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Arsenic trioxide-induced cytotoxicity in small cell lung cancer via altered redox homeostasis and mitochondrial integrity

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Abstract. Arsenic trioxide (ATO) has demonstrated anti-cancer activity in different malignancies, especially acute promyelocytic leukemia, with a wide array of putative mechanisms. In this study, we aimed to elucidate the activity and mechanisms of ATO in small cell lung cancer (SCLC). A panel of SCLC cell lines (H841, DMS79, H526, H69 and H187) was employed to demonstrate the activity of ATO. Cell viability, apoptosis and mitochondrial membrane depolarization were assessed. Western blotting was performed to determine the alteration of pro-apoptotic and anti-apoptotic mediators. Reactive oxygen species (ROS) (hydrogen peroxide and superoxide) and intracellular glutathione (GSH) were measured. Antioxidants, N-acetyl-L-cysteine (NAC) and butylated hydroxyanisole (BHA), were applied to restore GSH content and reduce production of ROS. All SCLC cell lines were relatively sensitive to ATO with IC_{50} values below 10 μM. ATO induced cell death mainly through apoptosis in H841 cells in a dose-dependent manner. Hydrogen peroxide was the major ROS in SCLC cells induced by ATO. Along with GSH depletion and Bcl-2 downregulation, mitochondrial membrane permeabilization was enhanced, followed by release of AIF and SMAC from mitochondria to initiate different cell death pathways. NAC reversed cell death and molecular changes induced by ATO via restoring GSH and reducing ROS content. BHA inhibited hydrogen peroxide production completely and partially restored GSH content accounting for partial reversal of cell inhibition and mitochondrial dysfunction. Nonetheless, ATO reduced both reduced and oxidized form of thioredoxin 1 (Trx1) with no effect on Trx1 redox potential. ATO led to cell death in SCLC mainly through mitochondrial dysfunction, resulting from altered cellular redox homeostasis, namely, hydrogen peroxide generation, GSH depletion and Trx1 down-regulation.

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Abbreviations: ATO, arsenic trioxide; BHA, butylated hydroxyanisole; GSH, glutathione; NAC, N-acetyl-L-cysteine; SMAC/DIABLO, second mitochondria-derived activator of caspases; Trx1, thioredoxin 1; XIAP, X-linked inhibitor of apoptosis protein

Key words: arsenic trioxide, apoptosis, mitochondrial membrane depolarization, glutathione, hydrogen peroxide, thioredoxin 1

Introduction

SCLC, accounting for 15-20% of newly diagnosed lung cancer, is an extremely aggressive malignancy with early metastasis and poor prognosis. Despite a prompt response to chemotherapy, relapses occur in the majority of patients with SCLC. Therefore the development of an alternative therapy against SCLC becomes imperative (1).

ATO has been proven to be an effective therapeutic agent in acute promyelocytic leukemia (APL) with high complete remission rate and prolonged survival (2,3). ATO can induce apoptosis through PML-RARα-independent pathways in APL or other cancer cells via p53 activation (4,5), Bcl-2 down-regulation (6,7), mitochondrial membrane depolarization and cytochrome *c* release (8-10), depletion of intracellular reduced glutathione (GSH) content and elevation of reactive oxygen species (11,12). More recently, the application of ATO in lung cancer treatment has been explored in preclinical models, mainly in non-small cell lung cancer (NSCLC). ATO induces growth inhibition and apoptosis in NSCLC cells through G2/M cell cycle arrest (13,14), Bcl-2 downregulation and GSH depletion (15). Recently, downregulation of thymidylate synthase and E2F1 were observed in lung adenocarcinoma (16,17) and mesothelioma (18). Synergistic effects have been observed when combining ATO with cisplatin (19), buthionine sulfoximine (BSO) (15), suberoylanilide hydroxamic acid (20), and sulindac (21). The effect of ATO in SCLC is less reported. In a panel of lung cancer cell lines, ATO is highly cytotoxic to SCLC preferentially mediated through necrosis rather than apoptosis (22).

Biologically, ATO is known to bind to proteins with sulfhydryl (-SH) groups (23). It targets GSH which is a thiol molecule involved in detoxification of arsenicals (11,24). Evidently, loss of GSH and its cytotoxic effect is not directly caused by damage of GSH-related enzymes (glutathione peroxidase, glutathione reductase and glutathione transferase) (25). Nonetheless, thioredoxin (Trx) system serves as a key

antioxidant target of ATO in breast cancer cells (26). Trx system, composed of thioredoxin reductase (TrxR), Trx and NADPH, plays an important role in maintaining somatic redox homeostasis. Moreover, TrxR and Trx are overexpressed in many cancers (27-29), which take part in redox regulation of transcription factors (30). Therefore, Trx system has become a new target in cancer therapy (31).

In the present study, we aimed to elucidate the mechanisms of ATO in SCLC using a cell line model, which may provide a scientific base for future application of ATO in treatment of SCLC.

Materials and methods

Chemicals and reagents. ATO, N-acetyl-L-cystine (NAC), buthionine sulphoximine (BSO) and butylated hydroxyanisole (BHA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Apoptosis inducing factor (AIF), Bcl-2, cleaved caspase-3, cytochrome *c*, PARP, SMAC, thioredoxin and XIAP antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell lines and culture. Five SCLC cell lines (H187, DMS79, H526, H69 and H841) were obtained from the American Type Culture Collection (Manassas, VA, USA). H187, DMS79, H526 and H69 cells were cultured in RPMI-1640 medium (Gibco®, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), while H841 cells were grown in HETIS medium supplemented with 5% FBS. Cells were incubated in 37˚C with a humidified atmosphere of 5% CO_2.

Cell proliferation assay. The cytotoxicity of ATO in SCLC cell lines was measured using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (ATCC) as previously performed (32).

Annexin V-phycoerythrin (PE)/7-aminoactinomycin D (AAD) staining. Apoptosis was determined by PE-conjugated Annexin V/7-AAD kit (BD Biosciences, CA, USA). Briefly, treated cells were collected, washed and re-suspended in binding buffer. Cells were stained for 15 min at room temperature with PE-Annexin V (Ex/Em = 488/578 nm)/7-AAD (Ex/Em = 546/647 nm), then read-out by Cytomics FC 500 analyzer with FL2/FL4 channels (Beckman Coulter, CA, USA). The cell populations in $PE^+/7\text{-}AAD^-$ and $PE^+/7\text{-}AAD^+$ quadrants were calculated.

Measurement of mitochondrial membrane depolarization (MMD). Briefly, cells after treatment were collected, washed, and stained with JC-1 (5 μg/ml) at 37˚C for 15 min before reading with FL1/FL2 channels of Cytomics FC 500 analyzer.

