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$_2$O$_3$. Thus, molecular or pharmacological targeting of these kinases may provide a novel approach to enhance the generation of arsenic-dependent antileukemic responses.
Introduction

Arsenic trioxide (As$_2$O$_3$) 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 (Miller et al., 2002; Chen 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$_2$O$_3$ has also been shown to inhibit the growth of various other types of malignant cells in vitro, including chronic myelogenous leukemia (CML), multiple myeloma, prostate carcinoma, and neuroblastoma cells (Miller et al., 2002; Chen et al., 2002; O’Dwyer et al., 2002; Douer and Tallman, 2005; Platanias, 2009). The effects of As$_2$O$_3$ are known to be dose-dependent, with low doses ($\leq 0.5\mu$M) inducing differentiation in APL cells, while higher doses ($\geq 2\mu$M) are required for apoptosis (Miller et al., 2002; Chen 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$_2$O$_3$ 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$_2$O$_3$ (Verma et al., 2002; Giafis et al., 2006). The engagement of the p38 pathway appears to occur in a negative-feedback regulatory manner, with enhanced pro-apoptotic and/or antiproliferative effects seen following 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 (MSK1) (Kannan-Thulasiraman et al., 2006) and the MAPK-interacting kinases 1 and 2 (Mnk1/2) (Dolniak et al.,
2008) have been identified and their involvement in the negative control of generation of arsenic responses established (Kannan-Thulasiraman et al., 2006; Dolniak et al., 2008). Others have also recently shown 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.

As 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$_2$O$_3$. Our data demonstrate that such phosphorylation occurs downstream of As$_2$O$_3$-induced reduction/oxidation reactions, and that phosphorylation of p38 by As$_2$O$_3$ is regulated by upstream engagement of these kinases. In addition, the suppressive effects of As$_2$O$_3$ on primitive leukemic progenitors are enhanced by knockdown of TAO2 and TAK1, suggesting that these kinases negatively regulate generation of As$_2$O$_3$-mediated antileukemic responses.
Materials and Methods

Cells and Reagents. The NB4 human acute promyelocytic leukemia, the U937 acute myelomonocytic leukemia, and the KT-1 CML-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) has been provided by Dr. Saverio Minucci (European Institute of Oncology) and was also grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. As$_2$O$_3$, dithiothreitol (DTT), and N-acetylcysteine (NAC) were purchased from Sigma (St. Louis, MO). An antibody against the phosphorylated form of TAO2 (Ser181) was purchased from Abcam (Cambridge, MA). Antibodies against p38 MAPK as well as the phosphorylated forms of both TAK1 (Ser412) and p38 MAPK (Thr180/Tyr182) were purchased from Cell Signaling (Danvers, MA). An antibody against TAO2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as well as the p38 substrate ATF2 were obtained from Millipore (Billerica, MA). The TAK1 inhibitor 5Z-7-Oxozeaenol was purchased from Calbiochem (La Jolla, CA).

Cell Lysis and Immunoblotting. Cells were incubated with the indicated doses of As$_2$O$_3$ for the indicated times and subsequently lysed in phosphorylation lysis buffer as previously described (Uddin et al., 1995). Immunoblotting using an enhanced chemiluminescence (ECL) method was done as previously described (Uddin et al., 1995).

Kinase Assays. Cells were incubated with As$_2$O$_3$ for the indicated times. Total cell lysates were immunoprecipitated with an antibody against TAO2 or non-immune rabbit IgG. In vitro kinase
assays were performed as previously described (Verma et al., 2002; Kannan-Thulasiraman et al., 2006).

**Generation of Reactive Oxygen Species (ROS).** Generation of ROS was measured by monitoring the oxidation of 2’7’-dichlorofluorescein diacetate (DCFDA; Molecular Probes, Carlsbad, CA) to 2’7’-dichlorofluorescein (DCF) as previously described (Evens et al., 2005). DCFDA is a nonfluorescent compound that permeates cells and interacts with intracellular oxidants to form the fluorescent compound DCF. Briefly, following treatment with As$_2$O$_3$ in the absence or presence of the reducing agent DTT, cells were incubated in 5µM DCFDA for 30 minutes at 37°C. Cells were then analyzed for fluorescent intensity by flow cytometry.

