

# Arsenic trioxide induces human pulmonary fibroblast cell death via increasing ROS levels and GSH depletion

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**Abstract.** Arsenic trioxide (ATO;  $\text{As}_2\text{O}_3$ ) induces apoptotic cell death in various cancer cells including lung cancer via the induction of reactive oxygen species (ROS). However, little is known about the toxicological effects of ATO on normal primary lung cells. Here, we investigated the effects of N-acetyl cysteine (NAC) and vitamin C (well-known antioxidants) or L-buthionine sulfoximine (BSO; an inhibitor of GSH synthesis) on ATO-treated human pulmonary fibroblast (HPF) cells in relation to cell death, ROS and glutathione (GSH). ATO induced growth inhibition and death in HPF cells, accompanied by the loss of mitochondrial membrane potential (MMP;  $\Delta\Psi_m$ ). ATO increased ROS levels including  $\text{O}_2^{\cdot-}$  and GSH depleted cell numbers. NAC attenuated the growth inhibition, death and MMP ( $\Delta\Psi_m$ ) loss in ATO-treated HPF cells and also decreased the ROS levels in these cells. However, vitamin C enhanced the growth inhibition, death, MMP ( $\Delta\Psi_m$ ) loss and GSH depletion by ATO and even strongly increased mitochondrial  $\text{O}_2^{\cdot-}$  levels in ATO-treated HPF cells. BSO showed a strong increase in ROS levels in ATO-treated HPF cells and intensified the growth inhibition, cell death, MMP ( $\Delta\Psi_m$ ) loss and GSH depletion. Moreover, superoxide dismutase (SOD2)

or thioredoxin (TXN) siRNAs attenuated HPF cell death by ATO, which was not correlated with ROS and GSH level changes. In conclusion, ATO induced the growth inhibition and death of HPF cells, accompanied by increasing ROS levels and GSH depletion. NAC attenuated HPF cell death by ATO whereas vitamin C and BSO enhanced the death.

## Introduction

Reactive oxygen species (ROS) including hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), superoxide anion ( $\text{O}_2^{\cdot-}$ ) and hydroxyl radical ( $\cdot\text{OH}$ ) regulate many essential cellular events such as gene expression, differentiation, cell proliferation and cell death (1). A change in the redox state of tissues and cells affects an alteration in the generation or metabolism of ROS. They are mainly generated as by-products of mitochondrial respiration or are specifically made by oxidases such as nicotine adenine diphosphate (NADPH) oxidase and xanthine oxidase (XO) (2). The principal metabolic pathways include superoxide dismutases (SODs) [cytoplasmic (SOD1), mitochondrial (SOD2) or extracellular (SOD3) isoforms], which metabolize  $\text{O}_2^{\cdot-}$  to  $\text{H}_2\text{O}_2$  (3). Further metabolism by catalase (CAT) or glutathione (GSH) peroxidase (GPX), yields  $\text{O}_2$  and  $\text{H}_2\text{O}$  (4). Especially, thioredoxin (TXN) system consisting of TXN, TXN reductase (TXNR) and NADPH is critically involved in maintaining cellular redox homeostasis (5). TXN as a thiol reductase is a potent anti-oxidant and acts as a scavenger of ROS (5). Oxidative stress may be the result of either overproduction of ROS or accumulation of it. This stress can initiate events that lead to cell death depending on cell types (6-8).

Arsenic trioxide (ATO;  $\text{As}_2\text{O}_3$ ) has long been used as therapeutic agents for some severe diseases including leukemia in East Asia, especially China (9). Recently, ATO has been reported to cure patients with relapsed acute promyelocytic leukemia (APL) without severe marrow suppression (10,11). The antiproliferative effect of ATO is not restricted to APL cells but can also be implicated in a variety of hematological malignancies (12,13). Furthermore, ATO may be active against other malignancies such as solid tumor since the mechanisms of action of ATO are mainly the induction of apoptosis and the cell cycle arrest (14). In fact, accumulating literature has demonstrated that ATO regulate many biological functions of cell proliferation, differentiation and angiogenesis as well as apoptosis in the solid tumor cells of renal (15), head and

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**Abbreviations:** HPF, human pulmonary fibroblast; ATO; arsenic trioxide ( $\text{As}_2\text{O}_3$ ); ROS, reactive oxygen species; MMP ( $\Delta\Psi_m$ ), mitochondrial membrane potential; NADPH oxidase, nicotine adenine diphosphate oxidase; XO, xanthine oxidase; SOD, superoxide dismutase; CAT, catalase; GPX, GSH peroxidase; TXN, thioredoxin; TXNR, TXN reductase; FBS, fetal bovine serum; PI, propidium iodide; FITC, fluorescein isothiocyanate;  $\text{H}_2\text{DCFDA}$ , 2,2'-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; GSH, glutathione; CMFDA, 5-chloromethylfluorescein diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; siRNA, small interfering RNA; NAC, N-acetyl cysteine; BSO, L-buthionine sulfoximine

**Key words:** arsenic trioxide, cell death, human pulmonary fibroblast, reactive oxygen species, glutathione

neck (16), ovarian (17), prostate (17), hepatoma (18), bladder (19), colon (20), lung (21), breast (22), cervical (23) and gastric cancer cells (24). ATO as a mitochondrial poison can induce the failure of the mitochondrial transmembrane potential (MMP;  $\Delta\Psi_m$ ) and, as such, it generates high amounts of ROS (14,25,26). ATO also stimulates ROS generation via the activation of NADPH oxidase (27) or the inhibition of GPX and TXNR (28,29). These phenomena can trigger the apoptosis of target cells. In addition, it has been reported that the intracellular GSH content has a decisive effect on ATO-induced apoptosis (26,30–32). Furthermore, a combination of ATO and L-buthionine sulfoximine (BSO; an inhibitor of GSH synthesis) induces synergistic cytotoxicity in several cancer cells of renal cell carcinoma (30), bladder cancer (19), leukemia (32), lung cancer (33), hepatocellular carcinoma (34) and solid tumors (35).

