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Arsenic Trioxide-Dependent Activation of TAO2 and TAK1

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Running Title Page

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Abbreviations: As_2O_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

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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 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 TAO2 and TAK1 are rapidly activated in response to treatment of acute leukemia cells with As_2O_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_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.

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Introduction

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 (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_2O_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_2O_3 are known to be dose-dependent, with low doses ($\leq$0.5μM) inducing differentiation in APL cells, while higher doses ($\geq$2μM) 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_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 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.,

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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_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 generation of As_2O_3-mediated antileukemic responses.

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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_2O_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_2O_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_2O_3 for the indicated times. Total cell lysates were immunoprecipitated with an antibody against TAO2 or non-immune rabbit IgG. *In vitro* kinase

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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_2O_3 in the absence or presence of the reducing agent DTT, cells were incubated in 5μM DCFDA for 30 minutes at 37^{o}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_2O_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

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leukemic progenitor colony formation from KT-1 leukemic cells were assessed by clonogenic assays in methylcellulose as in previous studies (Kroczynska et al., 2009).

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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). Similarly, 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 treatment of various acute leukemia cell lines with As_2O_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_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.,

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1999; Jing et al., 1999; Miller et al., 2002; Platanias, 2009). As expected, treatment of cells with As_2O_3 also resulted in generation of ROS in our system (Fig. 3A). Such ROS induction appears to be necessary for As_2O_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_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). 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_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. 4B 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, 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;

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Choo et al., 2006). Pre-treatment of cells with 5Z-7-Oxozeaenol inhibited 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 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 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_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. 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_2O_3 responses.

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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 (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_2O_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_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 following 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), 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

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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_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 (MAPKKKK) levels of the cascade is also of considerable interest, as 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; 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-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

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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_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 as 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. 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

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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.

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References

Altman JK, Yoon P, Katsoulidis E, Kroczynska B, Sassano A, Redig AJ, Glaser H, Jordan A, Tallman MS, Hay N, and Platanias LC (2008) Regulatory effects of mammalian target of rapamycin-mediated signals in the generation of arsenic trioxide responses. *J Biol Chem* **283:**1992-2001.

Chen Z, Chen GQ, Shen ZX, Sun GL, Tong JH, Wang ZY, and Chen SJ (2002) Expanding the use of arsenic trioxide: leukemias and beyond. *Semin Hematol* **39:**22-26.

Chen Z and Cobb MH (2001) Regulation of stress-responsive mitogen-activated protein (MAP) kinase pathways by TAO2. *J Biol Chem* **276:**16070-16075.

Chen Z, Hutchison M, and Cobb MH (1999) Isolation of the protein kinase TAO2 and identification of its mitogen-activated protein kinase/extracellular signal-regulated kinase kinase binding domain. *J Biol Chem* **274:**28803-28807.

Chen Z, Raman M, Chen L, Lee SF, Gilman AG, and Cobb MH (2003) TAO (Thousand-and-One Amino Acid) protein kinases mediate signaling from carbachol to p38 mitogen-activated protein kinase and ternary complex factors. *J Biol Chem* **278:**22278-22283.

Cheung PC, Campbell DG, Nebreda AR, and Cohen P (2003) Feedback control of the protein kinase TAK1 by SAPK2a/p38alpha. *EMBO J* **22:**5793-5805.

Choo MK, Kawasaki N, Singhirunnusorn P, Koizumi K, Sato S, Akira S, Saiki I, and Sakurai H (2006) Blockade of transforming growth factor-beta-activated kinase 1 activity enhances TRAIL-induced apoptosis through activation of a caspase cascade. *Mol Cancer Ther* **5:**2970-2976.

Dai J, Weinberg RS, Waxman S, and Jing Y (1999) Malignant cells can be sensitized to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system. *Blood* **93:**268-277.

Davison K, Mann KK, Waxman S, and Miller WH Jr (2004) JNK activation is a mediator of arsenic trioxide-induced apoptosis in acute promyelocytic leukemia cells. *Blood* **103:**3496-3502.

Dermime S, Grignani F, Clerici M, Nervi C, Sozzi G, Talamo GP, Marchesi E, Formelli F, Parmiani G, and Pelicci PG (1993) Occurrence of resistance to retinoic acid in the acute promyelocytic leukemia cell line NB4 is associated with altered expression of the pml/RAR alpha protein. *Blood* **82:**1573–1577.

