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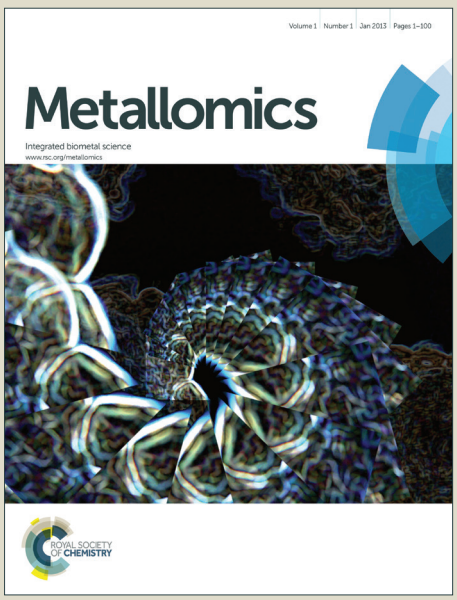

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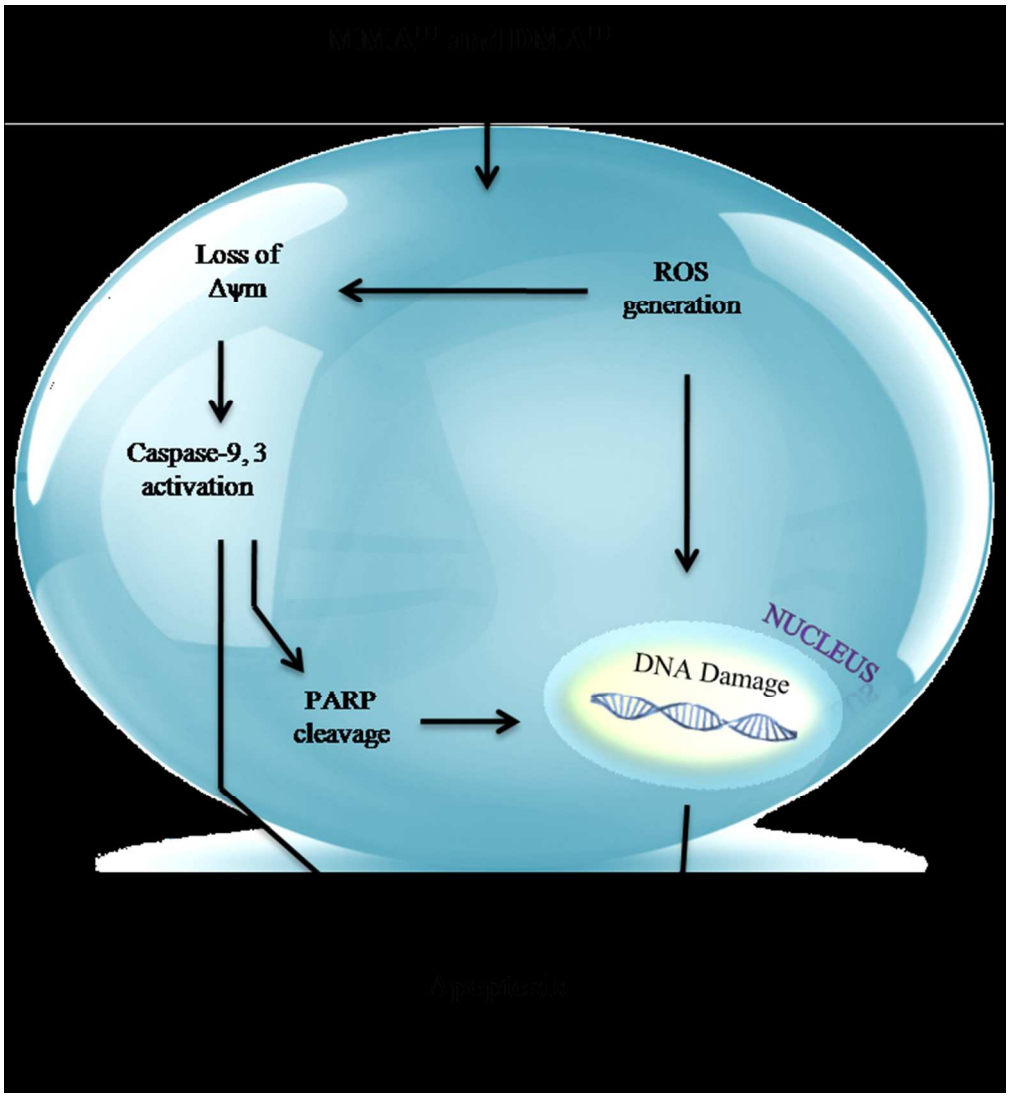

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Trivalent Methylated Arsenic Metabolites Induce Apoptosis in Human Myeloid Leukemic HL-60 Cells through Generation of Reactive Oxygen Species

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Abstract

Arsenic trioxide (As_2O_3) has remarkable therapeutic efficacy against leukemia. However, after As_2O_3 biotransformation, the role of arsenic metabolites in the clinical efficacy against leukemia still needs to be elucidated. Thereby, to explore contribution of trivalent methylated arsenicals in therapeutic effects, we investigated and compared the toxic effects of arsenite (iAs^{III}), monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) on HL-60 cells. Methylated arsenic species MMA^{III} and DMA^{III} showed potentially reduced cell survival with IC_{50} values of 3 and 2 μM, respectively. We found that methylated metabolites caused apoptosis through oxidative stress and loss of mitochondrial membrane potential. Furthermore, we found that the caspase-9 and -3 were markedly activated by exposure to methylated metabolites, with cleavage of poly ADP ribose polymerase (PARP). Conversely, cellular apoptosis, generation of ROS, activation of caspase-3, -9 as well as PARP cleavage were significantly attenuated by pretreatment with antioxidant, N-acetylcysteine (NAC).DNA damage was also markedly observed in HL-60 cells exposed to either MMA^{III} or DMA^{III}, while iAs^{III} did not show any relevant effects in HL-60 cells. Likewise, phosphorylation of histone H2A variant (γ-H2AX), a biomarker of DNA damage, significantly occurred in cellular nuclei following exposure to two methylated species which was reduced in the presences of NAC, suggesting that the induction of DNA damage was predominantly caused by the two metabolites via oxidative stress. In conclusion, we suggest that arsenic intermediate metabolites; MMA^{III} and DMA^{III} might prove to be of clinical relevance in future as such approaches may help in leukemia or other types of cancer treatments.

Keywords Arsenite; Dimethylarsinous acid; Monomethylarsonous acid; HL-60 cells; Reactive oxygen species; DNA laddering; γ-H2AX.

