Combination treatment with arsenic trioxide and phytosphingosine enhances apoptotic cell death in arsenic trioxide–resistant cancer cells

Moon-Taek Park,[1] Young-Hee Kang,[1] In-Chul Park,[2] Chun-Ho Kim,[3] Yun-Sil Lee,[4] Hee Yong Chung,[5] and Su-Jae Lee[1]

[1]Laboratory of Radiation Experimental Therapeutics, [2]Laboratory of Radiation Functional Genomics, [3]Laboratory of Tissue Engineering, and [4]Laboratory of Radiation Effect, Korea Institute of Radiological and Medical Sciences; and [5]Department of Microbiology, College of Medicine, Hanyang University, Seoul, Korea

Abstract

Resistance to anticancer drugs can sometimes be overcome by combination treatment with other therapeutic drugs. Here, we showed that phytosphingosine treatment in combination with arsenic trioxide (As_2O_3) enhanced cell death of naturally As_2O_3-resistant human myeloid leukemia cells. The combination treatment induced an increase in intracellular reactive oxygen species level, mitochondrial relocalization of Bax, poly(ADP-ribose) polymerase-1 (PARP-1) activation, and cytochrome *c* release from the mitochondria. *N*-acetyl-L-cysteine, a thiol-containing antioxidant, completely blocked Bax relocalization, PARP-1 activation, and cytochrome *c* release. Pretreatment of 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2*H*)-isoquinolinone, a PARP-1 inhibitor, or PARP-1/small interfering RNA partially attenuated cytochrome *c* release, whereas the same treatment did not affect Bax relocalization. The combination treatment induced selective activation of p38 mitogen-activated protein kinase (MAPK). Inhibition of p38 MAPK by treatment of SB203580 or expression of dominant-negative forms of p38 MAPK suppressed the combination treatment–induced Bax relocalization but did not affect PARP-1 activation. In addition, antioxidant *N*-acetyl-L-cysteine completely blocked p38 MAPK activation. These results indicate that phytosphingosine in combination with As_2O_3 induces synergistic apoptosis in As_2O_3-resistant leukemia cells through the p38 MAPK–mediated mitochondrial translocation of Bax and the PARP-1 activation, and that p38 MAPK and PARP-1 activations are reactive oxygen species dependent. The molecular mechanism that we elucidated in this study may provide insight into the design of future combination cancer therapies to cells intrinsically less sensitive to As_2O_3 treatment. [Mol Cancer Ther 2007;6(1):82–92]

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Note: M-T. Park and Y-H. Kang contributed equally to this work.

Requests for reprints: Su-Jae Lee, Laboratory of Experimental Therapeutics, Korea Institute of Radiological and Medical Sciences, 215-4 Gongneung-Dong, Nowon-Ku, Seoul 139-706, Korea. Phone: 82-2-970-1324; Fax: 82-2-970-2402. E-mail: sjlee@kcch.re.kr

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Introduction

Arsenic agents have been successfully employed in treatment of acute promyelocytic leukemia (APL), and its efficacy has been confirmed even in patients resistant to conventional chemotherapy (1, 2). It has become evident that apoptotic effects of arsenic trioxide (As_2O_3) are not only restricted to APL cells but can also be observed in other malignant cells, including non-APL acute myeloid leukemia cells, myeloma cells, and chronic myeloid leukemia cells, as well as in various solid tumor cells (3–5).

Recent studies showed that clinically achievable concentration of As_2O_3 (1–2 μmol/L) induces apoptosis through a reactive oxygen species (ROS)–dependent pathway in APL-derived NB4 cells. Accumulation of intracellular ROS by As_2O_3 led to dissipation of the mitochondrial membrane potential, release of cytochrome *c* from mitochondria, subsequent activation of the caspases, and ultimately to apoptotic cell death (6, 7). Arsenic disturbs the natural oxidation and reduction equilibrium in cells via various mechanisms, which involve complex reduction/oxidation reactions with endogenous oxidants and cellular antioxidant systems. Recently, it has been shown that arsenic exposure leads to an increase of ROS by activation of the NADPH oxidase (8–10). The intracellular glutathione reduction/oxidation system also has been proposed to be responsible for the As_2O_3 sensitivity. Compared with other leukemia cells that are less sensitive to As_2O_3, APL-derived NB4 cells contain lower level of the glutathione peroxidase and catalase and relatively higher levels of the intracellular hydrogen peroxide (H_2O_2; ref. 11). In certain APL cells, As_2O_3 decreases glutathione and increases intracellular ROS level (12).

The proapoptotic proteins Bax, Bid, and Bad, which reside in the cytosol, translocate to mitochondrial outer membrane when triggered by certain stimuli. The translocation of Bax from the cytoplasm to the mitochondria is both necessary and sufficient to induce apoptotic cell death (13–15). Bax reduces the mitochondrial membrane potential, causes the release of cytochrome *c* from the mitochondria, and activates caspases (16–20). Recent observations show that p38 mitogen-activated protein kinase (MAPK)

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induces apoptosis by regulating the translocation of Bax from the cytoplasm to the mitochondria in response to various stimuli (21–23). These results are consistent with the fact that p38 MAPK acts at an early step before dysfunction of mitochondria and caspase activation in several cell types (23–25).

Poly(ADP-ribose) polymerase (PARP-1) is a nuclear enzyme that catalyzes covalent attachment of long branched chains of poly(ADP-ribose), with NAD^+ as its substrate to a variety of nuclear DNA-binding proteins, including PARP-1 itself, and facilitates DNA repair in response to genomic DNA damage (26, 27). In several pathologic situations that involve massive DNA damage, excessive activation of PARP-1 depletes the cellular reserve of both NAD and its precursor ATP, leading to cell death via energy failure (28, 29). Recently, it has been shown that ROS-mediated DNA damage triggers activation of the PARP-1 and subsequent cell death (30, 31). Although PARP-1–mediated cell death is thought to be necrotic (32, 33), recent reports have shown that PARP-1–mediated cell death also has many features in common with the apoptotic forms of cell death (30, 31, 34, 35).

Sphingolipid metabolites, such as ceramides, sphingosines, and phytosphingosine, have been implicated as an important component of some anticancer drug–induced apoptosis of human cancer cells (36, 37). This apoptotic pathway is initiated by hydrolysis of sphingomyelin, a membrane lipid, attributable to the activation of sphingomyelinases to generate ceramide. Ceramide, in turn, can activate several pathways important for the induction of apoptosis (38). Moreover, direct exposure of cells to the cell-permeable ceramide analogues also has been shown to sensitize cancer cells to some anticancer drug and ionizing radiation, which lends further support to the notion that ceramide generation might be an important step for some anticancer drug–induced apoptosis in human cancer cells (30, 39, 40). Although many reports emphasized the contributions of ceramide in anticancer drug–induced cell death, the role of other sphingolipid metabolites in the modulation of anticancer drug sensitivity and their precise action mechanisms were largely unknown.

In this study, we investigate the mechanisms underlying synergistic enhancement of As_2O_3-induced apoptotic cell death by phytosphingosine in intrinsically As_2O_3-resistant human myeloid leukemia cells. We show that enhancement of cell death of As_2O_3-resistant cancer cells by phytosphingosine in combination with As_2O_3 is mediated by the p38 MAPK–mediated Bax relocalization and PARP-1 activation via intracellular ROS level increase.

