The Effect of MAPK Inhibitors on Arsenic Trioxide-treated Calu-6 Lung Cells in Relation to Cell Death, ROS and GSH Levels

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Abstract. *Arsenic trioxide (ATO) can regulate many biological functions such as apoptosis and differentiation. We recently demonstrated that ATO-induced apoptosis in Calu-6 lung cancer cells is correlated with glutathione (GSH) content. Here, the effects of ATO and/or mitogen-activated protein kinase (MAPK) inhibitors on Calu-6 cells were investigated in relation to cell growth, cell death, reactive oxygen species (ROS) and GSH levels. Treatment with ATO inhibited the growth of the Calu-6 cells at 72 hours. ATO induced apoptosis, which was accompanied by the loss of mitochondrial membrane potential (MMP; $\Delta\Psi_m$). While general nonspecific ROS decreased in the ATO-treated Calu-6 cells, the intracellular superoxide anion ($O_2^{\bullet-}$) level including mitochondrial $O_2^{\bullet-}$ increased. ATO also induced GSH depletion in the Calu-6 cells. The treatment with MAP kinase kinase (MEK), c-Jun N-terminal kinase (JNK) and p38 inhibitors intensified the cell growth inhibition, cell death, MMP ($\Delta\Psi_m$) loss, and GSH depletion in the ATO-treated Calu-6 cells. In addition, the JNK and p38 inhibitors significantly increased the ROS levels including $O_2^{\bullet-}$ in the ATO-treated Calu-6 cells. In conclusion, all the MAPK inhibitors slightly intensify cell death in the ATO-treated Calu-6 cells and the changes of ROS and GSH brought about by ATO and/or MAPK inhibitor treatment partially influence cell growth and death in Calu-6 cells.*

Abbreviations: ATO, Arsenic trioxide (As_2O_3); ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; MEK, MAP kinase kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun *N*-terminal kinase; SOD, superoxide dismutase; MMP ($\Delta\Psi_m$), mitochondrial membrane potential; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; GSH, glutathione; CMFDA, 5-chloromethylfluorescein diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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Reactive oxygen species (ROS) include hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$). These molecules regulate many important cellular events, including transcription factor activation, gene expression, differentiation and cell proliferation (1, 2). ROS are formed as by-products of mitochondrial respiration or the action of oxidases, including nicotine adenine diphosphate (NADPH) oxidase, xanthine oxidase (XO) and certain arachidonic acid oxygenases (3). A change in the redox state of a cell or tissue implies a change in ROS generation or metabolism. The principal metabolic pathways include superoxide dismutase (SOD), which is expressed as extracellular, intracellular and mitochondrial isoforms. These isoforms metabolize $O_2^{\bullet-}$ to H_2O_2. Further metabolism by peroxidases, which include catalase and glutathione (GSH) peroxidase, yields O_2 and H_2O (4). GSH is the main non-protein antioxidant in the cell and provides electrons for enzymes such as glutathione peroxidase, which reduce H_2O_2 to H_2O. GSH is crucial for cell proliferation, cell cycle progression and apoptosis (5, 6) and is known to protect cells from toxic insult by detoxifying toxic metabolites of drugs and ROS (7). Although cells possess antioxidant systems to control their redox state, excessive production of ROS can be induced and gives rise to the activation of events that lead to death or survival in different cell types (8, 9).

Arsenic trioxide (ATO; As_2O_3) has been reported to induce complete remission in patients with relapsed or refractory acute promyelocytic leukemia (APL) without severe marrow suppression (10). The antiproliferative effect of ATO is not limited to APL cells, but can also be observed in a variety of hematological malignancies (11, 12). Accumulating evidence has indicated that ATO can regulate many biological functions such as cell proliferation, apoptosis, differentiation and angiogenesis in various cell lines such as renal (13), head and neck (14), hepatoma (15),

lung (16) and gastric (17) cancer cells. ATO as a mitochondrial toxin induces a loss of mitochondrial membrane potential (MMP; $\Delta\Psi_m$) (11, 13) and, as such, it induces the generation of ROS (18). These phenomena could trigger the apoptosis of target cells, since it is known that apoptosis is closely related to the collapse of MMP ($\Delta\Psi_m$) (19). Therefore, it is thought that ATO induces apoptosis in tumor cells by affecting the mitochondria and ROS generation. In addition, it has been reported that the intracellular GSH content has a decisive effect on ATO-induced apoptosis (20, 21).