Detection of glutathione (GSH) and reactive oxygen species (ROS). Cellular GSH level was measured by using 5-chloromethylfluorescein diacetate (CMFDA, Ex/Em = 522/595 nm, Invitrogen, CA, USA) fluorescence as described previously (33). After treatment, cells were washed and incubated for 30 min at 37˚C with 5 μM CMFDA in FBS-free medium, followed by incubation for 40 min at 37˚C with complete medium. CMF fluorescence intensity was determined using a Cytomics FC500 flow cytometer. Hydrogen peroxide (H_2O_2) and superoxide were measured by 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Ex/Em = 500/520 nm) and dihydroethidium (DHE, Ex/Em = 518/605 nm) (Invitrogen) staining, respectively. Treated cells were washed and incubated for 20 min at 37˚C with 1 μM of H_2DCFDA or 1 μM DHE in FBS-free medium following flow cytometry.

Immunofluorescence. Oxidative DNA damage was assessed using 8-hydroxy-2-deoxyguanosine (8-OHdG) immunofluorescence. Cells were seeded on slides before treatment. Slides were washed and blocked for 60 min with 10% normal goat serum in PBS, followed by incubation at 4˚C overnight with monoclonal anti-8-OHdG antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The slides were then incubated for 2 h with FITC-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Ex/Em = 500/520 nm), coverslipped with UltraCruz™ Mounting Medium (Santa Cruz Biotechnology) and observed with Eclipse E-800 (Nikon, Tokyo, Japan). DAPI (Santa Cruz Biotechnology) staining was used to visualize the nucleus.

Western blotting. Total protein was extracted with RIPA buffer (PBS, 1% Nonidet-P-40, 0.1% deoxycholate, 0.1% sodium dodecyl sulfate) containing protease inhibitors. Nuclear protein and mitochondrial protein were collected with NE-PER Nuclear and Cytoplasmic Extraction kit and Mitochondria Isolation kit respectively (Pierce Biotech, Rockford, IL, USA) as described by the manufacturer. Proteins (40 μg) were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK), immunoblotted with corresponding primary and secondary antibodies, and detected using ECL detection kit (GE Healthcare). Image analysis was carried out with ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA).

DNA fragmentation assay. Cellular DNA was extracted with standard DNA phenol/chloroform extraction method (34). DNA (0.1 μg/sample) was separated in 1.5% agarose gel and bands were visualized on a UV transilluminator at 302 nm.

Redox western blot analysis. Redox forms of Trx1 were separated as previously described (26,35). Trx1 was carboxymethylated in guanidine-Tris solution (6 M guanidine-HCl, 50 mM Tris, 1 mM EDTA, 30 mM iodoacetic acid) at pH 7.5. After incubation at 37˚C for 30 min, proteins were desalted and concentrated using centrifugal filter device (Millipore, Billerica, MA, USA). Proteins were separated with native gel and western blotting was performed. Redox potential was calculated with Nernst equation: Eh, Trx1 = 240 mV + 30 x ln (ratio of $Trx1^{Ox}$ and $Trx1^{Red}$) (36).

Statistical analysis. Data were presented as mean ± standard deviation (SD), with between-group differences analyzed by two-tailed Student's t-test. A p-value <0.05 was considered statistically significant. Linear regression was used to calculate IC_{50} values. All statistical analyses were performed using Prism 5 (GraphPad Software, La Jolla, CA, USA).