Lung cancer is a main cause of cancer death in developed countries. Various novel remedial strategies including new drug development are currently under consideration due to intrinsic or acquired resistant and toxicity of conventional drugs (36). Studies of the molecular mechanisms of cytotoxic drug action have shed light on the treatment of lung cancer. It has been reported that ATO alone or its combination with other agents inhibits the growth of lung cancer cells (33,37,38). We also reported that ATO induces apoptosis in Calu-6 lung cancer cells via GSH depletion (39). However, little is known about the toxicological effects of ATO on normal primary lung cells. Because we observed that ATO induces the growth inhibition and death in human pulmonary fibroblast (HPF) cells (40), in the present study we investigated the effects of N-acetyl cysteine (NAC) and vitamin C (well-known antioxidants) or L-buthionine sulfoximine [BSO; an inhibitor of GSH synthesis (41)] on ATO-treated HPF cells in relation to cell growth, cell death, ROS and GSH levels. Furthermore, we examined the effects of antioxidant-related siRNAs on cell death and ROS levels in ATO-treated HPF cells.

## Materials and methods

**Cell culture.** The human pulmonary fibroblast (HPF) cells from PromoCell GmbH (Heidelberg, Germany) were maintained in humidified incubator containing 5% CO<sub>2</sub> at 37°C. HPF cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco-BRL, Grand Island, NY). HPF cells were used between passages 4 and 8.

**Reagents.** ATO was purchased from Sigma-Aldrich Chemicals (St. Louis, MO) and was dissolved in 1.65 M NaOH at 1x10<sup>-1</sup> M as a stock solution. NAC and BSO were obtained from Sigma-Aldrich Chemicals. NAC was dissolved in the buffer [20 mM HEPES (pH 7.0)]. BSO was dissolved in water. Vitamin C purchased from Riedel-de Haen (Hannover, Germany) was also dissolved in water. Cells were pretreated with 2 mM NAC or 10  $\mu$ M BSO or 0.4 mM vitamin C for 1 h prior to ATO treatment.

**Detection of intracellular ROS levels.** Intracellular ROS were detected by a fluorescent probe dye, 2',7'-Dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA, Ex/Em = 495 nm/529 nm;

Invitrogen Molecular Probes, Eugene, OR) as previously described (42). H<sub>2</sub>DCFDA is poorly selective for superoxide anion radical (O<sub>2</sub><sup>•-</sup>). In contrast, dihydroethidium (DHE, Ex/Em = 518 nm/605 nm; Invitrogen Molecular Probes) is a fluorogenic probe that is highly selective for O<sub>2</sub><sup>•-</sup> among ROS as previously described (42). Mitochondrial O<sub>2</sub><sup>•-</sup> level was detected using MitoSOX<sup>TM</sup> Red mitochondrial O<sub>2</sub><sup>•-</sup> indicator (Ex/Em=510 nm/580 nm; Invitrogen Molecular Probes) as previously described (42). In brief, 1x10<sup>6</sup> cells in 60-mm culture dish (Nunc, Roskilde, Denmark) were incubated with the indicated doses of ATO in the presence or absence of NAC, BSO, vitamin C or antioxidant-related siRNA duplex for the indicated times. Cells were then washed in PBS and incubated with 20  $\mu$ M H<sub>2</sub>DCFDA, 20  $\mu$ M DHE or 5  $\mu$ M MitoSOX<sup>TM</sup> Red at 37°C for 30 min. DCF, DHE and MitoSOX<sup>TM</sup> Red fluorescences were detected using a FACStar flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). ROS and O<sub>2</sub><sup>•-</sup> levels were expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software (Becton-Dickinson).

**Detection of the intracellular glutathione (GSH).** Cellular GSH levels were analyzed using a 5-chloromethylfluorescein diacetate dye (CMFDA, Ex/Em = 522 nm/595 nm; Invitrogen Molecular Probes) as previously described (42). In brief, 1x10<sup>6</sup> cells in 60-mm culture dish (Nunc) were incubated with the indicated doses of ATO in the presence or absence of NAC, BSO or vitamin C for the indicated times. Cells were then washed with PBS and incubated with 5  $\mu$ M CMFDA at 37°C for 30 min. CMF fluorescence intensity was determined using a FACStar flow cytometer (Becton-Dickinson). Negative CMF staining (GSH depleted) cells were expressed as the percent of (-) CMF cells.

**Cell growth assay.** The effect of drugs on HPF cell growth was determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Chemicals Co.) dye absorbance as previously described (12). In brief, 5x10<sup>3</sup> cells per well were seeded in 96-well microtiter plates (Nunc). After exposure to 50 ATO  $\mu$ M with or without NAC, BSO or vitamin C for 24 h, 20  $\mu$ l of MTT solution [2 mg/ml in phosphate-buffered saline (PBS)] were added to each well of the 96-well plates. The plates were incubated for an additional 3 h at 37°C. Media in wells were withdrawn by pipetting, and 200  $\mu$ l of DMSO was added to each well to solubilize the formazan crystals. Optical density was measured at 570 nm using a microplate reader (SpectraMAX 340, Molecular Devices, Sunnyvale, CA).