The mitogen-activated protein kinases (MAPKs) are a large family of proline-directed, serine/threonine kinases, which are major components of signaling pathways in cell proliferation, differentiation, embryogenesis and cell death and respond to the activation of receptor tyrosine kinase, protein tyrosine kinases, receptors of cytokines and growth factors and heterotrimeric G protein-coupled receptors (22, 23). There are currently four known MAPKs: the extracellular signal regulated kinase (ERK1/2), the c-Jun *N*-terminal kinase/stress-activated protein kinase (JNK/SAPK) and the p38 (22). Each MAP kinase pathway has relatively different upstream activators and specific substrates (24). Much evidence demonstrates that JNK and p38 are strongly activated by ROS or by a mild oxidative shift of the intracellular thiol/disulfide redox state, leading to apoptosis (25, 26). ROS also are known to induce ERK phosphorylation and activate the ERK pathway (27). In most instances, ERK activation has a pro-survival function rather than pro-apoptotic effects (28). It has also been reported that ATO-induced apoptosis involves stress-mediated pathways such as the activation of ERK (29, 30), JNK (30-32) and/or p38 (32, 33) depending on cell type. Since different ROS levels and diverse functions of MAPKs by ROS may have opposite effects even in the same type of cells, the relationship between ROS and MAPKs in relation to cell survival or cell death signaling needs to be more clarified in the future.

We recently demonstrated that ATO inhibited the growth of Calu-6 lung cancer cells by inducing the cell cycle arrest as well as triggering apoptosis (34) and ATO-induced cell death of Calu-6 is correlated with GSH depletion rather than ROS changes (35). Therefore, in the present study the effects of ATO and/or MAPK inhibitors on Calu-6 lung cancer cells were investigated in relation to cell growth, cell death, ROS and GSH levels.

Materials and Methods

Cell culture. The human pulmonary adenocarcinoma Calu-6 cell line was obtained from American Type Culture Collection (ATCC) and maintained in a humidified incubator containing 5% CO_2 at 37˚C. The Calu-6 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco Brl, Grand Island, NY, USA). The cells were routinely grown in 100-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) and harvested with a solution of trypsin-EDTA while in a logarithmic phase of growth. Cells were maintained in these culture conditions for all the experiments.

Reagents. ATO was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and dissolved in 1.65 M NaOH at 100 mM as a stock solution. JNK inhibitor (SP600125), MEK inhibitor (PD98059) and p38 inhibitor (SB203580) were purchased from Calbiochem (San Diego, CA, USA). These agents were dissolved in DMSO solution at 10 mM as a stock solution. The cells were pretreated as indicated with the MAPK inhibitors for 30 min prior to 72 hours' treatment with ATO. Based on previous experience, 5 μM ATO was chosen as a suitable dose to differentiate the levels of cell growth inhibition and death in the presence or absence of each MAPK inhibitor. The optimal dose of 10 μM MEK inhibitor, JNK inhibitor and p38 inhibitor determined as that, which alone did not strongly affect the growth of the Calu-6 cells was used for all the experiments. NaOH and DMSO were used as a control vehicle. All stock solutions were wrapped in foil and kept at –20˚C.

Cell growth assay. The effect of the drugs on Calu-6 cell growth was determined by trypan blue exclusion cell counting or measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance of living cells as previously described (11). In brief, 5×10^4 cells per well were seeded in 24-well plates (Nunc) for cell counting, and 5×10^3 cells per well were seeded in 96-well microtiter plates for the MTT assay. After exposure to ATO with or without inhibitor, the cells were collected with trypsin digestion for trypan blue exclusion cell counting and for the MTT assay. Twenty μl of MTT (Sigma Aldrich) solution (2 mg/ml in PBS) was added to each well of the 96-well plates. The plates were incubated for 4 additional hours at 37˚C. MTT solution in the medium was aspirated off and 200 μl of DMSO were added to each well to solubilize the formazan crystals formed in the viable cells. The optical density was measured at 570 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co, Sunnyvale, CA, USA).