Introduction

Arsenic trioxide (As_2O_3) has been well-known for its effectiveness against acute promyelocytic leukemia (APL),[1] however, relapse has been a major cause of death in patients with leukemia and thereby, appropriate leukemia treatment requires determination of specific apoptotic pathways involved in its pathophsiology.[2] On the other hand, arsenic has also been known as a human carcinogen, and chronic ingestion of arsenic contaminated drinking water and food or inhalation of arsenic containment air can cause skin, lung, and urinary bladder cancer in humans.[3,4]

Generally, inorganic arsenic can be metabolized into different active methylated forms in body after injection. Thus, the exact participation of arsenic species produced via biotransformation in arsenic-induced carcinogenesis as well as its anti-cancer effects is still partially understood. In fact, the toxicity associated with inorganic arsenic is usually related to its methylated metabolites biotransformed in body.[5] Nevertheless, the mechanism involved in arsenic metabolism can be an important pathway to elucidate the partially understood phenomena of arsenic induced cytotoxicity or anticancer effects. Conversely, toxic and clinical aspects related to arsenic may be greatly predicted in terms of arsenic biotransformation; however, the precise metabolic pathway involved in arsenic metabolism is still under debate.[6]

Correspondingly, it is still ambiguous that which arsenic methylated metabolite plays vital role in inducing major cytotoxic effects, as after ingestion or administration inorganic arsenic is known to rapidly metabolize in liver into methylated metabolites through consecutive reductive methylations in a protein-bound form via arsenic methyltransferase (AS3MT). iAs^{III} has shown to undergo methylation by AS3MT in the presence of S-adenosylmethionine (SAM), a methyl donor and glutathione (GSH) as a co-factor for the formation of monomethylarsonous acid (MMA^{III}), monomethylarsonic acid (MMA^{V}) dimethylarsinous acid (DMA^{III}) and dimethylarsinic acid (DMA^{V}).[7-10] These metabolites are then stated to be released into body fluids and excreted to urine mostly as pentavalent forms.[11] As far as the matter of toxicity is concerned, when compared to iAs^{V} and pentavalent methylated arsenic metabolites, iAs^{III} and its trivalent methylated arsenic metabolites are considered to be more potent which may influence the activities of various enzymes and proteins. [12-15] MMA^{III} and DMA^{III} have also found

to target different organelles including mitochondria and endoplasmic reticulum respectively, for the induction of their cytotoxic effects.[14,16]

This difference in cytotoxicity between the trivalent and pentavalent arsenicals could be because of more efficient cellular uptake and accumulation of iAs^{III}[17-19] as well as high binding affinity of trivalent arsenicals to sulfhydryl groups of proteins.[20] Several investigations have suggested trivalent arsenicals to be potentially more cytotoxic as compared to pentavalents either via induction of chromosomal abnormalities and/or oxidative DNA damage.[21,22] Even though, arsenite has not been completely declared mutagenic but it has been recognized for induction of oxidative stress involving DNA strand breaks and interfering with DNA repair mechanisms including enzymatic incision/ligation.[23,24]

Likewise, As_2O_3 has also known to be toxic towards mitochondria along with disturbing cellular balance of oxidation and reduction at high dose leading to generation of intracellular reactive oxygen species (ROS).[25,26] Still, a very little is known about the type of arsenic species which particularly contributes for the anticancer effects of arsenic because when As_2O_3 is given intravenously to patients, it immediately undergo biotransformation within the body to mono- and dimethylated arsenic metabolites.[9,27] Moreover, trivalent methylated arsenic metabolites (i.e., MMA^{III} and DMA^{III}) have shown to induce pronounce mitochondrial toxicity as compared to iAs^{III}. In particular, DMA^{III} has been suggested to directly interact with voltage-dependent anion channel and induce Cyt c release from mitochondria.[28] Though, trivalent methylated metabolites are documented to be more toxic metabolites of arsenic,[29] yet, only a small number of investigations have investigated the genotoxic effects of trivalent methylated metabolites along with the mechanism involved in these toxic events.[22,30,31] Moreover, there is still a need to elucidate that in particular which arsenic specie is involved in effectively targeting the apoptotic pathways exerting anticancer effects.

The current study has been aimed to understand the mode of antileukemic activity of iAs^{III}, MMA^{III} and DMA^{III} using HL-60 cells. The probable targets for arsenicals that could be involved in the induction of toxicity in HL-60 cells were explored by using various cellular and sub cellular assays after treating the cells with sub-cytotoxic concentrations of trivalent arsenicals. The effects of these arsenicals were investigated in

terms of their pro-apoptotic effects on HL-60 cells. We observed oxidative stress to be the critical transitional mechanisms involved that lead to apoptosis in HL-60 cells after exposure to MMA^{III} and DMA^{III}, which can induce loss of mitochondrial membrane potential, DNA damage and activation of caspase cascade. These results give a clear indication that the trivalent arsenic intermediate metabolites possess greater toxic potential against leukemia cells as compared to their precursor; inorganic arsenite.

Materials and methods

Reagents

All reagents were of analytical grade. Milli-Q water (Millipore) was used for all experiments. Trizma® HCl and Trizma® Base were purchased from Sigma (St. Louis, MO, USA). Nitric acid, hydrogen chloride, ammonium acetate, acetic acid, 28 % ammonia solution, L-cysteine sodium, iAs^{III} and DMA^{V} were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). MMA^{V} was obtained from Tri Chemicals (Yamanashi, Japan). Stock solutions of all arsenic compounds (10 mM/L) were prepared from the respective standard compounds. All stock solutions were stored in dark place at 4°C. Diluted standard solutions for analysis were prepared fresh daily. The 6-carboxy-2',7' dichloro- dihydrofluorescein diacetate (DCFH-DA), MTT and N-acetyl-L- cysteine (NAC) were purchased from Beyotime (Jiangsu, China); Annexin V-FITC Apoptosis Detection Kit was purchased from Invitrogen (USA). Antibodies for γ-H2AX, caspase-9 were purchased form Cell Signaling Technology (MA, USA), while that of caspase-3 and PARP were purchased from Santa Cruz Biotechnology (CA, USA). Caspase-3 activity kit was obtained from Beyotime Institute of Biotechnology (Haimen, China).

Preparation of Monomethylarsonous Acid (MMA^{III}) and Dimethylarsinous Acid (DMA^{III})

MMA^{III} and DMA^{III} were prepared by reducing MMA^{V} and DMA^{V} respectively with 5 molar equivalents of L-cysteine in distilled water at 90 °C for 1 h. The trivalent forms were confirmed by comparison of the respective retention times on a GS 220 gel filtration column by HPLC–ICP MS with those prepared from their iodide forms in distilled water under nitrogen atmosphere. Purity of MMA^{III} (98%, with 2% of MMA^{V}) and DMA^{III} (95%, and with 5% of DMA^{V}) was confirmed by HPLC-ICP MS and then used further for experimental purpose.

Cell and Culture Condition

Human myeloid leukemia HL-60 cells were obtained from type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were grown in RPMI 1640 containing 10% fetal calf serum (Invitrogen, Grand Island, NY, USA), 100 U/ml penicillin and 100 µg/ml streptomycin, at 37° C under a 5% CO_2 atmosphere. The

exponentially growing cells were further seeded and treated with iAs^{III}, MMA^{III} and DMA^{III} (in presence or absence of 2 mM NAC) according to the experiments performed.