**siRNA-mediated Knockdown of TAO2 or TAK1 in Human Leukemic Cells.** Cells were transfected with SMARTpool pre-designed TAO2- or TAK1-specific siRNAs from Dharmacon (Lafayette, CO), using Amaxa Biosystems Nucleofector Kits, as recommended by the manufacturer (Walkersville, MD). 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. The effects of As$_2$O$_3$ on leukemic progenitor colony formation (CFU-L) were assessed by clonogenic assays in methylcellulose as previously described (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 (Kroczyńska et al., 2009).
Results

We first determined whether As$_2$O$_3$ 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$_2$O$_3$ 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$_2$O$_3$ treatment resulted in phosphorylation of TAO2 in both U937 (Fig. 1A) and NB4 cells (Fig. 1B). Similarly, As$_2$O$_3$ 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$_2$O$_3$ on the phosphorylation/activation of TAK1, a similar pattern of phosphorylation of TAK1 on serine 412 was seen in response to treatment of various acute leukemia cell lines with As$_2$O$_3$ (Fig. 2A-C). Interestingly, more baseline TAK1 phosphorylation was detected in NB4.306 cells as compared to NB4 cells (Fig. 2A, C). Phosphorylation of both kinases was rapid, occurring within 5 minutes of treatment of cells, with signal intensity peaking at 30-60 minutes (Fig. 1A-C and Fig. 2A-C). In time- and dose-response experiments, we found that low concentrations of As$_2$O$_3$ 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$_2$O$_3$ 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$_2$O$_2$ 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$_2$O$_3$ also resulted in generation of ROS in our system (Fig. 3A). Such ROS induction appears to be necessary for As$_2$O$_3$-dependent phosphorylation/activation of TAO2 or TAK1, as pre-treatment of cells with the reducing agents DTT or NAC resulted in inhibition of arsenic-inducible phosphorylation of both TAO2 (Fig. 3B) and TAK1 (Fig. 3C). Thus, similar to what was previously observed in the case of the p38 MAPK pathway (Verma et al., 2002), phosphorylation of TAO2 and TAK1 occurs downstream of As$_2$O$_3$-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). As 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 inhibition of expression of TAO2 or TAK1 results in defective activation of p38 in response to treatment of acute leukemia cell lines with As$_2$O$_3$. 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$_2$O$_3$ was examined. Knockdown of TAO2 or TAK1 blocked As$_2$O$_3$-induced p38 phosphorylation (Fig. 4B and C), establishing that these kinases act as upstream effectors of the As$_2$O$_3$-induced p38 MAPK pathway. We also performed experiments using the TAK1 inhibitor, 5Z-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;
Pre-treatment of cells with 5Z-7-Oxozeaenol inhibited phosphorylation of p38 (Fig. 4D), establishing that TAK1 is necessary for As$_2$O$_3$-induced p38 phosphorylation.

To assess the functional relevance of TAO2 and TAK1 in the generation of As$_2$O$_3$ antileukemic properties, we determined whether siRNA-mediated knockdown of TAO2 or TAK1 or pharmacological inhibition of TAK1 enhances the suppressive effects of As$_2$O$_3$ 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$_2$O$_3$-dependent suppression of CFU-L colony formation was clearly enhanced in cells transfected with either TAO2 or TAK1 siRNA compared to controls (Fig. 5B and C). Concomitant treatment of KT-1 cells with the TAK1 inhibitor 5Z-7-Oxozeaenol also led to enhanced growth-suppressive effects of As$_2$O$_3$ 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$_2$O$_3$ 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$_2$O$_3$ suppressed the growth of primary leukemic CFU-L progenitors, and such growth inhibition was further enhanced by knockdown of either TAO2 or TAK1 (Fig. 6A, B) or by concomitant treatment of cells with 5Z-7-Oxozeaenol (Fig. 6C), underscoring the importance of these kinases in the regulation of As$_2$O$_3$ responses.
Discussion

The ability of As$_2$O$_3$ to induce apoptosis and inhibit the growth of malignant cells both in vitro and in vivo has been well-documented over the years (Miller et al., 2002; Chen 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$_2$O$_3$ exhibits its effects on target neoplastic cells involves 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 dependent upon 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 pre-treatment of malignant cells with the reducing agent DTT prevents loss of mitochondrial inner transmembrane potential and limits arsenic-induced apoptosis, while the effects of arsenic are augmented by pre-treatment 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 JNK pathway (Davison et al., 2004; Mann et al., 2005) and inhibition of the NF-κB pathway (Mathas et al., 2003; Kerbauy et al., 2005; Wei et al., 2005) playing roles in As$_2$O$_3$-induced cell death.

