

Role of the p38 Mitogen-Activated Protein Kinase Pathway in the Generation of Arsenic Trioxide–Dependent Cellular Responses

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Abstract

Arsenic trioxide (As₂O₃) induces differentiation and apoptosis of leukemic cells *in vitro* and *in vivo*, but the precise mechanisms that mediate such effects are not known. In the present study, we provide evidence that the kinases MAPK kinase 3 (Mkk3) and Mkk6 are activated during treatment of leukemic cell lines with As₂O₃ to regulate downstream engagement of the p38 mitogen-activated protein kinase. Using cells with targeted disruption of both the *Mkk3* and *Mkk6* genes, we show that As₂O₃-dependent activation of p38 is defective in the absence of Mkk3 and Mkk6, establishing that these kinases are essential for As₂O₃-dependent engagement of the p38 pathway. Pharmacologic inhibition of p38 enhances As₂O₃-dependent activation of the c-jun NH₂-terminal kinase (JNK) and subsequent induction of apoptosis of chronic myelogenous leukemia (CML)– or acute promyelocytic leukemia (APL)–derived cell lines. In addition, in APL blasts, inhibition of p38 enhances myeloid cell differentiation in response to As₂O₃, as well as suppression of Bcl-2 expression and loss of mitochondrial membrane potential. Similarly, induction of As₂O₃-dependent apoptosis is enhanced in mouse embryonic fibroblasts (MEF) with targeted disruption of both the *Mkk3* and *Mkk6* genes, establishing a key role for this pathway in the regulation of As₂O₃-induced apoptosis. In other studies, we show that the small-molecule p38 inhibitors SD-282 and SCIO-469 potentiate As₂O₃-mediated suppression of myeloid leukemic progenitor growth from CML patients, indicating a critical regulatory role for p38 in the induction of antileukemic responses. Altogether, our data indicate that the Mkk3/6-p38 signaling cascade is activated in a negative regulatory feedback manner to control induction of As₂O₃-mediated antileukemic effects. (Cancer Res 2006; 66(13): 6763-71)

Introduction

Arsenic trioxide (As₂O₃) exhibits potent antitumor effects *in vitro* and *in vivo* and is widely used in the treatment of acute promyelocytic leukemia (APL) in humans (1–4). The effects of As₂O₃ are dose dependent, with higher concentrations leading to apoptosis and lower concentrations (<0.5 μmol/L) inducing differentiation (1–4). In addition to APL, arsenic is of potential

therapeutic value for the treatment of other hematologic malignancies, including chronic myelogenous leukemia (CML; refs. 5, 6). CML is a clonal myeloproliferative disorder of stem cells, the hallmark of which is the presence of the abnormal BCR-ABL oncogenic tyrosine kinase, which transforms the cells (7). There is evidence that As₂O₃ can induce apoptosis of BCR-ABL-expressing cell lines (8) and inhibit the proliferation of CML leukemic blasts (9, 10). These findings have suggested a potential role for this agent in the treatment of CML (5, 6) but the precise mechanisms by which it induces its antileukemic effects remain to be defined.

Mitogen-activated protein kinases (MAPK) are a family of enzymes that transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli, and modulate several important biological functions including gene expression, mitosis, proliferation, motility, and apoptosis (reviewed in refs. 11–13). Three major groups of MAPKs exist, the extracellular signal-regulated kinase (ERK), c-jun NH₂-terminal kinase (JNK), and p38 MAPK families (11–13). The involvement of these kinases in the generation of stress responses, as well as in signaling for various cytokines and growth factors, has been extensively studied and documented in previous studies (reviewed in refs. 11–13). However, little is known on the putative roles of MAPKs in leukemogenesis or their involvement in pathways that mediate antileukemic responses (12).

Previous work from our laboratory has shown that As₂O₃ induces activation of the p38 MAPK in various neoplastic cell lines (14), suggesting a role for this pathway in the regulation of arsenic-responses in malignant cells. In the present study, we sought to define the functional relevance of activation of p38 by As₂O₃. Our data show that activation of p38α exhibits a negative regulatory role on the induction of apoptosis by As₂O₃, as evidenced by the enhanced induction of apoptosis during pharmacologic inhibition of p38 or in cells with targeted disruption of the *p38α* gene. In efforts to define the sequence of events that lead to arsenic-dependent activation of p38, we found that the kinases MAPK kinase 3 (Mkk3) and Mkk6 are activated by As₂O₃ and act as the upstream effectors of p38. Furthermore, apoptosis and growth inhibition promoted by As₂O₃ were augmented in cells lacking both *Mkk3* and *Mkk6* genes compared with their wild-type counterparts. Finally, we show that the pharmacologic inhibitors of p38, SD-282 and/or SCIO-469, promote arsenic-mediated differentiation of APL blasts and enhance the suppressive properties of As₂O₃ on primitive leukemic granulocyte-macrophage colony-forming unit (CFU-GM) progenitors from patients with CML. Altogether, our results suggest that the p38 MAPK signaling cascade acts as a negative feedback regulator for the generation of antileukemic responses, and its activation may account for lack of sensitivity or resistance to As₂O₃ in different leukemia cell types.

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Materials and Methods

Cells and reagents. The CML-derived KT-1 cell line and the NB-4 human APL cell line were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics. Immortalized mouse embryonic fibroblasts (MEF) from Mkk3/6 knockout mice (15) and p38 α knockout mice (16) were cultured in DMEM containing 10% FBS. Similarly, Jnk1/Jnk2 double-knockout MEFs (17) were grown in DMEM-10% FBS. As₂O₃ was purchased from Sigma (St. Louis, MO). Antibodies against the phosphorylated forms of p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²), Mkk3/6 (Ser^{189/207}), and stress-activated protein kinase/JNK (Thr¹⁸³/Tyr¹⁸⁵) were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies against p38 α were obtained from Cell Signaling Technology and Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Bcl-2 antibody was obtained from Cell Signaling Technology. Antitubulin mouse monoclonal antibody was obtained from AbCam, Inc. (Cambridge, MA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse antibody was purchased from Chemicon International (Temecula, CA). The p38 MAPK inhibitor SB203580 was purchased from EMD Biosciences (San Diego, CA). The small-molecule pharmacologic inhibitors of p38 MAPK, SD-282 and SCIO-469, were from Scios, Inc. (Fremont, CA). SCIO-469 has an *in vitro* IC₅₀ of 9 nmol/L for inhibition of p38 α , ~10-fold selectivity for p38 α over p38 β , and at least 2,000-fold selectivity for p38 α over an *in vitro* panel of 20 other kinases, including other MAPKs. No significant affinity has been detected in a panel of 70 enzymes and receptors (18). SD-282 is a potent small-molecule inhibitor of p38 α and p38 β with IC₅₀ of 1.1 to 1.6 nmol/L and 22 to 23 nmol/L, respectively. It does not inhibit p38 δ , p38 γ , JNK, ERKs, or MAPK-activated protein kinase 2, or the p38 activating kinases Mkk3 and Mkk6 at concentrations of up to 50 μ mol/L *in vitro* (19).

Cell lysis, immunoprecipitations, and immunoblotting. Cells were stimulated with the indicated doses of As₂O₃ for the indicated times and subsequently lysed in phosphorylation lysis buffer as previously described (20). Immunoprecipitations and immunoblotting using an enhanced chemiluminescence method were done as described in previous studies (20).