Dhillon AS, Hagan S, Rath O, and Kolch W (2007) MAP kinase signalling pathways in cancer. *Oncogene* **26:**3279-3290.

Dolniak B, Katsoulidis E, Carayol N, Altman JK, Redig AJ, Tallman MS, Ueda T, Watanabe-Fukunaga R, Fukunaga R, and Platanias LC (2008) Regulation of arsenic trioxide-induced cellular responses by Mnk1 and Mnk2. *J Biol Chem* **283:**12034-12042.

Douer D and Tallman MS (2005) Arsenic trioxide: new clinical experience with an old medication in hematologic malignancies. *J Clin Oncol* **23**:2396-2410.

Evens AM, Lecane P, Magda D, Prachand S, Singhal S, Nelson J, Miller RA, Gartenhaus RB, and Gordon LI (2005) Motexafin gadolinium generates reactive oxygen species and induces apoptosis in sensitive and highly resistant multiple myeloma cells. *Blood* **105:**1265-1273.

Frazier DP, Wilson A, Dougherty CJ, Li H, Bishopric NH, and Webster KA (2007) PKC-alpha and TAK-1 are intermediates in the activation of c-Jun NH2-terminal kinase by hypoxia-reoxygenation. *Am J Physiol Heart Circ Physiol* **292:**H1675-H1684.

Giafis N, Katsoulidis E, Sassano A, Tallman MS, Higgins LS, Nebreda AR, Davis RJ, and Platanias LC (2006) Role of the p38 mitogen-activated protein kinase pathway in the generation of arsenic trioxide-dependent cellular responses. *Cancer Res* **66:**6763-6771.

Hanafusa H, Ninomiya-Tsuji J, Masuyama N, Nishita M, Fujisawa J, Shibuya H, Matsumoto K, and Nishida E (1999) Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression. *J Biol Chem* **274:**27161-27167.

Huangfu WC, Omori E, Akira S, Matsumoto K, and Ninomiya-Tsuji J (2006) Osmotic stress activates the TAK1-JNK pathway while blocking TAK1-mediated NF-kappaB activation: TAO2 regulates TAK1 pathways. *J Biol Chem* **281:**28802-28810.

Inagaki M, Omori E, Kim JY, Komatsu Y, Scott G, Ray MK, Yamada G, Matsumoto K, Mishina Y, and Ninomiya-Tsuji J (2008) TAK1-binding protein 1, TAB1, mediates osmotic stress-induced TAK1 activation but is dispensable for TAK1-mediated cytokine signaling. *J Biol Chem* **283:**33080-33086.

Jing Y, Dai J, Chalmers-Redman RM, Tatton WG, and Waxman S (1999) Arsenic trioxide selectively induces acute promyelocytic leukemia cell apoptosis via a hydrogen peroxide-dependent pathway. *Blood* **94:**2102-2111.

Kannan-Thulasiraman P, Katsoulidis E, Tallman MS, Arthur JS, and Platanias LC (2006) Activation of the mitogen- and stress-activated kinase 1 by arsenic trioxide. *J Biol Chem* **281:**22446-22452.

Kerbauy DM, Lesnikov V, Abbasi N, Seal S, Scott B, and Deeg HJ (2005) NF-kappaB and FLIP in arsenic trioxide (ATO)-induced apoptosis in myelodysplastic syndromes (MDSs). *Blood* **106:**3917-3925.

Kroczynska B, Kaur S, Katsoulidis E, Majchrzak-Kita B, Sassano A, Kozma SC, Fish EN, and Platanias LC (2009) Interferon-dependent engagement of eukaryotic initiation factor 4B via S6 kinase (S6K)- and ribosomal protein S6K-mediated signals. *Mol Cell Biol* **29:**2865-2875.

Mahieux R, Pise-Masison C, Gessain A, Brady JN, Olivier R, Perret E, Misteli T, and Nicot C (2001) Arsenic trioxide induces apoptosis in human T-cell leukemia virus type 1- and type 2-infected cells by a caspase-3-dependent mechanism involving Bcl-2 cleavage. *Blood* **98:**3762-3769.