Cell Viability by MTT Assay

HL-60 cells were seeded at a density of 2×10^4 cells per 100 µL per well in 96-well microtiter plates (Promega Corporation). The cultures were exposed to various concentrations of iAs^{III}, MMA^{III} and DMA^{III} (1, 2, 5, 10, 20, 50 and 100 µM) in FBS-free medium and incubated for 24 hr at 37 °C in 5% CO_2. For control, cells were incubated only in culture medium (untreated wells). Later, 20 µL of MTT solution (5 mg/ml in PBS) was added to each well and the plates were incubated for further 3 hr at 37 °C. After incubation, the medium was then gently aspirated from test cultures and 200 µL of DMSO was added to each well to dissolve the formazan crystals. The plate was then wrapped in foil paper and incubated on shaker for 10 min to dissolve air bubbles. Cell viability was measured as the absorbance at 490 nm after a 2 min shaking with a microplate reader (Beckman Coulter, Multimode Detector DTX-880). Cell viability rate was related to the percentage of MTT absorption and was calculated as: % survival = (mean experimental absorbance/mean control absorbance) x100.

Assessment of Cellular Apoptosis

Cellular apoptosis was measured by Annexin V-FITC and propidium iodide (PI) staining. HL-60 cells were treated with 1µM of iAs^{III}, MMA^{III} and DMA^{III} with and without 2 mM NAC for 24 h. Later the cells were washed with PBS and resuspended (1×10^6 /mL). Cells were then stained with 5 µL Annexin V-FITC and were incubated for 15 min in dark at 37 ºC. Afterward, PI (20 µg/mL) was added and the samples were immediately analyzed on flow cytometry (Beckmancoulter).

Measurement of intracellular ROS

The oxidation-sensitive fluorescent probe (DCFH-DA) was used to detect the intracellular ROS level. HL-60 cells were treated with 1µM of iAs^{III}, MMA^{III} and DMA^{III} with and without 2 mM NAC for 12 hr. After incubation, cells were washed with PBS and then incubated with 10µM/L DCFH-DA at 37 ºC for 20 min. Fluorescence was detected by flow cytometry (Beckmancoulter).

Apoptotic DNA Ladder Determination

HL-60 cells (1×10^6) were treated with 1μM of iAs^{III}, MMA^{III} and DMA^{III} with and without 2 mM NAC for 24 hr. After incubation, cells were collected and 5 μL proteinase K (1mg/mL) was added to 1 ml DNA lysis buffer (Beyotime, Jiangsu, China). Further, this mixture was added to the cells which were allowed to digest overnight at 50 °C. Later, cells were treated with Tris-phenol, vortex and centrifuged to separate the water and organic phase. The upper aqueous phase was collected and treated with chloroform, vortex and centrifuged. Further, ammonium acetate and absolute ethyl alcohol was added to the supernatant to precipitate DNA fragments and incubated overnight at -20 °C. Afterward, cells were treated with 70% ethanol to remove salts. They were centrifuged and the supernatant ethanol was discarded, residual was completely dried and was re-suspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to dissolve DNA. Subsequently, DNA samples were mixed with loading buffer and loaded on 1% agarose gel (0.5 mg/ml ethidium bromide) and electrophoresed at 25 V. The DNA ladder was visualized with Quantity One analyzer by UV light.

Detection of γ-H2AX by Immunofluorescence

HL-60 cells (1×10^5) were treated with 5 μM of iAs^{III}, MMA^{III} and DMA^{III} with and without 2 mM NAC for 3 hr. After incubation, cells were seeded on glass slides (Shandon cytospin; Runcorn, UK) and were fixed with 4% paraformaldehyde (PFA) for 15 min. After wiping extra PFA from the slides, cold PBS was used to wash the cells. Later, for enhanced permealization, 0.1% Triton X-100 was added for further 15 min at room temperature. After incubation, the cells were washed and blocked using 3% bovine serum albumin (BSA) for 1 hr at room temperature. Further, primary antibody against phospho-H2AX (1:200) was added and the cells were incubated overnight at 4 ºC. Cells nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue) for 5 min at 4 °C. After washing with PBS, phycoerythrin-conjugated streptavidin was added for 30 min before washing, mounting in Aquamount, and examination under a Zeiss (Göttingen, Germany) 510 confocal microscope. Confocal scanning parameters were set up so that the cells in the well without the compounds had no fluorescent signal.

Detection of Mitochondrial Membrane Potential

To observe the effect of arsenicals on the mitochondrial membrane potential of HL-60 cells, the mitochondria were loaded with JC-I probe to observe the shift of color from green to red. When the mitochondrial membrane potential is low, JC-1 remains as monomer (green) and when the mitochondrial membrane potential is high it exists as aggregate (red). Cultured HL-60 cells (2×10^5 cells/mL) were treated with 1 μM of iAs^{III}, MMA^{III} and DMA^{III} for 6 hr. After incubation, cells were collected and washed with PBS, loaded with 10 μg/mL JC-1 dye and incubated at 37 ºC in dark for 15 min. After incubation, the cells were analyzed on Zeiss (Go¨ttingen, Germany) 510 confocal microscope.

Cellular Uptake of iAs^{III} and its Methylated Trivalent Metabolites

HL-60 cells were seeded at a density of 1.0×10^6 and then exposed to the three trivalent arsenic species at 1μM for 24h. In addition, the cell monolayer was then collected and washed twice with cold PBS. The concentrations of arsenic in the whole cells were determined by ICP MS after wet-ashing with concentrated nitric acid for 2 days.

Western Blot Analysis

HL-60 cells (3×10^6) were treated with 5 μM of iAs^{III}, MMA^{III} and DMA^{III} with and without 2 mM NAC for 3 hr to determine γ-H2AX expression, while to determine expressions of Caspase-9, Caspase-3 and PARP; HL-60 cells (5×10^4) were treated with 1 μM of iAs^{III}, MMA^{III} and DMA^{III} with and without 2 mM NAC for 12 hr. The cells were then lysed in lysis buffer in the presence of protease inhibitors [50 mM Tris–HCl buffer, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM phenylmethylsulphonyl fluoride (PMSF)]. The Protein concentrations were determined by Bio-Rad microprotein assay using BSA as the standard. The samples containing whole cell protein were separated on 12.5% polyacrylamide gels (SDS-PAGE), and blotted onto polyvinylidene fluoride (PVDF) transfer membranes. These membranes were then blocked using 5% non-fat milk, and were then incubated with relevant primary antibodies against phospho-H2AX (1:000), caspase-9, caspase-3 and PARP (1:1000) overnight at 4 ºC. Afterwards, these membranes were washed twice with T-TBS followed by incubation with secondary antibody conjugated with HRP (1:10,000) (Jackson Immno Research, West Grove, PA)

for 1 hr at room temperature. After incubation, using enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Arlington Heights, IL, USA), the protein expressions were visualized.