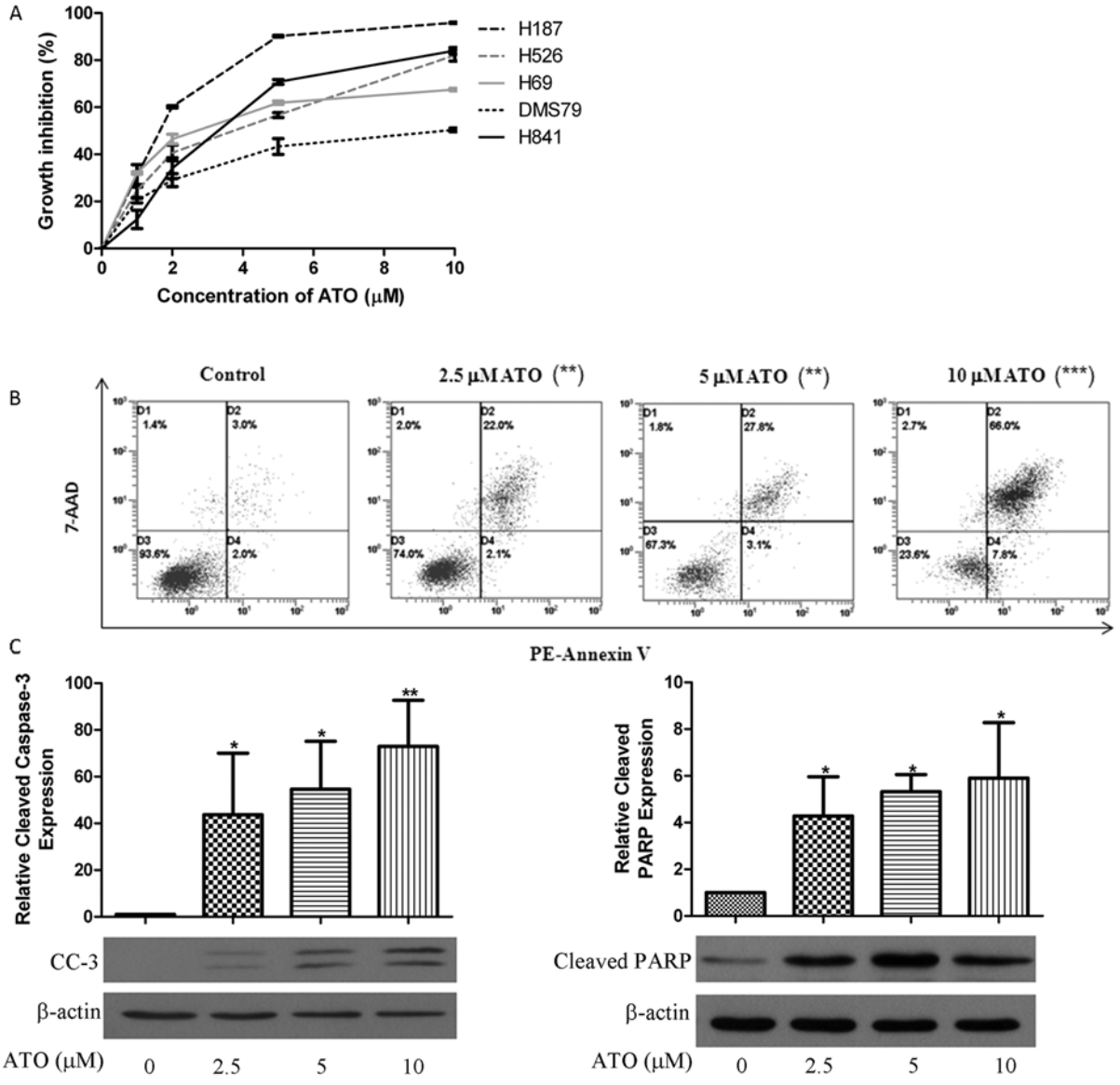

Figure 1. Cytotoxicity of ATO in SCLC cell lines. (A) Dose-dependent growth inhibition in five SCLC cell lines (H187, H526, H69, DMS79 and H841) with ATO treatment. (B) Dose-dependent increase in apoptotic cell death, (C) cleaved caspase-3 and cleaved PARP with ATO treatment in H841 cells. Statistical significance ($^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.001$) indicates comparison with control.

Results

Cell growth inhibition and apoptosis induced by ATO. Five SCLC cell lines were incubated for 48 h with different concentrations of ATO. Cell proliferation was suppressed in a dose-dependent manner with IC_{50} values in clinically reachable levels (Fig. 1A). H841 cell line was chosen due to its adherent property for apoptotic assay. ATO induced dose-dependent apoptosis (Fig. 1B) in H841 cells accompanied by increased cleaved caspase-3 and PARP (Fig. 1C).

ATO-induced MMD, GSH depletion and ROS elevation. ATO prompted a dose-dependent shift from red to green fluorescence that indicated MMD in H841 cells (Fig. 2A). This was associated with downregulation of Bcl-2 and XIAP as well as release of AIF and SMAC to cytosol (Fig. 2B). Low concentration of ATO (2.5 μM) reduced 60% of GSH content (Fig. 2C) and increased H_2O_2 by >1.5-fold compared with control H841 cells (30500±6150 vs 13522±3146) (Fig. 2D). Nonetheless, superoxide content remained unchanged except at the highest concentration (10 μM) of ATO treatment (Fig. 2D).

ATO induced oxidative DNA damage and fragmentation. Treatment with ATO (5 μM) resulted in stronger FITC fluorescence (green) than in the control group, in keeping with increased 8-OHdG content in H841 cells upon exposure to ATO (Fig. 3A). This is associated with appearance of lower molecular weight DNA fragments with ATO treatment (Fig. 3B).

Changes in MAPKs expression during ATO treatment. Elevation of p-Erk1/2 was detected after 30 min of ATO treatment and it was sustained for ≥48 h (Fig. 4A) while p-P38 and p-JNK were unaltered (data not shown). Nonetheless, application of specific p-Erk1/2 inhibitor (PD98059) (20 μM) decreased p-Erk1/2

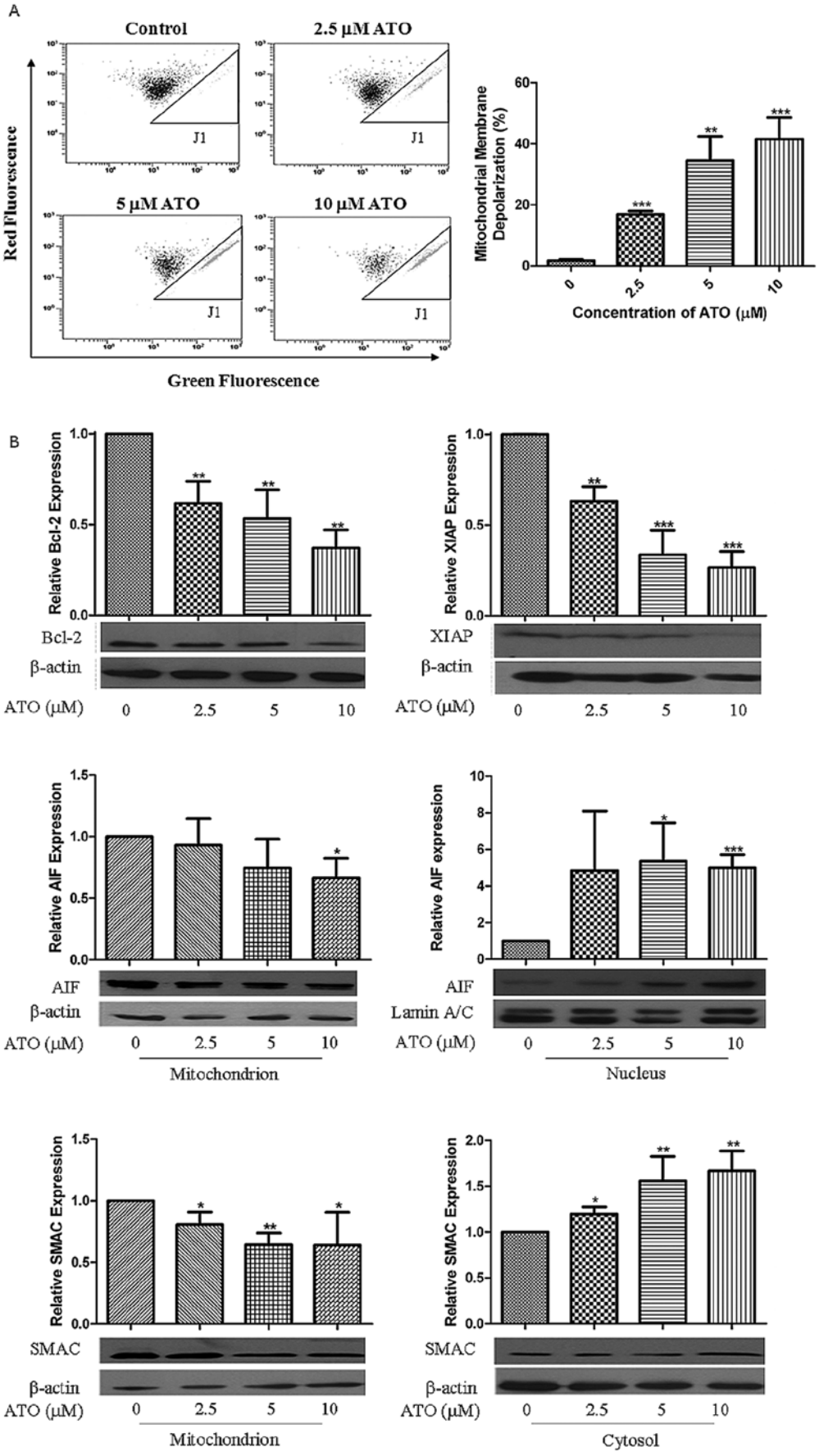

Figure 2. Effect of ATO on mitochondrial membrane permeability, apoptotic mediators or inhibitors as well as GSH depletion and H_2O_2 accumulation. (A) Increased mitochondrial membrane depolarization (MMD) with ATO treatment as indicated by JC-1 staining. J_1 represents the proportion of the cells with MMD. (B) Downregulation of apoptotic inhibitors (Bcl-2 and XIAP) with ATO treatment, associated with translocation of AIF and Smac from mitochondria to nucleus and cytosol respectively.