***In vitro* kinase assays.** These assays were done as previously described (20, 21). Briefly, cells were treated for the indicated times with the indicated doses of As₂O₃ and lysed in phosphorylation lysis buffer. Cell lysates were then immunoprecipitated with antibodies against Mkk3 or Mkk6. The immunoprecipitates were then washed thrice with phosphorylation lysis buffer followed by two washes with kinase buffer (25 mmol/L HEPES, 25 mmol/L MgCl₂, 25 mmol/L β -glycerophosphate, 100 μ mol/L sodium orthovanadate, 2 mmol/L DTT, 20 μ mol/L ATP). They were then resuspended in 30 μ L of kinase buffer containing 3 μ g of glutathione S-transferase (GST)-p38 α (for the Mkk3 and Mkk6 kinase assays) and 10 μ Ci of [γ -³²P]ATP. The reaction was subsequently incubated for 20 minutes at room temperature and terminated by addition of SDS sample buffer. Proteins were analyzed by SDS-PAGE and the phosphorylated form of p38 α was detected by autoradiography.

Cell proliferation assays. Mkk3/6^{+/+} and Mkk3/6^{-/-} MEFs were treated with the indicated doses of As₂O₃ for the designated time periods. Subsequently, cell proliferation assays using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method were done as previously described (22).

Evaluation of apoptosis. Cells were treated with the indicated doses of As₂O₃ for the indicated time periods. Flow cytometric assays to evaluate apoptosis by Annexin and propidium iodide staining were done essentially as previously described (23).

Mitochondrial membrane potential detection. Tetramethylrhodamine, ethyl ester, perchlorate (TMRE; Molecular Probes, Carlsbad, CA) was used to assess loss of mitochondrial membrane potential. The uptake and accumulation of TMRE in the mitochondria is driven by membrane potential ($\Delta\Psi_m$); therefore, depolarization of $\Delta\Psi_m$ can be represented by the loss of TMRE staining. Briefly, TMRE working solution was added directly to the medium to reach 100 nmol/L and then incubated at 37°C for 15 minutes. Cells were then washed with cold PBS once and kept on ice for immediate TMRE detection by flow cytometry.

Flow cytometric analysis. Flow cytometric studies to detect granulocytic differentiation of APL cells were done as previously described (22).

Briefly, NB-4 cells were treated with As₂O₃ for the indicated times and cell differentiation was determined by staining with the anti-CD11b monoclonal antibody. The anti-CD11b monoclonal antibody and a matched isotype control were purchased from BD Biosciences (San Jose, CA).

Hematopoietic progenitor cell assays. Bone marrow or peripheral blood from patients with CML were collected after obtaining consent approved by the Institutional Review Board of Northwestern University. Mononuclear cells were isolated by Ficoll-Hypaque centrifugation and used for clonogenic assays in methylcellulose as previously described (23). The cells were cultured in the presence or absence of As₂O₃ (1 μ mol/L), with or without SB203580 (10 μ mol/L), SD-282 (400 nmol/L), or SCIO-469 (400 nmol/L). Leukemic CFU-GM colonies were scored on day 14 of culture (23).

Results

We first determined whether As₂O₃ treatment induces phosphorylation/activation of p38 MAPK in the CML-derived KT-1 cell line. Cells were incubated in the absence or presence of As₂O₃ (2 μ mol/L) for various times and total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated/activated form of p38. Treatment of KT-1 cells with As₂O₃ induced strong phosphorylation/activation of p38, which was detectable after 30 minutes of treatment and persisted up to 120 minutes of incubation of the cells with As₂O₃ (Fig. 1A). On the other hand, the levels of p38 protein expression were similar before and after As₂O₃ treatment of the cells (Fig. 1A). Thus, the p38 MAPK is phosphorylated/activated in an As₂O₃-inducible manner in BCR-ABL-expressing cells, suggesting that its function may be important for the induction of the antileukemic effects of As₂O₃.

In subsequent studies, we sought to identify upstream kinases that regulate phosphorylation and activation of the p38 MAPK in response to As₂O₃. As MAPK kinases 3 and 6 (Mkk3 and Mkk6) have previously been shown to phosphorylate p38 in response to stress (15, 24, 25), we examined their involvement in the regulation of the arsenic-activated p38 MAPK pathway. Treatment with As₂O₃ resulted in phosphorylation of Mkk3/Mkk6 in both KT-1 cells (1B) and the APL-derived NB-4 cell line (1C), in which p38 is also activated in response to As₂O₃ or retinoids (14, 22). Consistent with this, in experiments in which immunocomplex kinase assays were done in anti-Mkk3 and anti-Mkk6 immunoprecipitates (Fig. 1D), there was detectable As₂O₃-dependent Mkk kinase activity. Thus, the kinases Mkk3 and Mkk6 are activated in an As₂O₃-dependent manner in sensitive cells, suggesting that they may play regulatory roles in the activation of the p38 pathway by As₂O₃.

To directly define the roles that Mkk3 and Mkk6 may play in the activation of p38 by As₂O₃, we examined the phosphorylation/activation of p38 in double-knockout MEFs for both the *Mkk3* and *Mkk6* genes (15). Mkk3^{+/+}Mkk6^{+/+} and Mkk3^{-/-}Mkk6^{-/-} MEFs were incubated in the presence or absence of As₂O₃. The cells were subsequently lysed and cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-p38 antibody. As₂O₃ treatment resulted in phosphorylation/activation of p38 in Mkk3/6^{+/+} cells whereas such phosphorylation was defective in the MEFs with targeted deletion of both the *Mkk3* and *Mkk6* genes (Fig. 1E), establishing that activation of the p38 MAPK cascade by As₂O₃ requires the upstream functions of Mkk3 and Mkk6.

We subsequently sought to determine the functional relevance of activation of the Mkk3/6-p38 MAPK pathway during treatment of BCR-ABL-expressing cells with As₂O₃. We first examined the effects of a p38 inhibitor, the pyridinyl imidazole compound SB203580, on the induction of apoptosis by As₂O₃. Treatment of