Measurement of Cellular Caspase-3 Activity

Caspase-3 specific substrate (Ac-DEVD-*p*NA) was used to measure the activity of cellular caspase-3. Cleavage of this substrate resulted in release of *p*NA (chromophore, p-nitroaniline). Cellular caspase-3 activity was determined in concentration and time-course dependent manners. Moreover, caspase-3 activity was also evaluated in the presence and absence of NAC. For concentration dependent, the cultured HL-60 cells were treated with various concentrations (1, 2, 5 and 10 μM) of iAs^{III}, MMA^{III} and DMA^{III} for 12 hr. For time-coarse, the cells were treated with 1 μM of iAs^{III}, MMA^{III} and DMA^{III} for 6, 12 and 24 hrs. Moreover, the effect of antioxidant on cellular caspase-3 activity was also evaluated using NAC. Briefly, the cells were pretreated with 2 mM NAC for 1 h and latter, 1 μM of iAs^{III}, MMA^{III} and DMA^{III} was added for 12 hr. Assays were performed according to the manufacturer's instruction. Briefly, after obtaining the cell lysates, 10 μL protein of cell lysate from each sample and 80 μL of reaction buffer [1% NP-40, 20 mM Tris–HCl (pH 7.5), 137 mM Nad and 10% glycerol] was added in 96-well plate along with the addition of 10 μL of caspase-3 specific substrate (2 mM Ac-DEVD-*p*NA) in each well. The plate was then incubated for 2 hr at 37° C. Later, the samples were measured at an absorbance of 405 nm with a microplate reader (Beckman Coulter, Multimode Detector DTX-880)

Statistics

Each experiment was performed at least three times and the values were calculated as mean ± S.D. Data were statistically analyzed using one-way ANOVA followed by Dunnett's test and/or Newman-Keuls posthoc pair-wise multiple comparison *tests* (GraphPad Prism 5, Software Inc., USA). The value of significant difference was adjusted at $P < 0.05$.

Results

Effect of iAs^{III}, MMA^{III} and DMA^{III} on Viability of Leukemic Cells

The cytotoxic potential of iAs^{III}, MMA^{III} and DMA^{III} against HL-60 cells was measured by MTT assay, as shown in Figure 1, iAs^{III} showed no considerable effect on cell viability at low doses and depicted an IC_{50} value at 10 µM (Fig.1A). However, the methylated forms, namely, MMA^{III} and DMA^{III} significantly reduced cell survival at much lower concentrations (Fig.1B and C), and the IC_{50} values for MMA^{III} and DMA^{III} were calculated to be 3 and 2 µM, respectively.

Effect of iAs^{III}, MMA^{III} and DMA^{III} on Cellular Apoptosis

As at lower doses, MMA^{III} and DMA^{III} were capable of decreasing cell viability, thus, to observe whether these methylated arsenic metabolites can induce cell death via apoptosis, HL-60 cells were analyzed for apoptosis by Annexin V and propidium iodide assay as shown in Figure 2. The treatment with arsenic methylated metabolites MMA^{III} and DMA^{III} showed higher rate of apoptosis in HL-60 cells than that of iAs^{III}. Moreover, there were fewer cells found stained with PI alone hence, it was observed that treatment with these methylated arsenicals might not have activated necrotic cell death. Moreover, the apoptosis induced by MMA^{III} and DMA^{III} was effectively rescued in cells pretreated with antioxidant N-acetylcysteine (NAC), suggesting that generation of ROS might probably be a factor involved in MMA^{III} and DMA^{III}-induced apoptosis in HL-60 cells.

Effect of iAs^{III}, MMA^{III} and DMA^{III} on Intracellular ROS Generation

To find the relationship between MMA^{III} and DMA^{III}-induced cellular apoptosis and oxidative stress, we examined intracellular level of ROS generation after exposing HL-60 cells to iAs^{III}, MMA^{III} and DMA^{III}. Cells were stained with intracellular indicator, DCFH-DA and were analyzed by flow cytometry. Figure 3A shows the effect of three arsenic species on ROS generation in HL-60 cells. Cells exposed to MMA^{III} and DMA^{III} showed a remarkable increase in intracellular ROS generation as compared to inorganic iAs^{III}, suggesting arsenic intermediate metabolites as strong inducers of ROS in HL-60 cells. Moreover, the generation of ROS was significantly reduced in the cells pretreated with NAC, this clearly indicated that oxidative stressed induced by the two methylated metabolite was responsible for the induction of apoptosis in HL-60 cells (Fig. 3B).

MMA^{III} and DMA^{III} can induces loss of Mitochondrial Membrane Potential

It has been suggested that the loss of mitochondrial membrane potential is associated with cell apoptosis and in particular, it has been reported that high levels arsenic may induce mitochondria dysfunction. To explore that which trivalent arsenic specie can potentially target the mitochondrial membrane potential, cells were exposed to iAs^{III} and its two methylated metabolites at a concentration of 1μM for 12 hr. No appreciable effect was observed on mitochondrial membrane potential in HL-60 cells after exposure to iAs^{III} when compared to control (red florescence) as shown in Figure 3C. Unlike arsenite, methylated MMA^{III} and DMA^{III} showed a decrease in red florescence exhibiting green florescence which is an indicator of depolarized mitochondria with loss of membrane potential. This result clearly showed that both MMA^{III} and DMA^{III} can disrupt mitochondrial membrane potential in HL-60 cells.

Cellular Uptake of iAs^{III} and its Methylated Trivalent Metabolites

To observe the uptake of trivalent arsenicals by HL-60 cells, HL-60 cells were incubated with iAs^{III}, MMA^{III} and DMA^{III} at sub-toxic concentration (1μM) for up to 24 h. As shown in Figure 4, the methylated metabolites were found to be taken up by cells more efficiently than that of iAs^{III}.

MMA^{III} and DMA^{III} Induce Activation of Apoptosis-related proteins

We further opt to determine the apoptosis-related proteins such as caspase-3, -9 and poly (ADP-ribose) polymerase (PARP) in HL-60 cells after exposure to the three arsenic species in presence or absence of antioxidant NAC, as shown in Figure 5. Cleaved caspase-9, caspase-3 and poly (ADP-ribose) polymerase (PARP) were found in HL-60 cells by exposure to MMA^{III} and DMA^{III} (i.e., without NAC), while the cells exposed to iAs^{III} did not show activation of caspase-9, -3 and PARP at the same exposure dose (Fig.5A and B). Moreover, it was found that cleavage of caspase-9, -3 and PARP induced by both MMA^{III} and DMA^{III} were significantly reverted in cells pretreated with NAC (Fig. 5A and B).

Time-Course and Concentration Dependent Effect of iAs^{III}, MMA^{III} and DMA^{III} on Cellular Caspase-3 Activity

Additionally, we further determined activity of caspase-3 in HL-60 cells in time and concentration dependent manners (Fig. 6A and B). The caspase-3 is known to play a vital role in induction of final phases of cellular apoptosis. From our result we found that MMA^{III} and DMA^{III} showed a significant increase in the caspase-3 activity in both; time- and concentration dependent manner. However, iAs^{III} started showing a slight increase in caspase-3 activity minimally somewhere between 12 to 24 hr at 1μM. In addition, we also investigated the effects of NAC on arsenic-induced caspase-3 activation. Cells were pre-treated with 2 mM NAC for 1h and then exposed to arsenicals for 12 hr to determine the activity of caspase-3, as shown in Figure 6C, a significant decrease in caspase-3 activity was observed in cultured HL-60 cells pre-treated with NAC as compared to cells treated with MMA^{III} and DMA^{III} alone.