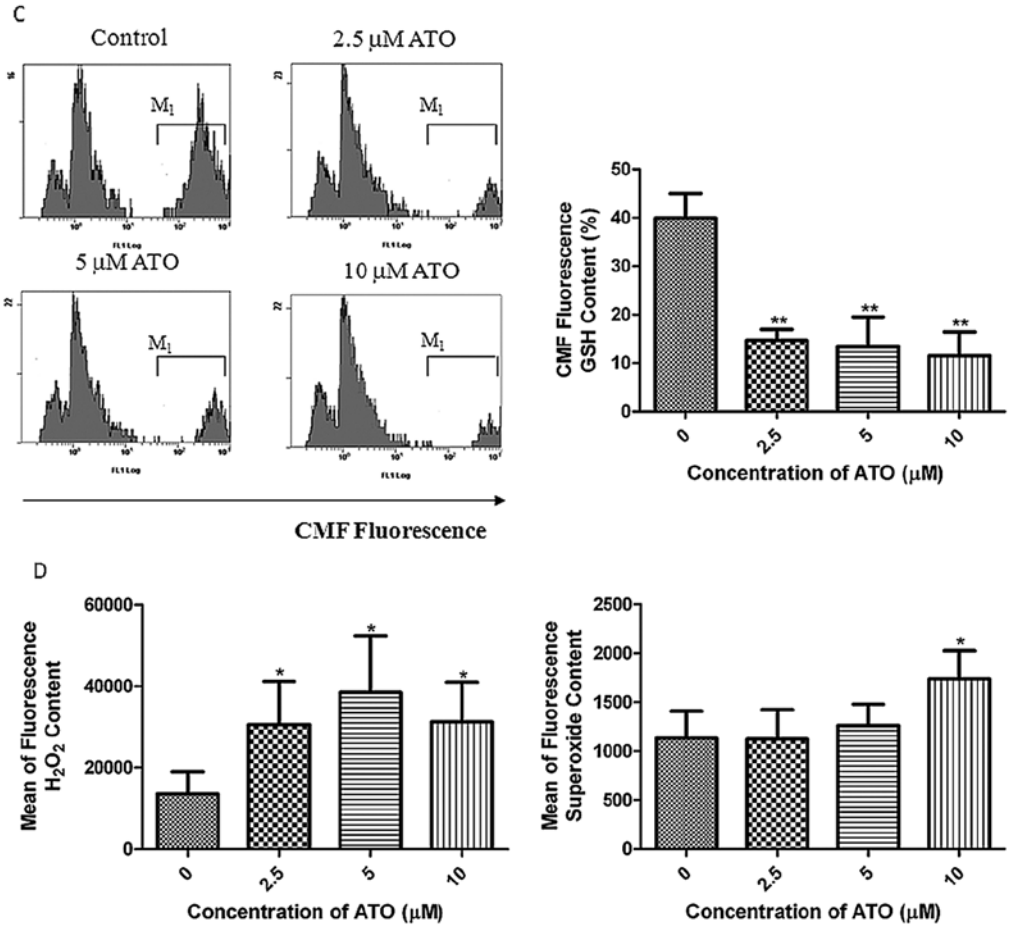

Figure 2. Continued. (C) ATO depleted GSH at 2.5 μM, with similar degree of suppression up to 10 μM. M_1 represents the GSH content. (D) ATO significantly increased H_2O_2 at 2.5-10 μM, while superoxide was significantly raised only at the highest concentration of ATO at 10 μM. Statistical significance ($^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.001$) indicates comparison with control.

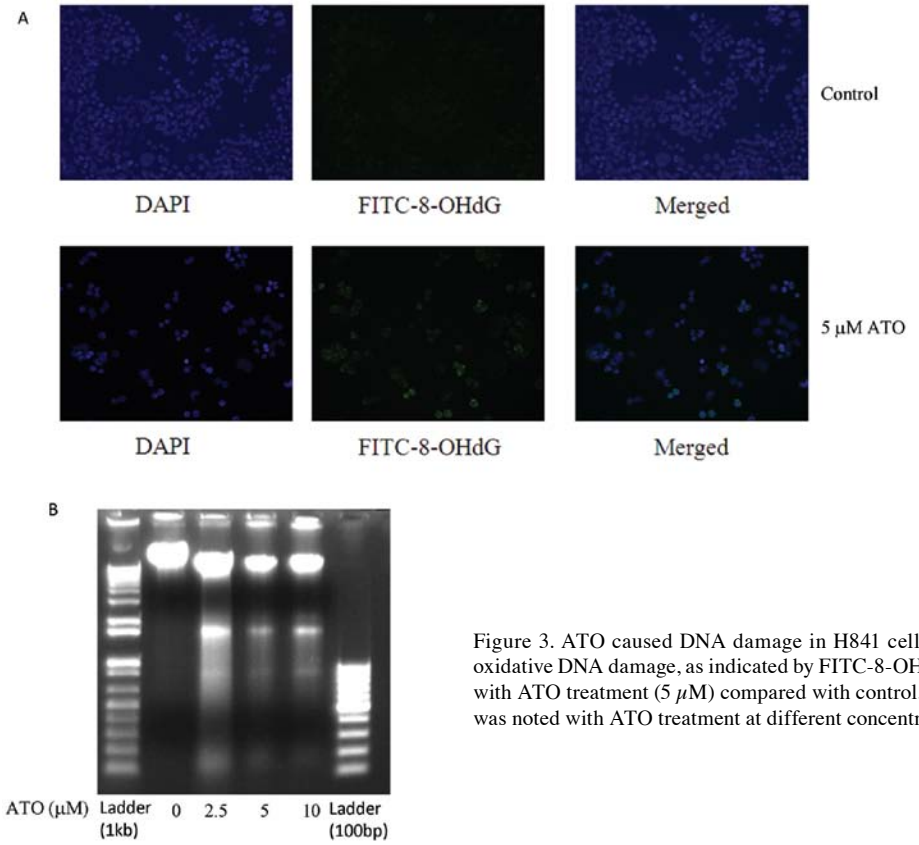

Figure 3. ATO caused DNA damage in H841 cell line. (A) The degree of oxidative DNA damage, as indicated by FITC-8-OHdG staining, was evident with ATO treatment (5 μM) compared with control. (B) DNA fragmentation was noted with ATO treatment at different concentrations.