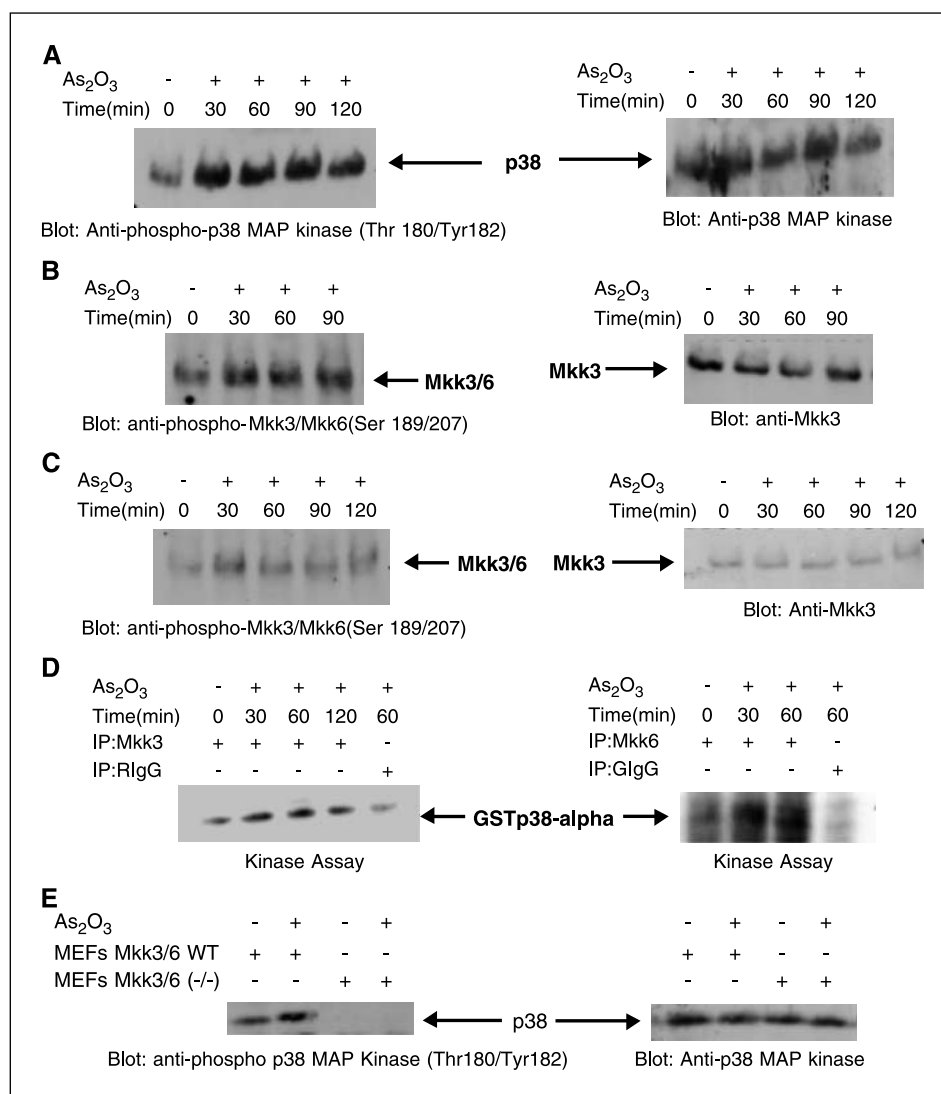


Figure 1. As₂O₃-dependent phosphorylation/activation of the p38 MAPK and its upstream effectors Mkk3 and Mkk6. **A**, KT-1 cells were incubated in the absence or presence As₂O₃ (2 μmol/L) for the indicated times. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an anti-phospho-p38 antibody (*left*). The same blot was stripped and re probed with anti-p38 antibody to control for equal loading (*right*). **B**, KT-1 cells were incubated in the absence or presence As₂O₃ (2 μmol/L) for the indicated times. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an anti-phospho-Mkk3/6 antibody (*left*). The same blot was stripped and re probed with anti-Mkk3 antibody to control for equal loading (*right*). **C**, NB-4 cells were incubated in the absence or presence of As₂O₃ (2 μmol/L) for the indicated times. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-Mkk3/6 antibody (*left*). The same blot was stripped and re probed with an anti-Mkk3 antibody to control for protein loading (*right*). **D**, KT-1 cells were incubated for the designated times in the absence or presence of As₂O₃ (2 μmol/L). Cell lysates were immunoprecipitated with an anti-Mkk3 antibody or control nonimmune rabbit immunoglobulin (RlgG), and immunoprecipitated proteins were subjected to *in vitro* kinase assays using GST-p38α as an exogenous substrate. Proteins were resolved by SDS-PAGE and phosphorylated proteins were detected by autoradiography (*left*). NB-4 cells were incubated for the indicated times in the absence or presence of As₂O₃ (2 μmol/L). Cell lysates were immunoprecipitated with an anti-Mkk6 antibody or control nonimmune goat immunoglobulin (GlgG), and immunoprecipitated proteins were subjected to *in vitro* kinase assays using GST-p38α as an exogenous substrate. Proteins were then resolved by SDS-PAGE and phosphorylated proteins were detected by autoradiography (*right*). **E**, Mkk3/6^{+/+} and Mkk3/6^{-/-} MEFs were incubated in the absence or presence of As₂O₃ (2 μmol/L) for 120 minutes. The cells were lysed and equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with anti-phospho-p38 antibody (*left*). The same blot was stripped and re probed with an anti-p38 antibody to control for protein loading (*right*).

KT-1 cells with As₂O₃ resulted in induction of apoptosis, as determined by Annexin V/propidium iodide staining, whereas the SB203580 inhibitor alone did not induce apoptosis of the cells (Fig. 2A). However, concomitant addition of the p38 inhibitor together with As₂O₃ in the cultures strongly enhanced the induction of apoptosis as compared with cells treated with As₂O₃ alone (Fig. 2A). We also did similar studies using the SD-282 and SCIO-469 specific inhibitors of p38. The activity of these inhibitors against the p38 MAPK has been extensively docu-

mented previously (18, 19, 26). As in the case of the SB203580 inhibitor, both SD-282 and SCIO-469 potentiated the induction of apoptosis by As₂O₃ (Fig. 2B). We did experiments in which the expression of p38α was blocked in CML-derived cells using the small interfering RNA (siRNA) method and the induction of apoptosis by As₂O₃ in KT-1 cells was compared in the presence or absence of p38α. The p38α siRNA inhibited expression of p38α (Fig. 2C) whereas the induction of apoptosis was enhanced in cells transfected with p38α siRNA as compared with cells