Mann KK, Padovani AM, Guo Q, Colosimo AL, Lee HY, Kurie JM, and Miller WH Jr (2005) Arsenic trioxide inhibits nuclear receptor function via SEK1/JNK-mediated RXRalpha phosphorylation. *J Clin Invest* **115:**2924-2933.

Mathas S, Lietz A, Janz M, Hinz M, Jundt F, Scheidereit C, Bommert K, and Dorken B (2003) Inhibition of NF-kappaB essentially contributes to arsenic-induced apoptosis. *Blood* **102:**1028-1034.

Miller WH Jr, Schipper HM, Lee JS, Singer J, and Waxman S (2002) Mechanisms of action of arsenic trioxide. *Cancer Res* **62:**3893-3903.

Moriguchi T, Kuroyanagi N, Yamaguchi K, Gotoh Y, Irie K, Kano T, Shirakabe K, Muro Y, Shibuya H, Matsumoto K, Nishida E, and Hagiwara M (1996) A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J Biol Chem* **271:**13675-13679.

Ninomiya-Tsuji J, Kajino T, Ono K, Ohtomo T, Matsumoto M, Shiina M, Mihara M, Tsuchiya M, and Matsumoto K (2003) A resorcylic acid lactone, 5Z-7-oxozeaenol, prevents inflammation by inhibiting the catalytic activity of TAK1 MAPK kinase kinase. *J Biol Chem* **278:**18485-18490.

Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue J, Cao Z, and Matsumoto K (1999) The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature* **398:**252-256.

O'Dwyer ME, La Rosee P, Nimmanapalli R, Bhalla KN, and Druker BJ (2002) Recent advances in Philadelphia chromosome-positive malignancies: the potential role of arsenic trioxide. *Semin Hematol* **39**:18-21.

Park WH, Seol JG, Kim ES, Hyun JM, Jung CW, Lee CC, Kim BK, and Lee YY (2000) Arsenic trioxide-mediated growth inhibition in MC/CAR myeloma cells via cell cycle arrest in association with induction of cyclin-dependent kinase inhibitor, p21, and apoptosis. *Cancer Res* **60:**3065-3071.

Platanias LC (2009) Biological responses to arsenic compounds. *J Biol Chem* **284:**18583-18587.

Raman M, Earnest S, Zhang K, Zhao Y, and Cobb MH (2007) TAO kinases mediate activation of p38 in response to DNA damage. *EMBO J* **26:**2005-2014.

Sakurai H, Shigemori N, Hasegawa K, and Sugita T (1998) TGF-beta-activated kinase 1 stimulates NF-kappa B activation by an NF-kappa B-inducing kinase-independent mechanism. *Biochem Biophys Res Commun* **243:**545-549.

Sassano A, Katsoulidis E, Antico G, Altman JK, Redig AJ, Minucci S, Tallman MS, and Platanias LC (2007) Suppressive effects of statins on acute promyelocytic leukemia cells. *Cancer Res* **67:**4524–4532.

Shim JH, Xiao C, Paschal AE, Bailey ST, Rao P, Hayden MS, Lee KY, Bussey C, Steckel M, Tanaka N, Yamada G, Akira S, Matsumoto K, and Ghosh S (2005) TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. *Genes Dev* **19:**2668-2681.

Takaesu G, Surabhi RM, Park KJ, Ninomiya-Tsuji J, Matsumoto K, and Gaynor RB (2003) TAK1 is critical for IkappaB kinase-mediated activation of the NF-kappaB pathway. *J Mol Biol* **326:**105-115.

Uddin S, Yenush L, Sun XJ, Sweet ME, White MF, and Platanias LC (1995) Interferon-alpha engages the insulin receptor substrate-1 to associate with the phosphatidylinositol 3'-kinase. *J Biol Chem* **270:**15938-15941.

Verma A, Mohindru M, Deb DK, Sassano A, Kambhampati S, Ravandi F, Minucci S, Kalvakolanu DV, and Platanias LC (2002) Activation of Rac1 and the p38 mitogen-activated protein kinase pathway in response to arsenic trioxide. *J Biol Chem* **277:**44988-44995.

Wang ZG, Rivi R, Delva L, Konig A, Scheinberg DA, Gambacorti-Passerini C, Gabrilove JL, Warrell RP Jr, and Pandolfi PP (1998) Arsenic trioxide and melarsoprol induce programmed cell death in myeloid leukemia cell lines and function in a PML and PML-RARalpha independent manner. *Blood* **92:**1497-1504.

