Arsenic compounds induce apoptosis by activating the MAPK and caspase pathways in FaDu oral squamous carcinoma cells

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Abstract. For a number of years, oral cancer has remained in the top ten most common types of cancer, with an incidence rate that is steadily increasing. In total, ~75% oral cancer cases are associated with lifestyle factors, including uncontrolled alcohol consumption, betel and tobacco chewing, and the excessive use of tobacco. Notably, betel chewing is highly associated with oral cancer in Southeast Asia. Arsenic is a key environmental toxicant; however, arsenic trioxide has been used as a medicine for the treatment of acute promyelocytic leukemia, highlighting its anticancer properties. The present study aimed to investigate the role of arsenic compounds in the treatment of cancer, using FaDu oral squamous carcinoma cells treated with sodium arsenite ($NaAsO_2$) and dimethyl arsenic acid (DMA). The results demonstrated that FaDu cells exhibited membrane blebbing phenomena and high levels of apoptosis following treatment with 10 μM $NaAsO_2$ and 1 mM DMA for 24 h. The results of cell viability assay demonstrated that the rate of FaDu cell survival was markedly reduced as the concentration of arsenic compounds increased from 10 to 100 μM $NaAsO_2$, and 1 to 100 mM DMA. Moreover, flow cytometry was carried out to further examine the effects of arsenic compounds on FaDu cell cycle regulation; the results revealed that treatment with $NaAsO_2$ and DMA led to a significant increase in the percentage of FaDu cells in the sub-G1 and G2/M phases of the cell cycle. An Annexin V/PI double staining assay was subsequently performed to verify the levels of FaDu cell apoptosis following treatment with arsenic compounds. Furthermore, the results of the western blot analyses revealed that the expression levels of caspase-8, -9 and -3, and poly ADP-ribose polymerase, as well the levels of phosphorylated JNK and ERK1/2 were increased following treatment with $NaAsO_2$ and DMA in the FaDu cells. On the whole, the results of the present study revealed that treatment with $NaAsO_2$ and DMA promoted the apoptosis of FaDu oral cancer cells, by activating MAPK pathways, as well as the extrinsic and intrinsic apoptotic pathways.

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Introduction

Head and neck squamous cell carcinomas (HNSCC) develop from the mucosal linings of the upper aerodigestive tract, including the nasal cavity and paranasal sinuses, nasopharynx, hypopharynx, larynx, trachea, oral cavity and oropharynx. Notably, squamous cell carcinoma (SCC) is the most frequent malignant tumor of the head and neck region (1). The incidence rate of HNSCC is higher among males and individuals aged >50 years (2), and is often associated with a number of environmental and lifestyle risk factors, such as alcohol consumption, UV light exposure, tobacco smoking and human papillomavirus infection (3). Moreover, betel chewing is also associated with the development of oral cancer in individuals in Southeast Asia (4). The treatment of SCC includes surgery, radiation, chemotherapy, immunotherapy and gene therapy (5); however, the incidence rates continue to increase (6).

Arsenic is a natural element found on the Earth's crust that exhibits both metallic and non-metallic properties (7), which is further classified based on its valence state. Notably, inorganic arsenic is considered more toxic than organic arsenic (8), and for numerous years, it has been used as a

pesticide due to its high toxicity. Moreover, arsenic trioxide (ATO), originally used as an ingredient in Traditional Chinese Medicine, was shown to exert antitumor effects in patients with acute promyelocytic leukemia (APL) in 1997 (9). Further studies have demonstrated that ATO induces malignant cell apoptosis in numerous types of cancer, including APL (10), multiple myeloma (11) and lung cancer (12). The inorganic arsenic compound, arsenic hexoxide, has also demonstrated anticancer properties in MCF-7 breast cancer cells (13). In addition, this organic arsenic derivative has been shown to be safe and effective in the treatment of hematologic and solid tumors in preclinical models (14). Thus, both inorganic and organic arsenic compounds have exhibited potential in the treatment of tumor progression both *in vitro* and *in vivo*.

There are a number of mechanisms underlying cell death, including autophagy, apoptosis and necrosis, which occur as cells sense environmental stresses or intracellular signals (15-17). Both autophagy and apoptosis are characterized as programmed cell death (18), and defective apoptosis is considered a major causative factor in the progression of cancer (19). The features of apoptosis are predominantly morphological, such as cell shrinkage, plasma membrane blebbing, DNA fragmentation and chromatin condensation (20,21). At the molecular level, extrinsic and intrinsic apoptotic pathways are activated in response to original stimuli, such as granyzme and perforin, and the regulatory aspartate-specific cysteine protease (caspase) cascade (22).

The extrinsic apoptotic pathway is also referred to as the death receptor pathway, which is induced by death receptor (DR)3, DR4, DR5, tumor necrosis factor (TNF) receptor 1 and Fas/CD95 when bound to a specific ligand, such as TNF (23). After binding, the trimerized receptor recruits associated signaling molecules by interacting with the death domain to induce the cleavage of procaspase-8, initiating the protease cascade to cleave targets inside cells, causing apoptotic cell death (22,24). On the other hand, the intrinsic pathway is dependent on the decreasing mitochondrial membrane potential (25). Under conditions of stress, such as DNA damage, UV exposure or hypoxia, cytochrome c is released from the mitochondrial intermembrane space to the cytosol. In turn, cytochrome c binds to apoptotic protease activating factor 1 to form a complex referred to as the apoptosome, which recruits procaspase-9 (26). Active caspase-9 subsequently cleaves procaspase-3, which is released to the cytosol, affecting proteolytic degradation upon target substrates (26). Both apoptotic pathways stimulate effector caspases, which initiate poly ADP-ribose polymerase (PARP) cleavage and delay cellular DNA repair function. Moreover, numerous studies have demonstrated that ATO stimulates tumor cell apoptosis by downregulating Bcl-2 expression and activating the caspase cascade (22,27,28).

It has previously been established that MAPKs play crucial roles in regulating cell death associated with apoptosis (29). MAPKs contain three family members: ERK1 and 2, p38 MAPKs and c-Jun NH2-terminal kinase (JNK1, 2 and 3) (29). Moreover, MAPKs promote either cell survival or death, depending on the cell type and stimulus (29,30). It has previously been reported that the activation of JNK and ERK enhances ovarian carcinoma cell apoptosis with cisplatin (30). Furthermore, the results of a previous study demonstrated that ATO activated JNK and p38 to induce human cervical cancer cell death through the mitochondrial apoptotic cascade (31).

Of note, the authors have previously published a study on the anticancer effects of arsenic compounds on OEC-M1 gingival epidermal carcinoma cells (32). It was found that the arsenic compounds induced the apoptosis of OEC-M1 cells via the MAPK and caspase pathways (32). The present study aimed to examine the potential anticancer properties of the arsenic compounds in a different type of oral cancer, hypopharyngeal SCC, using FaDu cells (33). FaDu cells were treated with both sodium arsenite ($NaAsO_2$) and dimethyl arsenic acid (DMA), and cell viability, cell cycle progression, signaling pathways and apoptosis were investigated. The findings of the present study may provide a novel theoretical basis for the treatment of oral cancers.

Materials and methods

Chemicals, reagents and antibodies. $NaAsO_2$, DMA, PI, high-glucose DMEM, staurosporine, penicillin-streptomycin, MTT and RNase A were purchased from Sigma-Aldrich; Merck KGaA. Trypsin-EDTA and FBS and were purchased from AG Scientific, Inc. Tris base, potassium chloride, HEPES and sodium chloride were obtained from J.T. Baker. Potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$) and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Honeywell Riedel-de Haen. An Annexin V-FITC apoptosis detection kit was purchased from Strong Biotech Corporation. Tween-20, sodium hydroxide, DMSO, hydrochloric acid and SDS were purchased from Sigma-Aldrich; Merck KGaA. Donkey anti-rabbit IgG (cat. no. NEF81200-1EA) conjugated to HRP was purchased from PerkinElmer, Inc. An ECL detection kit was purchased from MilliporeSigma. A Micro BCA protein assay kit was purchased from Thermo Fisher Scientific, Inc. Antibodies against phosphorylated (p)-p38 (cat. no. 9215), p38 (cat. no. 9212), p-ERK1/2 (cat. no. 9101), ERK1/2 (cat. no. 9102), p-JNK (cat. no. 9251), JNK (cat. no. 9252), cleaved PARP (cat. no. 9542), cleaved caspase-8 (cat. no. 9429), cleaved caspase-3 (cat. no. 9661), cleaved caspase-9 (cat. no. 9509) PARP and β-actin (cat. no. 58169; 1:5,000) were obtained from Cell Signaling Technology, Inc.