**Annexin V/PI staining for cell death detection.** Apoptosis was determined by staining cells with Annexin V-fluorescein isothiocyanate (FITC, Ex/Em = 488 nm/519 nm; Invitrogen Molecular Probes) and propidium iodide (PI, Ex/Em = 488 nm/617 nm; Sigma-Aldrich Chemicals). In brief, 1x10<sup>6</sup> cells in 60-mm culture dish (Nunc) were incubated with 50  $\mu$ M ATO in the presence or absence of NAC, BSO, vitamin C or antioxidant-related siRNA duplex for 24 h. Cells were washed twice with cold PBS and then resuspended in 500  $\mu$ l of binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>) at a concentration of 1x10<sup>6</sup> cells/ml. Annexin V-FITC (5  $\mu$ l) and PI (1  $\mu$ g/ml) were then added to

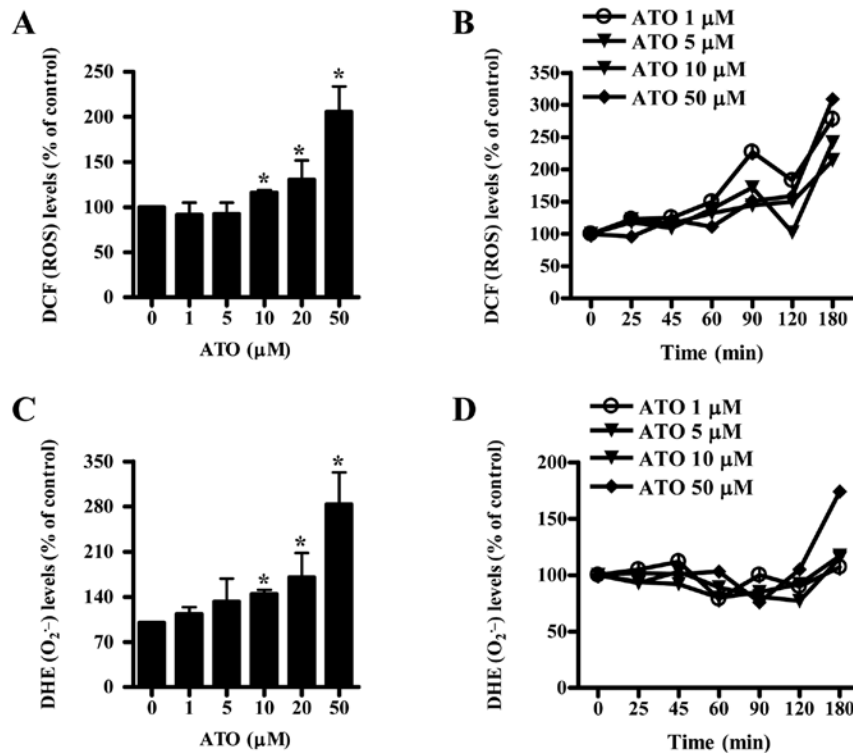


Figure 1. Effects of ATO on cell growth and ROS levels in HPF cells. Exponentially growing cells were treated with the indicated amounts of ATO for the indicated times. Intracellular ROS levels including O<sub>2</sub><sup>-</sup> were measured using a FACStar flow cytometer. (A and B) Graphs indicate DCF (ROS) levels (%) in HPF cells at 24 h (A) and the early times (B). (C and D) Graphs indicate DHE (O<sub>2</sub><sup>-</sup>) levels (%) in HPF cells at 24 h (C) and the early times (D). \*P<0.05 compared with the control group.

these cells, which were analyzed with a FACStar flow cytometer (Becton-Dickinson).

**Measurement of MMP ( $\Delta\Psi_m$ ).** MMP ( $\Delta\Psi_m$ ) levels were measured using a rhodamine 123 fluorescent dye (Ex/Em = 485 nm/535 nm; Sigma-Aldrich Chemical) as previously described (43). In brief,  $1 \times 10^6$  cells in 60-mm culture dish (Nunc) were incubated with 50  $\mu$ M ATO in the presence or absence of NAC, BSO or vitamin C for 24 h. Cells were washed twice with PBS and incubated with the rhodamine 123 (0.1  $\mu$ g/ml) at 37°C for 30 min. Rhodamine 123 staining intensity was determined by flow cytometry (Becton-Dickinson). An absence of rhodamine 123 from cells indicated the loss of MMP ( $\Delta\Psi_m$ ) in HPF cells.

**Western blot analysis.** The changes of antioxidant-related protein in ATO-treated cells were determined by western blotting as previously described (12). In brief,  $1 \times 10^6$  cells in 60-mm culture dish (Nunc) were incubated with 50  $\mu$ M ATO in the presence or absence of 2 mM NAC for 24 h. The cells were then washed in PBS and suspended in five volumes of lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% NP40, 0.5 mM DTT, 1% protease inhibitor cocktail). Supernatant protein concentrations were determined using the Bradford method. Samples containing 10  $\mu$ g total protein were resolved by 12.5% SDS-PAGE gels, transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA) by electroblotting and then probed with anti-SOD1, anti-SOD2, anti-TXN, anti-TXNR1 and anti- $\beta$ -actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Blots were developed using an ECL kit (Amersham, Arlington Heights, IL).

**Transfection of cells with antioxidant-related siRNAs.** Gene silencing of SOD1, SOD2, CAT, GPX and TXN was performed as previously described (44). A non-specific control siRNA duplex [5'-CCUACGCCACCAAUUUCGU(dTdT)-3'], SOD1 siRNA duplex [5'-GAAACACGGUGGGCCAAA(dTdT)-3'], SOD2 siRNA duplex [5'-CUGGGAGAAUGUACUGAA(dTdT)-3'], CAT siRNA duplex [5'-CACUGAUUUCACAACAGAU(dTdT)-3'], GPX siRNA duplex [5'-CAAGCUCAUCCUGGUCU(dTdT)-3'] and TXN siRNA duplex [5'-GCAUGCCAACAUUCCA GUU(dTdT)-3'] were purchased from the Bioneer Corp. (Daejeon, South Korea). In brief,  $2.5 \times 10^5$  cells in 6-well plates (Nunc) were incubated in RPMI-1640 supplemented with 10% FBS. The next day, cells (~30-40% confluence) in each well were transfected with the control or each siRNA duplex [80 pmol in Opti-MEM (Gibco-BRL)] using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen, Brandford, CT). Two days later, cells were treated with or without 50  $\mu$ M ATO for additional 24 h. The transfected cells were collected and used for the measurement of Annexin V-FITC/PI staining cells and ROS levels.