Materials and Methods

Materials

As_2O_3 was purchased from Sigma (St. Louis, MO). Phytosphingosine was purchased from Doo-San Bio-Tech (Seoul, Korea). Antibodies specific for polyclonal anti-caspase-9, Bcl2, Bax, Bid, heat shock protein 60, phosphorylated c-Jun NH_2-terminal kinase, c-Jun NH_2-terminal kinase, p38, and α-tubulin and monoclonal anti–phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) and ERK were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody specific for polyclonal anti-β-actin was from Sigma. Monoclonal antibody to cytochrome *c* was obtained from PharMingen (San Diego, CA). Polyclonal antibodies to cleaved caspase-3, PARP, and phosphorylated p38 were from Cell Signaling Technology (Beverly, MA). Polyclonal anti-PAR antibody was from Calbiochem (Cambridge, MA). The specific PARP inhibitor 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2*H*)-isoquinolinone (DPQ) was from Calbiochem. The caspase-3 inhibitor z-DEVD-fmk and the caspase-9 inhibitor z-LEHD-fmk were from Calbiochem. Other chemicals were obtained from Calbiochem.

Cell Culture and Transfection

Human leukemia cell line U937, human multiple myeloma cell line IM9, human chronic myelogenous leukemia cell line K562, and human myeloma cell line U266B1 were obtained from the American Type Culture Collection (Rockville, MD); grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin; and incubated at 37°C in 5% CO_2. The method of constructing the MFG retroviral vector by replacing the green fluorescent protein (GFP) sequence of MFG.GFP.IRES.puro was also used to construct MFG-DN-p38 MAPK puro. The MFG.GFP.IRES.puro itself was used as a negative control throughout the experiment. The retroviral plasmids were introduced into the 293gpg retrovirus packaging cell line by transient transfection with LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). After 72 h, the supernatants were harvested and used for retroviral infection. The virus titers, measured in NIH3T3 cell line by puromycin-resistant colony formation, were between 10^5 and 5×10^5 per mL. Target cells were infected with 1 mL of MFG-DN-p38 supernatants in the presence of 8 μg/mL polybrene (Sigma Chemical Co., St. Louis, MO).

Small Interfering RNA Transfection

RNA interferences of Bax or PARP-1 were done using 21-bp (including a 2-deoxynucleotide overhang) small interfering RNA (siRNA) duplexes purchased from Ambion (Austin, TX). The sense strand nucleotide sequence for PARP-1 siRNA was GGCCAGGAUGGAAUUGGUAdTdT. The coding strand for Bax siRNA was AACATGGAGCTGCAGAGGATGAdTdT. A control siRNA specific to the GFP DNA sequence CCACTACCTGAGCACCCAG was used as a negative control. For transfection, U937 cells were seeded in six-well plates at 30% confluency, and siRNA duplexes (200 nmol/L) were introduced into the cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's recommendations. Assays were done 48 h after transfection. The protein levels of Bax and PARP-1 were detected in the cell lysate by Western blot.

Quantification of Cell Death

For the cell death assessment, the cells were plated in six-well plates with cell density of 1×10^5 per well, and then As_2O_3 and phytosphingosine were treated. At indicated time points, cells were centrifuged, washed in PBS, and then incubated in propidium iodide (2.5 mg/mL) for 5 min

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at room temperature and analyzed with a flow cytometer. When necessary, *N*-acetyl-L-cysteine (NAC), specific PARP inhibitor DPQ (50 μmol/L), or pan-caspase inhibitor z-VAD-fmk (30 μmol/L) and caspase-3 inhibitor z-DEVD-fmk (30 μmol/L) were applied 30 min before As_2O_3 and phytosphingosine treatments and kept in the medium during and after As_2O_3 and phytosphingosine treatments, until the cells were analyzed.

Clonogenic Survival Assay in Soft Agar

Cells were plated onto 60-mm dishes at a density of 2×10^6 per dish and exposed to a range of doses of radiation at 0 to 3 Gy. After irradiation, cells were harvested, and 10^4 cells per dish were suspended in 2 mL of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 2 mL of 0.8% agar-medium base layer in 60-mm dishes and followed by incubation for 14 days at 37°C in 5% CO_2 incubator. Before counting colonies, the culture medium was decanted, and the cells were fixed in 95% methanol and stained with 0.5% crystal violet, and the numbers of colonies (>50 cells) from triplicate dishes were counted. Mean colony numbers relative to unirradiated colony numbers were plotted.

Measurement of Mitochondrial Membrane Potential and ROS Generation

The measurement of mitochondrial membrane potential ($\Delta\Psi_m$) and ROS generation were done as described by Chen et al. (10). Briefly, cells (5×10^5 per mL) were exposed to 2 μmol/L As_2O_3 and 3 μg/mL phytosphingosine for the indicated times. After exposure, cells were incubated in 30 nmol/L 3,3′-dihexyloxacarboxyanine iodide and 10 μmol/L DCFH-DA (Molecular Probes, Eugene, OR) at 37°C for 30 min and harvested by trypsinization and washed with cold PBS solution for three times. ROS and $\Delta\Psi_m$ were determined by fluorescence-activated cell sorting analysis.

Confocal Microscopy

U937 cells treated with As_2O_3 and phytosphingosine were washed twice with ice-cold PBS before fixation with ice-cold methanol. After blocking with 2% bovine serum albumin in PBS containing 0.2% Triton X-100, cells were incubated with the primary antibody against Bax for 1 h. Cells were washed with blocking solution three times and incubated with the secondary antibody conjugated with FITC (Molecular Probes) for 1 h. Nuclei were stained with propidium iodide (Sigma) for 10 min after secondary antibody incubation and two rinses with PBS. After washing three times with PBS, coverslips were mounted onto microscope slides using ProLong antifade mounting reagent (Molecular Probes). The slides were analyzed by a confocal laser-scanning microscope (Leica Microsystems, Bannockburn, IL).

Western Blot Analysis

Western blot analysis was done as described (22). Briefly, cell lysates were prepared by extracting proteins with lysis buffer [40 mmol/L Tris-HCl (pH 8), 120 mmol/L NaCl, 0.1% NP40] supplemented with protease inhibitors. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA). The membranes were blocked with 5% nonfat dry milk in TBS and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence procedures (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's recommendations.

Preparation of Cytosolic and Mitochondrial Fractions for Cytochrome *c* and bax Translocation Measurement

Cells were collected and washed twice in ice-cold PBS, resuspended in S-100 buffer [20 mmol/L HEPES (pH 7.5), 10 mmol/L KCl, 1.9 mmol/L $MgCl_2$, 1 mmol/L EGTA, 1 mmol/L EDTA, mixture of protease inhibitors], and incubated on ice for 20 min. After a 20-min incubation on ice, the cells were homogenized with a Dounce glass homogenizer and a loose pestle (Wheaton, Millville, NJ) for 70 strokes. Cell homogenates were spun at $1,000 \times g$ to remove unbroken cells, nuclei, and heavy membranes. The supernatant was re-spun at $14,000 \times g$ for 30 min to collect the mitochondria-rich (the pellet) and the cytosolic (the supernatant) fractions. The mitochondria-rich fraction was washed once with the extraction buffer, followed by a final resuspension in lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 1 mmol/L EGTA] containing protease inhibitors for Western blot analysis.