Cells and cell culture. FaDu human oral cancer cells (hypopharyngeal SCC; HTB-43) purchased from ATCC (33) were used in the present study. FaDu cells were maintained in high-glucose DMEM supplemented with $NaHCO_3$ (24 mM), HEPES (25 mM), 10% heat-inactivated FBS and 100 U/ml penicillin plus 100 μg/ml streptomycin (pH 7.4) in a humidified atmosphere at 37˚C containing 95% air with 5% CO_2 (34).

Morphological analysis. A total of $4.5x10^5$ FaDu cells were plated in a 6-cm Petri dish in 2 ml culture medium. At ~70% confluency, the cells were treated with $NaAsO_2$ (0.1, 1, 10, 25, 50 and 100 μM) or DMA (0.1, 1, 2, 5, 10, 25, 50 and 100 mM) for 24 h. All the aforementioned concentrations have been used in previous studies to exert apoptotic effects on testicular and oral cancer cells (32,35). Changes in cell morphology were examined using an Olympus CK40 light microscope, and recorded using an Olympus DP20 digital camera (Olympus Corporation).

MTT assay. A total of $8x10^3$ FaDu cells were plated in 96-well plates with 100 μl culture medium per well. At ~80% confluence,

cells were treated with $NaAsO_2$ (0.1, 1, 10, 25, 50 and 100 μM) or DMA (0.1, 1, 2, 5, 10, 25, 50 and 100 mM) for 24 h. MTT was added at a final concentration of 0.5 mg/ml and incubated at 37˚C for 4 h. The medium was subsequently discarded and 50 μl DMSO were added to each well to dissolve the crystals for 20 min in the dark by shaking the plate (36-38). The absorbance values were confirmed at λ=570 nm using the VersaMax ELISA reader (Molecular Devices, LLC).

Cell cycle progression analysis. To determine the effects of $NaAsO_2$ and DMA on FaDu cell apoptosis, cell cycle progression was determined using flow cytometry with PI staining. A total of $4.5x10^5$ FaDu cells were plated in a 6-cm Petri dish in 2 ml culture medium. At ~70% confluency, the cells were treated with $NaAsO_2$ (0.1, 1, 10, 25, 50 and 100 μM) or DMA (0.1, 1, 2, 5, 10, 25, 50 and 100 mM) for 24 h. The cells were subsequently collected using trypsin and centrifuged at 400 x g and 4˚C for 12 min. Following centrifugation, the cells were washed with isoton II and fixed with 70% ethanol at -20˚C for ~2 h. The cells were then washed with isoton II again and subsequently harvested by centrifugation at 400 x g for 12 min at 4˚C. Isoton II mixed with 100 μg/ml RNase and 40 μg/ml PI were used to resuspend the cell pellets for 30 min at 25˚C. A flow cytometer (FACScan; Becton, Dickinson and Company) was used to analyze the stained cells with excitation set at λ=488 nm, which would highlight the G1 phase DNA content in normal cells that are diploid, as DNA synthesis increases in the G2/M phase. However, sub-G1 phase cells exhibit a reduced DNA content and are hypodiploid, which indicates cell apoptosis (39-41). The percentages of cells in the sub-G1, S and G2/M phase were further analyzed using FACStation v6.1x and Modfit LT v3.3 software (BD Biosciences).

Annexin V/PI double staining assay. Following treatment with $NaAsO_2$ or DMA as aforementioned, the FaDu cells were collected using trypsin and subsequently washed with 2 ml medium. Following centrifugation at 160 x g for 10 min at 4˚C, cold isoton II was used to resuspend pellets prior to centrifugation again at 400 x g for 12 min at 4˚C. The pellets were subsequently mixed for 15 min with 100 μl staining solution (Annexin V-FITC apoptosis detection kit; Strong Biotech). A FACScan flow cytometer (Becton, Dickinson and Company) was used to analyze the stained cells at >600-nm band pass filter for PI detection, and λ=488 nm excitation using 515-nm band pass filter for FITC detection. The plots comprise four quadrants, which include negative cells, PI-positive cells (necrosis), Annexin V-positive cells (early apoptosis) and Annexin V/PI double-positive cells (late apoptosis) (42,43). The percentage of cells in the four quadrants were analyzed using FACStation v6.1x software. In addition, cells were also treated with staurosporine (Sigma-Aldrich; Merck KGaA) and these were considered as a positive control.

Western blot analysis. A total of $6x10^5$ FaDu cells were plated in a 60-mm dish. At ~70% confluency, the cells were treated with 10 and 25 μM $NaAsO_2$, or 10 and 25 mM DMA for 3, 6, 12 and 24 h. The cell medium was transferred to a 15-ml tube and centrifuged at 1,500 x g for 10 min at 4˚C. The attached FaDu cells were lysed with 100 μl lysis buffer containing proteinase inhibitor (cat. no. P8340; Sigma-Aldrich; Merck KGaA). The pellets were subsequently resuspended with 10 μl lysis buffer, blended into cell lysates and centrifuged again at 12,000 x g for 12 min at 4˚C. The supernatants were then harvested and stored at -80˚C until further use. The protein concentration of the cell lysates was determined using a Micro BCA assay (44,45). For western blot analysis, ~30 μg lysates per lane were resolved on a 12% SDS-PAGE gel with standard running buffer (24 mM Tris/HCl, 0.19 M glycine, 0.5% SDS, pH 8.3) at 25˚C, and were subsequently transferred to PVDF membranes at 4˚C. The membranes were then blocked with 4% milk at room temperature for 60 min, and incubated with the following primary antibodies against p-p38 (1:1,000), p38 (1:4,000), p-ERK1/2 (1:4,000), ERK1/2 (1:4,000), p-JNK (1:4,000), JNK (1:1,000), cleaved PARP (1:1,000), cleaved caspase-8 (1:1,000), cleaved caspase-3 (1:1,000), cleaved caspase-9 (1:1,000) and β-actin (1:5,000) (all antibody details are as aforementioned) overnight at 4˚C. The membranes were then washed with 0.1% TBS Tween-20 and incubated with HRP-conjugated secondary antibodies (donkey anti-rabbit IgG; 1:2,000) for 1 h at room temperature. The membranes were visualized using an ECL detection kit and UVP EC3 BioImaging Systems (Analytik Jena AG) (33,34). Band semi-quantification was performed using ImageJ software version 1.50 (National Institutes of Health).

Statistical analysis. Data are expressed as the mean ± SEM of three independent experiments. Significantly statistical differences between control and treatment groups were examined using one-way ANOVA followed by Tukey's post hoc test, using GraphPad Prism 6 software (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Arsenic compounds induce morphological changes in FaDu cells. The FaDu cells were plated in a 6-cm Petri dish with either 0, 0.1, 1, 10, 25, 50 and 100 μM $NaAsO_2$ or 0, 0.1, 1, 2, 5, 10, 25, 50 and 100 mM DMA for 24 h. The morphological differences were subsequently examined under a light microscope. In the control group, the FaDu cells were firmly attached to the Petri dish and formed healthy polygonal shapes (Fig. 1A). However, following treatment with $NaAsO_2$, cells floated in the medium and acquired a more rounded shape in a concentration-dependent manner. Notably, the shapes of the attached cells were irregular, indicating cell death (Fig. 1B-E).

Moreover, cells that were treated with ≤50 and 100 μM $NaAsO_2$ exhibited notable blebbing in the plasma membrane, which is a characteristic of cell apoptosis (Fig. 1F and G). Treatment with 0.1 to 2 mM DMA also caused the morphological rounding of the FaDu cells (Fig. 1H-J), and increasing concentrations of DMA at 5 and 10 mM induced the rounding of the majority of cells (Fig. 1K and L). Furthermore, following treatment with 50 and 100 mM DMA, cells floated in the cell medium (Fig. 1N and O). Notably, following treatment with 25 mM DMA, the morphology of the FaDu cells was reversed, and the cells exhibited a shriveled membrane (Fig. 1M). The results of the present study demonstrated that treatment with both $NaAsO_2$ and DMA induced abnormal morphological changes of the FaDu cells in a concentration-dependent manner.