Annexin V staining. Apoptosis was determined by staining cells with annexin V-fluorescein isothiocyanate (FITC) (Ex/Em=488 nm/519 nm) as previously described (36). In brief, 1×10^6 cells were incubated with or without the reagents, washed twice with cold PBS and then resuspended in 500 μl of binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. Five microliters of annexin V-FITC (PharMingen, San Diego, CA, USA) were then added to these cells, which were analyzed with a FACStar flow cytometer (Becton Dickinson Biosciences, CA, USA).

Measurement of MMP ($\Delta\Psi_m$). MMP ($\Delta\Psi_m$) levels were measured by rhodamine 123 fluorescent dye (Ex/Em=485 nm/535 nm), as previously described (37). In brief, 1×10^6 cells were incubated with or without the agents, washed twice with PBS and incubated with rhodamine 123 (0.1 μg/ml; Sigma) at 37˚C for 30 min. Rhodamine 123 staining intensity was determined by flow cytometry. Rhodamine 123-negative cells indicate the loss of MMP ($\Delta\Psi_m$). The MMP ($\Delta\Psi_m$) levels in the cells, except the MMP ($\Delta\Psi_m$) loss cells, were expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software (Becton Dickinson).

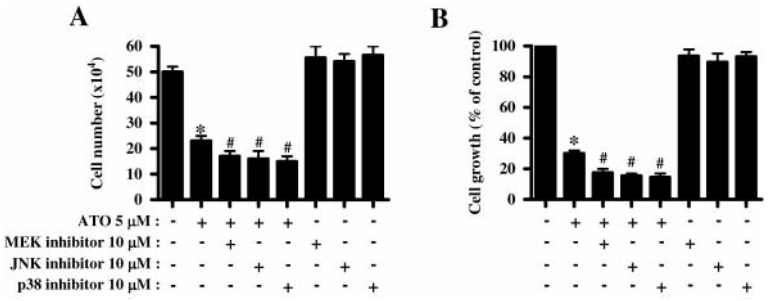

Figure 1. *Effects of MAPK inhibitors on cell growth in ATO-treated Calu-6 cells, after 72 hours' incubation. A, Cell number assessed by trypan blue exclusion cell counting and B, cell growth assessed by MTT assay. *p<0.05 compared with the control group. #p<0.05 compared with cells treated with ATO only.*

Detection of intracellular ROS and $O_2^{\bullet-}$ levels. Intracellular ROS including H_2O_2, $^{\bullet}OH$ and $ONOO^{\bullet}$ were detected by means of an oxidation-sensitive fluorescent probe dye, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen Molecular Probes, Eugene, OR, USA) as previously described (38). H_2DCFDA (Ex/Em=495 nm/529 nm) is poorly selective for the superoxide anion radical ($O_2^{\bullet-}$). Additionally, dihydroethidium (DHE) (Ex/Em= 518 nm/605 nm) (Invitrogen Molecular Probes) a fluorogenic probe that in contrast to H_2DCFDA is highly selective for $O_2^{\bullet-}$ among ROS as previously described was also used (38). Mitochondrial $O_2^{\bullet-}$ levels were detected using MitoSOX™ Red mitochondrial $O_2^{\bullet-}$ indicator (Invitrogen Molecular Probes) as previously described (38). MitoSOX™ Red (Ex/Em=510 nm/580 nm) is a fluorogenic dye for highly selective detection of $O_2^{\bullet-}$ in the mitochondria of cells. In brief, 1×10^6 cells were incubated with or without the reagents and then washed in PBS and incubated with 20 μM H_2DCFDA, 20 μM DHE or 5 μM MitoSOX™ Red at 37˚C for 30 min according to the instructions of the manufacturer. DCF, DHE and MitoSOX™ Red fluorescences were detected using a FACStar flow cytometer (Becton Dickinson). The ROS and $O_2^{\bullet-}$ levels were expressed as MFI, which was calculated by CellQuest software.