Cellular ELISA Method for Detection of PARP-1 Activation

PARP enzyme activity was measured using a commercial kit (Oncogene, San Diego, CA). Briefly, cell extracts were incubated in reaction buffer (56 mmol/L HEPES, 28 mmol/L KCl, 28 mmol/L NaCl, 2 mmol/L $MgCl_2$) containing 0.01% digitonin and 10 μmol/L biotinylated NAD^+. After incubating at 37°C for 30 min, reactions were developed with TACS-Saphire (Trevigen, Gaithersburg, MD) substrate (100 μL per well). The absorbance was measured with a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Data were expressed as mean ± SD of quadruplicate samples.

Results

We first determined the As_2O_3 response in various human myeloma cell lines. As shown in Fig. 1A, clonogenic survival assay revealed that U937 and K562 cells showed resistance to AS_2O_3 compare with IM9 and U266B1 cells. We next investigated whether the As_2O_3-resistant cells that showed an increased clonogenic survival following As_2O_3 also had resistance to the As_2O_3-induced apoptotic cell death. To examine As_2O_3-induced apoptosis, the cells were treated with 2 μmol/L As_2O_3 and were stained with Hoechst 33258 at individual times. As_2O_3 effectively killed IM9 and U266B1 cells but failed to induce cell death in U937 and K562 cells after As_2O_3 treatment (Fig. 1B). We further examined whether phytosphingosine treatment in combination with As_2O_3 had a sensitizing effect on cell death in As_2O_3-resistant U937 and K562 cells. As shown in

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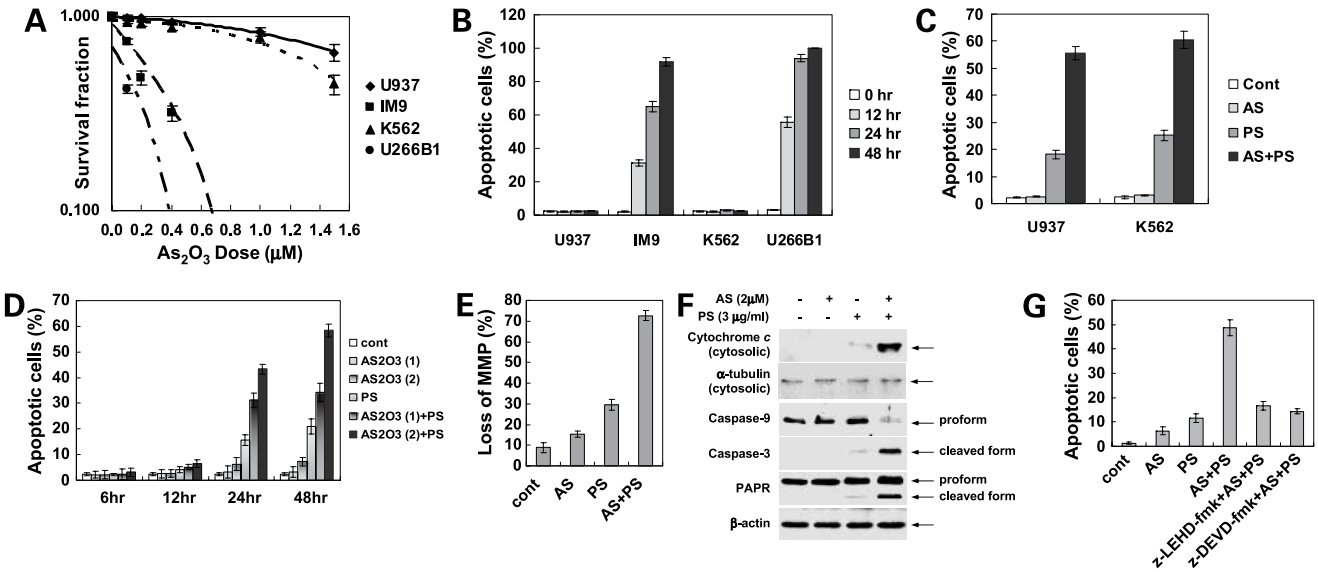

Figure 1. Phytosphingosine in combination with As_2O_3 enhances apoptotic cell death in As_2O_3-resistant cancer cells. **A,** human myeloma cell lines U937, IM9, K562, and U266B1 were treated with increasing dose of As_2O_3. Cells were allowed to grow on soft agar for 15 to 20 d, stained with 0.5% crystal violet, and scored for colony formation. *Points,* mean of three independent experiments; *bars,* SE. **B,** human myeloma cell lines were treated with 2 μmol/L As_2O_3 and incubated for individual times. After 12, 24, and 48 h, cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. *Columns,* mean of three independent experiments; *bars,* SE. **C,** As_2O_3-resistant U937 and K562 cells were treated with 2 μmol/L As_2O_3 alone (*AS*), 3 μg/mL phytosphingosine alone (*PS*), or combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL; *AS + PS*). After 48 h, cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. *Columns,* mean of three independent experiments; *bars,* SE. **D,** phytosphingosine sensitizes As_2O_3-resistant U937 leukemia cells to As_2O_3-induced apoptotic cell death. As_2O_3-resistant U937 cells were treated with 1 or 2 μmol/L As_2O_3 alone, 3 μg/mL phytosphingosine alone, or combination of As_2O_3 (1 or 2 μmol/L) and phytosphingosine (3 μg/mL). After 6, 12, 24, and 48 h, cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. *Columns,* mean of three independent experiments; *bars,* SE. **E,** U937 cells were treated with 2 μmol/L As_2O_3 alone, 3 μg/mL phytosphingosine alone, or combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL). After 48 h, mitochondrial transmembrane potential (*MMP*) was determined by retention of 3,3′-dihexyloxacarboxyanine iodide added during the last 30 min of treatment. After removal of the medium, the amounts of retained 3,3′-dihexyloxacarboxyanine iodide were measured by flow cytometry. *Columns,* mean of three independent experiments; *bars,* SE. **F,** cells were treated with 2 μmol/L As_2O_3 alone, 3 μg/mL phytosphingosine alone, or combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL). After 24 h, cytosolic fraction was obtained and was subjected to Western blot analysis with anti–cytochrome *c* and anti-α-tubulin antibodies. Total cell extract was subjected to Western blot analysis with anti-caspase-9, anti-caspase-3, anti-PARP, and anti-β-actin antibodies. α-Tubulin and β-actin were used as a cytosolic marker protein and a loading control, respectively. Typical experiment conducted three times with similar results. **G,** cells were treated with 2 μmol/L As_2O_3 alone, 3 μg/mL phytosphingosine alone, or combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL) in the presence of caspase-9–specific inhibitor z-LEHD-fmk (30 μmol/L) or caspase-3–specific inhibitor z-DEVD-fmk (30 μmol/L). After 24 h, cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. *Columns,* mean of three independent experiments; *bars,* SE.

Fig. 1C, the combination treatment indeed synergistically enhanced the apoptotic cell death of As_2O_3-resistant U937 and K562 cells. In addition, the combination treatment synergistically enhanced the cell death of U937 cells in dose- and time-dependent manner (Fig. 1D). However, treatment of cells with As_2O_3 alone (1 or 2 μmol/L) did not show any effect on cell death. In addition, phytosphingosine treatment alone (3 μg/mL) showed a subtle cytotoxic effect. To determine whether the mitochondrial pathway is involved in induction of the apoptotic cell death seen after combination treatment, we examined changes in mitochondrial membrane potential and cytochrome *c* release from the mitochondria into the cytosol after combination treatment. Phytosphingosine in combination with As_2O_3 significantly disrupted the mitochondrial membrane potential (Fig. 1E). At the same time, level of the cytosolic cytochrome *c* was markedly increased (Fig. 1F), coinciding with changes in the mitochondrial membrane potential. Combination treatment with phytosphingosine and As_2O_3 also caused activation of caspase-9 and caspase-3 and cleavage of PARP (Fig. 1F). However, we failed to detect caspase-8 and Bid cleavage (data not shown). Moreover, pretreatment of a caspase-9–specific inhibitor z-LEHD-fmk or a caspase-3–specific inhibitor z-DEVD-fmk completely attenuated the combination treatment-induced apoptotic cell death (Fig. 1G). These results indicate that phytosphingosine treatment in combination with As_2O_3 induces synergistic apoptotic cell death through mitochondrial dysfunction–dependent manner.