Arsenic compounds suppress FaDu cell viability. Following treatment with the arsenic compounds, the levels of FaDu cell

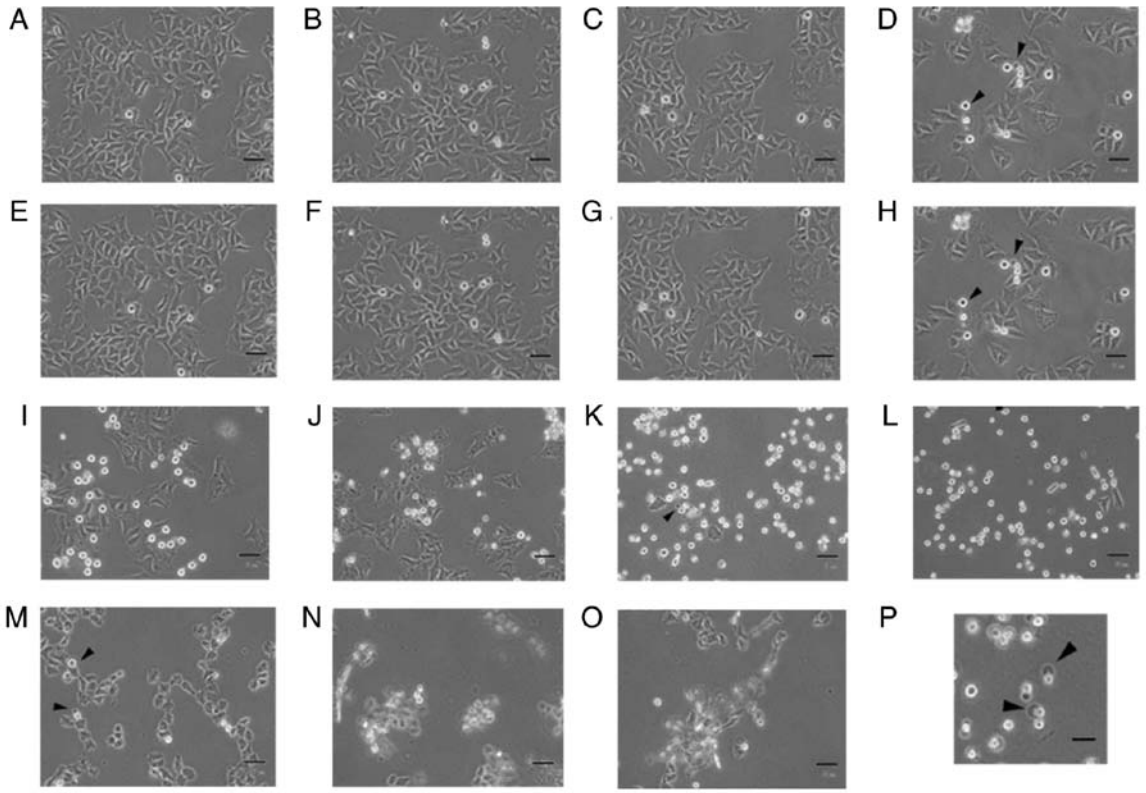

Figure 1. Arsenic compounds induce morphological changes in FaDu cells. FaDu cells were treated with (A) plain medium, (B) 0.1, (C) 1, (D) 10, (E) 25, (F) 50 and (G) 100 μM sodium arsenite or (H) 0.1, (I) 1, (J) 2, (K) 5, (L) 10, (M) 25, (N) 50 and (O) 100 mM dimethyl arsenic acid for 24 h, respectively. Morphological differences were examined by light microscopy (scale bar, 50 μm). Arrowheads indicate cells with membrane blebbing. (P) Enlarged image (4-fold) from (G) of cells with membrane blebbing (scale bar, 200 μm).

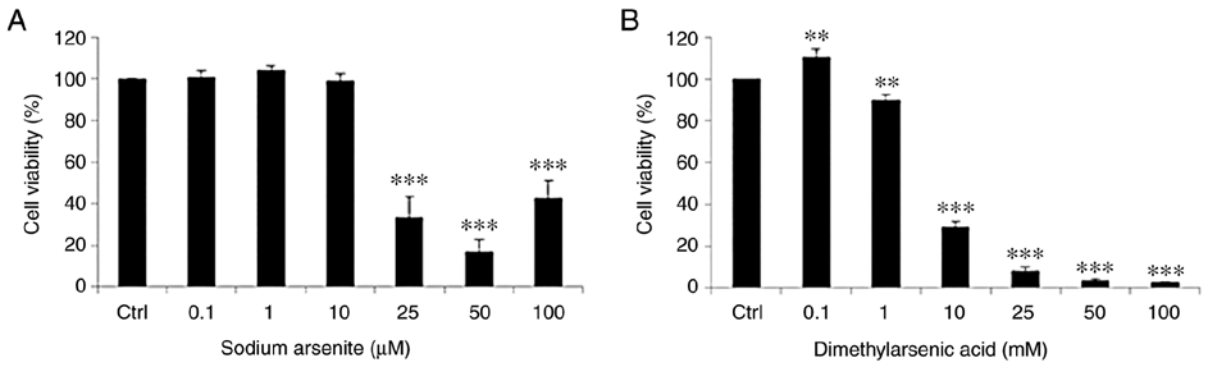

Figure 2. Arsenic compounds regulate FaDu cell viability. FaDu cells were challenged with (A) 0.1-100 μM sodium arsenite or (B) 0.1-100 mM dimethyl arsenic acid for 24 h. MTT assay was then used to determine cell viability. Data are illustrated as percentages of cell growth compared to the control. The results are expressed as the mean ± SEM of three separate experiments (**P<0.01 and ***P<0.001, significant differences compared to the control group). Ctrl, control.

viability were determined using MTT assays. FaDu cells were treated with 0, 0.1, 1, 10, 25, 50 and 100 μM $NaAsO_2$ or 0.1, 1, 2, 5, 10, 25, 50 and 100 mM DMA for 24 h. The results of the present study demonstrated that treatment with 25, 50 and 100 μM $NaAsO_2$ markedly suppressed FaDu cell viability, and treatment with DMA significantly decreased FaDu cell viability in a concentration-dependent manner. The survival rate of the FaDu cells was significantly decreased following treatment with $NaAsO_2$ from 25-100 μM, and following treatment with DMA from 1-100 mM (Fig. 2A and B).

The concentration of DMA that was required to reduce the level of FaDu cell viability to 50% was ~1,000-fold higher than the concentration of $NaAsO_2$. Therefore, $NaAsO_2$ exerted an increased level of cytotoxicity in FaDu cells than DMA.

Arsenic compounds modulate FaDu cell cycle progression. To investigate the effects of $NaAsO_2$ and DMA on cell apoptosis, FaDu cells were treated with these arsenic compounds and subsequently examined using flow cytometric analysis. Briefly, the FaDu cells were treated with $NaAsO_2$ (0, 0.1, 1, 10, 25, 50 and 100 μM) or DMA (0, 0.1, 1, 10, 25, 50 and 100 mM) for 24 h, and the effects of the compounds on cell cycle regulation were determined (Figs. 3 and 4). The results of previous