Wei LH, Lai KP, Chen CA, Cheng CH, Huang YJ, Chou CH, Kuo ML, and Hsieh CY (2005) Arsenic trioxide prevents radiation-enhanced tumor invasiveness and inhibits matrix

metalloproteinase-9 through downregulation of nuclear factor kappaB. *Oncogene* **24:**390-398.

Wen J, Cheng HY, Feng Y, Rice L, Liu S, Mo A, Huang J, Zu Y, Ballon DJ, and Chang CC (2008) P38 MAPK inhibition enhancing ATO-induced cytotoxicity against multiple myeloma cells. *Br J Haematol* **140:**169-180.

Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, and Matsumoto K (1995) Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. *Science* **270:**2008-2011.

Zhu XH, Shen YL, Jing YK, Cai X, Jia PM, Huang Y, Tang W, Shi GY, Sun YP, Dai J, Wang ZY, Chen SJ, Zhang TD, Waxman S, Chen Z, and Chen GQ (1999) Apoptosis and growth inhibition in malignant lymphocytes after treatment with arsenic trioxide at clinically achievable concentrations. *J Natl Cancer Inst* **91:**772-778.

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Footnotes

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Figure Legends

Figure 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 (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_2O_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_2O_3-dependent phosphorylation of TAK1 in leukemic cell lines. A, NB4 cells were incubated in the absence or presence of As_2O_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_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 (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).*

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Figure 3. As_2O_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_2O_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_2O_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_2O_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_2O_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

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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_2O_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_2O_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_2O_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_2O_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_2O_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_2O_3 (0.5μM), in the absence or presence of 5Z-7-Oxozeaenol (100nM) as indicated, and leukemic CFU-L colony

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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 As_2O_3 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 As_2O_3-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 As_2O_3 (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 As_2O_3 (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 As_2O_3 in the absence or presence of 5Z-7-Oxozeaenol showed a paired *p* value = 0.01009.