Effect of iAs^{III}, MMA^{III} and DMA^{III} on DNA Ladder

DNA damage is recognized to be one of the hallmarks of apoptosis. Thus, we observed and compared the effects of arsenicals on DNA damage taking DNA laddering as a parameter. The DNA ladders were determined after treating HL-60 cells with arsenicals in the presence or absence of NAC, as shown in Figure 7. Correspondingly, we found that MMA^{III} and DMA^{III} induced significant DNA fragmentation in HL-60 cells as determined by DNA laddering assay, whereas treatment with iAs^{III} did not result in DNA damage, and DNA fragmentation was effectively inhibited by pretreatment with NAC.

Effect of iAs^{III}, MMA^{III} and DMA^{III} on γ-H2AX generation

As generation of γ-H2AX has been recognized as a marker of DNA damage, we used immunofluorescence staining to evaluate the generation of γ-H2AX in nucleus of HL-60 cells exposed to 5 μM of iAs^{III}, MMA^{III} and DMA^{III} for short time (3 hr) either with or without NAC, as shown in Figure 8. Both MMA^{III} and DMA^{III} treated cells showed significant generation of γ-H2AX foci in their nucleus, however, no significant effect on the generation of γ-H2AX foci was observed in HL-60 cells exposed to iAs^{III}. Interestingly, NAC prevented the generation of γ-H2AX in nucleus (Fig.8A), suggesting that the DNA damage might be caused predominantly by the generation of oxidative stress. In addition, the generation of γ-H2AX in cells was also confirmed by western blot analysis, as shown in Figure 8B.

Discussion

It has been suggested that apoptosis may play a vital role as a response to chemotherapy in leukemia patients, and there probably exists an association between clinical efficacy and therapy-induced apoptosis. The HL-60 leukemia cell line was initially obtained from acute myeloid leukemia patient, and a few studies have indicated that HL-60 cells are resistant to arsenic trioxide in clinical treatment.[32] Indeed, many toxicologists together with our group have suggested iAs^{III} and its trivalent methylated metabolites to be more cytotoxic and genotoxic as compared to the pentavalent arsenicals.[22,30,33] Additionally, MMA^{III} and DMA^{III} being potent cyto- and genotoxic arsenicals have been suggested as treatment for various malignancies.[21,34]

On observing and comparing the IC_{50} values of our results obtained by MTT assay, it became evident that MMA^{III} and DMA^{III} are able to induce cell death at much lower concentrations in HL-60 cells than that of their precursor iAs^{III} (Fig.1). This result clearly indicates that trivalent methylated arsenic metabolites are more toxic to leukemia cells than inorganic iAs^{III}. In addition, our further results found trivalent methylated arsenic metabolites (i.e., MMA^{III} and DMA^{III}) to be more potent inducers of oxidative stress as compared to arsenite. Moreover in current study, we also found both MMA^{III} and DMA^{III} that can biotransformed in body from iAs^{III} have strong effects on induction of apoptosis in leukemia HL-60 cells. These results indicate that arsenic intermediate metabolites have potential cytotoxicity against HL-60 cells and may contribute to anti-tumor therapy in near future.

To estimate the toxic abilities of these arsenicals against HL-60 cells, we investigated and compared the apoptotic effects of iAs^{III}, MMA^{III} and DMA^{III} on cells. We observed that the cells exposed to MMA^{III} and DMA^{III} alone underwent marked apoptosis as compared with iAs^{III}, which was significantly prevented in cells pretreated with NAC (Fig.2). Thus, our work verified the propensity of trivalent methylated arsenic metabolites to induced apoptosis in HL-60 cells,

Subsequently, to delineate the cause of apoptotic death, we further decided to elucidate the probable involvement of oxidative stress in apoptosis.[25,26,34,35,36] Interestingly, we found that cells exposed to MMA^{III} and DMA^{III} showed significant increase in the levels of ROS, however, iAs^{III} failed to trigger this event (Fig.3A). This

evaluation further supported the differences found among the three arsenic species (i.e., iAs^{III}, MMA^{III}, and DMA^{III}) regarding cell survival. These observations clearly indicated that the methylated trivalent arsenic metabolites may effectively induce apoptosis as compared to iAs^{III}, especially DMA^{III} being a strong inducer of apoptosis in HL-60 cells.

Different pathways responsible for apoptosis are recognized to be major cause of cell death, we thereby hypothesized that as trivalent methylated arsenic metabolites have shown to increase intracellular ROS level, they might cause disturbance of mitochondrial membrane potential further increasing oxidant stress burden. In present study, we noted that MMA^{III} and DMA^{III} significantly resulted in loss of mitochondrial membrane potential, while iAs^{III} did not show any significant effect on mitochondrial membrane potential (Fig.3C). In our previous studies, we have shown that MMA^{III} and DMA^{III} can target mitochondria by inhibiting complex-II and IV of electron transfer chain and endoplasmic reticulum (ER) respectively which may lead to apoptosis via the generation of ROS.[14,16] We also determined in the present work that the trivalent methylated metabolites were efficiently taken up by the cells (Fig.4) which was found to be similar to previously published reports using other cell lines[37,38], thus, by these studies the toxic nature of trivalent arsenicals can be explained at least in part by the efficient cellular uptake of these compounds.

Further, in current work, we measured the expression level of proteins that are probably known to be involved in cellular apoptosis including caspase-9, -3 and PARP . We found that both caspase-9 and -3 were potentially activated in cells exposed to MMA^{III} and DMA^{III}. Moreover, PARP cleavage was also observed following MMA^{III} and DMA^{III} exposure (Fig.5A and B). In addition, we investigated the effect of these arsenicals on intracellular activity of caspase-3 in time and dose-dependent manners (Fig.6). As expected, caspase-3 activation increased with the increase in both time and dose after exposure to MMA^{III} and DMA^{III}, however, iAs^{III} exhibited to be much weaker than its methylated metabolites (Fig.6A and B). In addition, the caspase-3 activation was found to be inhibited in cells pretreated with NAC, suggesting that caspase dependent pathway can be the underlying cause accompanying ROS generation which may lead to apoptosis in HL-60 cells (Fig.6C).

Arsenic is known to bind to various proteins changing their conformation which can alter the functioning and interaction of these proteins with DNA and other proteins.[39,40] In addition, MMA^{III} and DMA^{III} have been reported to considerably inhibit DNA repair enzymes, induce DNA strand breakage, genotoxic effects on bacteriophage φXX174 supercoiled DNA, DNA of cultured human lymphocytes and pBR322 plasmid DNA.[30,31,41,42,43] In current study, we opt to examine the effects of iAs^{III} and its trivalent methylated metabolites on DNA integrity in HL-60 cells (Fig.7). Interestingly, MMA^{III} and DMA^{III} showed to have more potential for inducing DNA damage as compared to iAs^{III}, whereas the antioxidant NAC significantly reduced the DNA damage which was induced by arsenic methylated metabolites. Hence the rescue of DNA damage by NAC pretreatment indicated that MMA^{III} and DMA^{III} might have induced DNA fragmentation via generation of ROS. Similarly, γ-H2AX, a biomarker of DNA break down, was also significantly generated (Fig.8) following exposure to the two methylated arsenic species, however, was markedly attenuated by pretreatment with NAC, suggesting that generation of ROS caused DNA damage in cells exposed to trivalent methylated arsenic metabolites.