**Statistical analysis.** The results represent the mean of at least three independent experiments (mean  $\pm$  SD). The data were analyzed using InStat software (GraphPad Prism 4, San Diego, CA). The Student's t-test or one-way analysis of variance (ANOVA) with post hoc analysis using Tukey's multiple

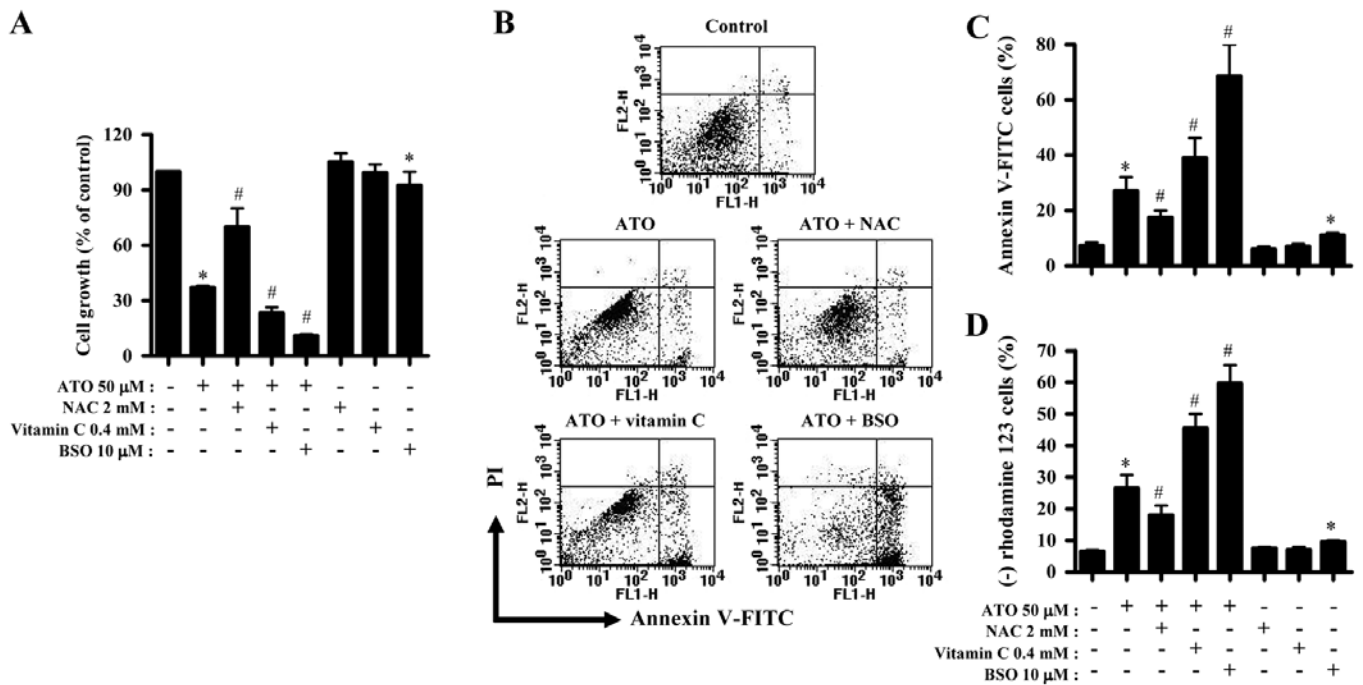


Figure 2. Effects of NAC, vitamin C or BSO on cell growth, cell death and MMP ( $\Delta\Psi_m$ ) in ATO-treated HPF cells. Exponentially growing cells were treated with 50  $\mu$ M ATO for 24 h following a 1 h pre-incubation with 2 mM NAC, 0.4 mM vitamin C or 10  $\mu$ M BSO. (A) The graph shows cell growth changes in HPF cells as assessed by MTT assays. (B) Annexin V-FITC/PI cells were measured using a FACStar flow cytometer. Each figure shows a representative for Annexin V and/or PI staining cells. (C) Graph shows the percents of Annexin V-FITC positive staining cells from B in HPF cells. (D) Graph shows the percents of rhodamine 123 negative [MMP ( $\Delta\Psi_m$ ) loss] cells in HPF cells, as measured with a FACStar flow cytometer. \* $P < 0.05$  compared with the control group. # $P < 0.05$  compared with cells treated with ATO only.

comparison test was used for parametric data. Statistical significance was defined as  $P < 0.05$ .

## Results

**ATO changes ROS levels in HPF cells.** We used doses of 1-50  $\mu$ M ATO to assess ROS levels in HPF cells. Treatment with 1 or 5  $\mu$ M ATO increased the growth of HPF cells at 24 h whereas 10-50  $\mu$ M ATO inhibited the growth (unpublished data). As shown in Fig. 1A, ROS (DCF) levels were not altered in 1 or 5  $\mu$ M ATO-treated HPF cells at 24 h but were increased in 10-50  $\mu$ M ATO-treated HPF cells. All the tested doses of ATO generally increased ROS (DCF) levels from the early time of 25 min and the gradual increases lasted for the tested times (25-80 min) although there was a transient decrease in ROS levels at 120 min (Fig. 1B). Intracellular  $O_2^{\cdot-}$  (DHE) level was increased in 1-50  $\mu$ M ATO-treated HPF cells in a dose-dependent manner (Fig. 1C). However,  $O_2^{\cdot-}$  levels in these cells were not clearly augmented at the early time of 25 or 45 min and the levels were instead gradually decreased until 120 min (Fig. 1D). At 180 min, ATO seemed to increase  $O_2^{\cdot-}$  level in HPF cells and 50  $\mu$ M ATO showed a strong increase in this level (Fig. D).