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Figure 1

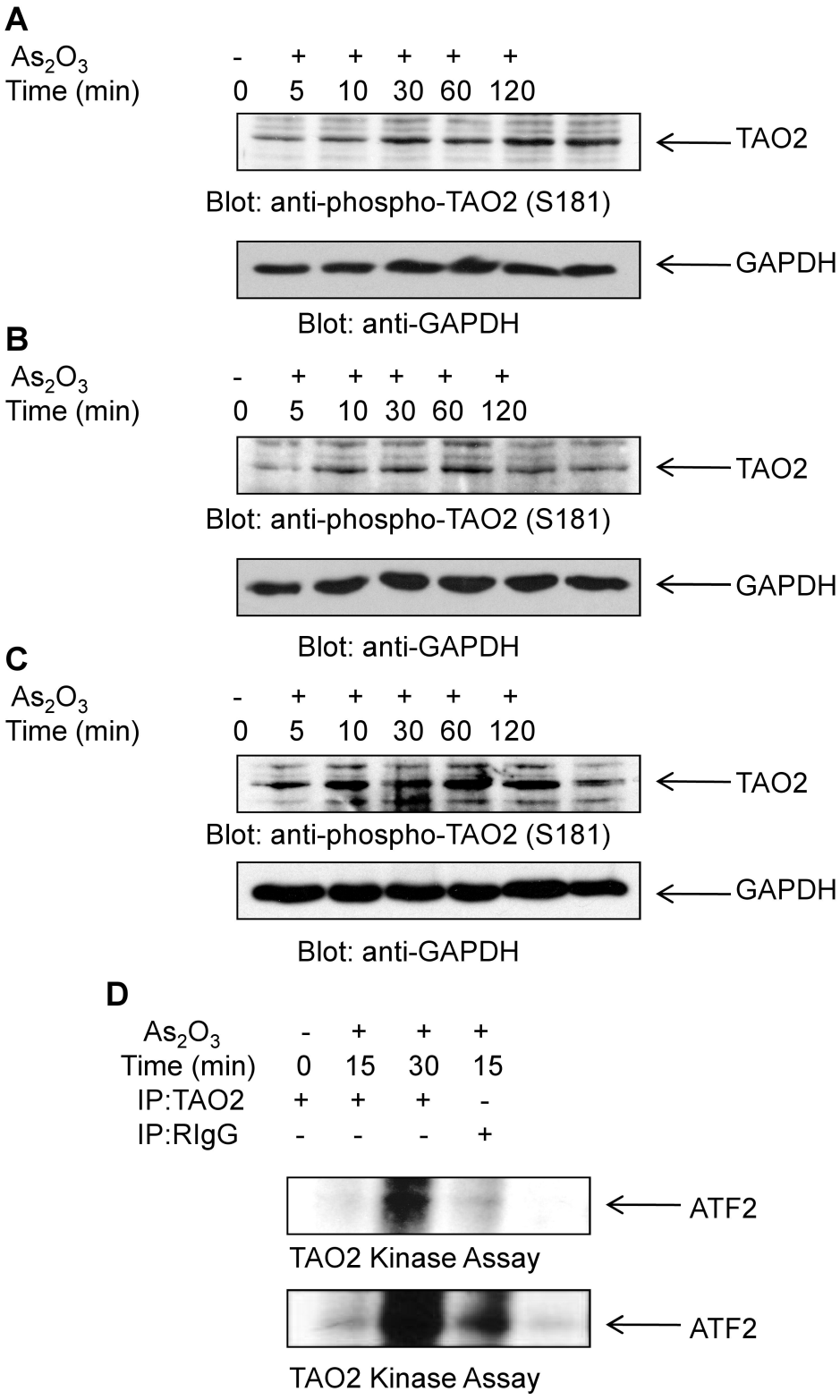

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Figure 2