Conclusion

In this work we investigated the uptake of three arsenic compounds by HL-60 cells, along with their ability to target mitochondrial stability, induce ROS generation, DNA damage and cellular apoptosis. To summarize, our current work provides a clear influential insight into the toxic measures of MMA^{III} and DMA^{III} against HL-60 leukemia cells as compared to their precursor (iAs^{III}). The two methylated arsenic species; MMA^{III} and DMA^{III} induced apoptosis presented in this study was found to occur mainly through the generation of ROS in cells. However, we could not find their precursor iAs^{III} accountable for such events at the low dose. Thus, We suggest that biomethylation of inorganic arsenite could be an important pathway to determine its clinical efficacy as in the present investigation that we have observed the apoptotic mode of cell death induced by trivalent methylated arsenic metabolites. Though this might prove to be of clinical relevance in future, however, more studies are required to unravel the signaling mechanisms targeted by trivalent arsenic metabolites.

Conflict of Interest

The authors declare that they do not have any conflict of interest for this article.

Acknowledgments

The authors wish to acknowledge the National Natural Science Foundation of China (No. 8127338 and 81274138), Zhejiang Provincial Natural Science Foundation of China (No. R2110231), Key Science and Technology Innovation Team of Zhejiang Province (2010R50047), and Zhejiang Provincial Program for the Cultivation of Medical Rookies.

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Figure Legends

Figure 1. Cell Viability of HL-60 cells after exposure to iAs^{III}, MMA^{III} and DMA^{III}. Cells were exposed to various concentrations of (A) iAs^{III}, (B) MMA^{III} and (C) DMA^{III} for 24 h. The IC_{50} values for iAs^{III} MMA^{III} and MMA^{III} were calculated to be 10 μM, 3 μM and 2 μM, respectively. Data were expressed as mean of three independent experiments.

Figure 2. Effect of iAs^{III}, MMA^{III} and DMA^{III} on induction of apoptosis in HL-60 cells. Cells were treated with 1 μM of iAs^{III}, MMA^{III}, and DMA^{III} in the presence and/or absence of NAC for 24 hr. (A) Apoptosis was measured by annexin-V and PI staining using flow cytometry. One representative experiment out of three is presented. (B) The histogram shows quantifications of all the experiments performed and data were shown as the mean ± S.D. (n=3). *; when compared with control. $^{\#}$; when compared with MMA^{III}. $^{\#\#}$; when compared with DMA^{III}.

Figure 3. Determination of reactive oxygen species (ROS) and mitochondrial membrane potential in HL-60 cells. Cells were exposed to 1 μM of iAs^{III}, MMA^{III}, and DMA^{III} without (A) or with (B) 2 mM NAC for 12h. After incubation in a drug-free medium, cells were exposed to 10 μM DCFH-DA for 10 min as specified in materials and methods. (C) HL-60 mitochondrial membrane potential loaded with mitochondria sensitive stain JC-1. The images of control and iAs^{III} show red florescence indicating highly energized mitochondria of cells, green florescence indicating depolarized mitochondria of HL-60 cells. Scale bars indicate 20 μm.

Figure 4. Concentration of arsenic in HL-60 cells exposed to iAs^{III}, MMA^{III}, and DMA^{III} at a sub-toxic concentration. Cells ($1x10^6$) were exposed to 1μM concentration of three arsenic compounds for 24h. After cells were washed twice with cold PBS, they were collected. Concentrations of arsenic in the whole cells were determined by ICP MS after ashing with HNO_3 for 2days.

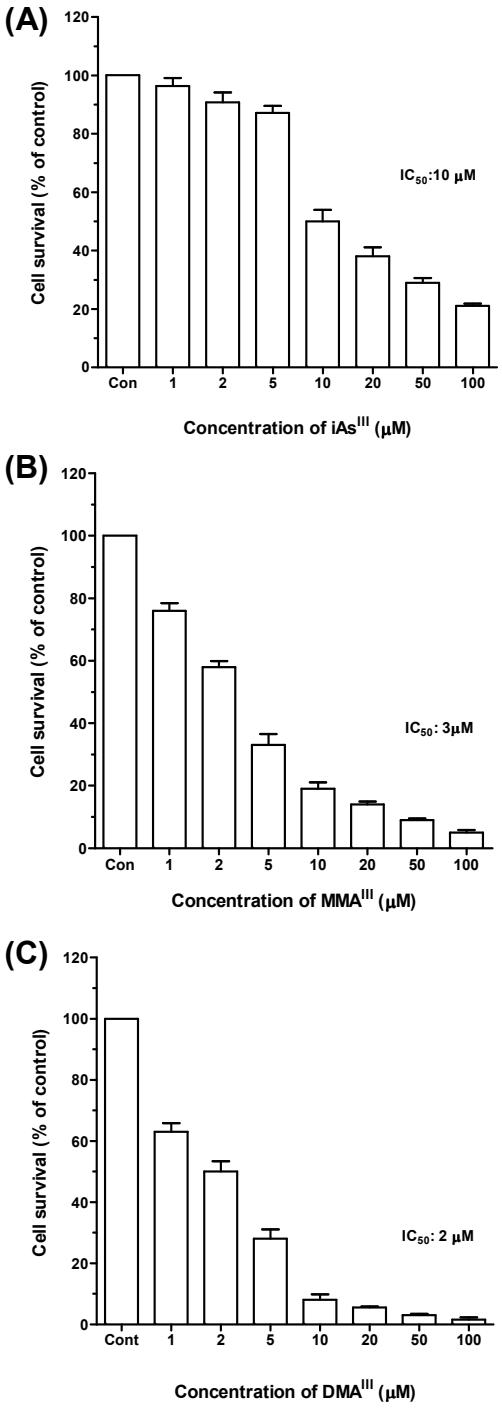
Figure 5. Effect of iAs^{III}, MMA^{III} and DMA^{III} on caspase-3, -9 and PARP. Cells were treated with 1 μM of iAs^{III}, MMA^{III}, and DMA^{III} in the presence and/or absence of NAC for 12 hr. (A) expression of cleaved caspase-3, -9 and (B) PARP induced in HL-60 cells was observed by immunoblotting. Actin was used as control for the equal loading of proteins.

Figure 6. Determination of activation of caspase-3 in HL-60 cells. Cells were exposed to 1 μM of iAs^{III}, MMA^{III}, and DMA^{III} for 12 hr. The HL-60 cells were observed for cellular caspase-3 activity in a concentration-dependent (A), time-course manner (B) and in the presence and/or absence of NAC (C) as described in materials and methods. Data represent means ± S.D. for three determinations with $P<0.05$. (*) when compared with control.

Figure 7. Effect of iAs^{III}, MMA^{III} and DMA^{III} on DNA damage in HL-60 cells. Cells were treated with 1 μM of iAs^{III}, MMA^{III}, and DMA^{III} in the presence and/or absence of NAC for 24 hr. Lane M; DNA marker, lane 1; control, lane 2; iAs^{III}, lane 3; MMA^{III}, lane 4; DMA^{III}, lane 5; NAC, lane 6; iAs^{III} + NAC, lane 7; MMA^{III} + NAC and lane 8; DMA^{III} + NAC. Data were representative of three similar experiments.