In previous work we have demonstrated that the p38 MAPK pathway is activated in a variety of leukemic cell lines following treatment with As$_2$O$_3$ (Verma et al., 2002; Giafis et al., 2006). Moreover, the upstream regulators Mkk3 and Mkk6 (Verma et al., 2002; Giafis et al., 2006), as well as the downstream regulators MSK1 (Kannan-Thulasiraman et al., 2006) and Mnk1/2 (Dolniak et al., 2008) were identified as arsenic-regulated kinases. Our previous work
has also suggested that the p38 MAPK pathway regulates arsenic responses in a negative-feedback regulatory manner, as we have found that the pro-apoptotic and antiproliferative effects of As$_2$O$_3$ 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$_2$O$_3$. Identifying upstream effectors at the MAPK kinase kinase (MAPKKK) or MAPK kinase kinase kinase (MAPKKKK) levels of the cascade is also of considerable interest, as the ability to enhance the antineoplastic effects of As$_2$O$_3$ could lead to broader uses of this agent at physiologically achievable concentrations.

TAO2 (Chen et al., 1999; Chen and Cobb, 2001; Chen et al., 2003; Dhillon et al., 2007) and TAK1 (Yamaguchi et al., 1995; Moriguchi et al., 1996; Hanafusa et al., 1999; Dhillon et al., 2007) have both 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 MAPKKK 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, as well as chemotherapy-induced stress by hydroxyurea (Chen and Cobb, 2001; Raman et al., 2007). TAK1 was initially identified as a mediator of TGF-$\beta$ 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-$\beta$ stimulation (Hanafusa et al., 1999). Besides TGF-$\beta$, other cytokines such as
tumor necrosis factor (TNF) and interleukin-1 (IL-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 JNK (Huangfu et al., 2006; Frazier et al., 2007) and NF-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$_2$O$_3$-generated redox reactions and that the function of both of them is required for engagement of p38. This finding is of substantial interest as it suggests either sequential linear or parallel function of these kinases in the regulation of As$_2$O$_3$-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. Importantly, 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, as 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.
References


Footnotes

This work was supported by the National Institutes of Health [Grants CA121192, CA100579, T32 CA079447 and T32 CA070085]; and a grant from the Department of Veterans Affairs.

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**Figure Legends**

**Figure 1.** As$_2$O$_3$-dependent phosphorylation of TAO2 in leukemic cell lines. A, U937 cells were incubated in the absence or presence of As$_2$O$_3$ (2µM) for the indicated times. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-TAO2 (S181) antibody (*upper panel*). The same blot was re-probed with an anti-GAPDH antibody to control for protein loading (*lower panel*). B, As in A, but using NB4 cells. C, As in A, but using NB4.306 cells. D, U937 cells were incubated with As$_2$O$_3$ (2µM) as indicated. Cell lysates were subjected to *in vitro* kinase assays using ATF2 as an exogenous substrate. Proteins were resolved by SDS-PAGE, and phosphorylated proteins were detected by autoradiography (*upper panel*). Longer exposure of the same membrane is also shown (*lower panel*).

**Figure 2.** As$_2$O$_3$-dependent phosphorylation of TAK1 in leukemic cell lines. A, NB4 cells were incubated in the absence or presence of As$_2$O$_3$ (2µM) for the indicated times. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-TAK1 (S412) antibody (*upper panel*). The same blot was re-probed with an anti-GAPDH antibody to control for protein loading (*lower panel*). 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$_2$O$_3$ 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 (S181) antibody (*upper panel*) or an anti-phospho-TAK1 (S412) antibody (*middle panel*). The same blot was re-probed with an anti-GAPDH antibody to control for protein loading (*lower panel*).
Figure 3. As$_2$O$_3$-induced phosphorylation of TAO2 and TAK1 is diminished by the reducing agents DTT and NAC. A, NB4 cells were pre-incubated for one hour with DTT (1mM) and subsequently incubated with As$_2$O$_3$ (2µM) for 30 minutes. Cells were then analyzed by flow cytometry for the presence of ROS as described in materials and methods. Data are expressed as 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 (1mM), NAC (10mM), and As$_2$O$_3$ (2µM) as indicated. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-TAO2 (S181) antibody (upper panel). The same blot was re-probed with an anti-GAPDH antibody to control for protein loading (lower panel). C, Similar experiment as in B, with the upper panel demonstrating immunoblotting with an anti-phospho-TAK1 (S412) antibody.