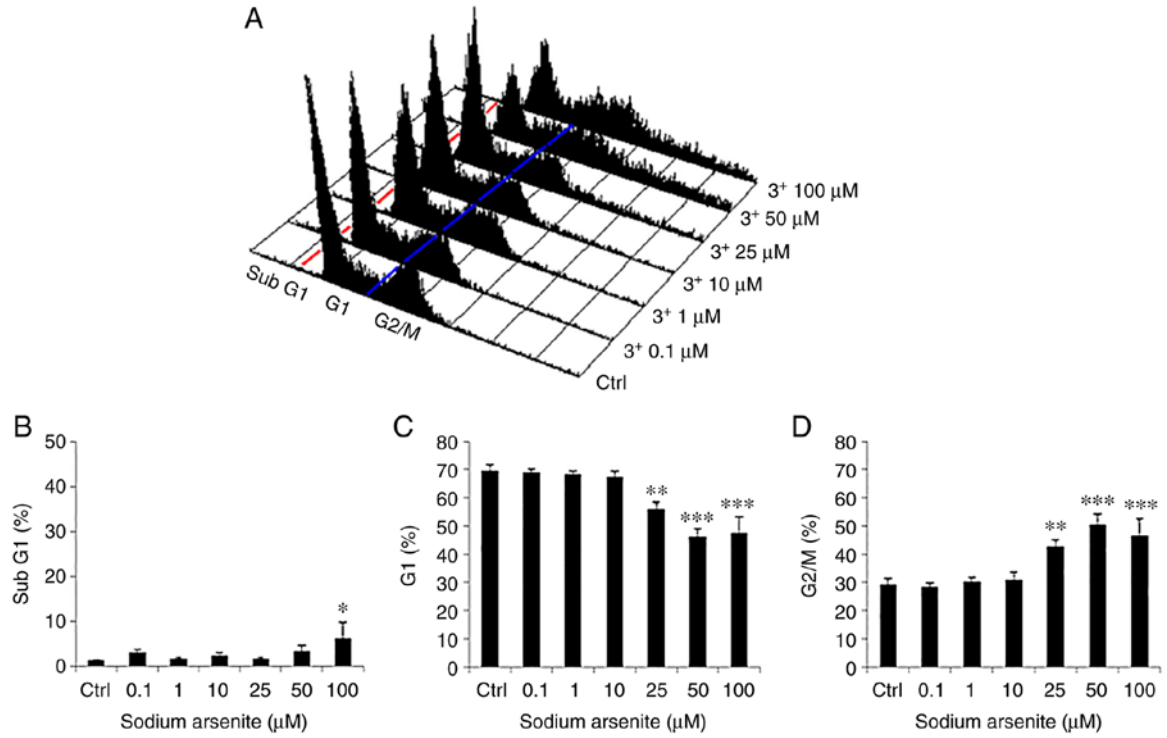

Figure 3. Sodium arsenite modulates the cell cycle progression of FaDu cells. (A) FaDu cells were treated with various concentrations of sodium arsenite (0-100 μM) for 24 h, and then fixed/stained with PI and evaluated using flow cytometry. Cells in the sub-G1 phase with less DNA content, compared to normal cells, indicate apoptosis. Red and blue lines are plotted to illustrate the changes of sub-G1 (left to red line), G0/G1 (between red and blue lines) and G2/M phases (right to blue line) in the different treatment groups. Percentages of (B) sub-G1, (C) G1, and (D) G2/M phase cells are illustrated, respectively. Results are presented as the mean ± SEM of three separate experiments (*P<0.05, **P<0.01 and ***P<0.001, significant differences compared to the control group). Ctrl, control.

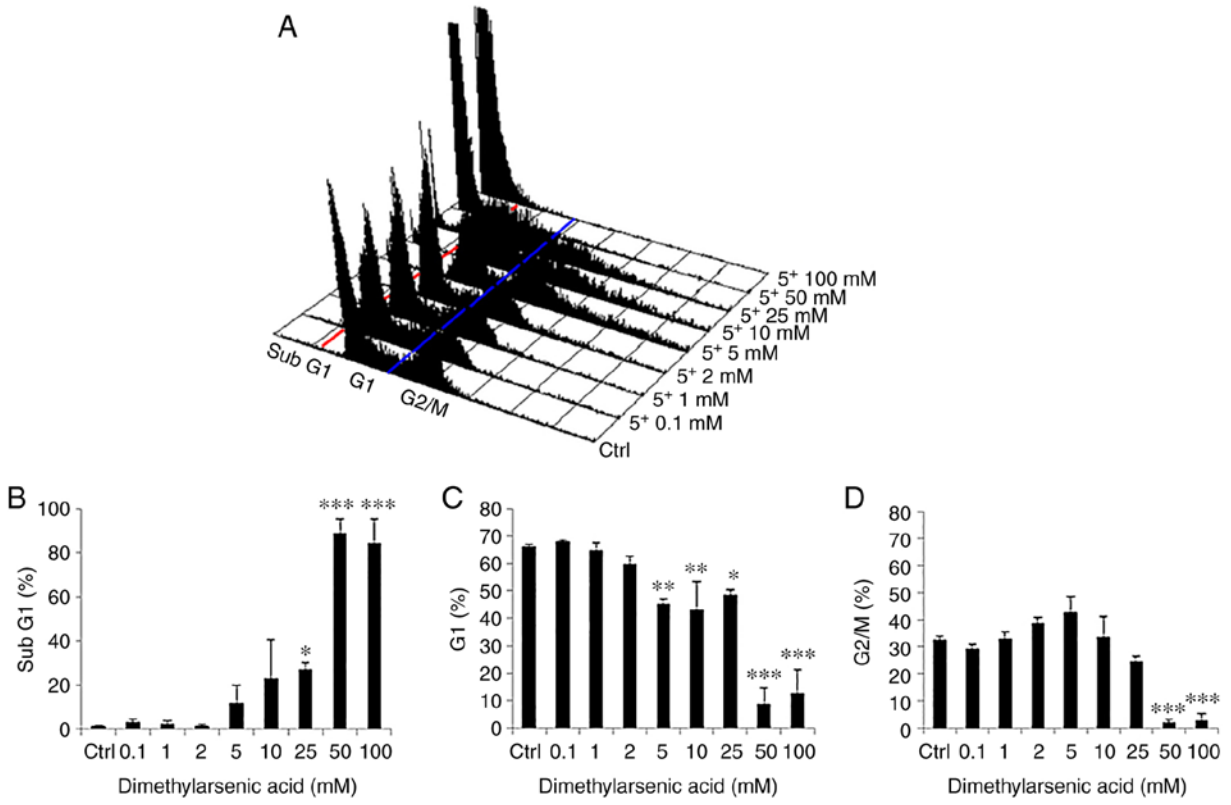

Figure 4. Dimethyl arsenic acid modulates the cell cycle progression of FaDu cells. (A) FaDu cells were treated with various concentrations of DMA (0-100 mM) for 24 h, and then fixed/stained with PI and evaluated using flow cytometry. Cells in the sub-G1 phase with less DNA content, compared to normal cells, indicate apoptosis. Red and blue lines were plotted to illustrate the changes of subG1 (left to red line), G0/G1 (between red and blue lines) and G2/M phases (right to blue line) in the different treatment groups. Percentages of (B) sub-G1, (C) G1, and (D) G2/M phase cells are illustrated, respectively. Results are presented as the mean ± SEM of three separate experiments (*P<0.05, **P<0.01 and ***P<0.001, significant differences compared to the control group). Ctrl, control.

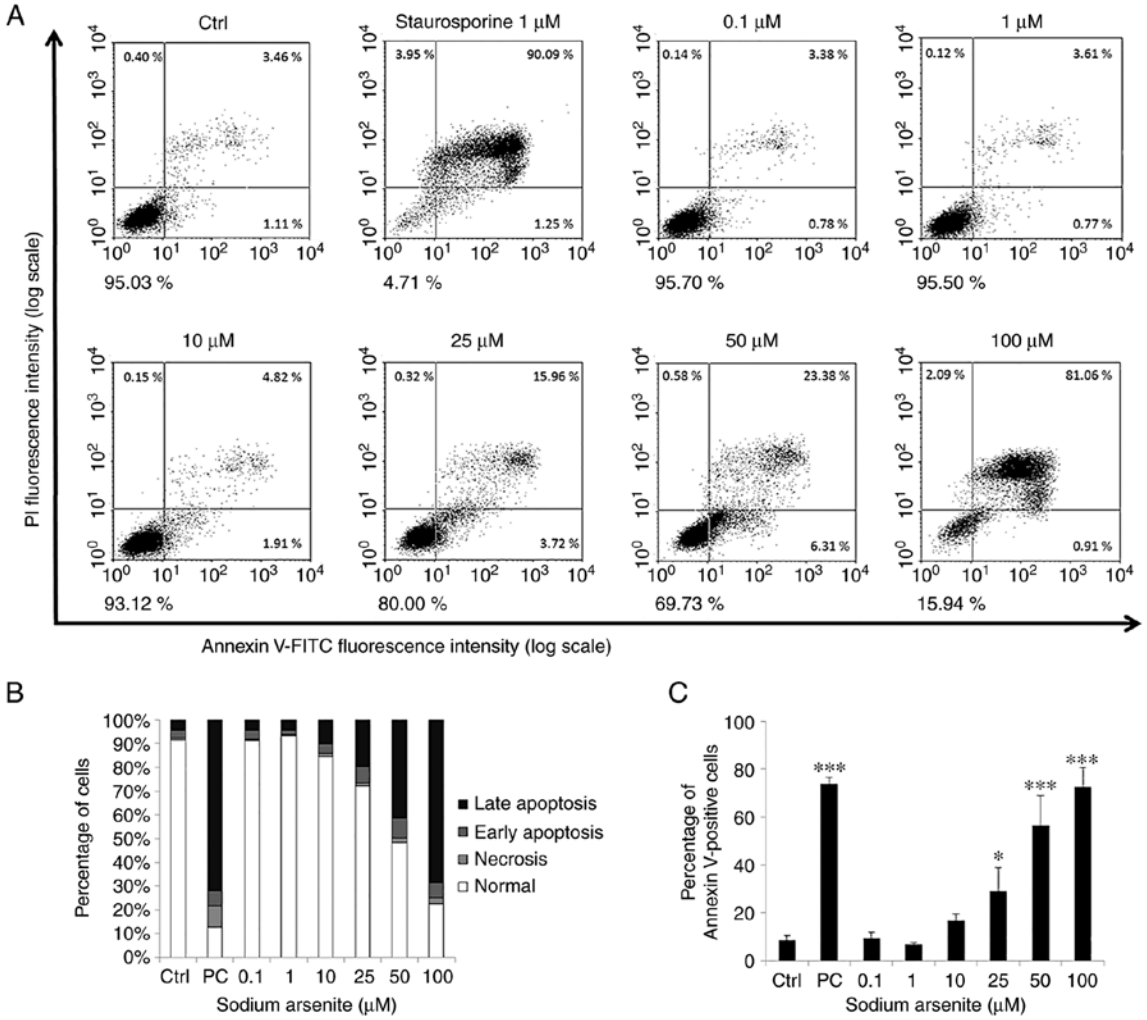

Figure 5. Sodium arsenite significantly induces FaDu cell apoptosis. FaDu cells were challenged with various concentrations of sodium arsenite (0-100 μM) for 24 h. Staurosporine-treated cells were considered as a positive control. (A) Apoptotic phenomena were examined using Annexin V/PI double staining assay. (B) Percentages of staining negative (viable), PI-positive (necrosis), Annexin V-positive (early apoptosis) and double-positive (late apoptosis) cells are shown, respectively. (C) Cells which are Annexin V-positive were analyzed and shown. Results are presented as the mean ± SEM of three separate experiments (*P<0.05 and ***P<0.001, significant differences compared to the control group). Ctrl, control; PC, staurosporine, positive control.

studies have revealed that DNA fragmentation in the sub-G1 phase cells is recognized as cell apoptosis (39,46).