Detection of the intracellular glutathione (GSH). The cellular GSH levels were analyzed using 5-chloromethylfluorescein diacetate (CMFDA, Invitrogen Molecular Probes) (Ex/Em=522 nm/595 nm) as previously described (38, 39). In brief, 1×10^6 cells were incubated with or without the reagents and then washed with PBS and incubated with 5 μM CMFDA at 37˚C for 30 min. Cytoplasmic esterases convert non-fluorescent CMFDA to fluorescent 5-chloromethylfluorescein, which can then react with GSH. The CMF fluorescence intensity was determined using a FACStar flow cytometer (Becton Dickinson). Negative CMF staining (GSH depleted) cells were expressed as the percentage of (–) CMF cells. The CMF levels in the cells, except the GSH depleted cells, were expressed as MFI, which was calculated by CellQuest software.

Statistical analysis. The results shown represent the mean of at least two independent experiments; bar, SD. The data were analyzed using Instat software (GraphPad Prism4, San Diego, CA, USA). The student's *t*-test or one-way analysis of variance (ANOVA) with *post hoc* analysis using Tukey's multiple comparison test was used for parametric data. The statistical significance was defined as $p<0.05$.

Results

Effects of MAPK inhibitors on cell growth. Treatment with 5 μM ATO alone inhibited cell growth about 58 and 70% using trypan blue cell counting and the MTT assay respectively at 72 hours (Figures 1A and B). At the 10 μM dose, the MEK, JNK and p38 inhibitors individually did not strongly affect the growth of the Calu-6 cells (Figure 1). MAPK inhibitors significantly inhibited the growth of Calu-6 cells at 20 μM (data not shown). All the MAPK inhibitors intensified the growth inhibition of the ATO-treated Calu-6 cells (Figure 1).

Effects of MAPK inhibitors on apoptosis and MMP ($\Delta\Psi_m$). ATO seemed to induce apoptosis in the Calu-6 cells at 72 hours, as evidenced by annexin V staining (Figure 2). All the MAPK inhibitors intensified the number of annexin V-FITC-positive cells in ATO-treated Calu-6 cells (Figure 2) and the increase was significant with the JNK and p38 inhibitors. A loss of MMP ($\Delta\Psi_m$) was observed in the ATO-treated Calu-6 cells at 72 hours (Figures 3A and B). All the MAPK inhibitors increased the MMP ($\Delta\Psi_m$) loss in the ATO-treated Calu-6 cells (Figures 3A and B). In relation to the MMP ($\Delta\Psi_m$) levels in cells, excluding the rhodamine 123-negative cells, ATO increased the MMP ($\Delta\Psi_m$) level in the Calu-6 cells (Figures 3A and C). All the MAPK inhibitors reduced the MMP ($\Delta\Psi_m$) level in the ATO-treated Calu-6 cells (Figures 3A and C). The MEK and p38 inhibitors also reduced the basal MMP ($\Delta\Psi_m$) level as shown in the Calu-6 control cells (Figure 3C).

Effects of MAPK inhibitors on ROS levels. The intracellular overall ROS (DCF) level decreased in the ATO-treated Calu-6 cells, but the treatment with JNK or p38 inhibitor increased the ROS level in the ATO-treated Calu-6 cells (Figures 4A and C). JNK and p38 inhibitors alone also increased the ROS level compared to that of the Calu-6

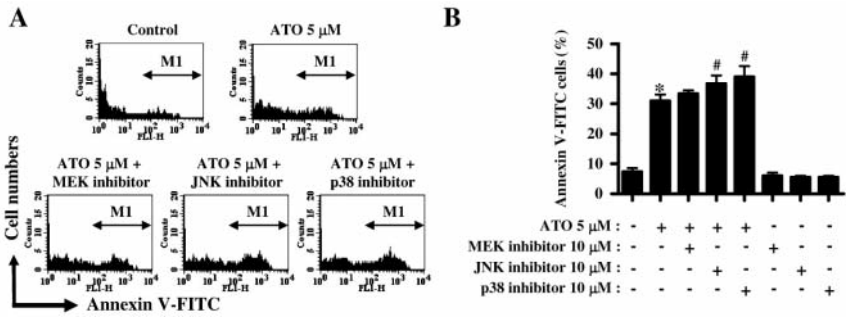

Figure 2. *Effects of MAPK inhibitors on apoptosis in ATO-treated Calu-6 cells after 72 hours' incubation. A, Annexin V-FITC-stained cells were measured by flow cytometry. B, Percentage of annexin V-positive cells (M1 region in A). Data are presented as means±SD of three replicates. *p<0.05 compared with the control group; #p<0.05 compared with cells treated with ATO only.*