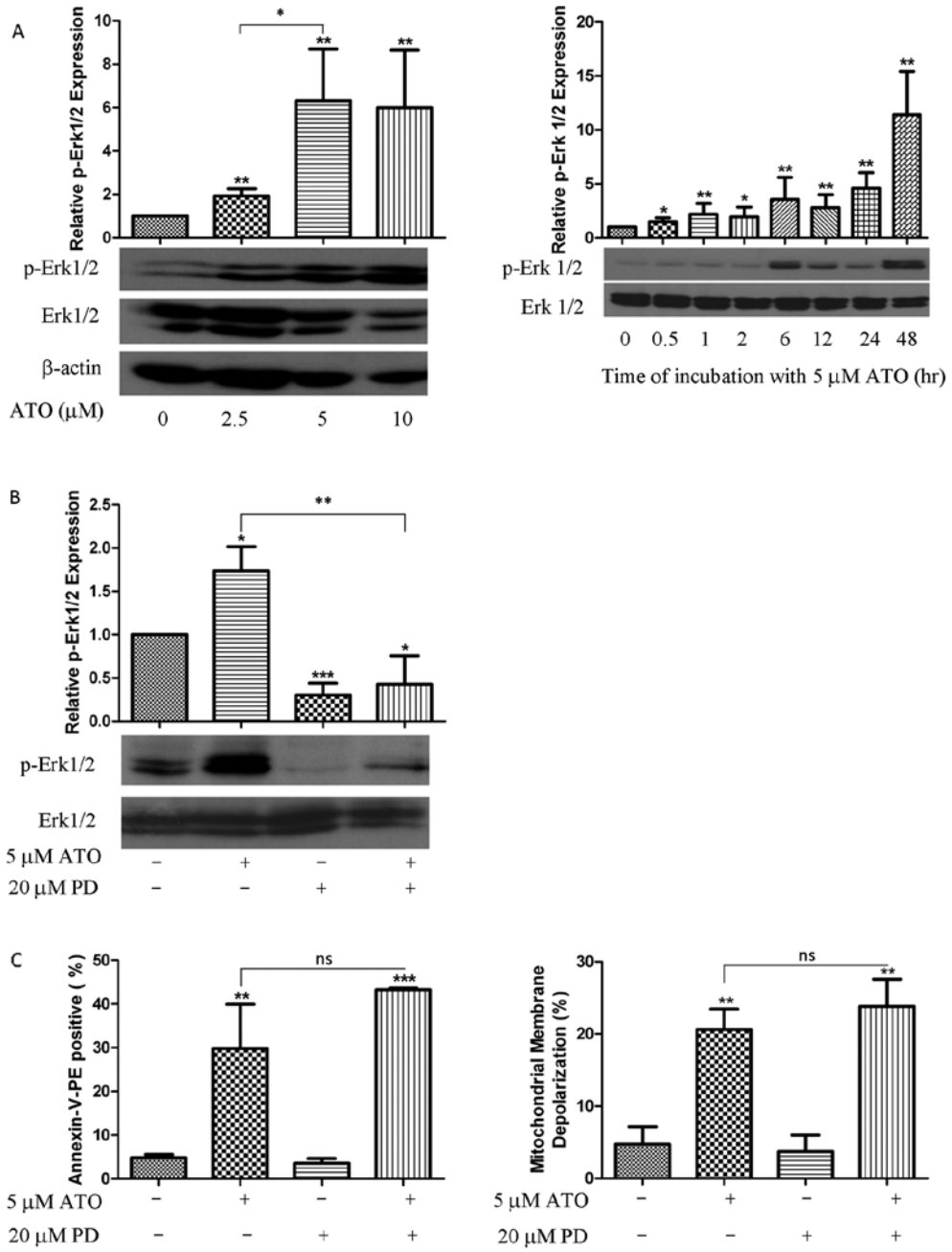

Figure 4. Upregulation of p-Erk1/2 with ATO in H841 cells had no cytotoxic effect. (A) Dose- and time-dependent induction of p-Erk1/2 upon ATO treatment. (B) PD-98059 (20 μM) (Erk inhibitor) effectively suppressed ATO-induced pErk1/2, without alleviating effect on mitochondrial membrane depolarization and apoptosis initiated by ATO. Statistical significance ($^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.001$; ns, not significant) indicates comparison with control.

expression induced by ATO (Fig. 4B) but did not reverse ATO-induced cell death and MMD (Fig. 4C).

Effects of antioxidants on cell death induced by ATO. NAC, a cysteine donor in GSH synthesis, was used to determine the role of GSH and ROS in ATO-induced cell death in H841 cells. NAC (10 mM) was shown to effectively restore GSH content and reduce H_2O_2 after ATO exposure (Fig. 5A). This was associated with reversal of apoptotic cell death (39.6±5.3 vs. 5.3±1.2% for ATO vs ATO + NAC) and MMD (34.5±4.5 vs. 5.6±0.4% for ATO vs. ATO + NAC) (Fig. 5B). In addition, NAC reversed the alterations in apoptosis-related proteins induced by ATO (Bcl-2, XIAP, cleaved caspase-3 and cleaved PARP) (Fig. 5C). On the other hand, a different antioxidant BHA suppressed H_2O_2 induced by ATO but failed to restore GSH content. Consequently, BHA only partially reversed apoptosis and MMD induced by ATO (Fig. 5D).