The results of the present study demonstrated a significant increase in the percentage of cells in the sub-G1 phase following treatment with $NaAsO_2$ at 100 μM for 24 h (Fig. 3A and B). Moreover, the percentage of cells in the G1 phase decreased with the increasing concentration of $NaAsO_2$ from 25-100 μM for 24 h (Fig. 3A and C). In addition, the percentage of FaDu cells undergoing G2/M phase arrest was markedly increased with the increasing concentration of $NaAsO_2$ from 25-100 μM for 24 h (Fig. 3A and D). The results of a previous study demonstrated that G2/M phase arrest led to cell apoptosis (47).

Moreover, treatment with increasing concentrations of DMA from 25-100 mM led to a notable increase in the percentage of cells in the sub-G1 phase (Fig. 4A and B). In addition, the increasing concentration of DMA from 5-100 mM significantly decreased the percentage of cells in the G1 phase (Fig. 4A and C). Furthermore, DMA at 50 and 100 mM significantly decreased the percentage of cells in the G2/M phase (Fig. 4A and D).

Arsenic compounds induce FaDu cell apoptosis. To investigate the effects of the arsenic compounds on FaDu cell apoptosis, an Annexin V and PI double staining assay was carried out in the present study. It has previously been established that the percentage of negative (viable), PI-positive (necrosis), Annexin V-positive (early apoptosis) and double-positive (late apoptosis) cells are shown in four quadrants to determine cell apoptotic phenomena (42).

The results of the present study demonstrated that treatment with $NaAsO_2$ (25-100 μM) and DMA (2-100 mM) for 24 h significantly promoted the apoptosis of the FaDu cells (early plus late apoptosis). Moreover, the number of Annexin V-positive cells increased following treatment with the arsenic compounds in a concentration-dependent manner (Figs. 5 and 6). Collectively, these results demonstrated that both $NaAsO_2$ and DMA promoted FaDu cell apoptosis.

Arsenic compounds activate extrinsic and intrinsic caspase pathways to induce FaDu cell apoptosis. To investigate whether arsenic compound-induced cell deaths are involved in

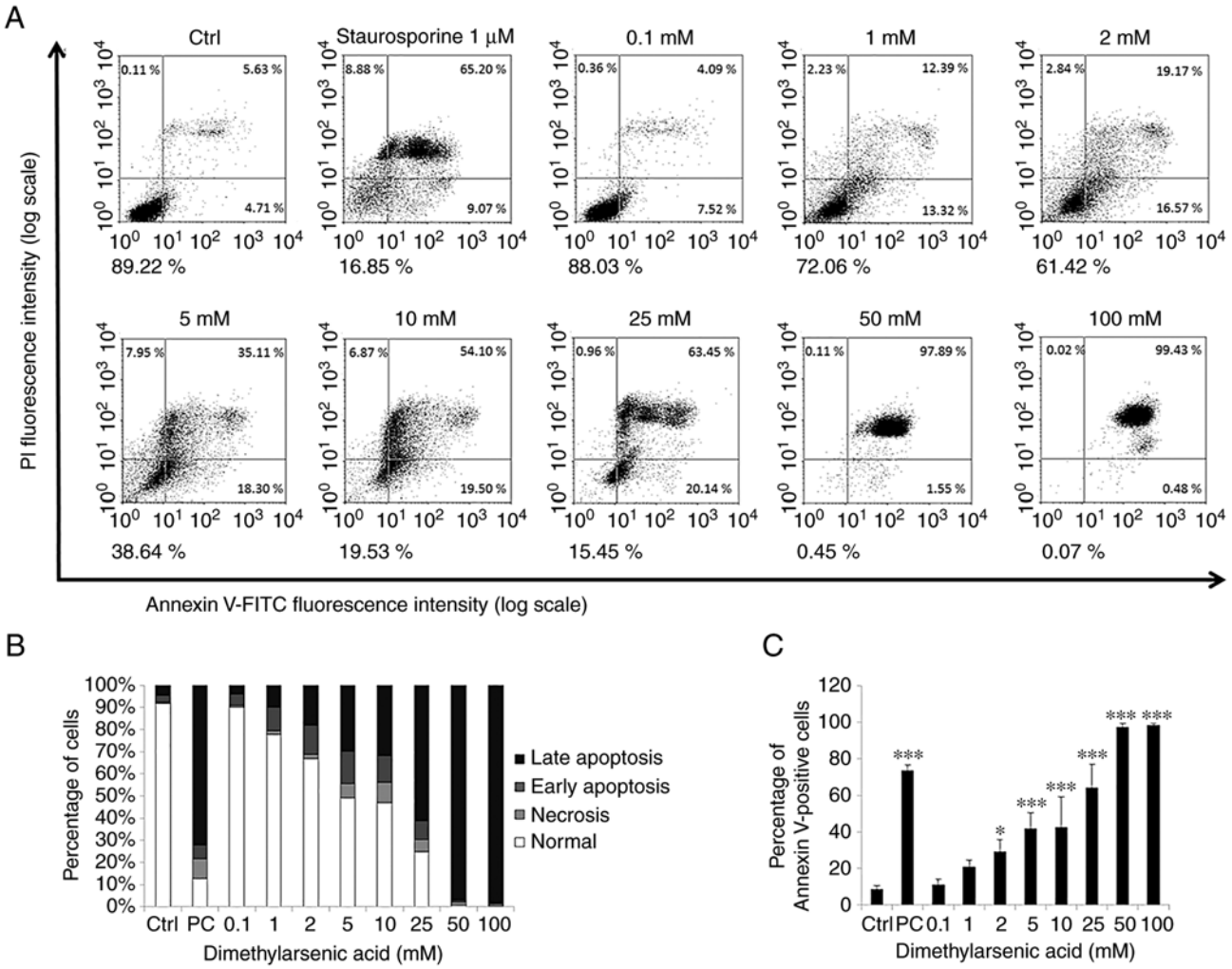

Figure 6. Dimethyl arsenic acid significantly induces FaDu cell apoptosis. FaDu cells were challenged with various concentrations of DMA (0-100 mM) for 24 h. Staurosporine-treated cells were considered as a positive control. (A) Apoptotic phenomena were examined using Annexin V/PI double staining assay. (B) Percentages of staining negative (viable), PI-positive (necrosis), Annexin V-positive (early apoptosis) and double-positive (late apoptosis) cells are shown, respectively. (C) Cells which are Annexin V-positive were analyzed and shown. Results are presented as the mean ± SEM of three separate experiments (*P<0.05 and ***P<0.001, significant differences compared to the control group). Ctrl, control; PC, staurosporine, positive control.

extrinsic (death receptor) or intrinsic (mitochondrial) apoptotic pathways, western blot analysis was performed to determine the expression levels of cleaved caspase-9, caspase-8, caspase-3 and cleaved PARP. The results of the present study demonstrated that treatment with 10 and 25 μM $NaAsO_2$ for 24 h induced the expression of cleaved caspase-8, -9 and -3, as well as the substrate of activated caspase, PARP, in the FaDu cells (Fig. 7). In addition, following treatment with 2 mM DMA for 12 h, the expression levels of cleaved caspase-3 and cleaved PARP were significantly increased, and treatment with 1 and 2 mM DMA for 24 h significantly increased the expression levels of cleaved caspase-9, -8, -3 and PARP, compared with the control group in the FaDu cells (Fig. 8). These data suggested that long-term treatment with $NaAsO_2$ and DMA may stimulate caspase-8, -9, -3 and PARP expression to activate both death receptor and mitochondrial apoptotic pathways in FaDu cells.