**NAC, vitamin C or BSO influences the growth inhibition and death of ATO-treated HPF cells.** We examined the effect of NAC, vitamin C and BSO on the growth and death of ATO-treated HPF cells. For this experiment, 50  $\mu$ M ATO was used as a suitable dose to differentiate the levels of cell growth inhibition and death. NAC significantly prevented the growth

inhibition by ATO whereas vitamin C and BSO enhanced the growth inhibition (Fig. 2A). BSO alone inhibited HPF cell growth (Fig. 2A). In relation to cell death, ATO induced cell death in HPF cells at 24 h, as evidenced by Annexin V staining cells (Fig. 2B and C). NAC significantly rescued HPF cells from ATO insult whereas vitamin C and BSO increased the cell death by ATO, and BSO alone also induced cell death in HPF control cells (Fig. 2B and C).

Apoptosis is closely related to the collapse of MMP ( $\Delta\Psi_m$ ) (45). As expected, loss of MMP ( $\Delta\Psi_m$ ) was observed in ATO-treated HPF cells (Fig. 2D). Similar to the results of Annexin V staining cells, NAC attenuated the loss of MMP ( $\Delta\Psi_m$ ) in ATO-treated HPF cells whereas vitamin C and BSO enhanced the loss in these cells (Fig. 2D). BSO alone induced MMP ( $\Delta\Psi_m$ ) loss in HPF control cells as well (Fig. 2D).

**NAC, vitamin C or BSO affects ROS and antioxidant-protein levels in ATO-treated HPF cells.** Next, ROS and antioxidant-protein levels in 50  $\mu$ M ATO-treated HPF cells were assessed in the presence or absence of NAC, vitamin C or BSO. As shown in Fig. 3A, ROS (DCF) level in ATO-treated HPF cells was significantly decreased by NAC and was also slightly attenuated by vitamin C. Both NAC and vitamin C decreased basal ROS (DCF) levels in HPF control cells (Fig. 3A). In contrast, BSO strongly increased ROS (DCF) levels in ATO-treated or -untreated HPF cells (Fig. 3A). Both NAC and vitamin C seemed to decrease  $O_2^{\cdot-}$  level in ATO-treated and -untreated HPF cells (Fig. 3B). However, BSO significantly increased  $O_2^{\cdot-}$  levels in ATO-treated or -untreated HPF cells

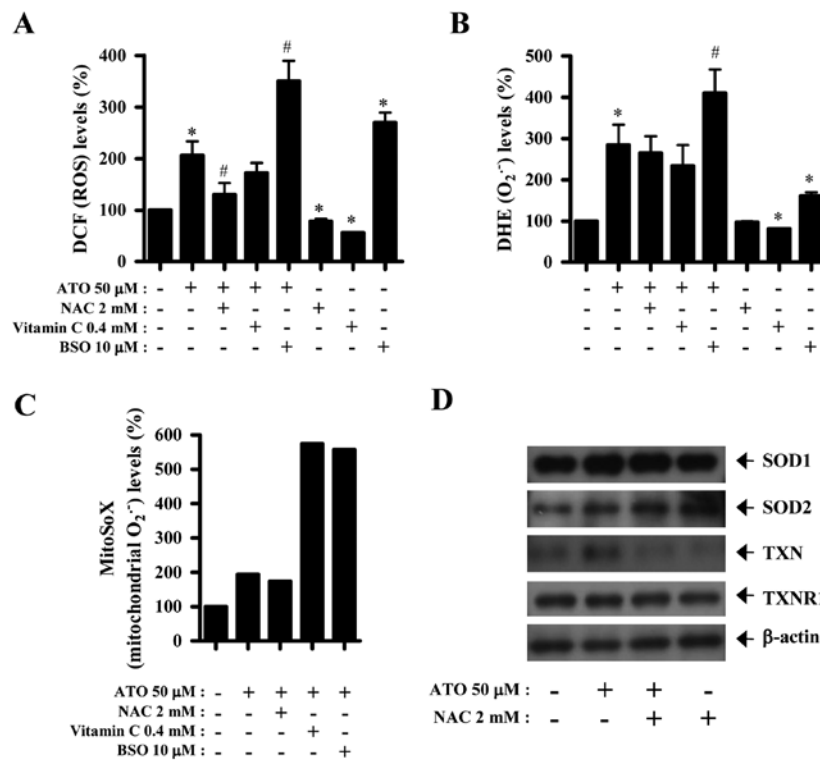


Figure 3. Effects of NAC, vitamin C or BSO on ROS and antioxidant-protein levels in ATO-treated HPF cells. Exponentially growing cells were treated with 50  $\mu$ M ATO for 24 h following a 1-h pre-incubation with 2 mM NAC, 0.4 mM vitamin C or 10  $\mu$ M BSO. ROS levels were measured with a FACStar flow cytometer. (A, B and C) Graphs indicate ROS (as determined by DCF) levels (%) (A), DHE ( $O_2^{\bullet-}$ ) levels (%) (B) and mitoSOX (mitochondrial  $O_2^{\bullet-}$ ) levels (%) compared with control cells (C). (D) Samples of protein extracts (10  $\mu$ g) were resolved by SDS-PAGE gel, transferred onto PVDF membranes and immunoblotted with the indicated antibodies against SOD1, SOD2, TXN, TXNR1 and  $\beta$ -actin. \*P<0.05 compared with the control group. #P<0.05 compared with cells treated with ATO only.