Figure 8. Effect of iAs^{III}, MMA^{III} and DMA^{III} on generation of γ-H2AX. Cells were treated with 5 μM of iAs^{III}, MMA^{III}, and DMA^{III} in the presence and/or absence of NAC for 3 hr. (A) Images of γ-H2AX foci generated in nucleus of HL-60 cells after treatment. Top panel, nuclei detected by DAPI staining (blue); middle panel, γ-H2AX foci detected by immunofluorescence staining (red); bottom panel, merge of DAPI and immunofluorescence images. Data were representative of three similar experiments. Scale bars indicate 5 μm (B) Western blot analysis of γ-H2AX generated after treatment. Actin was used as control for the equal loading of proteins.

Figure 1



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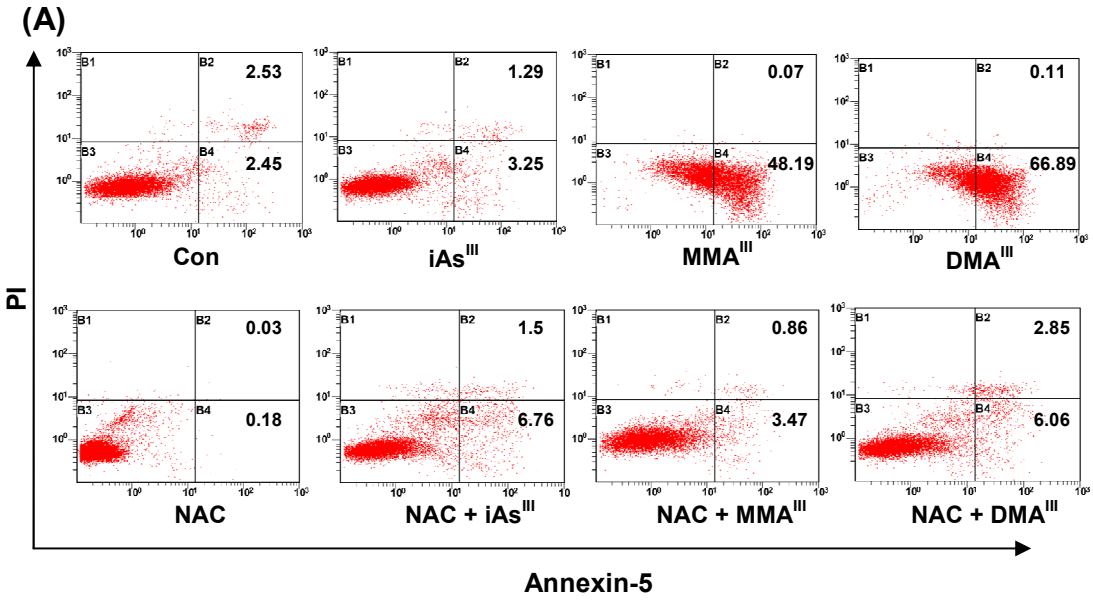

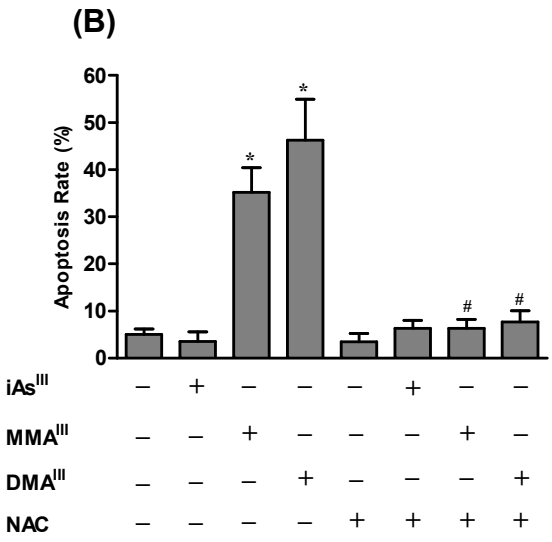

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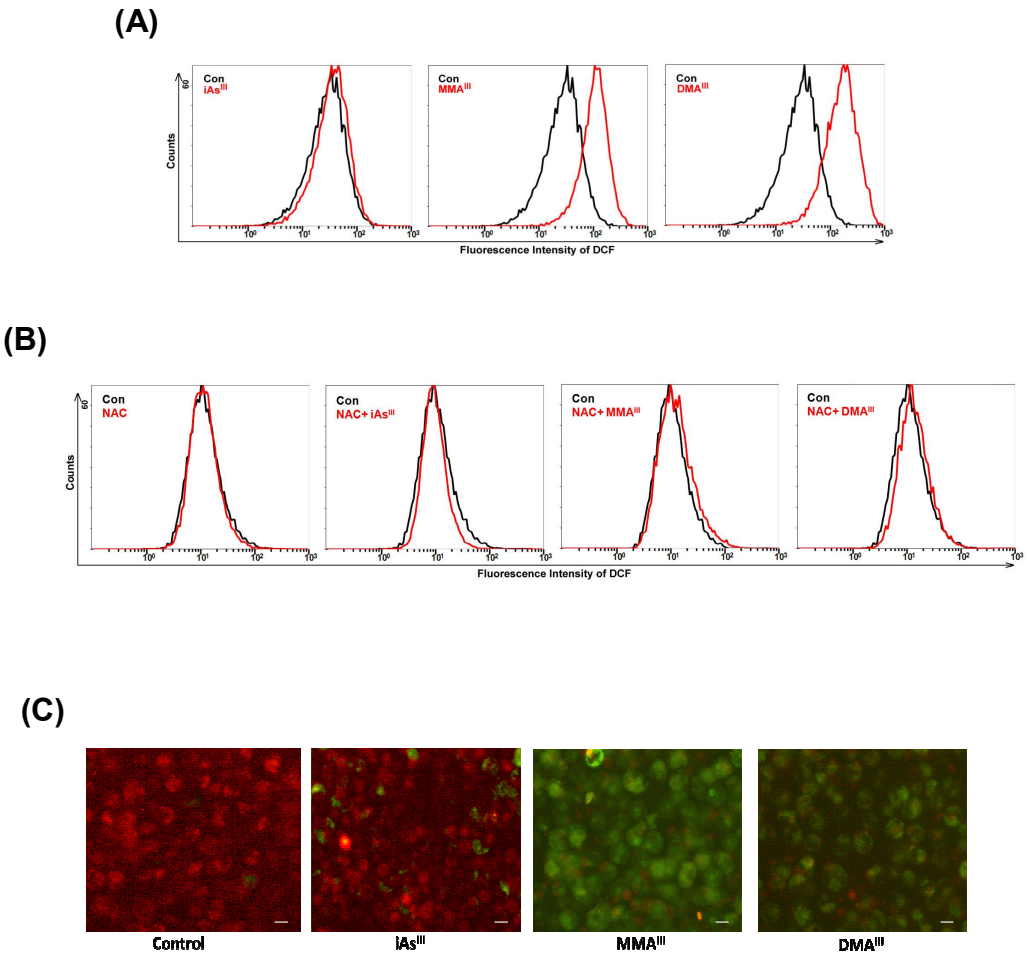