Arsenic compounds activate MAPK pathways to induce FaDu cell apoptosis. Numerous studies have demonstrated that MAPK pathways regulate cell mitosis, proliferation, survival, apoptosis, differentiation and gene expression (29,31,37). In the present study, to investigate the potential role of MAPK pathways in the induction of apoptosis following treatment with arsenic compounds, western blot analysis was performed to analyze the phosphorylation levels of JNK, ERK1/2 and p38.

The results revealed that treatment with 25 μM $NaAsO_2$ for 3 h significantly increased the phosphorylation levels of JNK, and treatment with 25 μM $NaAsO_2$ for 3, 12 and 24 h significantly increased the phosphorylation levels of ERK1/2 in FaDu cells (Fig. 9). Moreover, treatment with 1 and 2 mM DMA for 24 h significantly increased the phosphorylation levels of JNK, and treatment with 1 and 2 mM DMA for 12 and 24 h significantly increased the phosphorylation levels of ERK1/2 (Fig. 10). The phosphorylation status of p38 were not altered following treatment with both arsenic compounds in the FaDu cells.

In summary, the aforementioned results illustrate that $NaAsO_2$ and DMA activate JNK and ERK phosphorylation, but not p38, to induce caspase cascade and to thus stimulate FaDu cancer cell apoptosis (Fig. 11).

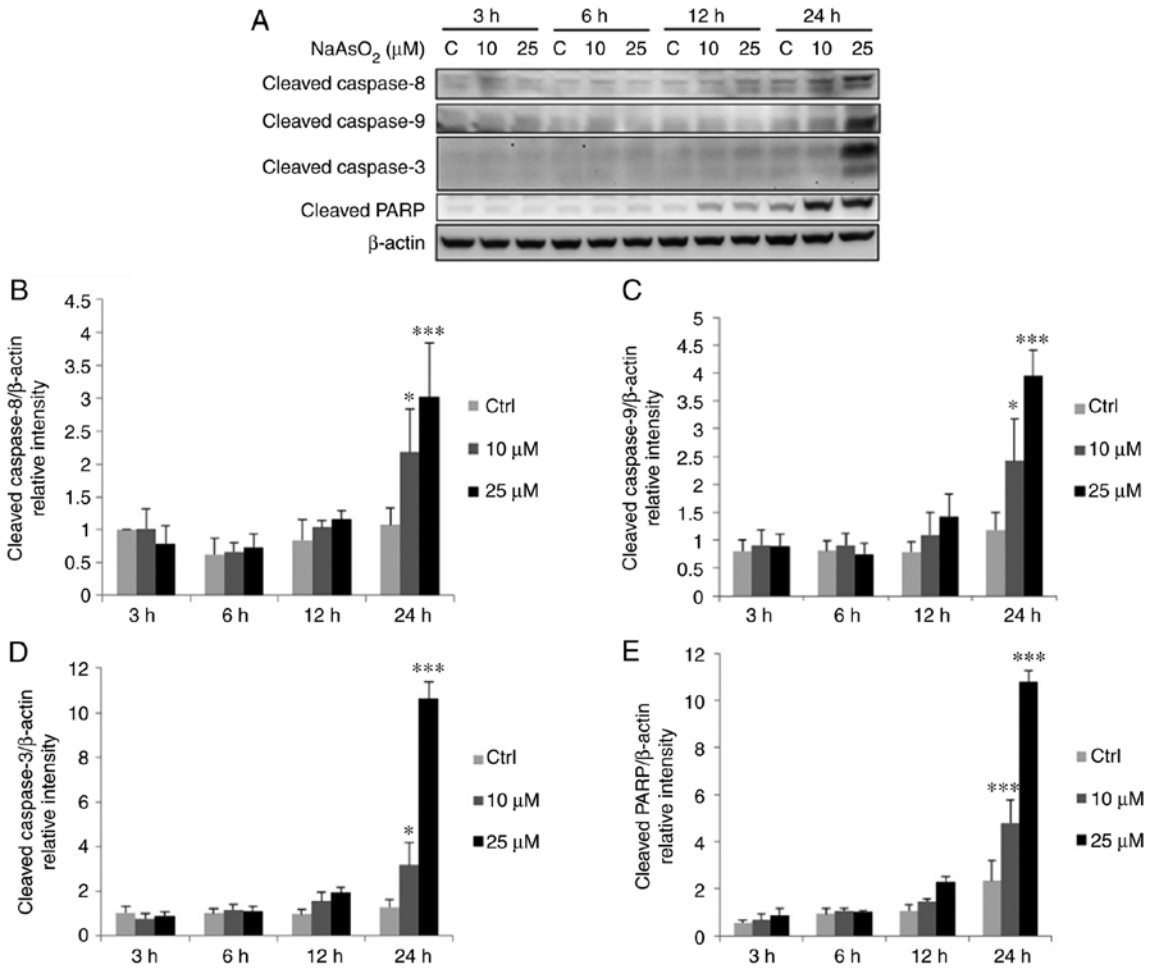

Figure 7. $NaAsO_2$ promotes the cleavage of caspase-8, -9, -3 plus PARP in FaDu cells. FaDu cells were treated with $NaAsO_2$ (0, 10 and 25 μM for 3, 6, 12 and 24 h, respectively). (A) The cleavage of caspase-8 (43 kDa), -9 (35/37 kDa), -3 (17/19 kDa) plus PARP (85-90 kDa) was examined using western blot analysis. Integrated optical intensities of cleaved (B) caspase-8, (C) caspase-9, (D) caspase-3, and (E) PARP were standardized by β-actin (43 kDa) among all lanes. Results are presented as the mean ± SEM of three separate experiments (*P<0.05 and ***P<0.001, significant differences compared to the control group). Ctrl, control. $NaAsO_2$, sodium arsenite.

Discussion

ATO initially demonstrated anticancer properties in patients with APL (48). Additionally, arsenic compounds have demonstrated a level of efficiency in treating numerous types of cancers, such as pancreatic, colon and breast cancers (13,49,50) by inducing cell apoptosis. However, the effects of arsenic compounds in the treatment of oral cavity cancers, and the underlying regulating mechanisms remain to be fully elucidated. Oral cancers are often refractory solid tumors, and the majority of oral cancers are classified as SCC. In Taiwan, the incidence and mortality rates increase each year (6). Treatment options remain limited; thus, the development of novel therapeutic strategies is required. In the present study, $NaAsO_2$ and DMA both demonstrated a capability to promote the apoptosis of FaDu oral cancer cells.

Numerous previous studies have demonstrated that the cytoskeleton is targeted by $NaAsO_2$ and DMA (51,52), and the degree of morphological alterations modulating the cell skeleton is associated with drug dosages and treatment time (53). The results of the present study revealed that treatment with $NaAsO_2$ and DMA induced a number of morphological changes in the FaDu cells. Following treatment with 25 and 50 μM $NaAsO_2$, and 1 and 2 mM DMA, the attached cells stretched along the border, forming large exposed cell surfaces. Compared with cells with abalone-like shapes following treatment with 25 and 50 μM $NaAsO_2$, the DMA-treated cells appeared to be similar to the control group, although they exhibited rough edges. Following treatment with 5 and 10 mM DMA, the cells were attached to the Petri dish, and the majority of cells appeared to be rounded in shape. Moreover, following treatment with 50 and 100 mM DMA, cells floated in clusters. However, the mechanisms underlying arsenic the regulatory effects of the compounds on cell morphology remain to be fully elucidated. Of note, these results indicate that changes associated with the cytoskeleton are dependent on both drug type and concentration.