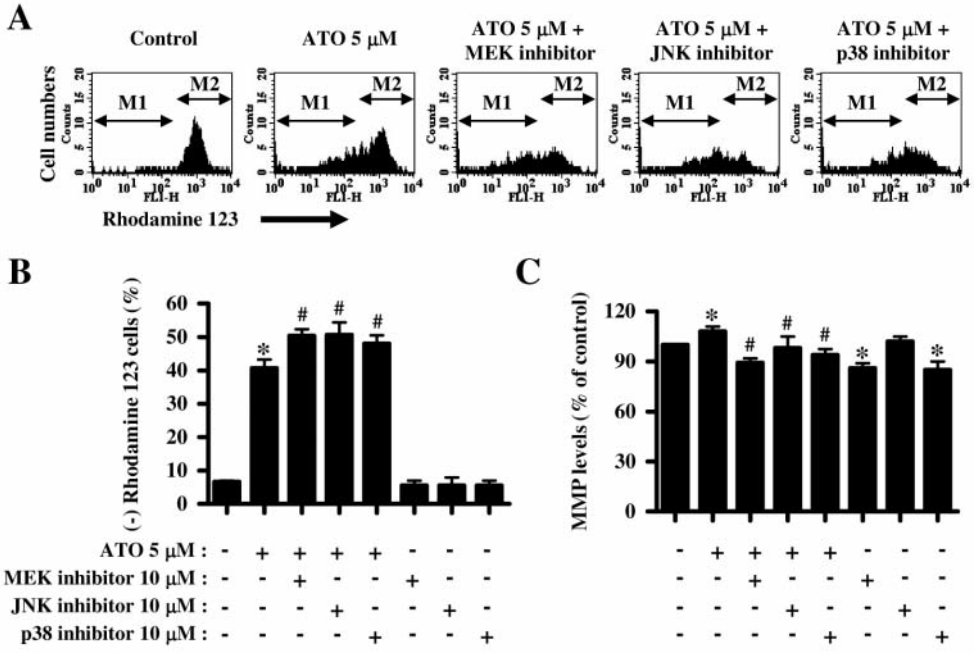

Figure 3. *Effects of MAPK inhibitors on MMP ($\Delta\Psi_m$) in ATO-treated Calu-6 cells after 72 hours' incubation. A, MMP ($\Delta\Psi_m$) measured by flow cytometry. B, Percentage of rhodamine 123-negative (MMP ($\Delta\Psi_m$) loss) cells (M1 region in A). C, MMP ($\Delta\Psi_m$) levels. Data are means±SD of three replicates. *p<0.05 compared with the control group; #p<0.05 compared with cells treated with ATO only.*

control cells (Figure 4C). The red fluorescence derived from DHE reflecting the intracellular $O_2^{\bullet-}$ level was increased in the ATO-treated Calu-6 cells and all the MAPK inhibitors intensified the $O_2^{\bullet-}$ levels in the ATO-treated Calu-6 cells (Figures 4B and D). None of inhibitors alone affected the $O_2^{\bullet-}$ levels compared to the Calu-6 control cells (Figure 4D). Furthermore, the MitoSOX Red fluorescence level, which specifically indicates $O_2^{\bullet-}$ levels in the mitochondria, increased in ATO-treated Calu-6 cells at 72 hours and all the MAPK inhibitors augmented the mitochondrial $O_2^{\bullet-}$ level (data not shown).