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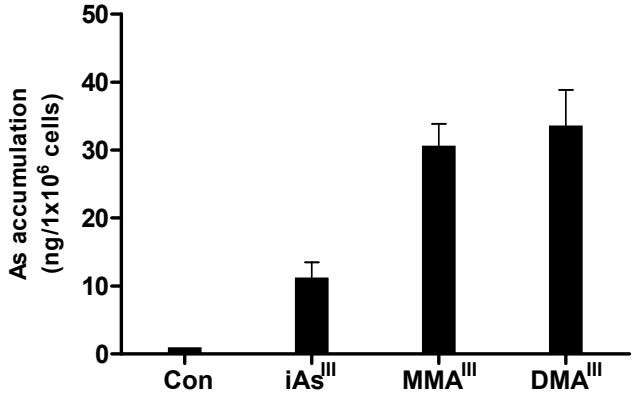

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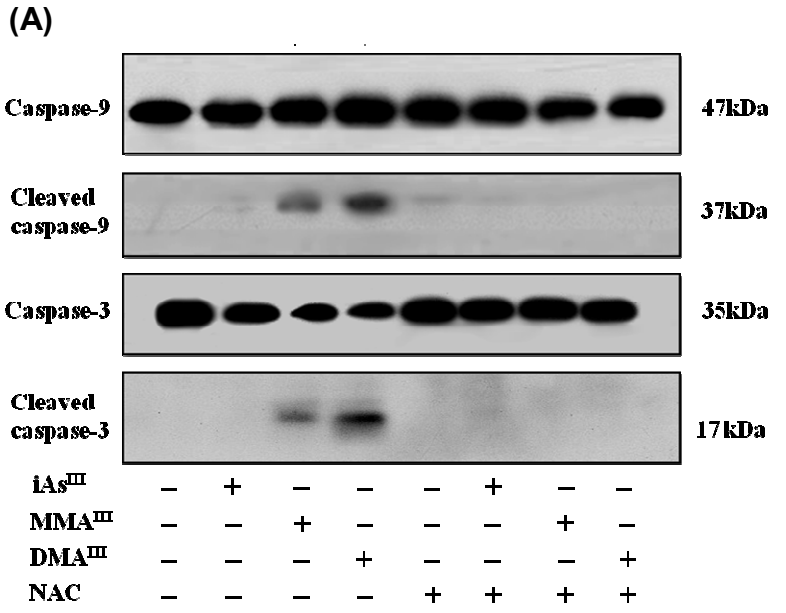

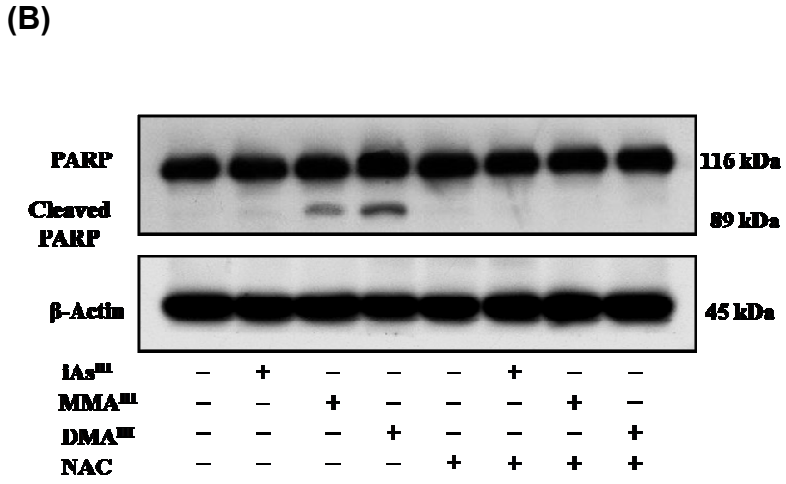

Figure 6

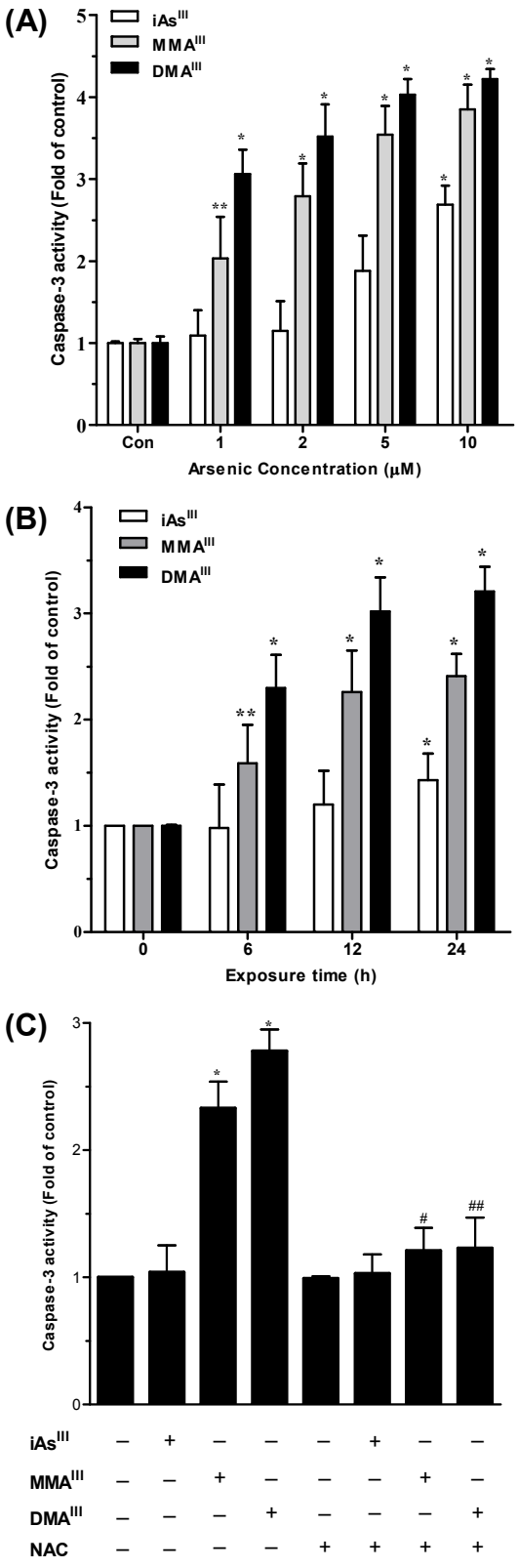

Figure 7

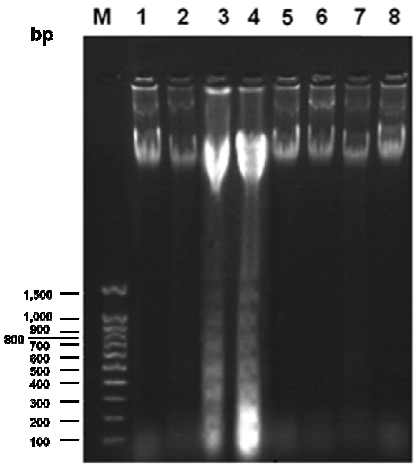

Figure 8