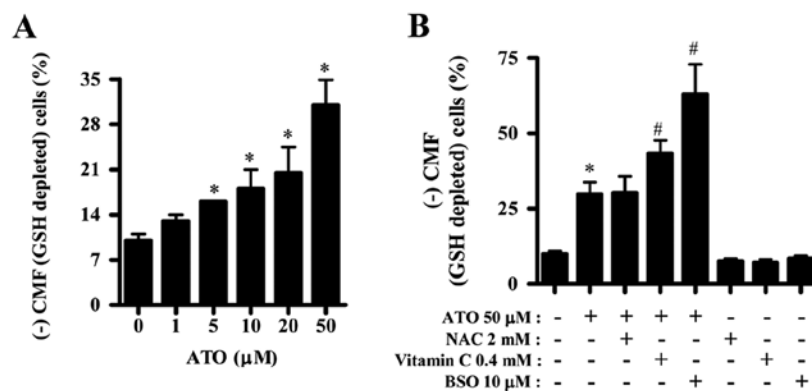


Figure 4. Effects of ATO and/or NAC, vitamin C or BSO on GSH levels in HPF cells. Exponentially growing cells were treated with 50  $\mu$ M ATO for 24 h following a 1-h pre-incubation with 2 mM NAC, 0.4 mM vitamin C or 10  $\mu$ M BSO. GSH levels were measured with a FACStar flow cytometer. (A and B) Graphs show the percents of (-) CMF (GSH-depleted) cells. \*P<0.05 compared with the control group. #P<0.05 compared with cells treated with ATO only.

(Fig. 3B). Furthermore, MitoSOX Red fluorescence levels, which specifically indicate  $O_2^{\bullet-}$  levels in the mitochondria, were strongly increased in 50  $\mu$ M ATO-treated HPF cells at 24 h (Fig. 3C). While NAC seemed to decrease mitochondrial  $O_2^{\bullet-}$  level in ATO-treated HPF cells, both vitamin C and BSO strongly enhanced the level (Fig. 3C). The expression of SOD1 was not changed by ATO and/or NAC whereas that of SOD2 was increased by both agents (Fig. 3D). In addition, ATO clearly upregulated the expression of TXN in HPF cells, which expression was completely downregulated by NAC (Fig. 3D).

Neither ATO nor NAC strongly affected the expression of TXNR1 (Fig. 3D).

*ATO and/or NAC, vitamin C or BSO affects GSH levels in HPF cells.* Next, we assessed the changes of GSH levels by ATO in the presence or absence of NAC, vitamin C or BSO. ATO increased GSH depleted cell number in HPF cells in a dose-dependent manner and even low dose of 1 or 5  $\mu$ M ATO induced GSH depletion (Fig. 4A). NAC did not affect GSH depleted cell number in ATO-treated HPF cells but vitamin