When specific checkpoint requirements are met by upstream events, the cell cycle continues to the next phase. Under conditions of cell damage or stress, cells can arrest in a specific phase and undergo programmed cell death (54). The results of a previous study demonstrated that arsenite induced microtubule network disruption, causing abnormalities in spindles to induce mitotic cell apoptosis (55). Moreover, arsenic-induced cellular mitotic arrest may be an essential

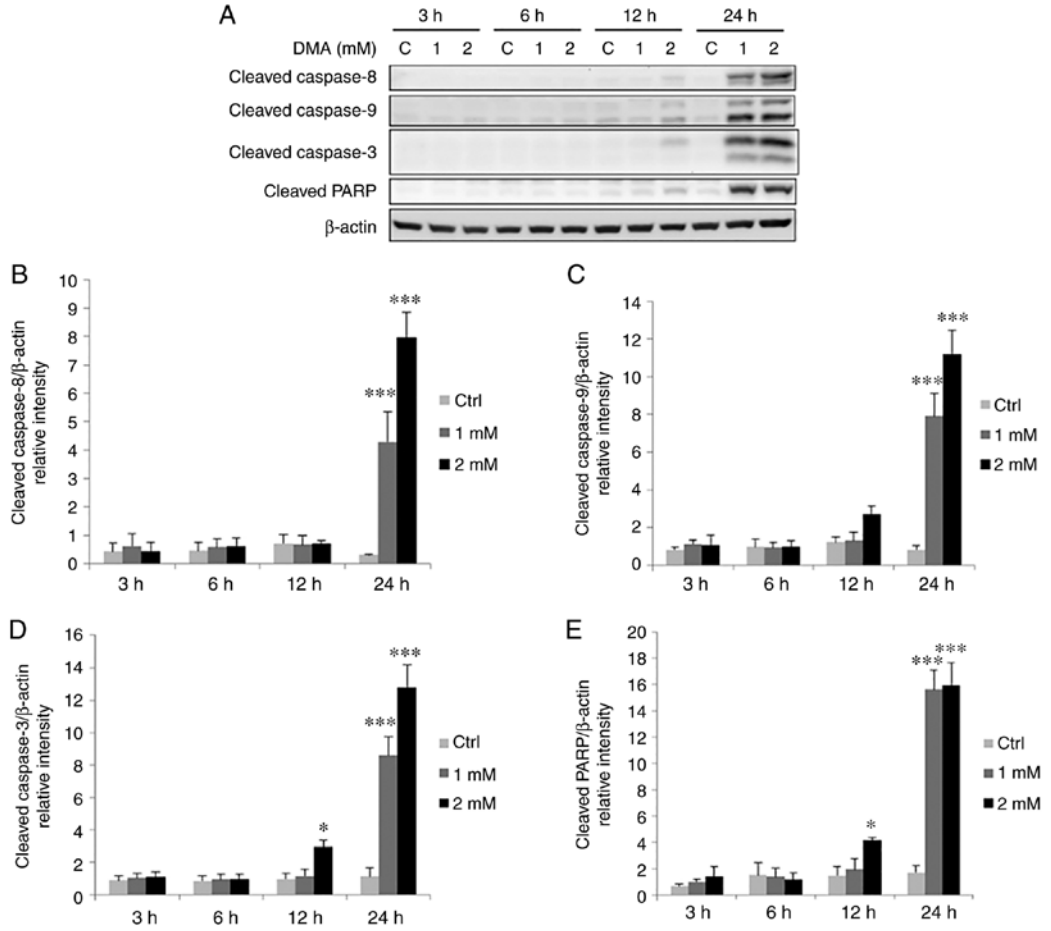

Figure 8. DMA promotes the cleavage of caspase-8, -9, -3 plus PARP in FaDu cells. FaDu cells were treated with DMA (0, 1 and 2 mM for 3, 6, 12 and 24 h, respectively). (A) The cleavage of caspase-8 (43 kDa), -9 (35/37 kDa), -3 (17/19 kDa) plus PARP (85~90 kDa) was examined using western blot analysis. Integrated optical intensities of cleaved (B) caspase-8, (C) caspase-9, (D) caspase-3, and (E) PARP were standardized by β-actin (43 kDa) among all lanes. Results are presented as the mean ± SEM of three separate experiments (*P<0.05 and ***P<0.001, significant differences compared to the control group). Ctrl, control; DMA, dimethyl arsenic acid.

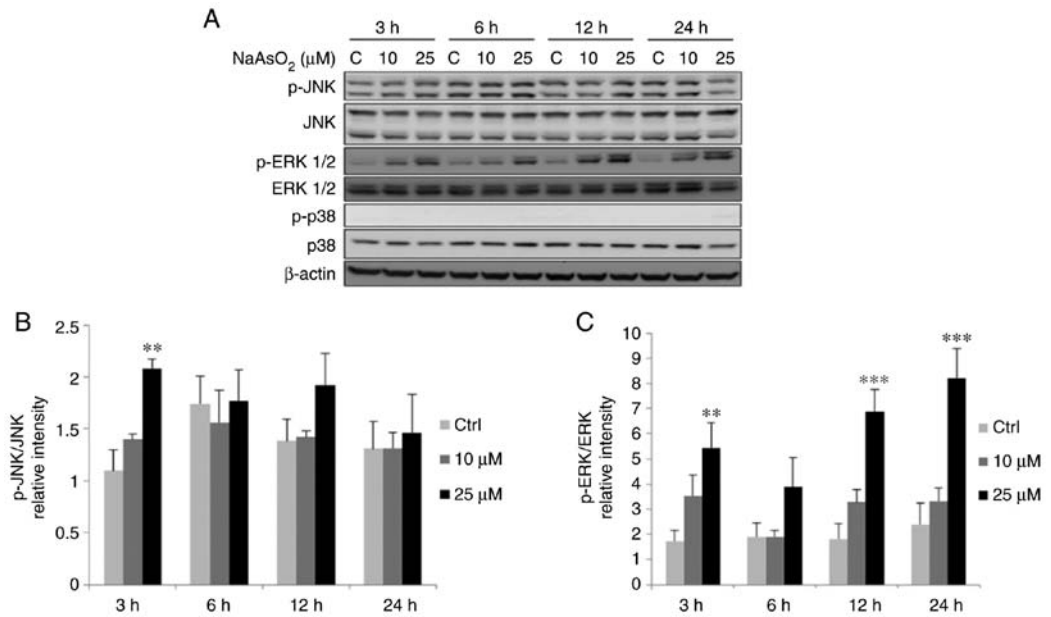

Figure 9. $NaAsO_2$ induces the phophorylation of MAPK pathways in FaDu cells. FaDu cells were treated with $NaAsO_2$ (0, 10 and 25 μM for 3, 6, 12 and 24 h, respectively). (A) Phosphorylated-JNK (46/54 kDa), total JNK (46/54 kDa), phosphorylated-ERK1/2 (42/44 kDa), total ERK1/2 (42/44 kDa), phosphorylated-p38 (43 kDa) and total p38 (43 kDa) were examined using western blot analysis. Integrated optical intensities of (B) p-JNK and (C) p-ERK proteins were standardized with total forms of JNK and ERK, respectively. Results are presented as the mean ± SEM of three separate experiments (**P<0.01 and ***P<0.001, significant differences compared to the control group). Ctrl, control; $NaAsO_2$, sodium arsenite.