Because it has been shown that translocation of Bax from the cytosol to the mitochondria causes loss of the mitochondrial membrane potential (16, 18, 41), we investigated whether the combination treatment with As_2O_3 and phytosphingosine induces mitochondrial translocation of Bax. As shown in Fig. 2A, the combination treatment redistributed Bax from cytosol to the mitochondria without changing the protein expression levels of Bcl-2 and Bax (Fig. 2A). Confocal microscopy also clearly revealed that Bax was translocated to the mitochondria (Fig. 2B). Furthermore, siRNA targeting of Bax effectively attenuated

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combination treatment-induced mitochondrial membrane potential loss and cell death (Fig. 2C and D). We also showed that siRNA targeting of Bax effectively inhibited cytochrome *c* release induced by the combination treatment (Fig. 2E). These results suggest that combination treatment with As_2O_3 and phytosphingosine induces Bax redistribution to mitochondria and subsequently promotes the loss of mitochondrial membrane potential and cytochrome *c* release.

To investigate a potential involvement of MAPK in combination treatment-induced apoptotic cell death, we first analyzed the activation status of ERK, c-Jun NH_2-terminal kinase, and p38 MAPK. As shown in Fig. 3A, combination treatment of cells with As_2O_3 and phytosphingosine resulted in a dramatic increase of the phosphorylated form of p38 MAPK, indicating its activation in human leukemia cells, whereas the phosphorylated ERK1/2 was down-regulated. However, level of the c-Jun NH_2-terminal kinase did not alter over the time course examined. The total expression level of MAPKs also remained constant. These results suggest that combination treatment with As_2O_3 and phytosphingosine can selectively induce activation of p38 MAPK during the apoptotic process of human leukemia cells. Pretreatment of these cells with SB203580, p38 MAPK–specific inhibitor, or forced expression of a dominant-negative form of p38 MAPK markedly suppressed combination treatment–induced apoptotic cell death (Fig. 3B). However, treatment of PD98059, a specific inhibitor of the MAP/ERK kinase/ERK pathway or overexpression of ERK, failed to show any changes in

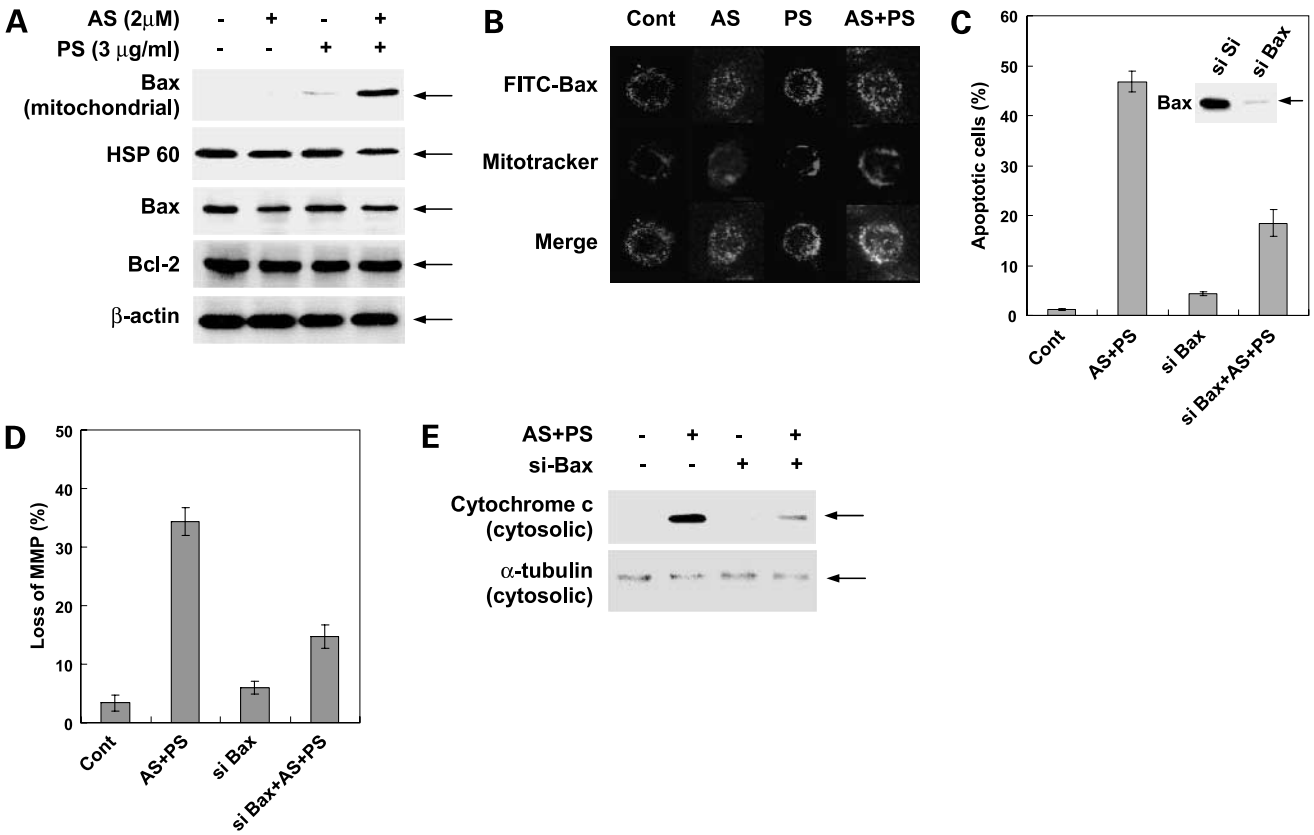

Figure 2. Combination treatment of As_2O_3 with phytosphingosine enhances Bax translocation to mitochondria. **A,** U937 cells were treated with combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL). After 24 h, mitochondrial fraction was obtained and subjected to Western blot analysis with anti-Bax and anti–heat shock protein 60 (*HSP60*) antibodies. Total cell extract was subjected to Western blot analysis with anti-Bax, anti-Bcl-2, and anti-β-actin antibodies. Heat shock protein 60 and β-actin were used as a mitochondrial marker protein and a loading control, respectively. Typical experiment conducted three times with similar results. **B,** representative confocal images for translocation of Bax to the mitochondria in response to the combination treatment. The mitochondrial translocation (*yellow color*) of Bax is shown by the overlap of Bax (*green*) and mitochondrial staining with Mitotracker (*red*). Typical experiment conducted three times with similar results. **C,** siRNA targeting of Bax attenuates the combination treatment–induced cell death. U937 cells transfected with Bax siRNA were treated with combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL). After 24 h, cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. *Columns,* mean of three independent experiments; *bars,* SE. **D,** cells transfected with Bax siRNA were treated with combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL). After 24 h, mitochondrial membrane potential of these cells was determined by retention of 3,3′-dihexyloxacarboxyanine iodide added during the last 30 min of treatment. After removal of the medium, the amount of retained 3,3′-dihexyloxacarboxyanine iodide were measured by flow cytometry. **E,** after 24 h, cytosolic fraction was obtained and subjected to Western blot analysis with anti–cytochrome *c* and anti-α-tubulin antibodies. α-Tubulin was used as a cytosolic marker protein. Typical experiment conducted three times with similar results.