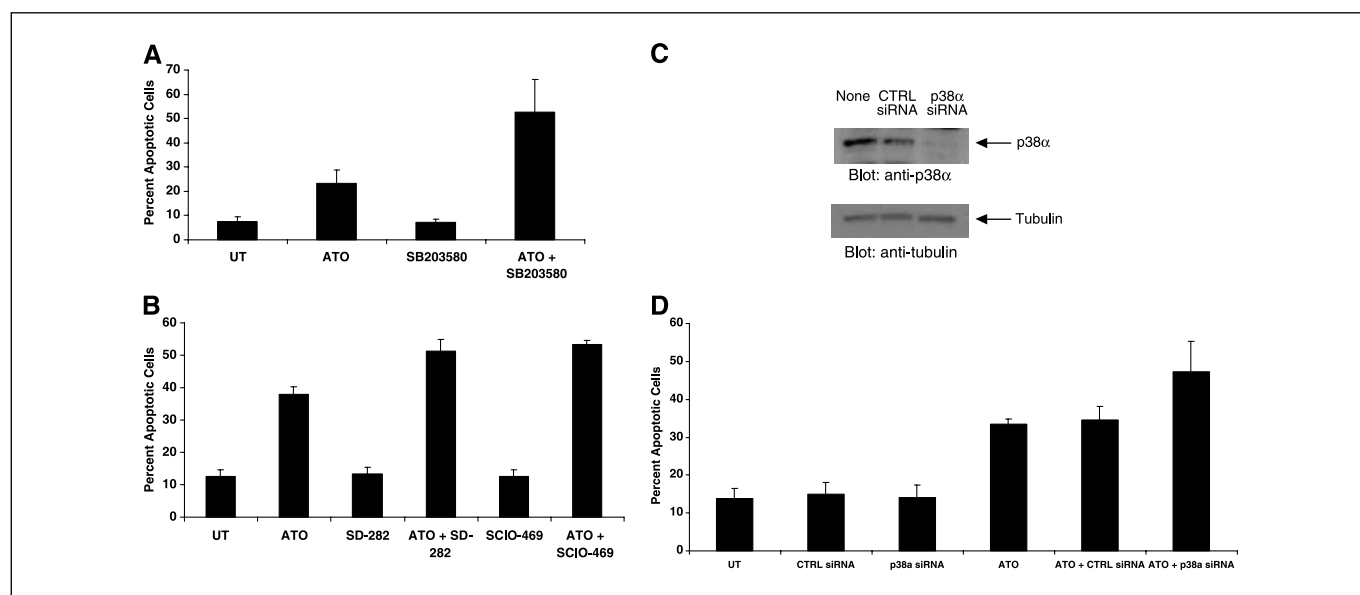


Figure 2. Pharmacologic inhibition of p38 MAPK activity or gene knockdown of *p38α* potentiates As_2O_3 -induced apoptosis. **A**, KT-1 cells were treated for 48 hours in the absence or presence of As_2O_3 (1 μ mol/L), in the presence or absence of SB203580, as indicated. Induction of apoptosis was evaluated by flow cytometry after Annexin V/propidium iodide staining. *Columns*, mean percent early and late apoptotic cells of three experiments; *bars*, SE. **B**, KT-1 cells were incubated for 48 hours in the absence or presence of As_2O_3 (1 μ mol/L), in the presence or absence of SD-282 (1 μ mol/L) or SCIO-469 (1 μ mol/L), as indicated. The cells were subsequently analyzed for apoptosis in the same manner as in (A). *Columns*, mean of three experiments; *bars*, SD. **C**, KT-1 cells were transfected with either p38 α -specific siRNA or control GAPDH siRNA. After 24 hours, these cells were lysed in phosphorylation lysis buffer and cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against p38 α (*top*). The same blot was stripped and reprobed with an antitubulin antibody to control for equal protein loading (*bottom*). **D**, KT-1 cells were transfected with either p38 α -specific siRNA (100 nmol/L) or control GAPDH siRNA (100 nmol/L). Twenty-four hours posttransfection, cells were treated for 48 hours with As_2O_3 (1 μ mol/L). Induction of apoptosis was evaluated by flow cytometry after Annexin V/propidium iodide staining. *Columns*, mean percent early and late apoptotic cells of three experiments; *bars*, SE.

transfected with control siRNA (Fig. 2D). Taken altogether, these findings strongly suggested a putative role for p38, acting as a negative regulator of As_2O_3 -induced apoptosis in BCR-ABL-expressing cells.

To further establish the role of the p38 MAPK pathway on the regulation of As_2O_3 -induced apoptosis, studies using immortalized MEFs with targeted deletion of gene-components of the p38 MAPK pathway were subsequently done. *Mkk3*^{+/+}*Mkk6*^{+/+} and *Mkk3*^{-/-}*Mkk6*^{-/-} MEFs were treated with As_2O_3 for 48 hours and the percentage of apoptotic cells was determined by flow cytometry. Treatment of *Mkk3*^{-/-}*Mkk6*^{-/-} cells with As_2O_3 resulted in significantly enhanced apoptosis as compared with *Mkk3*^{+/+}*Mkk6*^{+/+} cells (Fig. 3A). Similarly, when the antiproliferative effects of As_2O_3 were compared in the wild-type MEFs and the double *Mkk3*^{-/-}*Mkk6*^{-/-} knockout MEFs, there was a substantial augmentation of the inhibitory effects of different concentrations of As_2O_3 in the absence of *Mkk3* and *Mkk6* (Fig. 3B). Altogether, these studies provided firm evidence implicating *Mkk3* and *Mkk6* as negative regulators of As_2O_3 -induced apoptosis and growth inhibition. We also did experiments using MEFs with targeted disruption of the *p38α* gene (16). As shown in Fig. 3C, treatment of wild-type p38 α ^{+/+} MEFs with As_2O_3 resulted in phosphorylation/activation of p38 α whereas p38 α protein expression was absent in cells with targeted deletion of the *p38α* gene (p38 α ^{-/-}; Fig. 3C). In experiments in which the induction of apoptosis by As_2O_3 was compared between p38 α ^{+/+} and p38 α ^{-/-} MEFs, the numbers of cells undergoing apoptosis after arsenic treatment were higher in p38 α ^{-/-} cells as compared with parental p38 α ^{+/+} cells (Fig. 3D). However, as the increase in As_2O_3 -mediated apoptosis was not as pronounced in the p38 α cells when compared with the *Mkk3/6*^{-/-}

MEFs, we speculated that compensatory activities of other p38 isoforms might account for such differences in the MEFs. Consistent with such a hypothesis, pretreatment of p38 α ^{-/-} MEFs with the p38 inhibitor SB203580 further potentiated As_2O_3 -induced apoptosis (Fig. 3D). On the other hand, when similar studies were done in double-knockout MEFs for both *Jnk1* and *Jnk2* (17), there was no enhancement of As_2O_3 -dependent apoptosis (Fig. 3E). On the contrary, there was decreased apoptosis in the *Jnk1*^{-/-}*Jnk2*^{-/-} MEFs as compared with *Jnk1*^{+/+}*Jnk2*^{+/+} MEFs (Fig. 3E), confirming and expanding on the results of previous studies in which it was shown that pharmacologic inhibition of *Jnk* reverses As_2O_3 -dependent apoptosis (27).

As_2O_3 -induced apoptosis in malignant cells has previously been shown to involve opening of the mitochondrial permeability transition pore, which is dependent on a balance between the prosurvival and proapoptotic family members of the Bcl-2 family (28). It is also well established that Bcl-2 plays an important role in protecting against As_2O_3 -induced apoptosis in certain malignant cell types (29–32). We examined the effects of pharmacologic inhibition of the p38 on the suppression of Bcl-2 expression by As_2O_3 treatment in the NB-4 APL cell line. Cells were exposed to As_2O_3 for 24 hours, in the absence or presence of SB203580, and the expression of Bcl-2 protein was determined. As expected, treatment of cells with As_2O_3 resulted in down-regulation of Bcl-2 (Fig. 4A). However, such As_2O_3 -dependent down-regulation of Bcl-2 was further enhanced by the addition of SB203580 in the cultures as compared with treatment with As_2O_3 alone (Fig. 4A). Consistent with the enhanced suppression of Bcl-2 expression, inhibition of p38 with either SB203580 or SCIO-469 potentiated arsenic-induced loss of mitochondrial membrane potential ($\Delta\Psi_m$; Fig. 4B).

Altogether, these findings suggest a putative mechanism by which p38 inhibition enhances arsenic-dependent apoptosis in cells of promyelocytic origin, involving Bcl-2 down-regulation and enhanced loss of mitochondrial membrane potential.