BSO potentiated cytotoxic effect of ATO by further disturbing the redox status. BSO, an inhibitor of γ-glutamylcysteine synthetase, was used as a GSH inhibitor. BSO potentiated the cytotoxic effect of ATO in H841 cells, as shown by increased apoptotic death (PE^+ by flow cytometry in 17 vs. 44%: ATO vs. ATO + BSO), enhanced MMD (17 vs. 42%: ATO vs. ATO + BSO), increased cleavage of caspase-3 and downregulated XIAP (Fig. 6A). Nonetheless, administration of 10 mM NAC

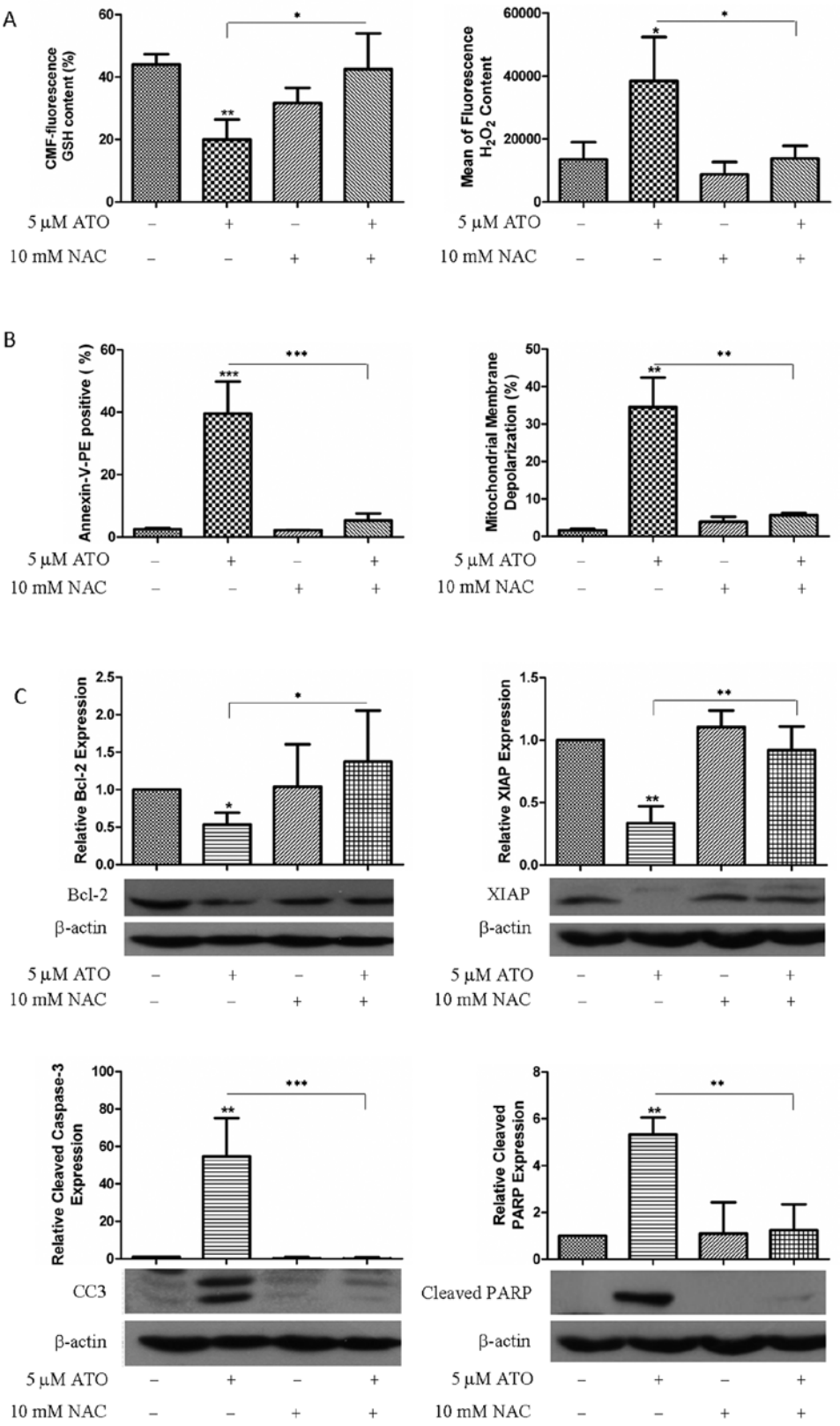

Figure 5. Role of oxidative stress in ATO-mediated apoptosis in H841 cell line. Addition of NAC (10 mM) to ATO (5 μM) resulted in (A) complete reversal of ATO-induced alterations in GSH and H_2O_2 content, (B) protection from mitochondrial membrane depolarization and apoptotic cell death induced by ATO, and (C) normalization of downregulated anti-apoptotic molecules (Bcl-2 and XIAP) and upregulated apoptotic markers (cleaved caspase-3 and cleaved PARP) due to ATO.

only partially rescued the H841 cells from apoptosis and MMD (Fig. 6A). Interestingly, BSO caused more accumulation of H_2O_2 than further depletion of GSH when combined with ATO (Fig. 6B), resulting in increased oxidative DNA damage (8-OHdG-FITC signal) in BSO/ATO group compared with either ATO or BSO alone (Fig. 6C).

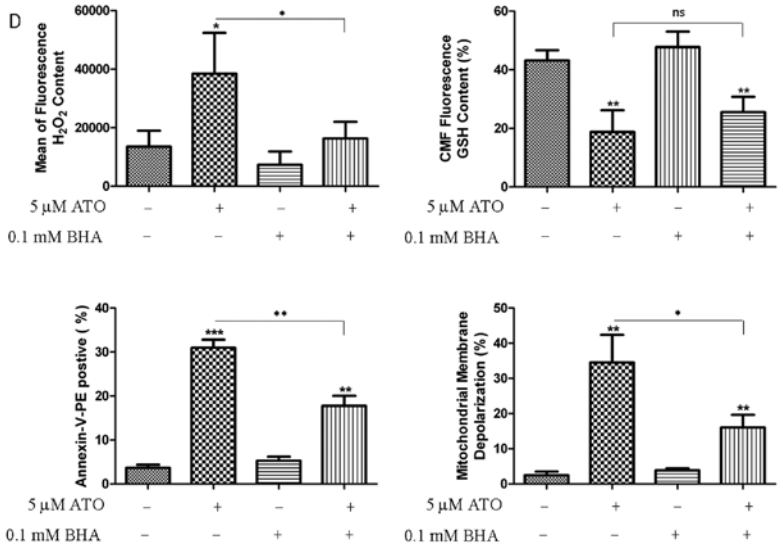

Figure 5. Continued. However, (D) BHA (0.1 mM) could suppress ATO-induced H_2O_2 accumulation without restoration of GSH, leading to only partial alleviation of mitochondrial membrane depolarization and apoptosis. Statistical significance ($^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.001$; ns, not significant) indicates comparison with control.