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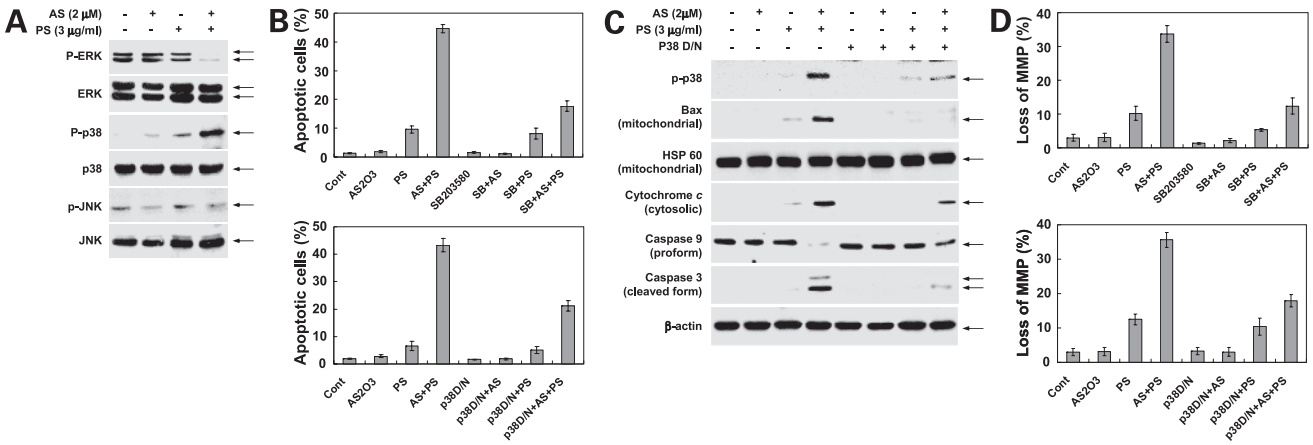

Figure 3. p38 MAPK activation is critical event for the combination treatment–induced Bax relocalization to the mitochondria. **A,** U937 cells were treated with 2 μmol/L As_2O_3 alone, 3 μg/mL phytosphingosine alone, or combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL). After 24 h, total cell extracts were subjected to Western blot analysis with anti–phosphorylated ERK (*P-ERK*), anti–phosphorylated p38 MAPK (*P-p38*), or anti–phosphorylated c-Jun NH_2-terminal kinase (*p-JNK*) antibodies, or anti-ERK, anti–p38 MAPK, or anti–c-Jun NH_2-terminal kinase antibodies. Typical experiment conducted three times with similar results. **B,** U937 cells were treated with combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL) in the presence or absence of 10 μmol/L SB203580 (*top*) or dominant-negative form of p38 MAPK (*p38 D/N*; *bottom*). After 24 h, cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. *Columns,* mean of three independent experiments; *bars,* SE. **C,** U937 cells were treated with combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL) in the presence or absence of the dominant-negative form of p38 MAPK. After 24 h, total cell extract was subjected to Western blot analysis with anti–p38 MAPK, anti-caspase-9, anti-caspase-3, and anti-β-actin antibodies. Mitochondrial fraction was obtained and subjected to Western blot analysis with anti-Bax and anti–heat shock protein 60 antibodies. Cytosolic fraction was subjected to Western blot analysis with anti–cytochrome *c* and anti-α-tubulin antibodies. α-Tubulin was used as a cytosolic marker protein. Heat shock protein 60 and β-actin were used as a mitochondrial marker protein and a loading control, respectively. Typical experiment conducted three times with similar results. **D,** U937 cells were treated with combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL) in the presence or absence of 10 μmol/L SB203580 (*top*) or the dominant-negative form of p38 MAPK (*bottom*). After 24 h, mitochondrial transmembrane potential was determined by retention of 3,3′-dihexyloxacarboxyanine iodide added during the last 30 min of treatment. After removal of the medium, the amounts of retained 3,3′-dihexyloxacarboxyanine iodide were measured by flow cytometry. *Columns,* mean of three independent experiments; *bars,* SE.

combination treatment-induced cell death (data not shown). Furthermore, pretreatment of SB203580 or expression of a dominant-negative form of p38 MAPK suppressed the combination treatment–induced mitochondrial translocation of Bax in U937 cells (Fig. 3C). Inhibition of p38 MAPK also efficiently blocked the combination treatment–induced mitochondrial membrane potential loss (Fig. 3D), cytochrome *c* release, and caspase-9 and caspase-3 activations (Fig. 3C). These results suggest that p38 MAPK acts as an important mediator of the Bax relocalization from the cytosol to the mitochondria during combination treatment–induced apoptotic cell death.

Both sphingolipid metabolites and As_2O_3 have been implicated in intracellular ROS accumulation, which, in turn, can activate several pathways important for the induction of apoptosis (23, 30, 35). We next examined involvement of ROS in the combination treatment–induced cell death of As_2O_3-resistant cells. As shown in Fig. 4A, ROS levels were dramatically increased after the combination treatment with As_2O_3 and phytosphingosine in As_2O_3-resistant U937 cells, and the increase in ROS level was effectively blocked by antioxidant NAC. Treatment of As_2O_3 or phytosphingosine alone failed to produce ROS in naturally As_2O_3-resistant U937 (Fig. 4A) and K562 (Fig. S1A)[6] cell lines in response to the As_2O_3. However, As_2O_3 alone markedly induced ROS generation in relatively As_2O_3-sensitive IM9 or U266B1 cell lines (Fig. S1B),[6] and antioxidant NAC efficiently suppressed As_2O_3-induced apoptotic cell death in IM9 and U266B1 cells (Fig. S1C),[6] suggesting that ROS generation might be an important factor to determine As_2O_3 sensitivity in human leukemia cells. To further examine the direct relationship between the increased intracellular ROS level and mitochondrial activation–mediated cell death pathway, cells were pretreated with NAC before the combination treatment. Pretreatment of NAC significantly attenuated the combination treatment–induced loss of the mitochondrial membrane potential (Fig. 4B), cytochrome *c* release, caspase-9 and caspase-3 activations (Fig. 4C), and apoptotic cell death (Fig. 4D), suggesting that ROS plays a crucial role in enhancement of As_2O_3-induced cell death by phytosphingosine. In addition, we found that pretreatment of rotenone, a mitochondrial ROS blocker, completely attenuated the combination treatment–induced ROS generation in U937 cells (Fig. S1D),[6] but pretreatment of DPI, a NADPH oxidase inhibitor, did not alter the ROS level (Fig. S1D),[6] suggesting that ROS generation in response to the combination treatment in naturally As_2O_3-resistant U937 cells is mitochondria dependent.

We next examined relationship between ROS and p38 MAPK activation in response to the combination treatment. We found that NAC effectively blocked p38 MAPK activation and mitochondrial translocation of Bax induced

[6] Supplementary material for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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by combination treatment with As_2O_3 and phytosphingosine in As_2O_3-resistant cells (Fig. 4E). However, inhibition of p38 MAPK with dominant-negative forms of p38 MAPK did not affect the combination treatment–induced ROS generation (Fig. 4F). These observations suggest that ROS contributes to p38 MAPK activation and subsequent mitochondrial cell death in response to the combination treatment.