To further understand the mechanisms by which p38 inhibition may promote As₂O₃-induced apoptosis of target cells, we examined the effects of p38 inhibitors on other signaling pathways of which the function is essential for the induction of apoptosis by As₂O₃. It is now well established by the work of others (27), and confirmed by our experiments using Jnk1/2 knockout MEFs (Fig. 3E), that activation of the Mkk4/JNK pathway is essential for the induction of As₂O₃-dependent apoptosis. When either NB-4 (Fig. 4C) or KT-1 (Fig. 4D) cells were concomitantly treated with SB203580, SCIO-469, or SD-282, we noticed a strong enhancement of Jnk1/Jnk2 phosphorylation in response to As₂O₃. Thus, pharmacologic inhibition of the p38 MAPK pathway seems to enhance activation of Jnk by As₂O₃, and such events may also contribute to the induction of apoptosis on target cells.

It is well established that, in contrast to high doses, low doses of As₂O₃ promote differentiation of APL blasts to granulocytes (1-4). There is also evidence from our previous work that pharmacologic inhibition of p38 enhances the induction of differentiation of APL cells in response to all-*trans*-retinoic acid (22). We examined the

effects of p38 inhibitors on the induction of As₂O₃-induced differentiation of NB-4 cells. As₂O₃ treatment of NB-4 cells resulted in granulocytic differentiation of a fraction of cells, as determined by the induction of CD11b expression on the surface of the cells (Fig. 5A). Cotreatment of cells with either SD-282 or SCIO-469 strongly enhanced the percentage of CD11b-expressing cells (Fig. 5A and B). On the other hand, cotreatment of cells with the MAPK/ERK kinase (MEK)/ERK inhibitor PD98059 reversed the induction of differentiation, suggesting that the MEK/ERK pathway promotes arsenic-mediated NB-4-cell differentiation (Fig. 5B) in a manner similar to what is seen in the case of retinoid-dependent differentiation of APL cells (22). As As₂O₃ is also known to inhibit the growth of NB-4 cells (27), we cannot absolutely exclude the possibility that the enhanced differentiation by the p38 inhibitors is not due in part to selective killing of undifferentiated cells by As₂O₃. However, this is unlikely as it has also previously been shown that arsenic does not induce significant apoptosis at the low concentrations used here (27). In addition, in further analysis in which the proportion of differentiated cells among 1×10^4 live cells analyzed by flow cytometry was compared with the fraction of differentiated cells present in the total population of cells (live and dead) counted, similar patterns of CD11b induction were observed in response to

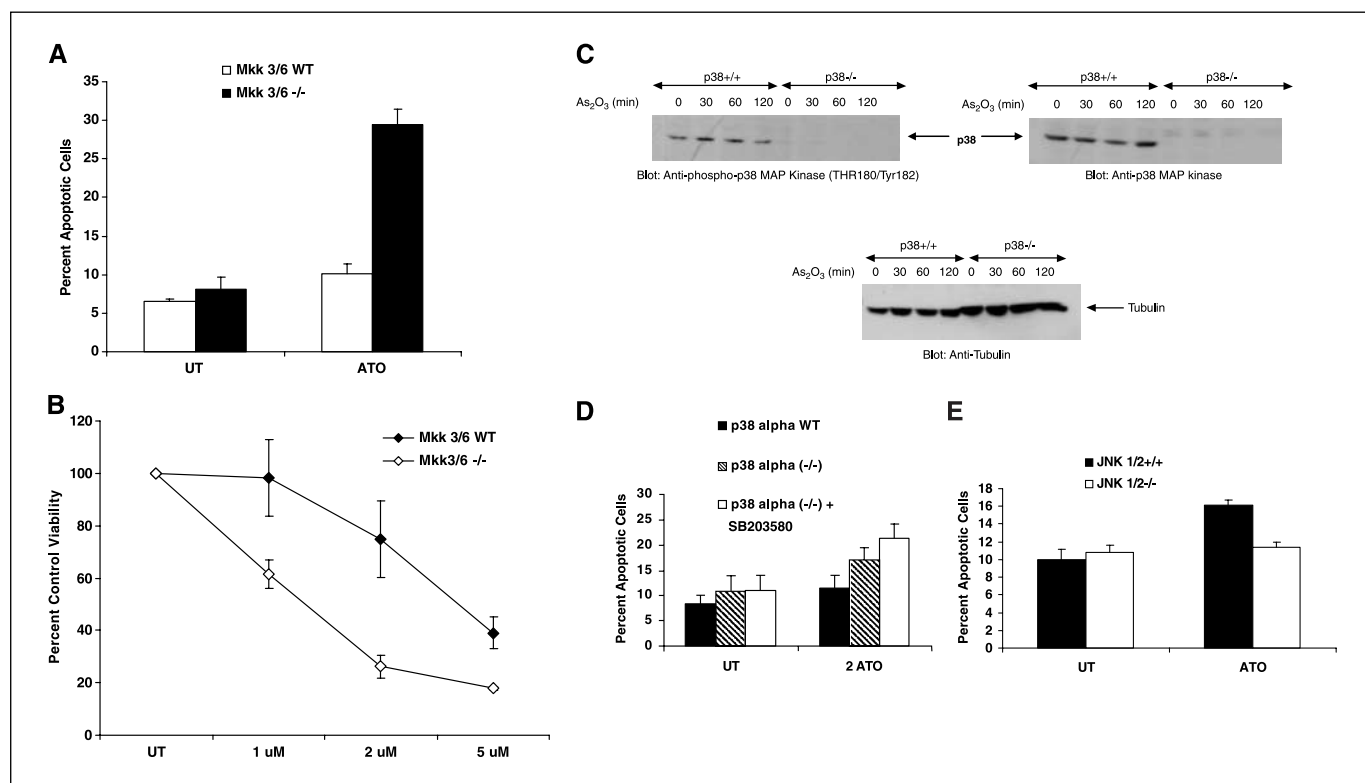


Figure 3. Targeted disruption of the Mkk3 and Mkk6, or p38 α , genes potentiates As₂O₃-induced apoptosis and growth inhibition. **A**, Mkk3^{+/+/+} and Mkk3^{-/-} MEFs were incubated in the absence or presence of As₂O₃ (2 μ mol/L) for 48 hours. The total percentage of apoptotic cells was determined by Annexin V/propidium iodide staining, followed by flow cytometry. **Columns**, mean of three experiments; **bars**, SE. **B**, Mkk3^{+/+/+} and Mkk3^{-/-} MEFs were incubated in the absence or presence of the indicated final concentrations of As₂O₃ for 3 days. Cell proliferation was assessed by MTT assay. **Points**, means of three experiments; **bars**, SE. **C**, p38 α ^{+/+} and p38 α ^{-/-} MEFs were incubated in the absence or presence of As₂O₃ (2 μ mol/L) for the indicated times. The cells were then lysed and total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-p38 MAPK antibody (*top left*). The same blot was subsequently stripped and reprobed with an anti-p38 MAPK antibody (*top right*). The same blot was subsequently stripped and reprobed with an antitubulin antibody to control for protein loading (*bottom*). **D**, p38 α ^{+/+} and p38 α ^{-/-} MEFs were incubated for 48 hours in the absence or presence of As₂O₃ (2 μ mol/L) for 48 hours, in the presence or absence of SB203580. The percentage of apoptotic cells was calculated by Annexin V/propidium iodide staining using flow cytometric analysis. **Columns**, mean of three experiments; **bars**, SE. **E**, Jnk1^{+/+/+} and Jnk1^{-/-}Jnk2^{-/-} MEFs were incubated for 48 hours in the absence or presence of As₂O₃ (2 μ mol/L) for 48 hours. Induction of apoptosis was evaluated by flow cytometry after Annexin V/propidium iodide staining. **Columns**, mean of three experiments; **bars**, SE.