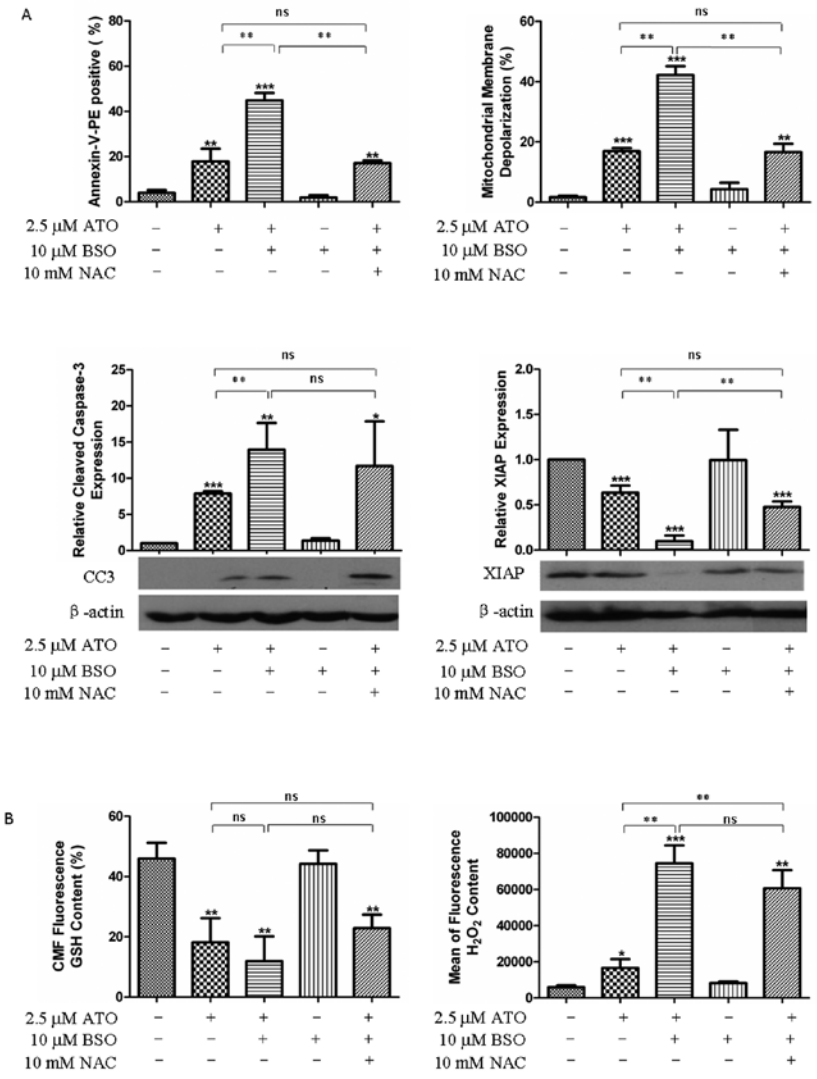

Figure 6. Effect of BSO on cytotoxicity of ATO in H841 cells. (A) BSO potentiated the mitochondrial membrane depolarization and apoptotic cell death due to ATO, accompanied by increased cleaved caspase-3 and decreased XIAP, which could only be partially reversed by NAC. (B) BSO increased H_2O_2 dramatically rather than depleting GSH further when combined with ATO, which could not be restored by NAC. Statistical significance ($^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.001$; ns, not significant) indicates comparison with control.

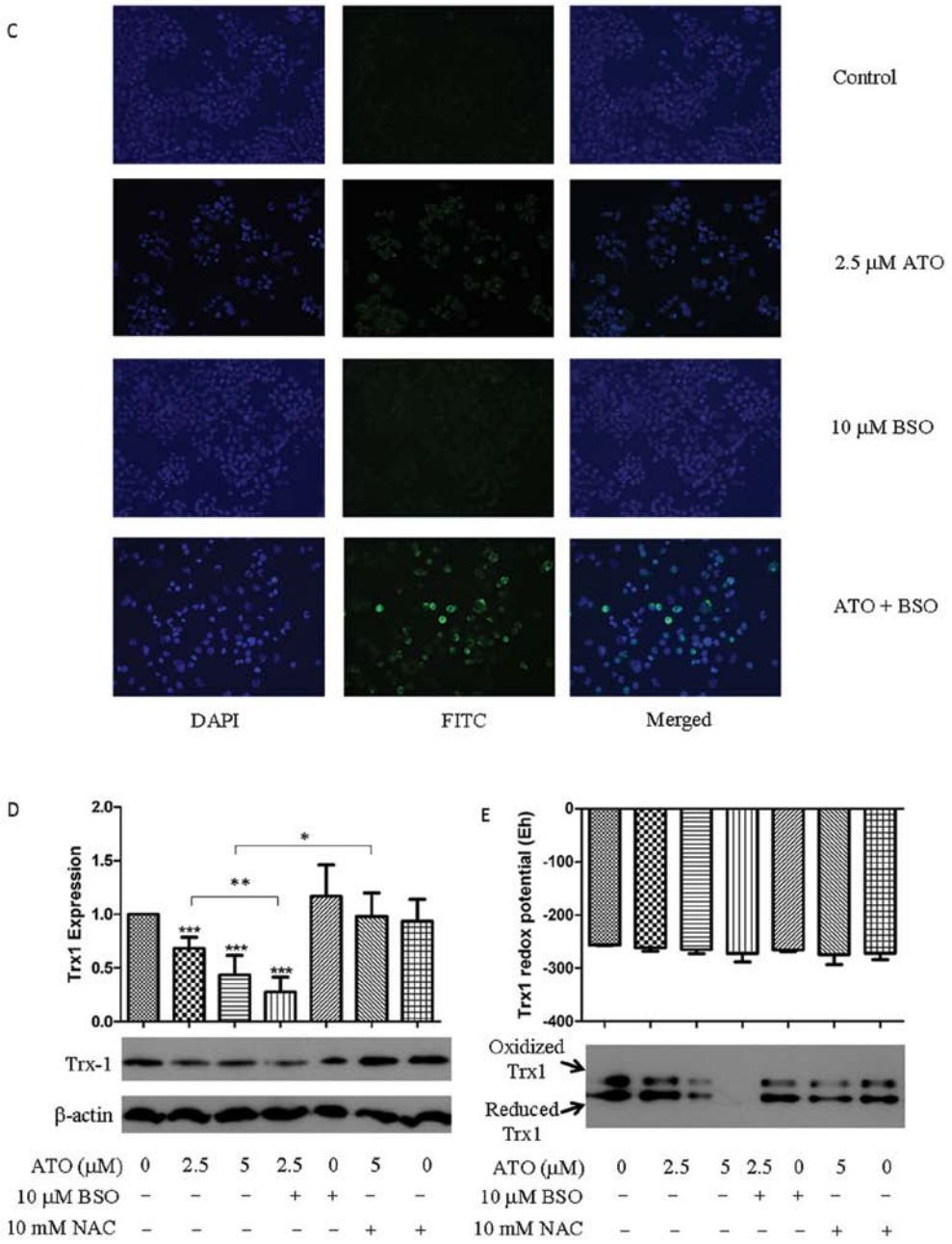

Figure 6. Continued. (C) BSO enhanced oxidative DNA damage when combined with ATO, as indicated by 8-OHdG-FITC staining. (D) ATO reduced Trx1 protein expression, which could be potentiated by BSO and reversed by NAC. (E) Despite reduction of both reduced and oxidized form of Trx1 by ATO (enhanced by addition of BSO), Trx1 redox potential was unaffected. Statistical significance ($^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.001$; ns, not significant) indicates comparison with control.