Because it has been shown that ROS-mediated DNA damage triggers activation of the PARP-1 and subsequent cell death (30, 31), we examined whether PARP-1 is involved in the combination treatment–induced apoptotic cell death in As_2O_3-resistant cancer cells. Exposure of U937 cells to phytosphingosine in combination with As_2O_3 induced a marked activation of PARP-1 (Fig. 5A). Pretreatment of cells with the PARP-1–specific inhibitor DPQ attenuated the combination treatment–induced cell death as well as PARP-1 activation (Fig. 5B and C). Furthermore, DPQ efficiently blocked loss of the mitochondrial membrane potential (Fig. 5D), cytochrome *c* release, and caspase-9 and caspase-3 activations induced by the combination treatment (Fig. 5E). However, inhibition of PARP-1 by DPQ did not affect the combination treatment–induced ROS generation (Fig. 5F), whereas thiol-containing antioxidant NAC completely blocked PARP-1 activation (Fig. 5G), indicating that PARP-1 activation is downstream of the increased ROS generation after phytosphingosine treatment in combination with As_2O_3. In addition, the PARP-1 inhibitor DPQ did not affect the combination treatment–induced p38 MAPK activation and Bax translocation (Fig. S2A);[6] conversely, inhibition of p38 MAPK with expression of a dominant-negative form of p38 MAPK has no effect on the combination treatment–induced PARP-1 activation (Fig. S2B).[6] These results indicate that PARP-1 is involved in the mitochondrial cell death in response to combination treatment, and that PARP-1 activation is ROS dependent but p38 MAPK independent.

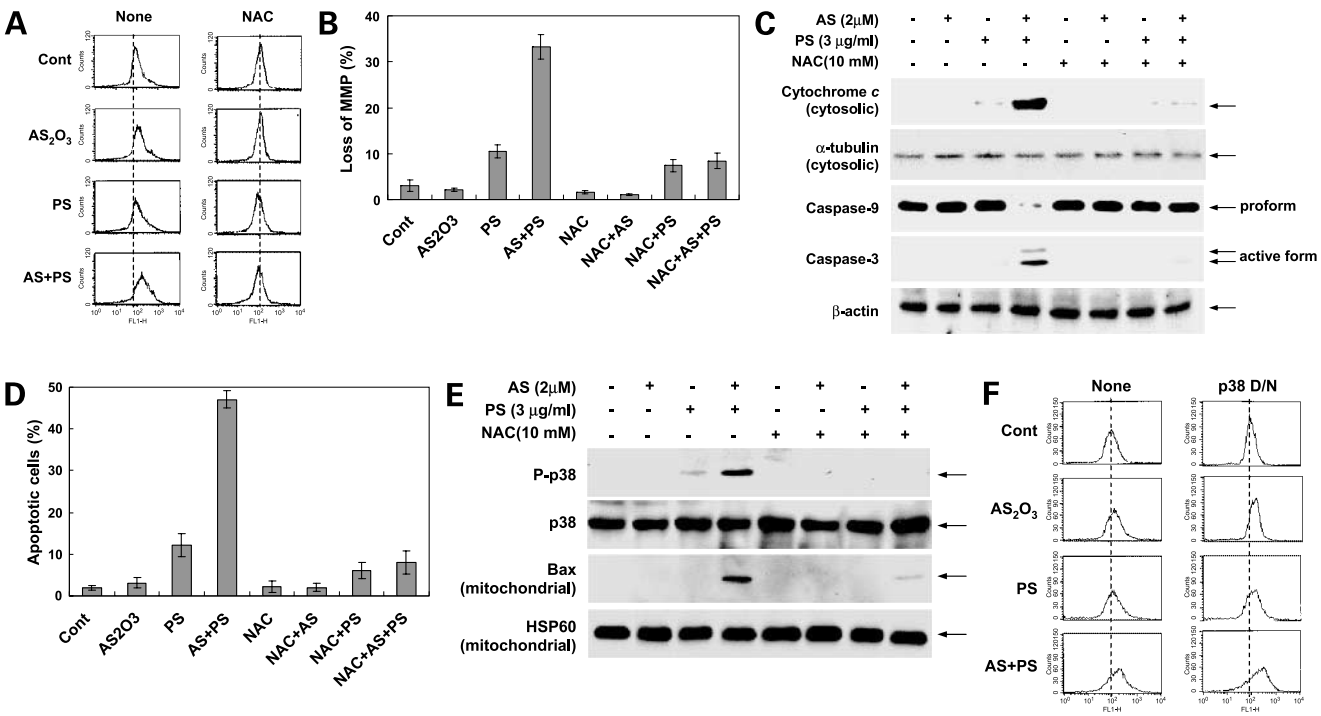

Figure 4. The combination treatment–induced apoptotic cell death is dependent on intracellular ROS level increase. U937 cells were treated with 2 μmol/L As_2O_3 alone, 3 μg/mL phytosphingosine alone, or combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL) in the presence or absence of NAC (10 mmol/L). **A,** elevation of the intracellular ROS level after the combination treatment. After 24 h, cells were incubated in 10 μmol/L DCFH-DA at 37°C during the last 30 min of treatment. The cells were harvested and washed with cold PBS solution three times, and the amount of fluorescence was measured using flow cytometry. Typical experiment conducted three times with similar results. **B,** after 24 h, mitochondrial transmembrane potential was determined by retention of 3,3′-dihexyloxacarboxyanine iodide added during the last 30 min of treatment. After removal of the medium, the amounts of retained 3,3′-dihexyloxacarboxyanine iodide were measured by flow cytometry. *Columns,* mean of three independent experiments; *bars,* SE. **C,** after 24 h, cytosolic fraction was obtained and subjected to Western blot analysis with anti–cytochrome *c* and anti-α-tubulin antibodies. Total cell extract was subjected to Western blot analysis with anti-caspase-9, anti-caspase-3, and anti-β-actin antibodies. α-Tubulin and β-actin were used as a cytosolic marker protein and a loading control, respectively. Typical experiment conducted three times with similar results. **D,** cells were treated with combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL) in the presence or absence of 10 mmol/L NAC. After 24 h, cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. *Columns,* mean of three independent experiments; *bars,* SE. **E,** after 24 h, total cell extract was subjected to Western blot analysis with anti–phosphorylated p38 MAPK and anti–p38 MAPK antibodies. Mitochondrial fraction was obtained and subjected to Western blot analysis with anti-Bax and anti–heat shock protein 60 antibodies. Heat shock protein 60 was used as a mitochondrial marker protein. Typical experiment conducted three times with similar results. **F,** cells were treated with combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL) in the presence or absence of the dominant-negative form of p38 MAPK. After 24 h, cells were incubated with 10 μmol/L DCFH-DA for 30 min and analyzed by flow cytometry to measure intracellular ROS level. Typical experiment conducted three times with similar results.