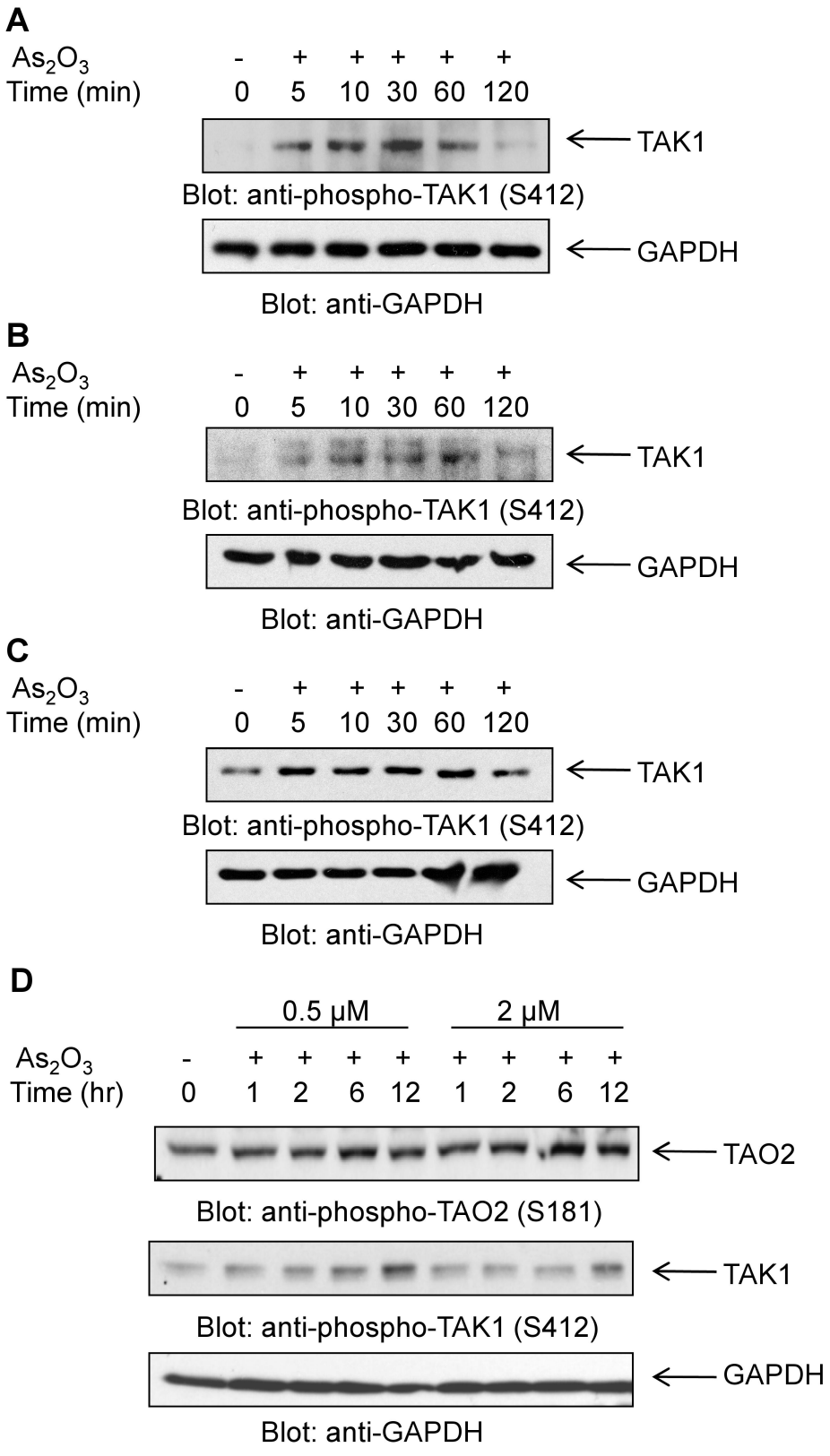

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Figure 3