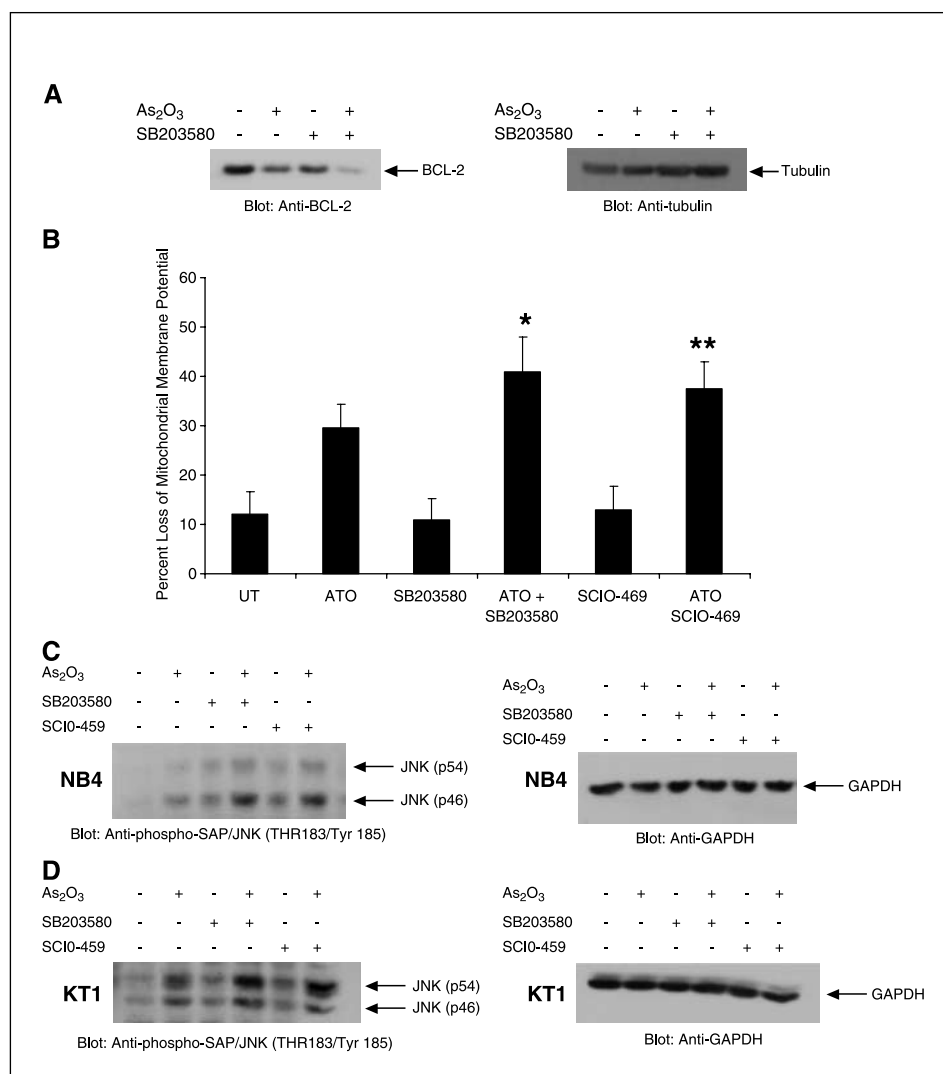


Figure 4. Inhibition of the p38 MAPK pathway enhances arsenic-induced suppression of Bcl-2 expression and activation of JNK. *A*, NB-4 cells were incubated in the absence or presence of As₂O₃ (1 μmol/L) for 24 hours, with or without SB203580 (10 μmol/L), as indicated. Following cell lysis, total lysates were resolved by SDS-PAGE and immunoblotted with an anti-Bcl-2 antibody (*left*). The same blot was stripped and reprobed with an antitubulin antibody to control for protein loading (*right*). *B*, NB-4 cells were incubated for 24 hours with As₂O₃ (1 μmol/L) in the presence or absence of SB203580 (10 μmol/L) or SCIO-469 (1 μmol/L), as indicated. The cells were subsequently stained with TMRE. The percentages of cells with loss of mitochondrial membrane potential are indicative of those with low TMRE intensity. *Columns*, mean of six experiments; *bars*, SE. Paired *t*-test analysis of the combinations of As₂O₃ + SB203580 or As₂O₃ + SCIO-469, as compared with As₂O₃ alone (columns marked with asterisks), showed *P* values of 0.0178 and 0.041, respectively. *C* and *D*, NB-4 (*C*) or KT-1 cells (*D*) were preincubated in the presence or absence of the indicated inhibitors for 1 hour and were subsequently treated with As₂O₃ (1 μmol/L) for 24 hours. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an anti-phospho-JNK antibody (*left*). The same blots were subsequently stripped and reprobed with an anti-GAPDH antibody (*right*).

treatment combinations of arsenic with the p38 inhibitors (data not shown). This makes it unlikely that the combination therapy selectively killed undifferentiated cells. Thus, pharmacologic inhibition of p38 enhances arsenic-induced differentiation of APL blasts to granulocytes, suggesting that the p38 pathway negatively regulates leukemic cell differentiation.

To obtain information on the role of p38 in the generation of As₂O₃-dependent responses in a more physiologically relevant system, studies were done with primary leukemic progenitors from patients with CML. Bone marrow or peripheral blood mononuclear cells from different patients with CML were treated with As₂O₃ in the absence or presence of different pharmacologic inhibitors of p38, including SB203580, SD-282, or SCIO-469, and subsequently assayed for leukemic CFU-GM progenitor colony formation. Concomitant treatment with As₂O₃ and the pyridinyl imidazole compound SB203580 enhanced suppression of leukemic CFU-GM progenitors (Fig. 6A; ref. 14). Importantly, the p38 inhibitors SD-282 and SCIO-469 also augmented the suppressive effects of As₂O₃ on leukemic CFU-GM colony formation (Fig. 6B). Using paired *t*-test statistical analysis, the effects of either SD-282 (two-tailed *P* = 0.0061) or SCIO-469 (two-tailed *P* = 0.0044) were found to be statistically significant. Thus, different classes of

pharmacologic inhibitors of p38 enhance the antileukemic properties of As₂O₃ *in vitro*, suggesting that such effects may also occur during concomitant administration of p38 inhibitors and As₂O₃ to leukemia patients *in vivo*.

Discussion

As₂O₃ is a heavy metal derivative that has potent antileukemic properties. This agent induces dramatic remissions in patients suffering from APL (1–4), whereas there is strong interest in its clinical development for other leukemias, and in particular, CML (5, 6). The important antitumor properties of As₂O₃ *in vitro* and *in vivo* have ignited extensive studies aimed to determine the mechanisms by which it induces apoptosis and antileukemic responses. It is now well established that arsenic exposure of target cells results in elevation of reactive oxygen species, loss of mitochondrial membrane potential, and release of cytochrome *c*, followed by activation of the caspase cascade and programmed cell death (32–35). There is also evidence that generation of reactive oxygen species, in particular H₂O₂, depends on cellular glutathione stores and that reduced cellular glutathione (GSH) is an inhibitor of As₂O₃-dependent cell death by conjugating arsenic in the form of

AS(GS)3 complexes and/or sequestering reactive oxygen species (36). Interestingly, GSH depletion by pretreatment of arsenic-resistant APL cells with buthionine sulfoximine has been shown to restore sensitivity to As_2O_3 -induced apoptosis (37).