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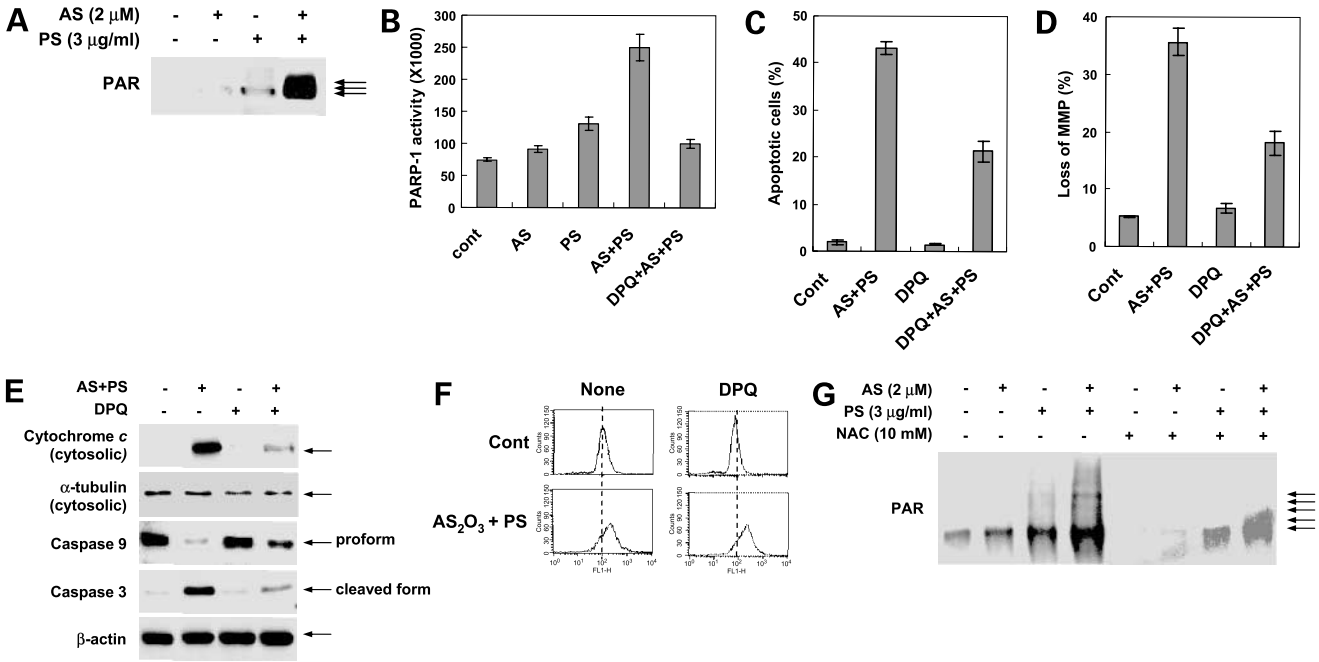

Figure 5. PARP-1 activation is required for the combination treatment – induced apoptotic cell death. **A,** analysis of PARP-1 activation in U937 cells after combination treatment with As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL). After 24 h, total cell extract was subjected to Western blot analysis with anti – poly(ADP-ribose) (*PAR*) antibody. **B,** U937 cells were treated with combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL) in the presence or absence of DPQ (50 μmol/L). After 24 h, PARP enzyme activity was measured using a commercial kit under guidance of manufacturer (see Materials and Methods). *Columns,* mean of three independent experiments; *bars,* SE. **C,** cells were treated with combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL) in the presence or absence of DPQ (50 μmol/L). After 24 h, cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. *Columns,* mean of three independent experiments; *bars,* SE. **D,** after 24 h, mitochondrial transmembrane potential was determined by retention of 3,3′-dihexyloxacarboxyanine iodide added during the last 30 min of treatment. After removal of the medium, the amounts of retained 3,3′-dihexyloxacarboxyanine iodide were measured by flow cytometry. *Columns,* mean of three independent experiments; *bars,* SE. **E,** after 24 h, cytosolic fraction was obtained and subjected to Western blot analysis with anti – cytochrome *c* and anti-α-tubulin antibodies. Total cell extract was subjected to Western blot analysis with anti-caspase-9, anti-caspase-3, and anti-β-actin antibodies. α-Tubulin and β-actin were used as a cytosolic marker protein and a loading control, respectively. Typical experiment conducted three times with similar results. **F,** U937 cells were treated with combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL) in the presence or absence of DPQ (50 μmol/L). After 24 h, cells were incubated in 10 μmol/L DCFH-DA at 37°C during the last 30 min of treatment. The cells were harvested and washed with cold PBS solution three times, and the amount of fluorescence was measured using flow cytometry. Typical experiment conducted three times with similar results. **G,** U937 cells were treated with combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL) in the presence or absence of NAC (10 mmol/L). After 24 h, total cell extract was subjected to Western blot analysis with anti – poly(ADP-ribose) antibody. Typical experiment conducted three times with similar results.

Taken together, these results indicate that phytosphingosine in combination with As_2O_3 induces synergistic apoptotic cell death, and that p38 MAPK–dependent mitochondrial translocation of Bax and p38 MAPK–independent PARP-1 activation are two separate pathways that induces mitochondrial cell death. To confirm the hypothesis that combination treatment with As_2O_3 and phytosphingosine uses two separate pathways to induce cell death in the As_2O_3-resistant U937 cells, we treated cells transfected with PARP-1 siRNA with the p38 MAPK inhibitor SB203580 before treatment with As_2O_3 and phytosphingosine. As shown in Fig. 6, the cells simultaneously treated with PARP-1 siRNA and SB203580 show more dramatic attenuation of the apoptotic cell death than the cells treated with either reagent alone.

Discussion

Resistance to anticancer drugs can sometimes be overcome by combination treatment with other therapeutic drugs. The aim of our investigation was to evaluate whether phytosphingosine was able to enhance As_2O_3-induced apoptotic cell death in naturally As_2O_3-resistant human cancer cells. We found that phytosphingosine treatment in combination with As_2O_3 dramatically enhanced apoptotic cell death of As_2O_3-resistant human myeloid leukemia cells through the p38 MAPK activation–mediated mitochondrial translocation of Bax and the PARP-1 activation that induces mitochondrial dysfunction, and that p38 MAPK and PARP-1 activations are ROS dependent.

Despite treatment of cells with As_2O_3 (1 or 2 μmol/L) or phytosphingosine alone (3 μmol/L) showed a subtle cytotoxic effect, combination treatment with As_2O_3 and phytosphingosine synergistically induced the apoptotic cell death of naturally As_2O_3-resistant U937 cells. The combination treatment also strongly enhanced disruption of the mitochondrial membrane potential, cytochrome *c* release, and caspase-9 and caspase-3 activations but did not caused any changes in caspase-8 and Bid (data not shown). These results suggest that the combination treatment enhances

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mitochondrial activation–mediated apoptotic cell death in U937 cells. In response to certain stimuli that require mitochondria-dependent pathway for apoptosis, Bax become activated, translocated to the outer membrane of mitochondria, and oligomerized therein (42). The mitochondrial membrane potential loss and the release of mitochondrial apoptogenic molecules into the cytosol ensue. We also found that combination treatment with As_2O_3 and phytosphingosine redistributed Bax from cytosol to the mitochondria, and that siRNA targeting of Bax effectively attenuated mitochondrial membrane potential loss and cytochrome *c* release to the cytosol, suggesting that mitochondrial redistribution of Bax may trigger dissipation of the mitochondrial membrane potential and cause subsequent cytochrome *c* release to the cytosol following the combined treatment with As_2O_3 and phytosphingosine.