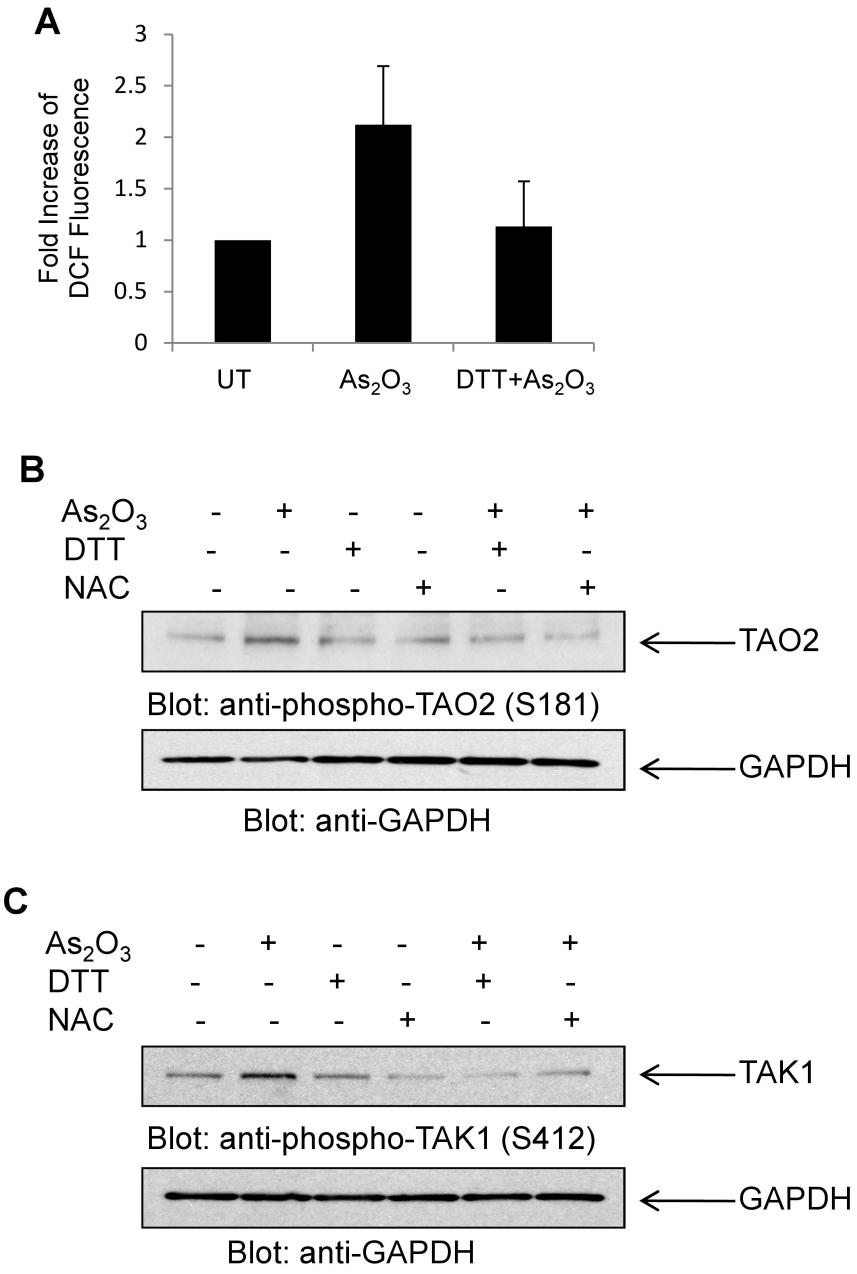

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Figure 4

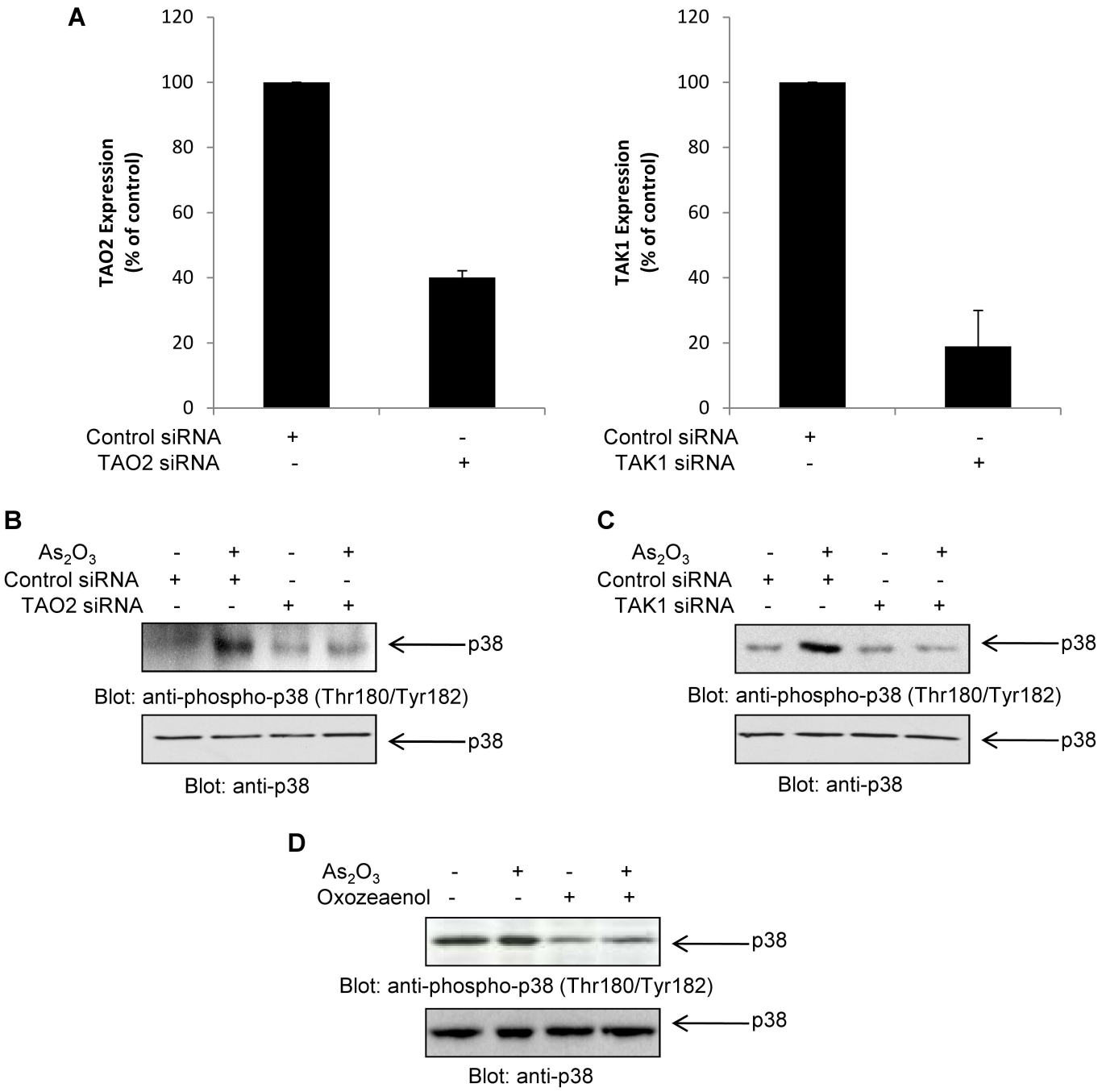