Despite the significant advances on the mechanisms of arsenic-induced apoptosis and the contribution of reactive oxygen species, very little is known on the roles that other signaling pathways play in the generation of As_2O_3 -mediated biological responses. There is some recent evidence that members of distinct MAPK families play differential roles in the generation of As_2O_3 -dependent effects. As_2O_3 has been shown to result in sequential activation of Mkk4 and Jnk (27, 38) whereas pharmacologic inhibition of Jnk was shown to inhibit arsenic-dependent apoptosis (27). Moreover, such apoptosis is defective in cells lacking Mkk4 (27), strongly suggesting that sequential activation of Mkk4 and Jnk is required for the induction of As_2O_3 -mediated apoptosis. On the other hand, ERK1/2 are also phosphorylated/activated in response to treatment of cells with As_2O_3 but inhibition of such activation with MEK1 inhibitors or siRNA-mediated blocking of MEK1 enhances arsenic-dependent

apoptosis (39). Thus, the MEK/ERK pathway seems to exhibit an opposing role to the JNK pathway in the regulation of arsenic-dependent apoptosis (39). The p38 MAPK pathway has also been shown to be activated in response to As_2O_3 (14) and the small GTPase Rac1 was identified as an upstream regulator of this pathway (14). However, the identity of the MAPK kinases that regulate p38 activation in response to As_2O_3 downstream of Rac1 has been unknown and the overall functional relevance of activation of this pathway by As_2O_3 has been unclear.

In the present study, we provide the first evidence that the protein kinases Mkk3 and Mkk6 are phosphorylated and activated in an As_2O_3 -inducible manner in human leukemic cell lines. The activation of these kinases seems to be critical for As_2O_3 -dependent activation of the p38 pathway as evidenced by the lack of phosphorylation/activation of p38 in double-knockout cells for both kinases (Mkk3^{-/-}Mkk6^{-/-}). Our data also show that arsenic-mediated induction of apoptosis and growth inhibition are strongly enhanced in Mkk3^{-/-}Mkk6^{-/-} cells as compared with parental cells. Moreover, As_2O_3 -dependent apoptosis is enhanced in cells with targeted disruption of the *p38 α* gene. Taken

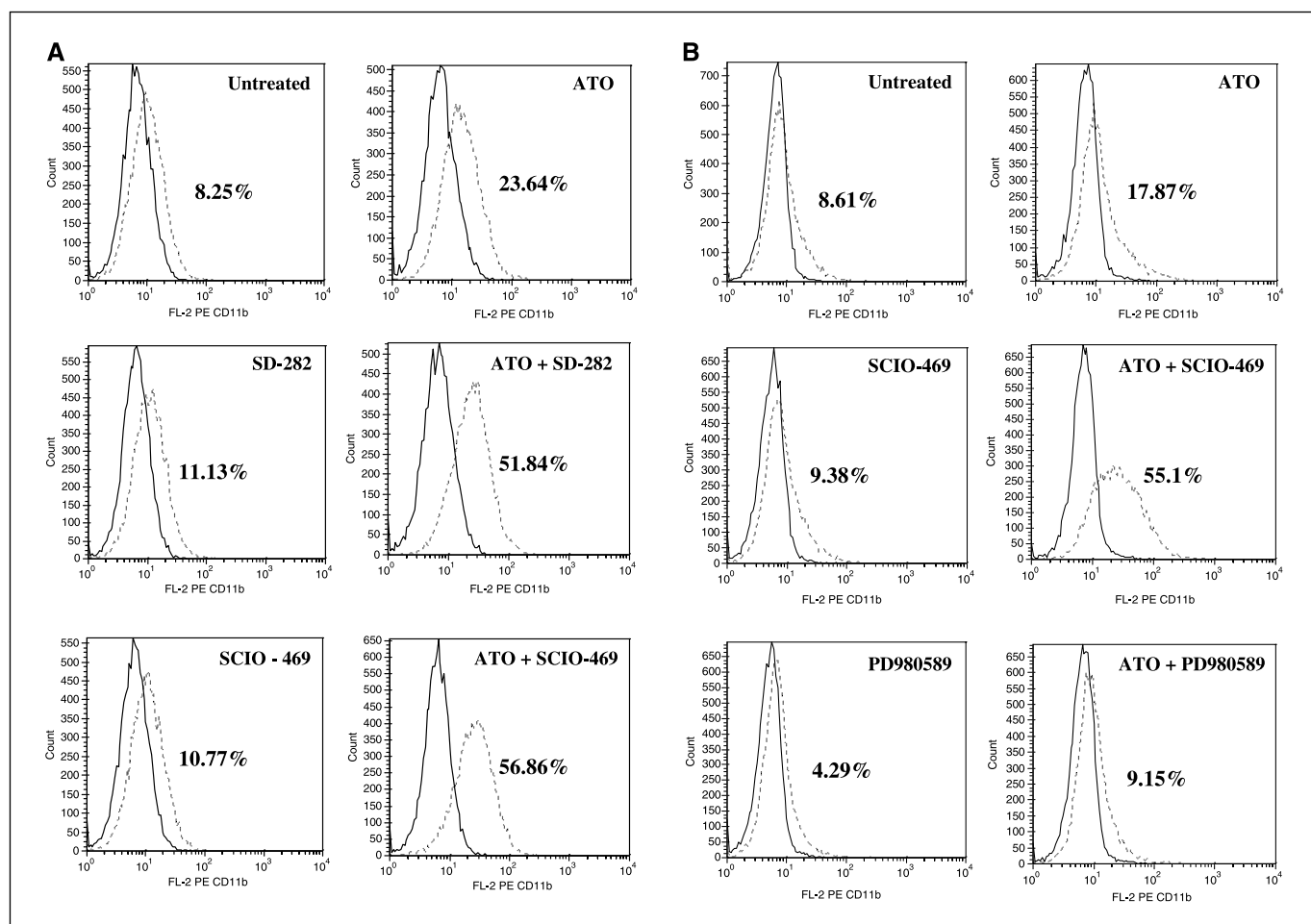


Figure 5. Inhibition of p38 MAPK activation enhances arsenic-induced cell differentiation of APL cells. **A**, NB-4 cells were incubated with As_2O_3 (0.5 μ mol/L) for 5 days in the presence or absence of SD-282 (1 μ mol/L) or SCIO-469 (1 μ mol/L) as indicated. The cells were subsequently stained with an anti-CD11b antibody and analyzed by flow cytometry. *Solid lines*, cells labeled with isotype control. *Dotted lines*, cells labeled with anti-CD11b. The percentage of cells positive for CD11b is indicated for each condition. **B**, NB-4 cells were incubated with As_2O_3 (0.5 μ mol/L) for 5 days, with or without SCIO-469 (1 μ mol/L) or PD980589 (2 μ mol/L), as indicated. The cells were subsequently stained with an anti-CD11b antibody and analyzed by flow cytometry. *Solid lines*, cells labeled with isotype control. *Dotted lines*, cells labeled with anti-CD11b. The percentage of cells positive for CD11b is indicated for each condition.