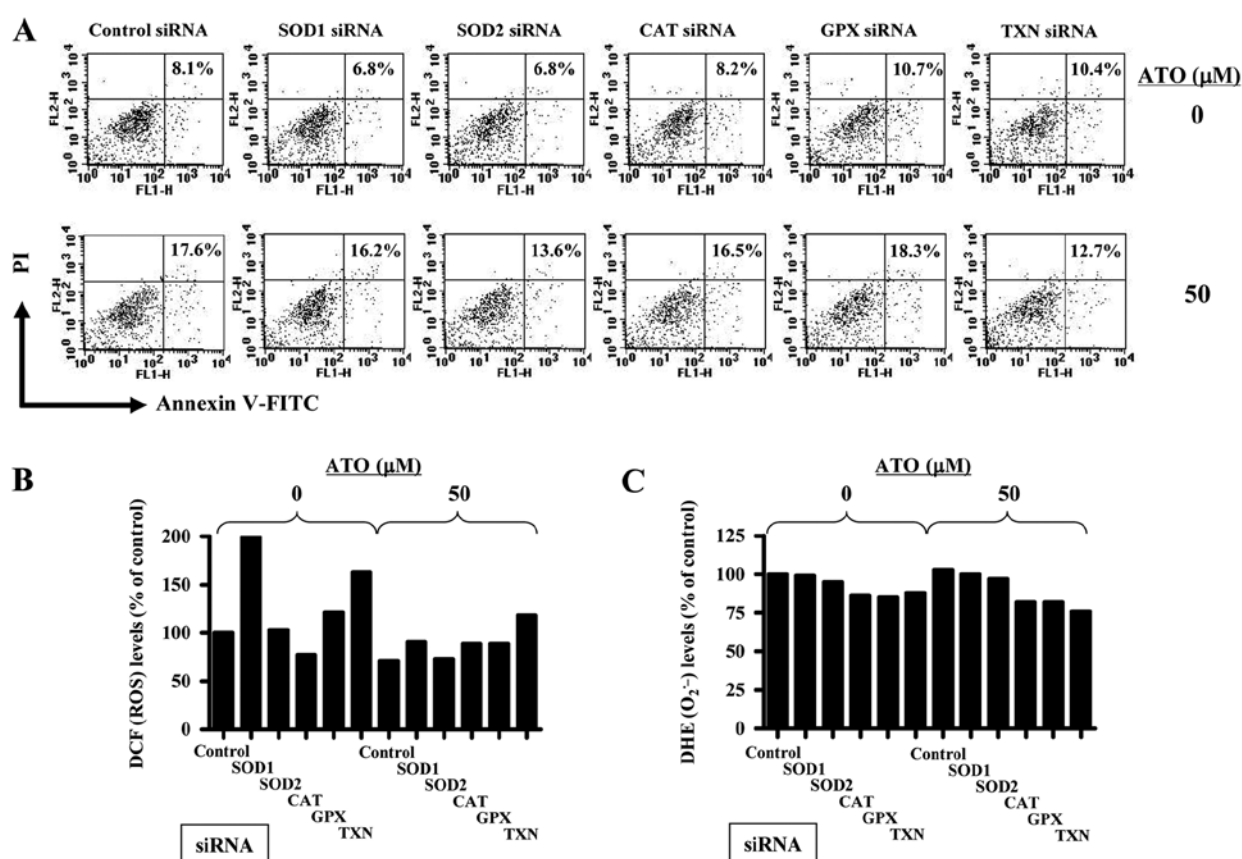


Figure 5. Effects of antioxidant-related siRNAs on cell death and ROS levels in ATO-treated HPF cells. HPF cells (~30-40% confluence) were transfected with either non-target control siRNA or each antioxidant-related siRNA. Two day later, cells were treated with 50  $\mu$ M ATO for additional 24 h. (A) Annexin V-FITC and PI cells were measured with a FACStar flow cytometer. The number (%) in each figure indicates Annexin V-FITC positive cells regardless of PI negative and positive cells. (B and C) Graphs indicate DCF (ROS) levels (%) (B) and DHE (O<sub>2</sub><sup>-</sup>) levels (%) compared with ATO-untreated control siRNA cells (C).

C and BSO significantly increased the number in these cells (Fig. 4B). NAC, vitamin C or BSO alone did not significantly affect the percent of GSH depletion in HPF control cells (Fig. 4B).

*Antioxidant-related siRNAs affect cell death, ROS and GSH depletion levels in ATO-treated HPF cells.* Furthermore, it was determined whether antioxidant (SOD1, SOD2, CAT, GPX or TXN)-related siRNAs changed cell death, ROS and GSH depletion levels in ATO-treated HPF cells. As shown in Fig. 5, 50  $\mu$ M ATO increased the proportion of Annexin V-stained cells about 9% compared with that in control siRNA treated HPF cells. Probably, the siRNA knockdown system with Lipofectamine 2000 attenuated the biological activity of ATO. The siRNAs of antioxidant-related proteins did not significantly alter Annexin V-stained cell number in HPF control cells for 72 h (Fig. 5A). Administration of SOD2 or TXN siRNA attenuated cell death in ATO-treated HPF cells, whereas SOD1, CAT or GPX siRNA did not (Fig. 5A). SOD1, GPX or TXN siRNA increased ROS (DCF) level in HPF control cells whereas CAT siRNA decreased the level for 72 h (Fig. 5B). CAT, GPX or TXN siRNA seemed to decrease O<sub>2</sub><sup>-</sup> level in HPF control cells (Fig. 5C). Treatment with 50  $\mu$ M ATO in this siRNA knockdown system did not increase ROS levels including O<sub>2</sub><sup>-</sup> in control siRNA treated HPF cells (Fig. 5B and C). Relatively, SOD1, CAT, GPX or TXN siRNA

upregulated the ROS (DCF) level in ATO-treated HPF cells (Fig. 5B). CAT, GPX or TXN siRNA reduced O<sub>2</sub><sup>-</sup> level in ATO-treated HPF cells (Fig. 5C). None of the antioxidant-related siRNAs affected GSH depleted cell number in HPF control cells for 72 h, and 50  $\mu$ M ATO did not clearly increase the number in control siRNA treated HPF cells at 24 h (data not shown). The tested siRNA did not strongly change GSH depleted cell number in ATO-treated HPF cells (data not shown).

## Discussion

ATO can disturb the natural oxidation and reduction equilibrium in cells via changing a variety of redox enzymes (14,27) and influencing MMP ( $\Delta\Psi_m$ ) (14,25,26). The increased intracellular ROS is observed in ATO-treated cervical cancer cells (46), APL cells (47), hepatocellular carcinoma HepG2 (26) and glioblastoma A172 cells (48). These results suggest that ATO-induced cell death is related to ROS accumulation. Therefore, in the present study we focused on the molecular mechanism of ATO-induced HPF cell death in relation to ROS and GSH.

ROS level (as determined by DCF) was increased in HPF cells treated with 10-50  $\mu$ M ATO and O<sub>2</sub><sup>-</sup> level (as determined by DHE) was also increased by all the tested doses of 1-50  $\mu$ M ATO at 24 h. ATO also ROS (DCF) levels from the early time

phases but did not increase  $O_2^{\bullet-}$  levels at these times. There was a transient decrease in ROS levels at 120 min. However, an increase in  $O_2^{\bullet-}$  level was observed from 120 min, which level was strongly increased at 180 min in 50  $\mu$ M ATO-treated HPF cells. Because ATO increases ROS levels including  $O_2^{\bullet-}$  via a variety of redox enzymes (14,27) as well as causing mitochondrial dysfunction (14,25,26), it is possible that ATO increased ROS (DCF) levels in HPF cells via affecting redox enzymes until 90 min and then ATO increased ROS levels including  $O_2^{\bullet-}$  via damaging mitochondria as well as changing the activities of redox enzymes from 120 min. In addition, the increased  $O_2^{\bullet-}$  levels in ATO-treated HPF cells at 24 h seemed to result from the enhanced production of  $O_2^{\bullet-}$  itself rather than the reduction of SOD activity since mitochondrial  $O_2^{\bullet-}$  level in HPF cells were increased by ATO and the expression SOD1 or 2 was not downregulated by ATO. Furthermore, ATO induced the loss of MMP ( $\Delta\Psi_m$ ) in HPF cells. Taken together, ATO induced growth inhibition and death in HPF cells accompanied by an increase in ROS levels including  $O_2^{\bullet-}$ . These results suggest the possibility that changes in ROS levels in ATO-treated HPF cells by NAC, vitamin C or BSO affect cell death in these cells. Thus, we assessed ROS and cell death levels in ATO-treated HPF cells with or without NAC, vitamin C or BSO.