Figure 4. Knockdown of TAO2 or TAK1 and pharmacological inhibition of TAK1 block As$_2$O$_3$-induced phosphorylation of p38 MAPK. A, (left panel) 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 panel) As in the left panel, 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$_2$O$_3$ (2µM) for 30 min. Total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-p38 (Thr180/Tyr182) antibody (upper panel). Equal amounts of cell lysates from the same experiment were resolved separately by SDS-PAGE and immunoblotted with an anti-p38 antibody (lower panel). C, Similar experiment as in B, but using TAK1 siRNA
instead of TAO2 siRNA. D, KT-1 cells were pre-treated for 60 min with 5Z-7-Oxozeaenol (500nM) and were subsequently incubated with As$_2$O$_3$ (2µM) for 30 min in the continuous absence or presence of 5Z-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 (upper panel). The same blot was re-probed with an anti-p38 antibody to control for protein loading (lower panel).

**Figure 5.** siRNA-mediated knockdown of TAO2 and TAK1 and pharmacological inhibition of TAK1 enhance the suppressive effects of As$_2$O$_3$ on leukemic progenitor (CFU-L) growth. A, (left panel) 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 % of control samples and represent means ± S.E. of three experiments; (right panel) As in the left panel, 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$_2$O$_3$ (0.5µM), and leukemic CFU-L colony formation was assessed. Data are expressed as percent of control colony formation of samples treated with control siRNA and represent means ± S.E. of 4 independent experiments as shown. Paired $t$ test analysis comparing the effects of As$_2$O$_3$ in the absence or presence of TAO2 siRNA showed a paired $p$ value = 0.0006. C, As in B, but using TAK1-specific siRNA. Paired $t$ test analysis comparing the effects of As$_2$O$_3$ in the absence or presence of TAK1 siRNA showed a paired $p$ value = 0.0087. D, KT-1 cells were incubated in methylcellulose with As$_2$O$_3$ (0.5µM), in the absence or presence of 5Z-7-Oxozeaenol (100nM) as indicated, and leukemic CFU-L colony formation was assessed.
formation was assessed. Data are expressed as percent of control colony formation of untreated samples and represent means ± S.E. of 5 independent experiments as shown. Paired t test analysis comparing the effects of As₂O₃ in the absence or presence of 5Z-7-Oxozeaenol showed a paired p value = 0.005935.

**Figure 6.** siRNA-mediated knockdown of TAO2 and TAK1 and pharmacological inhibition of TAK1 enhance As₂O₃-induced growth suppression of CFU-L colony formation from AML patients. A, Peripheral blood mononuclear cells from 2 AML patients were transfected with control siRNA or TAO2 siRNA and were subsequently incubated in methylcellulose in the absence or presence of As₂O₃ (0.5µM). CFU-L colony formation was assessed and data are expressed as means ± S.E. of the percent colony formation of samples treated with control siRNA only. B, As in A, but using TAK1-specific siRNA. C, Peripheral blood mononuclear cells from 3 AML patients were plated in a methylcellulose assay system with As₂O₃ (0.5µM), in the absence or presence of 5Z-7-Oxozeaenol (100nM), as indicated. CFU-L colony formation was assessed and data are expressed as means ± S.E. of the percent colony formation of untreated samples. Paired t test analysis comparing the effects of As₂O₃ in the absence or presence of 5Z-7-Oxozeaenol showed a paired p value = 0.01009.
Figure 1

A

\[ \text{As}_2\text{O}_3 \]
\[ \begin{array}{cccccc}
\text{Time (min)} & 0 & 5 & 10 & 30 & 60 & 120 \\
\end{array} \]

\[ \text{Blot: anti-phospho-TAO2 (S181)} \]
\[ \text{GAPDH} \]

B

\[ \text{As}_2\text{O}_3 \]
\[ \begin{array}{cccccc}
\text{Time (min)} & 0 & 5 & 10 & 30 & 60 & 120 \\
\end{array} \]

\[ \text{Blot: anti-phospho-TAO2 (S181)} \]
\[ \text{GAPDH} \]

C

\[ \text{As}_2\text{O}_3 \]
\[ \begin{array}{cccccc}
\text{Time (min)} & 0 & 5 & 10 & 30 & 60 & 120 \\
\end{array} \]

\[ \text{Blot: anti-phospho-TAO2 (S181)} \]
\[ \text{GAPDH} \]