Effects of MAPK inhibitors on GSH levels. The treatment with ATO increased the number of GSH depleted Calu-6 cells to about 50 % compared with 10 % in the control cells at 72 hours (Figures 5A and B). All the MAPK inhibitors increased GSH depletion in ATO-treated Calu-6 cells (Figures 5A and B). When the CMF (GSH) level in the Calu-6 cells was assessed, ATO increased the GSH level in the Calu-6 cells (Figures 5A and C). All the MAPK inhibitors slightly reduced the GSH level in these cells (Figures 5A and C). However, all the MAPK inhibitors individually increased the GSH level compared to that of the Calu-6 control cells (Figure 5C).

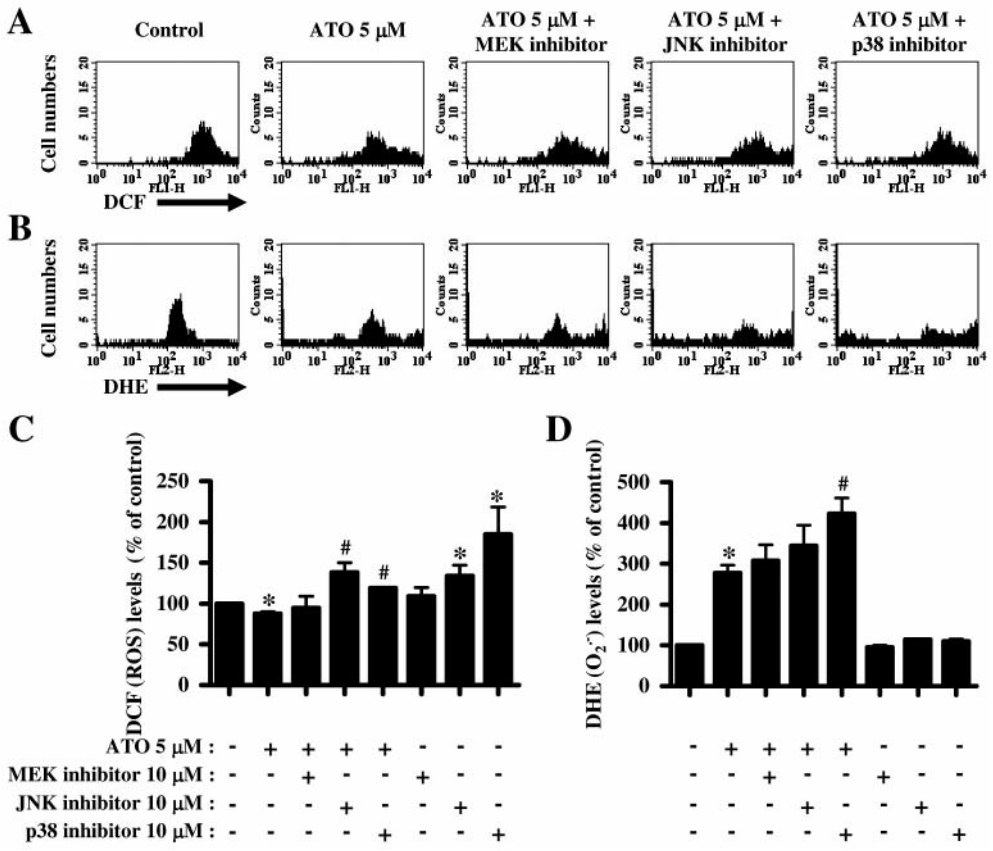

Figure 4. *Effects of MAPK inhibitors on ROS levels in ATO-treated Calu-6 cells after 72 hours' incubations. ROS levels in Calu-6 cells were measured by flow cytometry. A, DCF (ROS) intensities and B, DHE ($O_2^{\bullet-}$) intensities. C, DCF(ROS) levels and D, DHE ($O_2^{\bullet-}$) levels. *p<0.05 compared with the control group; #p<0.05 compared with cells treated with ATO only.*