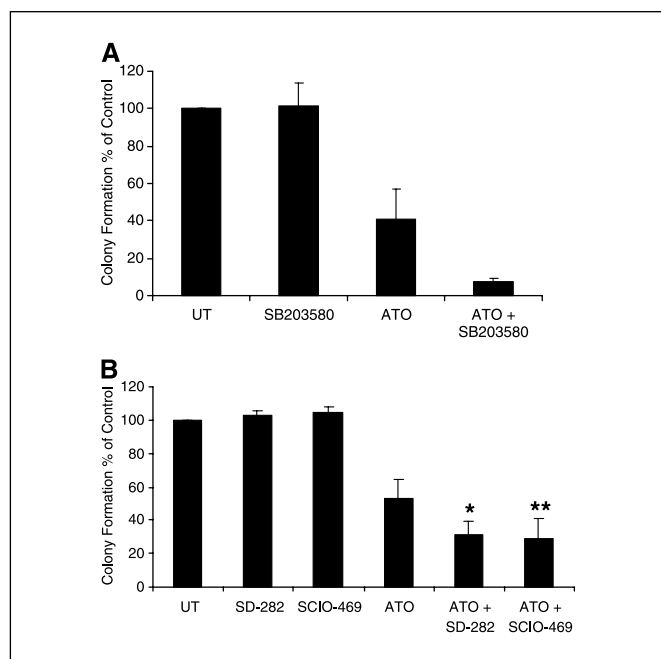


Figure 6. Pharmacologic inhibition of p38 enhances the suppressive effects of As_2O_3 on leukemic CFU-GM progenitors from patients with CML. *A*, peripheral blood mononuclear cells from three CML patients were plated in a methylcellulose culture assay system with As_2O_3 ($1 \mu\text{mol/L}$) in the presence or absence of SB203580 ($10 \mu\text{mol/L}$). Leukemic CFU-GM progenitor colonies were scored on day 14 of culture. *Columns*, mean percent control of CFU-GM colony numbers for untreated cells from three experiments using different patient samples; *bars*, SE values. *B*, bone marrow or peripheral blood mononuclear cells from four different patients with CML were plated in a methylcellulose culture assay system with As_2O_3 ($1 \mu\text{mol/L}$) and SD-282 (400 nmol/L) or SCIO-469 (400 nmol/L) as indicated. *Columns*, mean percent control of CFU-GM colony numbers for untreated cells from four experiments using different patient samples; *bars*, SE. Paired *t*-test analysis of the combinations of As_2O_3 + SD282 or As_2O_3 + SCIO-469, as compared with As_2O_3 alone (columns marked with asterisks), showed *P* values of 0.0061 and 0.0044, respectively.

altogether, these data provide direct and definitive evidence that the p38 MAPK pathway acts as a negative feedback regulator to counteract induction of As_2O_3 -induced cell death. On the other hand, our studies show that the induction of As_2O_3 -dependent apoptosis is defective in Jnk1/Jnk2 double-knockout MEFs, directly establishing that the function of the Jnk pathway is necessary for the generation of the effects of arsenic on target cells. In other studies, we show that, in addition to SB203580 (14), different pharmacologic inhibitors of p38 (SD282 and SCIO469) potentiate the antileukemic properties of As_2O_3 on primitive leukemic precursors derived from the peripheral blood or bone marrows of patients with CML, suggesting that activation of p38 is a physiologically relevant mechanism of resistance to the antileukemic properties of As_2O_3 .

Our studies also show that pharmacologic inhibition of p38 enhances the induction of cell differentiation of APL blasts at the low concentration of As_2O_3 ($0.5 \mu\text{mol/L}$) known to induce cell differentiation. Interestingly, in previous work, we had found that the p38 MAPK pathway is activated in response to treatment of NB-4 cells with all-*trans*-retinoic acid and negatively regulates all-*trans*-retinoic acid-induced cell differentiation (22). In those studies, we had found that all-*trans*-retinoic acid-dependent expression of CD11b was enhanced by pharmacologic inhibitors of p38 in a manner similar to the effects of p38 inhibitors on

arsenic-induced differentiation of NB-4 cells. Thus, it seems that the p38 MAPK pathway is a common negative regulator of cell differentiation of APL cells in response to either As_2O_3 or retinoids.

The p38 MAPK pathway has previously been shown to mediate a wide array of biological responses, including regulation of apoptosis, cell survival, and differentiation (11–13, 40). Interestingly, p38 has previously been shown to mediate both proapoptotic/growth inhibitory (41–44) and antiapoptotic/progrowth (45–47) signals in different systems, apparently depending on the stimulus and cell type involved. Although the precise mechanisms that account for the generation of such differential responses are unknown, one possibility is that they depend on the variable use of distinct isoforms, as there is evidence that different p38 isoforms have common and opposing effects (47). Our data also establish that in different leukemia cell types, inhibition of p38 activation results in enhanced As_2O_3 -dependent JNK kinase activity. Interestingly, previous studies had shown that phosphorylation/activation of JNK by stress-inducing stimuli, such as lipopolysaccharide and sorbitol, is also enhanced by the p38 α/β inhibitor SB203580 (48). Taken together with other studies that have shown that activation of the Mkk4/JNK cascade is essential for the generation of As_2O_3 -dependent apoptosis (27), our findings suggest that a major mechanism by which p38 may impede induction of apoptosis in response to As_2O_3 may involve direct or indirect inhibition of JNK activation.

Our findings also show that pharmacologic inhibition of p38 enhances arsenic-induced Bcl-2 down-regulation and loss of mitochondrial membrane potential in NB-4 APL cells, suggesting that, in this system, p38 up-regulates Bcl-2 expression and generation of antiapoptotic responses. Interestingly, previous studies have shown that sequential activation of Mkk6/p38 β /MAPK-activated protein kinase 3 regulates insulin-like growth factor I-dependent transcriptional induction of Bcl-2 by the nuclear transcription factor cyclic AMP response element-binding protein (45). Other studies have also shown that antisense oligodeoxynucleotides to Bcl-2 increase sensitivity of leukemic cells to As_2O_3 (49). Moreover, arsenic-inducible voltage-dependent anion channel homodimerization, which controls mitochondria membrane potential reduction and cytochrome *c* release, is blocked by Bcl-2 (50). Thus, it is conceivable that the enhanced As_2O_3 -dependent apoptosis seen by pharmacologic inhibition of p38, or in Mkk3/6 and p38 α knockout cells, reflects regulatory effects on Bcl-2 expression, but other mechanisms may be involved as well. Independently of the precise mechanisms involved, our findings have potential therapeutic implications. A major obstacle in the use of As_2O_3 as a therapeutic agent in the treatment of leukemias and solid tumors is the relative resistance of malignant cells to its proapoptotic effects. Our data raise the possibility that combinations of As_2O_3 with pharmacologic inhibitors of p38 may be one approach to overcome As_2O_3 resistance and raise the prospect of clinical-translational development of such combinations for the treatment of leukemias.

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