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Figure 5

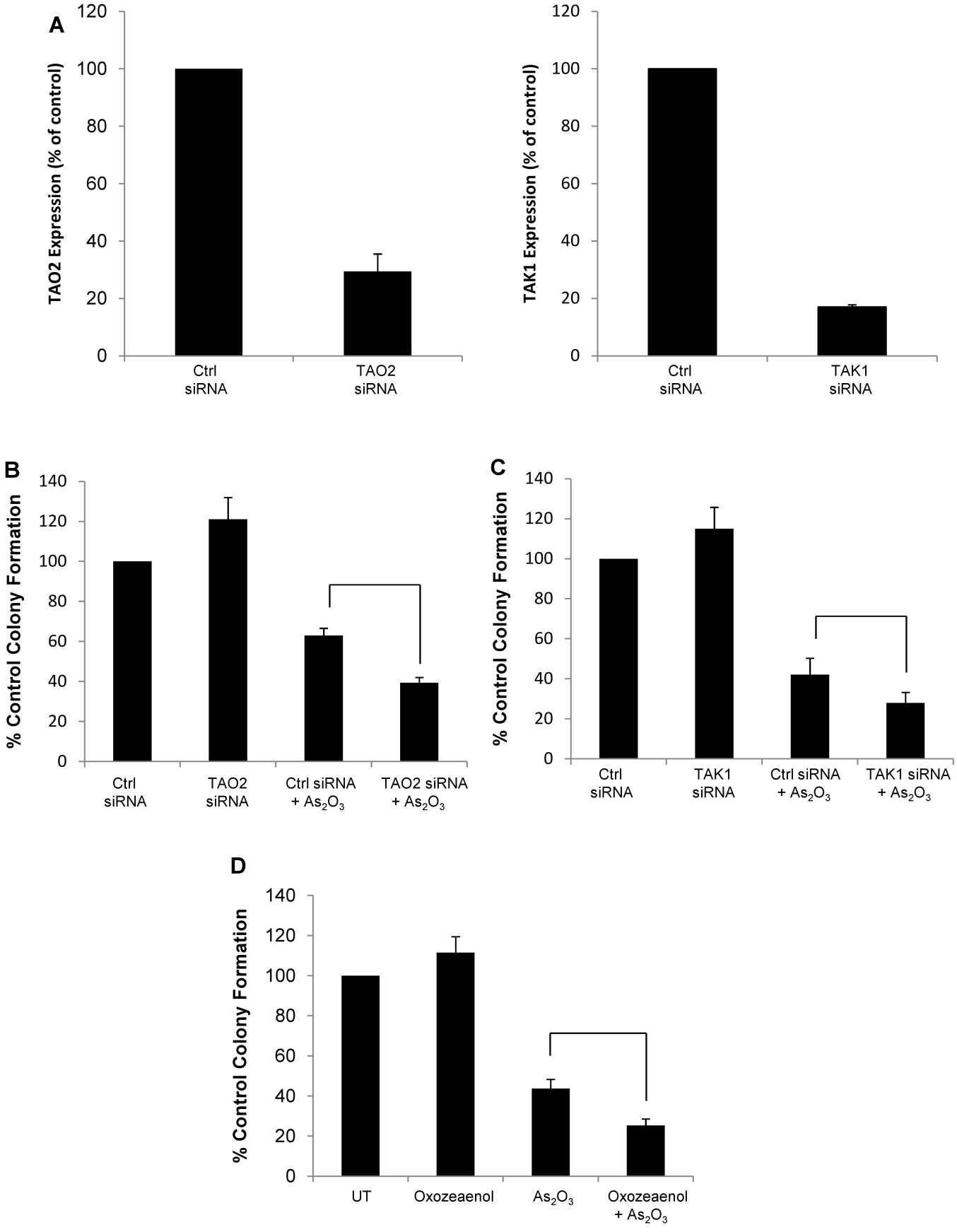

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Figure 6