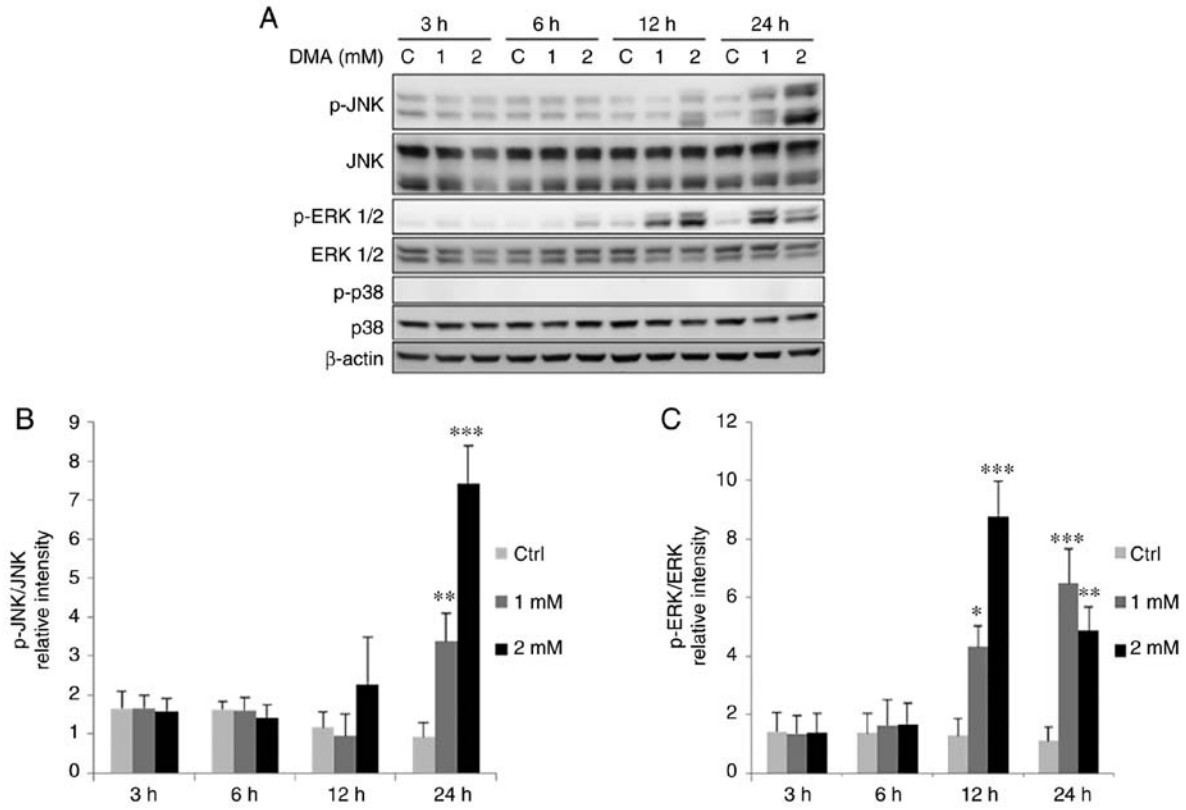

Figure 10. DMA induces the phosphorylation of MAPK pathways in FaDu cells. FaDu cells were treated with DMA (0, 1 and 2 mM for 3, 6, 12 and 24 h, respectively). (A) Phosphorylated-JNK (46/54 kDa), total JNK, phosphorylated-ERK1/2 (42/44 kDa), total ERK1/2 (42/44 kDa), phosphorylated-p38 (43 kDa) and total p38 were examined using western blot analysis. Integrated optical intensities of (B) p-JNK and (C) p-ERK proteins were standardized with total forms of JNK and ERK, respectively. Results are presented as the mean ± SEM of three separate experiments (*P<0.05, **P<0.01 and ***P<0.001, significant differences compared to the control group). Ctrl, control; DMA, dimethyl arsenic acid.

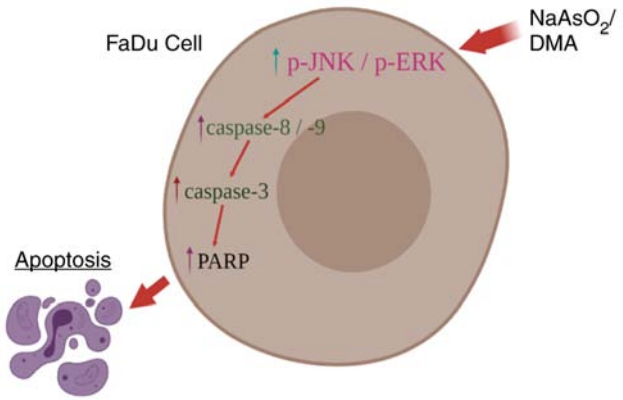

Figure 11. Schematic diagram of the possible molecular mechanisms through which $NaAsO_2$ and DMA induce the apoptosis in FaDu human oral cancer cells. Arsenic compounds induce JNK and ERK phosphorylation, but not that of p38, modulating the induction of the caspase cascade to promote the apoptosis of FaDu cancer cells. The figure was created with BioRender.com (https://biorender.com/, accessed November 6, 2021). $NaAsO_2$, sodium arsenite; DMA, dimethyl arsenic acid.

step in the activation of apoptotic pathways among different human tumor cells (56). Another study demonstrated a clear association between G2/M arrest and the apoptosis of ovarian carcinoma cells in response to DNA damage (57). In the present study, $NaAsO_2$ and DMA notably led to G2/M phase arrest, and increased the percentage of cells in the sub-G1 phase, indicating that both arsenic compounds promoted FaDu cell apoptosis. Moreover, the results of the present study demonstrated that arsenic-induced apoptosis was associated with aberrant cell cycle redistribution. The Annexin V/PI double staining assay further verified that apoptosis was induced by $NaAsO_2$ and DMA in a concentration-dependent manner.

Apoptosis is a crucial process for cell homeostasis, and resisting apoptosis leads to cancer development (58). Previous studies have demonstrated that apoptosis is initiated by an extrinsic or intrinsic death signal to activate caspase cascades (59,60). The results of a previous study revealed that ATO stimulated laryngeal cancer cell apoptosis by decreasing the mRNA expression of survivin and inhibiting caspase activation (61). Previous studies have also demonstrated that ATO induces apoptosis in myeloma and gastric cancers by stimulating caspase-9, -8 and -3 (11,62). The results of the present study demonstrated that $NaAsO_2$ and DMA significantly induced the expression of cleaved caspase-8, -9, 3 and PARP in FaDu cells. Consistent with the results of previous studies (11,32,35), these results indicate that arsenic compounds activate extrinsic and intrinsic apoptotic pathways in oral cavity cancer cells. The results of previous studies have also demonstrated that arginine-glycine-aspartate peptides, granzyme B and endoplasmic reticulum stress are responsible for direct caspase-3 activation, and the induction of apoptosis (63-65). Moreover, the results of the present study revealed that following treatment with 2 mM DMA for 12 h, caspase-3 activation occurred before the activation of both caspase-8 and -9, indicating that alternative signaling pathways require further investigation.

It has previously been established that apoptotic pathways are closely associated with numerous cellular mechanisms, and MAPK signaling pathways respond to different cellular stimuli to induce cell apoptosis (29,30). MAPK signaling pathways stimulate survival or induce apoptosis depending on the stimuli type, cell types and the latency of MAPK activation (29,30). JNKs (also known as stress-activated protein kinases) are ubiquitously expressed in response to growth factors or numerous types of stress (66,67). The results of a previous study demonstrated that JNK activation mediated ATO-induced APL cell apoptosis (68). The ERK pro-apoptotic function is also stimulated by various antitumor compounds (69,70). In addition, another study revealed that the activation of JNK1/2 and ERK was associated with the ATO-induced apoptosis of human mesothelioma cells (71). However, the involvement of p38 in cell apoptosis remains unclear, as it can reduce the expression levels of caspase-8 and -3 in human neutrophils (72). Furthermore, p38 activation mediates the apoptosis of endothelial cells by activating caspase-3 and suppressing Bcl-x(L) (73). The results of the present study demonstrated that the phosphorylation levels of JNK and ERK1/2 were increased following treatment with $NaAsO_2$ and DMA in FaDu cells. However, the levels of p-p38 were not detected in the FaDu cells following treatment with any arsenic compound. Notably, the results of the present study demonstrated that treatment with DMA activated caspases and PARP at 12 and 24 h, and stimulated MAPK at 3 h, highlighting that that MAPK activation was initially activated by DMA to induce the apoptosis of FaDu oral cancer cells.

The results of previous studies have revealed the anticancer effects of arsenic compounds through apoptotic cascades among different cell types (32,35,49,50,74). Previous studies have also demonstrated that arsenic compounds induce tumor cell deaths through cell cycle arrest, the production of reactive oxygen species, DNA methylation and the reduction of stem cell markers (75,76). Thus, further investigations into the anticancer mechanisms underlying arsenic compounds in FaDu cells are required. Moreover, the use of a control cell line for comparison with FaDu cells, *in vivo* experiments using an animal model and clinical trials are all required to increase the reliability of the results. Although further investigations are required, the present study provides a novel theoretical basis for the use of arsenic compounds in the clinical treatment on oral cancers.

Collectively, these results suggested that both $NaAsO_2$ and DMA stimulated FaDu cell apoptosis by activating apoptotic pathways, highlighting their antitumor effects in FaDu cells. Moreover, these arsenic compounds activated JNK and ERK phosphorylation, but not p38, modulating the induction of the caspase cascade to stimulate the apoptosis in FaDu cancer cells (Fig. 11). Thus, both arsenic compounds possess the potential for antitumor therapy in oral cancers and their efficiency is demonstrated in FaDu oral cancer cells.

In a previous study, the authors treated OEC-M1 cells with the arsenic compounds (32). Both $NaAsO_2$ and DMA induced cell apoptosis through extrinsic and intrinsic apoptotic pathways, exhibiting potential antitumor effects in the OEC-M1 oral cancer cells; similar effects were observed in the present study with the FaDu cells. In addition, the levels of phosphorylated JNK and ERK1/2 were elevated by $NaAsO_2$ and DMA in both the OEC-M1 (35) and FaDu cells. Of note, the levels of phosphorylated p38 were hardly detectable in the FaDu cells with either of the arsenic compounds; however, the expression of p-p38 did increase in the OEC-M1 cells following treatment with $NaAsO_2$ and DMA (32). These data illustrate that arsenic compounds activate different MAPK pathways to induce apoptosis between FaDu and OEC-M1 oral cavity cells. These differences activating MAPK pathways between FaDu and OEC-M1 are worthy of further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request, which adheres to the FAIR principles (https://www.go-fair.org/fair-principles/), including the fundamental principles of Findability, Accessibility, Interoperability, and Reusability.

Authors' contributions

SZW, YYL, CYC, YKW and YPL established and conducted the experiments, analyzed the results and wrote the manuscript. BMH and HYC participated in the study design and were also involved in the statistical analysis of the results, and revised the manuscript. SZW, HYC and BMH confirm the authenticity of all the raw data. All authors have read and approved the final version of manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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