Expectedly, a well-known antioxidant NAC seemed to attenuate ROS levels including mitochondrial  $O_2^{\bullet-}$  in ATO-treated or -untreated HPF cells. It also significantly prevented cell growth inhibition, cell death and MMP ( $\Delta\Psi_m$ ) loss in ATO-treated HPF cells. Furthermore, NAC upregulated the expression of SOD2 protein in HPF cells. These results are similar to other reports that NAC attenuated cell death and ROS increase in ATO-treated cells (26,49). In contrast, BSO showing a strong enhancement in cell death and MMP ( $\Delta\Psi_m$ ) loss in ATO-treated HPF cells intensified ROS level including mitochondrial  $O_2^{\bullet-}$  in these cells. Therefore, ATO seemed to induce HPF cell death through the induction of ROS. BSO alone induced cell growth inhibition, cell death and MMP ( $\Delta\Psi_m$ ) loss in HPF control cells and strongly increased ROS levels. Therefore, an increased ROS by BSO treatment seemed to be tightly related to HPF cell growth inhibition and death. However, although another antioxidant vitamin C seemed to decrease ROS levels including  $O_2^{\bullet-}$  in ATO-treated or -untreated HPF cells, this agent strongly intensified mitochondrial  $O_2^{\bullet-}$  level, cell growth inhibition and cell death in ATO-treated HPF cells. It was assumed that the enhancement of HPF cell death and mitochondrial  $O_2^{\bullet-}$  level by co-treatment with ATO and vitamin C resulted from the severe loss of MMP ( $\Delta\Psi_m$ ) by them. Our result is similar to the reports that vitamin C enhances ATO-induced cytotoxicity in multiple myeloma cells (50-52), leukemia cells (53,54) and hepatocellular carcinoma cells (26). Therefore, vitamin C plays a role as a prooxidant rather than antioxidant in ATO-treated cells including HPF cells and it can be used with ATO for treatment of cancer including hematological malignancies.

In relation to the administration of antioxidant-related siRNAs in ATO-treated HPF cells, 50  $\mu$ M ATO mildly increased Annexin V-stained cell number and did not increase ROS levels including  $O_2^{\bullet-}$  in control siRNA treated HPF cells. SOD2 or TXN siRNAs attenuated cell death in ATO-treated HPF cells without convincing changes in ROS levels. Therefore, the alteration of ATO-induced HPF cell death by

antioxidant-related siRNAs seemed not to be correlated with ROS changes. SOD2 or TXN as a potent antioxidant can stimulate cell proliferation or may confer resistance to anticancer drugs (55-57). Thus, the downregulation of SOD2 or TXN may render cells sensitive to several cytotoxic drugs. However, our results showed that SOD2 or TXN siRNA did not enhance HPF cell death by ATO but instead attenuated it. In addition, ATO clearly upregulated the expressions of SOD2 and TXN. The possibility that SOD2 and TXN are involved in HPF cell death in response to ATO via an unidentified mechanism is worthy of further study. Moreover, administration with SOD1, GPX or TXN siRNA increased ROS (DCF) level in HPF control cells but SOD2 or CAT siRNA did not. None of these siRNAs increased the  $O_2^{\bullet-}$  level in HPF control cells. Because a change in the generation or metabolism of ROS in the cells is influenced by various prooxidant or antioxidant enzymes as well as activities in various cellular organelles such as mitochondria and endoplasmic reticulum, our results suggest that the downregulation of each antioxidant protein by its corresponding siRNA does not simply increase ROS levels in HPF cells but can individually affect different ROS levels. Therefore, the role of ROS level change by antioxidant-related siRNAs in ATO-treated HPF cells need to be further studied in relation to cell death.

It has been reported that apoptotic effects are inversely comparative to GSH content (19,34,35,58-60). The intracellular GSH content has a decisive effect on ATO-induced apoptosis (26,30-32). In addition, BSO or vitamin C enhances GSH depletion in ATO-treated cells (26,33,50,54,61). Likewise, ATO dose-dependently increased the number of GSH-depleted cells in HPF cells. As expected, BSO as a GSH synthesis inhibitor increased the numbers of GSH depleted cells in ATO-treated HPF cells. Vitamin C showing an increase in HPF cell death by ATO augmented the numbers of GSH depleted cells in these cells. However, NAC did not affect GSH depletion in ATO-treated HPF cells. Although NAC is also known to be a GSH precursor, NAC did not seem to be a GSH precursor but be an antioxidant in HPF cells. Treatment with 10  $\mu$ M BSO showing a cell death effect in HPF control cells did not induce GSH depletion whereas the low dose of 1 or 5  $\mu$ M ATO induced GSH depletion in HPF cells without cell growth inhibition and death. Moreover, SOD2 or TXN siRNAs did not influence GSH depletion in ATO-treated HPF control cells. Taken together, our results suggest that the intracellular GSH content seem to be the decisive role on ATO-induced HPF cell death but changes of the content are not sufficient enough to predict cell death correctly.

In conclusion, ATO induced growth inhibition and death of HPF cells, which were accompanied by increasing ROS level and GSH depletion. NAC attenuated HPF cell death by ATO via decreasing ROS levels whereas vitamin C and BSO enhanced the death via increasing ROS and GSH depletion levels. Our present data provide useful information for understanding the cytotoxic or toxicological effects of ATO in normal lung cells in relation to ROS and GSH levels.

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