Trx1 is one of the important components of Trx redox system responsible for maintaining redox equilibrium in mammalian cells. ATO reduced total Trx1 protein expression in H841 cells, which could be further downregulated by BSO but was reversed by NAC (Fig. 6D). Based on Trx redox western blot analysis, both oxidized and reduced forms of Trx1 were downregulated by ATO, nevertheless, redox potential (Eh) remained the same in different treatment groups (Fig. 6E).

Discussion

In the past decade, ATO has been used as a highly effective anticancer therapy in leukemia (particularly APL) with or without all-trans retinoic acid and achieving high rate of complete remission (2,3). Nonetheless, it has been increasingly reported to exert cytotoxic effect in other forms of haematological malignancies and solid cancers such as breast (37), ovarian (38), cervical (39), and lung (20) cancers. The mechanisms and pathways involved are highly diverse and cell type-specific, though knowledge is scarce in SCLC.

In this study, a panel of five SCLC cell lines was tested for the effect of ATO on cellular proliferation, with IC_{50} values in clinical achievable concentrations (3).

Caspase-dependent apoptosis is classified into intrinsic (mitochondria-mediated) and extrinsic (death receptor-mediated) pathways (40). Both mechanisms converge to activate the

apoptotic executioner caspase-3 and -7, which consequently cleave polyADP-ribose polymerase (PARP) that impairs DNA repair. Classically, cleaved caspase-3 and cleaved PARP are widely adopted apoptotic markers. In this study, both markers were elevated after ATO treatment in H841 cells, indicating cell death via the caspase-dependent pathway (41).

AIF is one of the main mediators in cell death, which is liberated from mitochondria and translocated to nucleus resulting in DNA damage. It has been suggested that nuclear translocation of AIF accompanied by activation of receptor interacting protein 1 (RIP1) are markers of necroptosis (42). Nonetheless, the binding of AIF with DNA provokes caspase-independent chromatin condensation and DNA fragmentation (43-46). In our H841 cell line model, apart from triggering MMD accompanied by AIF release and nuclear translocation, ATO treatment also increased RIP1 expression (data not shown). Taken together, cell death initiated by ATO in H841 cells could be mediated through both caspase-dependent apoptosis and AIF-induced apoptosis or necroptosis.

On the other hand, MMD is associated with mitochondrial release of another apoptosis mediator SMAC, in which cytosolic SMAC can inhibit apoptosis inhibitor protein (XIAP) leading to subsequent release of caspases that initiate apoptosis (40). Our finding of SMAC release and XIAP downregulation during treatment with ATO in H841 cells suggests the possibility of caspase-3 activation via SMAC release/XIAP inhibition rather than cytochrome *c* release/caspase-9 activation. This is in keeping with previously reported mechanism of caspase-dependent apoptosis via SMAC/XIAP/caspase activation in other cancer cell line models with ATO treatment (47,48).

GSH depletion and H_2O_2 accumulation with ATO treatment in H841 cells detected in this study are in keeping with previous reports (11,49-52). Nonetheless, the type and amount of ROS needed to initiate cell death by ATO are still controversial, which can vary with different cell lines and dose of ATO (53). Indeed, the role of ROS in causing cell death induced by ATO was uncertain in other studies (54,55). Based on our findings in H841 SCLC model, oxidative stress with ATO treatment was due to both H_2O_2 production and GSH depletion, in which NAC (a GSH precursor) (56) could reverse these alterations resulting in restoration of mitochondrial membrane potential. In leukemia cell line U973, intracellular GSH depletion could trigger Bax translocation to mitochondria and provoke membrane permeabilization resulting in apoptosis (57). In H841 cell line, we demonstrated downregulation of Bcl-2 upon ATO treatment, abrogating the protection on mitochondria. The mechanism of ATO-induced Bcl-2 downregulation has not been clearly elucidated. Nonetheless Bcl-2 has manifested an antioxidant-like effect in response to either oxidative stress or GSH depletion (58,59). Reversal of ATO-induced Bcl-2 downregulation with NAC in our experiments has suggested the mechanistic role of oxidative stress and GSH depletion.

BHA is an effective scavenger of free radicals widely used in food industry. It has been shown to be effective in scavenging H_2O_2 to rescue cells from the DNA damage (60-63). In our study, adding BHA with ATO in H841 cells was capable of reducing H_2O_2 without effective restoration of GSH content compared with ATO alone, resulting in partial reversal of cell inhibition and apoptosis induced by ATO. It is possible that depletion of GSH with ATO treatment is not only due to consumption by H_2O_2 mediated via glutathione peroxidase (GPx) (64) but also direct consumption by ATO (65). In addition, oxidative DNA damage was more pronounced in ATO treatment with BSO compared with ATO alone, associated with significant GSH depletion due to increased H_2O_2 as previously reported (66,67). Taken together, GSH depletion plays a more important role in ATO-induced cell death in SCLC, similar to previous findings in NSCLC (66).

Thioredoxin system was reported to be an important target of ATO in some cancers (26,68). In H841 cell line model, ATO downregulated both reduced and oxidative form of Trx1 without affecting redox status. This is discrepant from previous report, which indicated that ATO could inhibit thioredoxin reductase and consequently decrease the reduced form of Trx1 (68). Although the activity or expression of thioredoxin reductase has not been measured, our findings suggest that ATO targets the total Trx1 rather than just the reduced form in H841 cells.

MAPKs are potential ROS response factors, nonetheless, p-JNK and p-p38 were not involved in ATO-induced cell death in our H841 cell line model (data not shown), which is different from other cancer models (69-71). Although upregulated p-Erk1/2 was detected after ATO treatment in H841 cells, specific Erk inhibitor PD98059 failed to revert the effects induced by ATO on MMD and cell death. It suggests that p-Erk1/2 is not an effective mediator leading to cell death induced by ATO in SCLC, which may serve in other biological actions (72,73).

In conclusion, the mechanisms of cell death induced by ATO in SCLC are mainly dependent on altered redox homeostasis that comprises GSH depletion, H_2O_2 accumulation and mitochondrial depolarization. Both caspase-dependent pathway mediated via Bcl-2/SMAC/XIAP/Caspase-3 and caspase-independent pathway mediated via AIF would take part in causing apoptosis and necroptosis in SCLC.

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