It has been reported that p38 MAPK is positively implicated in induction of apoptosis in response to various stimuli (43–46). Consistent with these findings, we also found that the p38 MAPK pathway plays an important role in the combination treatment–induced apoptotic cell death. The rapid phosphorylation of p38 MAPK after the combination treatment suggests that activation of p38 MAPK may play a key role in the early events of the combination treatment–induced cell death. This conclusion is further supported by observations made in this study of the role of p38 MAPK in apoptotic regulatory events. We showed that inhibition of p38 MAPK completely blocked Bax translocation and effectively attenuated mitochondrial membrane potential loss and subsequent cell death induced by the combination treatment, suggesting that p38 MAPK is involved in induction of cell death, and that it acts at a step before mitochondrial dysfunction–mediated apoptotic cell death. However, as the mitochondrial membrane potential loss and apoptotic cell death induced by the combination treatment are not fully inhibited by p38 MAPK inhibition or Bax-siRNA targeting, it is possible that undefined signals other than p38 MAPK-Bax signaling also play an important role in combination treatment–induced cytochrome *c* release and subsequent cell death. Recently, The phosphatidylinositol 3-kinase/Akt pathway is known to suppress translocation of Bax to the mitochondria (47). Therefore, we examined if the combination treatment suppresses the phosphatidylinositol 3-kinase/Akt pathway. However, we failed to detect any changes in the phosphatidylinositol 3-kinase/Akt pathway in response to the combination treatment in human leukemia cells (data not shown).

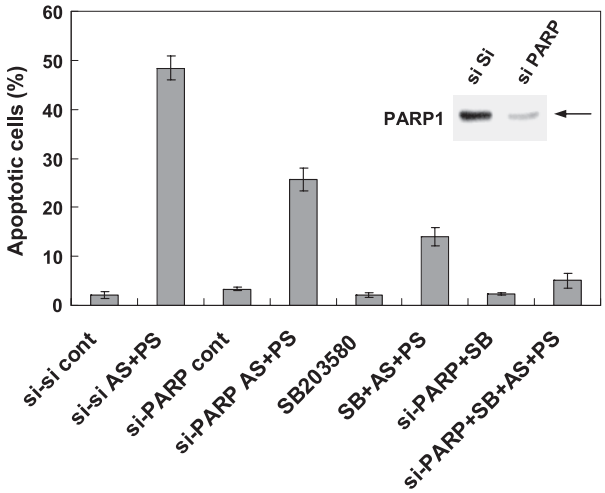

Figure 6. Enhancement of cell death after the combination treatment is mediated both by p38 MAPK and PARP-1 activation pathways. U937 cells were treated with 2 μmol/L As_2O_3 alone, 3 μg/mL phytosphingosine alone, or combination of As_2O_3 (2 μmol/L) and phytosphingosine (3 μg/mL) in the presence or absence of 10 μmol/L SB203580 or siRNA for PARP-1. After 24 h, cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. *Columns,* mean of three independent experiments; *bars,* SE.

Accumulation of intracellular ROS by diverse stimuli leads to disruption of the mitochondrial membrane potential, release of apoptogenic factors, and, ultimately, to caspase-dependent or caspase-independent apoptotic cell death (6, 7). Moreover, experiments using antioxidants indicated that ROS act upstream of Bax relocalization and mitochondrial membrane depolarization (21). In this study, we provided further evidence that increased level of ROS induced by combination treatment with As_2O_3 and phytosphingosine is essential for the p38 MAPK–mediated mitochondrial relocalization of Bax. Complete inhibition of the combination treatment–induced p38 MAPK activation, Bax translocation to the mitochondria, and mitochondrial apoptotic cell death by treatment with thiol-containing antioxidant NAC suggests that increased intracellular ROS level is critical for the p38 MAPK–mediated mitochondrial apoptotic cell death.

PARP-1 is a nuclear enzyme that facilitates DNA repair in response to DNA damage (26, 27). Recently, it has been shown that ROS-mediated DNA damage triggers activation PARP-1 and subsequent cell death (30, 31). Likewise, in this study, we also showed that PARP-1 activation is involved in the combination treatment–induced apoptotic cell death. Pretreatment of cells with PARP-1 inhibitors significantly attenuated loss of the mitochondrial membrane potential, cytochrome *c* release, and subsequent cell death induced by the combination treatment. However, PARP-1 inhibitors failed to block the combination treatment–induced ROS generation. In contrast, NAC completely inhibited PARP-1 activation, indicating that PARP-1 activation is mediated by ROS in response to the combination treatment. These results suggest that PARP-1 is involved in the combination treatment–induced mitochondrial apoptotic cell death, and that PARP-1 activation is initiated by the ROS generation. Although the biochemical mechanism underlying ROS-mediated PARP-1 activation and PARP-1–mediated mitochondrial cell death in cells treated with the combination treatment remains to be clarified, the regulatory role of ROS on PARP-1–mediated cytochrome *c* release that we have shown in this study may provide an important clue to understanding PARP-1–mediated cell death.

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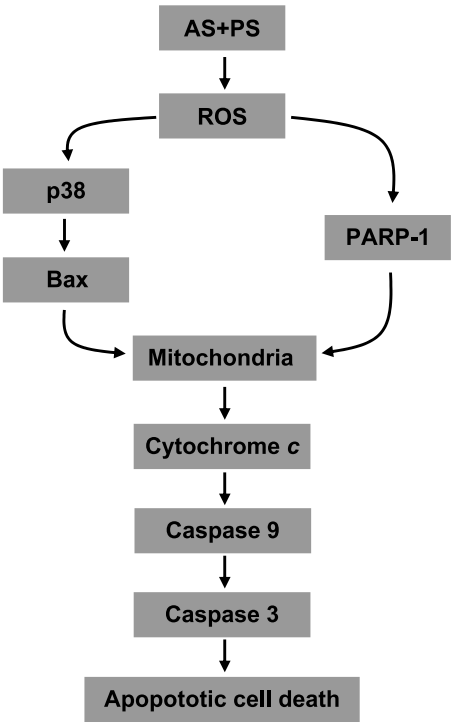

Figure 7. Schematic model of the combination treatment–induced cell death. Combination treatment of As_2O_3 and phytosphingosine increases intracellular ROS level, which induces p38 MAPK and PARP-1 activation. p38 activation mediated Bax translocation from cytosol to the mitochondria or PARP-1 activation reduces mitochondria membrane potential. This leads to cytochrome *c* release, which results in caspase-9 and caspase-3 activation and, eventually, apoptotic cell death. Since combination treatment of As_2O_3 and phytosphingosine enhances ROS level, which seems to use two different signaling pathways for overcoming the As_2O_3 resistance, understanding these signaling mechanisms that we elucidated in this study may provide insight into the design of future combination cancer therapies to cells intrinsically less sensitive to As_2O_3 treatment.

In summary, we report here that combination treatment with As_2O_3 and phytosphingosine induces synergistic cell death in intrinsically As_2O_3-resistant human myeloma cells through the mitochondrial activation–dependent cell death pathway by enhancing intracellular ROS generation. We show that the enhancement of intracellular ROS level by the combination treatment promotes the p38 MAPK–mediated mitochondrial translocation of Bax and the PARP-1 activation, leading to the collapse of mitochondria membrane potential and subsequent apoptotic cell death (Fig. 7). Thus, interestingly, the combination treatment seems to trigger two independent cell death pathways in a ROS-dependent fashion to overcome As_2O_3 resistance in As_2O_3-resistant human leukemia cells. Our results suggest that the combination treatment with As_2O_3 and phytosphingosine is potentially an effective way of treating cancers less sensitive to As_2O_3 treatment.

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Combination treatment with arsenic trioxide and phytosphingosine enhances apoptotic cell death in arsenic trioxide–resistant cancer cells

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