Discussion

Our recent (34, 39) and current results demonstrated that 5 μM ATO inhibited Calu-6 cell growth and induced apoptosis, which accompanied by the loss of MMP ($\Delta\Psi_m$). ATO can disturb the natural oxidation and reduction equilibrium in cells, leading to an increase of ROS by a variety of redox enzymes, including flavoprotein-dependent superoxide-producing enzymes such as NADPH oxidase (18, 40). In fact, it has been reported that increased intracellular H_2O_2 played an important role in ATO-induced cell death in cervical cancer cells (41), APL cells (42), hepatocellular carcinoma HepG2 (43), and glioblastoma A172 cells (44). However, our recent (34, 39) and current data demonstrated that 5 μM ATO reduced the ROS (DCF) level in Calu-6 cells, although 5 μM ATO increased the ROS (DCF) level without triggering apoptosis in A549 lung cancer cells (39). Data from Haga *et al*. also showed that H_2O_2 accumulation was detected in ATO-treated glioblastoma T98G cells, but apoptosis did not occur in these cells (44). Thus the apoptotic effects of ATO are not consistent with the intracellular ROS levels across different cancer cells types.

In relation to the MAPK inhibitors, the MEK inhibitor which enhanced cell death and cell growth inhibition in the ATO-treated Calu-6 cells did not change the ROS (DCF) level and alone did not alter the ROS level compared to the control cells. Thus, these results support the notion that ERK activation has a pro-survival function rather than pro-apoptotic effects (28) and also suggest that the signaling of MEK in ATO-treated Calu-6 cells does not tightly regulate ROS levels or is not tightly regulated by ROS. In addition, the present results were not consistent with the reports that ATO-induced apoptosis involves the activation of ERK (29, 30, 45). JNK and p38 inhibitor, which presumably inhibited JNK and p38 signaling respectively, increased the cell death and ROS level in ATO-treated Calu-6 cells, which was not consistent with the reports that the activation of JNK or p38 generally leads to apoptosis (25) and ATO-induced apoptosis involves activation of JNK (31, 32, 46) or p38 (32, 33, 46). All these data imply that each MAPK differently affects ATO-induced apoptosis depending on the cell type. In addition, the JNK and p38 inhibitors increased the ROS level without increasing cell death compared to the control cells. Collectively, the present data suggested that the changes of ROS

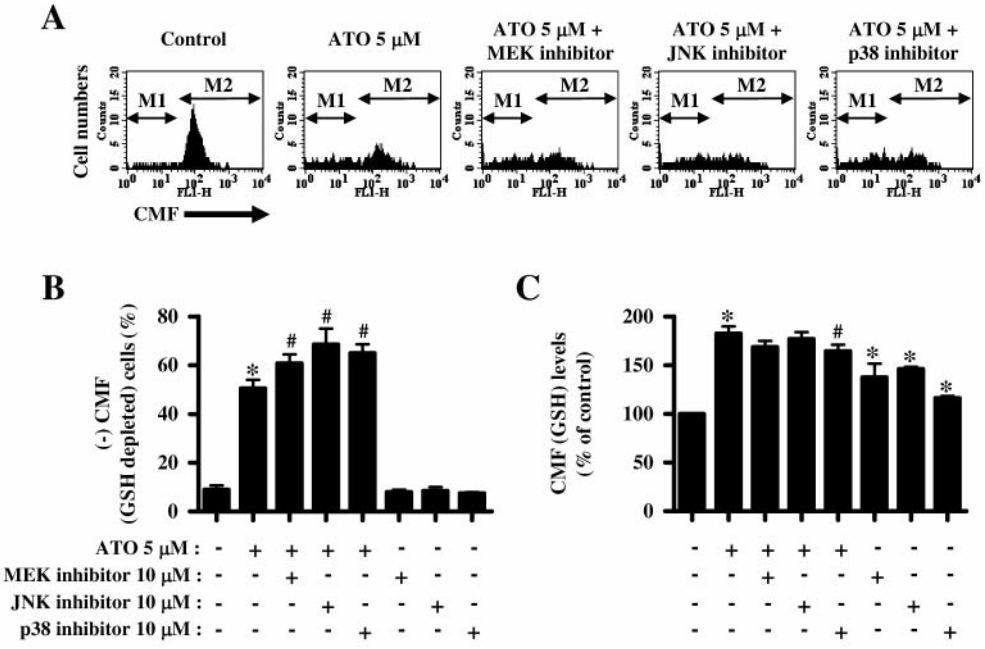

Figure 5. *Effects of MAPK inhibitors on GSH levels in ATO-treated Calu-6 cells, after 72 hours incubation. GSH levels were measured by flow cytometry. A: CMF intensity. B: Percent (-) CMF (GSH depleted) cells (M1 region in A). C: mean CMF (GSH) levels (M2 region in A). Data are presented as means±SD of three replicates. *p<0.05 compared with the control group. #p<0.05 compared with cells treated with ATO only.*

levels by ATO and/or MAPK inhibitors are not tightly correlated with cell death in Calu-6 cells. Therefore, the relationship between ROS, ATO and MAPK signaling in lung cell survival or cell death pathways needs further study.