D

\[ \text{As}_2\text{O}_3 \]
\[ \begin{array}{cccccc}
\text{Time (min)} & 0 & 15 & 30 & 15 \\
\end{array} \]

\[ \text{IP: TAO2} \]
\[ \text{IP: RlgG} \]

\[ \text{Blot: anti-GAPDH} \]
\[ \text{ATF2} \]

TAO2 Kinase Assay

ATF2

TAO2 Kinase Assay
Figure 2

A

\[
\begin{array}{ccccccc}
\text{As}_2\text{O}_3 & - & + & + & + & + \\
\text{Time (min)} & 0 & 5 & 10 & 30 & 60 & 120 \\
\end{array}
\]

Blot: anti-phospho-TAK1 (S412)

Blot: anti-GAPDH

B

\[
\begin{array}{ccccccc}
\text{As}_2\text{O}_3 & - & + & + & + & + \\
\text{Time (min)} & 0 & 5 & 10 & 30 & 60 & 120 \\
\end{array}
\]

Blot: anti-phospho-TAK1 (S412)

Blot: anti-GAPDH

C

\[
\begin{array}{ccccccc}
\text{As}_2\text{O}_3 & - & + & + & + & + \\
\text{Time (min)} & 0 & 5 & 10 & 30 & 60 & 120 \\
\end{array}
\]

Blot: anti-phospho-TAK1 (S412)

Blot: anti-GAPDH

D

\[
\begin{array}{cccccc}
\text{As}_2\text{O}_3 & 0.5 \mu\text{M} & & & 2 \mu\text{M} & \\
\text{Time (hr)} & 0 & 1 & 2 & 6 & 12 \\
\end{array}
\]

Blot: anti-phospho-DAO2 (S181)

Blot: anti-phospho-TAK1 (S412)

Blot: anti-GAPDH

Blot: anti-GAPDH
Figure 3

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>UT</th>
<th>As$_2$O$_3$</th>
<th>DTT + As$_2$O$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold Increase of DCF Fluorescence</td>
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</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Treatment</th>
<th>As$_2$O$_3$</th>
<th>DTT</th>
<th>NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>-</td>
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<tr>
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<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Blot: anti-phospho-TAO2 (S181)</td>
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<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>As$_2$O$_3$</th>
<th>DTT</th>
<th>NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>+</td>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Blot: anti-GAPDH</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>As$_2$O$_3$</th>
<th>DTT</th>
<th>NAC</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Blot: anti-phospho-TAK1 (S412)</td>
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</tbody>
</table>

<table>
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<tr>
<th>Treatment</th>
<th>As$_2$O$_3$</th>
<th>DTT</th>
<th>NAC</th>
</tr>
</thead>
<tbody>
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<tr>
<td>Blot: anti-GAPDH</td>
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Figure 4

A

<table>
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<tr>
<th>TAO2 Expression ( % of control )</th>
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</thead>
<tbody>
<tr>
<td>Control siRNA</td>
</tr>
<tr>
<td>TAO2 siRNA</td>
</tr>
</tbody>
</table>

B

| As$_2$O$_3$ | - | + | - | + |
| Control siRNA | + | + | - | - |
| TAO2 siRNA | - | - | + | + |

Blot: anti-phospho-p38 (Thr180/Tyr182) ← p38
Blot: anti-p38 ← p38

C

| As$_2$O$_3$ | - | + | - | + |
| Control siRNA | + | + | - | - |
| TAK1 siRNA | - | - | + | + |

Blot: anti-phospho-p38 (Thr180/Tyr182) ← p38
Blot: anti-p38 ← p38

D

| As$_2$O$_3$ | - | + | - | + |
| Oxozeaenol | - | - | + | + |

Blot: anti-phospho-p38 (Thr180/Tyr182) ← p38
Blot: anti-p38 ← p38
Figure 6

A

B

C

% Control Colony Formation

Ctrl siRNA  TAO2 siRNA  Ctrl siRNA +As$_2$O$_3$  TAO2 siRNA +As$_2$O$_3$

% Control Colony Formation

Ctrl siRNA  TAK1 siRNA  Ctrl siRNA +As$_2$O$_3$  TAK1 siRNA +As$_2$O$_3$

% Control Colony Formation

UT  Oxozaenol  As$_2$O$_3$  Oxozaenol + As$_2$O$_3$