As reported in esophageal cancer SHEE85 cells (47) and HeLa cervical cancer (48, 49), our recent and current data demonstrated that ATO increased the $O_2^{\bullet-}$ level in Calu-6 cells (34, 39). However, this pattern was not observed in ATO-treated acute myelogenous leukemia HL-60 cells (50) or in renal cell carcinoma ACHN cells (20). These discrepancies might result from different cell-types. The increase of $O_2^{\bullet-}$ level in ATO-treated Calu-6 cells was likely to be from the decreased activity of SOD as well as the production of $O_2^{\bullet-}$ at the mitochondria, since Cu/Zn-SOD activity is reduced by treatment with ATO (39) and an increase in mitochondrial $O_2^{\bullet-}$ level was observed in the ATO-treated Calu-6 cells. The collapse of MMP ($\Delta\Psi_m$) during apoptosis has been reported (19). Correspondingly, ATO induced the loss of MMP ($\Delta\Psi_m$) in the Calu-6 cells. The MAPK inhibitors showing enhancement of cell death in the ATO-treated Calu-6 cells magnified the MMP ($\Delta\Psi_m$) loss and the $O_2^{\bullet-}$ level including mitochondrial $O_2^{\bullet-}$. All the MAPK inhibitors individually did not induce cell death, the MMP ($\Delta\Psi_m$) loss, or ROS generation, including $O_2^{\bullet-}$ compared to the control cells. These results implied that the loss of MMP ($\Delta\Psi_m$) following treatment with ATO and/or MAPK inhibitors leads to apoptosis in Calu-6 cells and MAPK signaling positively affects MMP ($\Delta\Psi_m$) loss in ATO-treated Calu-6 cells. In addition, the present data supported the notion that the changes of the intracellular $O_2^{\bullet-}$ levels are at least partially correlated to apoptosis in ATO-treated HeLa cells (39). Interestingly, ATO increased the MMP ($\Delta\Psi_m$) level. Probably, the increased MMP ($\Delta\Psi_m$) level transiently appeared to compensate for the mitochondrial damage due to the ATO insult. All the MAPK inhibitors reduced the MMP ($\Delta\Psi_m$) level in the ATO-treated Calu-6 cells. The MEK and p38 inhibitors also reduced the MMP ($\Delta\Psi_m$) level compared to the control cells. The basal activity of each MAPK in Calu-6 cells might be differently involved in the maintenance of the MMP ($\Delta\Psi_m$).

The current data showed that ATO increased the number of GSH depleted Calu-6 cells and all the MAPK inhibitors magnified the number of GSH depleted ATO-treated Calu-6 cells. This result supports the notion that the intracellular GSH content has a decisive effect on ATO-induced apoptosis (20, 21). It is of note that the CMF (GSH) level in the ATO-treated Calu-6 cells was increased. The increased GSH level may have occurred in response to the increasing $O_2^{\bullet-}$ produced by the ATO treatment and the Calu-6 cells died if their capacity to resist $O_2^{\bullet-}$ insult was exceeded. All the MAPK inhibitors slightly reduced the GSH level in the ATO-treated Calu-6 cells. However, all the MAPK inhibitors increased the GSH level compared to the control cells. The signaling of each MAPK seems to regulate the GSH levels differently depending on the co-treatment agent and the intracellular ROS level.

In conclusion, all the MAPK inhibitors slightly intensified cell death in ATO-treated Calu-6 cells. The changes of ROS and GSH by ATO and/or MAPK inhibitors affect cell growth and death in Calu-6 cells.

Acknowledgements

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Conflict of Interest Statement

None declared.

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