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Metabolism of Arsenic Trioxide in Acute Promyelocytic Leukemia Cells

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ABSTRACT

Arsenic trioxide (As_2O_3) effectively induces complete clinical and molecular remissions in acute promyelocytic leukemia (APL) patients and triggers apoptosis in APL cells. The effect induced by As_2O_3 is also associated with extensive genomic-wide epigenetic changes with large-scale alterations in DNA methylation. We investigated the As_2O_3 metabolism in association with factors involved in the production of its methylated metabolites in APL-derived cell line, NB4. We used high performance liquid chromatography (HPLC) technique to detect As_2O_3 metabolites in NB4 cells. The effects of As_2O_3 on glutathione level, *S*-Adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) levels were investigated. Also, we studied the expression levels of arsenic methyltransferase (AS3MT) and DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) by real-time PCR. Our results show that after As_2O_3 entry into the cell, it was converted into methylated metabolites, monomethylarsenic (MMA) and dimethylarsenic (DMA). The glutathione (GSH) production was increased in parallel with the methylated metabolites formations. As_2O_3 treatment inhibited DNMTs (DNMT1, DNMT3a, and DNMT3b) in dose- and time-dependent manners. The SAH levels in As_2O_3-treated cells were increased; however, the SAM level was not affected. The present study shows that APL cell is capable of metabolizing As_2O_3. The continuous formation of intracellular methylated metabolites, the inhibition of DNMTs expression levels and the increase of SAH level by As_2O_3 biotransformation would probably affect the DNMTs-methylated DNA methylation in a manner related to the extent of DNA hypomethylation. Production of intracellular methylated metabolites and epigenetic changes of DNA methylation during As_2O_3 metabolism may contribute to the therapeutic effect of As_2O_3 in APL. J. Cell. Biochem. 115: 1729–1739, 2014. © 2014 Wiley Periodicals, Inc.

KEY WORDS: ARSENIC TRIOXIDE; ACUTE PROMYELOCYTIC LEUKEMIA; NB4; ARSENIC METHYLTRANSFERASE; DNA METHYLTRANSFERASES; MONOMETHYLARSENIC (MMA); DIMETHYLARSENIC (DMA)

Arsenic trioxide (As_2O_3), as a single agent, has been used successfully in the treatment of both newly diagnosed and relapsed patients with acute promyelocytic leukemia (APL) [Ghavamzadeh et al., 2006]. As_2O_3 effectively induces complete clinical and molecular remissions in vivo and triggers apoptotic death in APL cells [Ghaffari et al., 2006; Ghavamzadeh et al., 2006; Ravandi et al., 2009; Mathews et al., 2010; Ghavamzadeh et al., 2011]. Although, As_2O_3 has been widely used against APL, the molecular mechanisms underlying its anti-leukemic effects remain uncertain. So far, no single mechanism has been able to explain all of the effects seen with As_2O_3; it probably acts at multiple levels, with various modes of actions. Several mechanisms have been proposed for arsenic-induced apoptosis, including PML/RARA oncoprotein degradation through sumoylation [de The and Chen, 2010; Zhang et al., 2010], elevation of the intracellular ROS, disruption of mitochondrial membrane potential and down regulation of the anti-apoptotic protein Bcl-2 which lead to the release of cytochrome *c* and the activation of caspase cascade [Jiang et al., 2001; Zheng et al., 2004; Carney, 2008; Hassani et al., 2013]. Additionally, shortened telomere length and elevated telomerase activity have been demonstrated in

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APL patients which correlate with the disease progression and relapse [Ghaffari et al., 2008]; there is evidences that As_2O_3 might suppress growth and proliferation of tumor cells through the inhibition of telomerase and shortening of the telomere length [Zhang et al., 2003; Ghaffari et al., 2012b]. Moreover, there is ample evidence that the effect induced by As_2O_3 in APL cells is also associated with an extensive genomic-wide epigenetic changes. As_2O_3 by modulation of tumor and metastatic suppressor miRNAs may elicit cell cycle arrest and apoptosis in APL cells [Ghaffari et al., 2012a]. Exposure to As_2O_3 has been shown to cause hypomethylation in some tumor suppressor genes leading to their re-expression in cancer cells [Tong and Lin, 2002; Cui et al., 2006].

It is known that the PML-RARA fusion protein binds to target genes and then recruits repressor enzymes, such as histone deacetylase (HDACs) and DNA methyltransferase (DNMTs), the activity of them then leads to hypoacetylation of histone tails, DNA hypermethylation, and transcriptional silencing [Villa et al., 2006]. A growing body of evidence indicates that arsenic is associated with global DNA hypomethylation [Cui et al., 2006; Zhou et al., 2008]. In fact, recent findings indicate that the anticancer mechanisms of As_2O_3 are not only by induction of apoptosis as previously reported, but also by reactivation of the silenced tumor suppressor genes through DNA hypomethylation [Cui et al., 2008]. The effect induced by As_2O_3 is associated with extensive genomic-wide epigenetic changes with large-scale alterations in H_3 acetylation and DNA methylation [Martens et al., 2010]. The mechanism of DNA hypomethylation after arsenic exposure is not clear. However, the unique arsenic metabolism may play an essential factor in the DNA demethylation of CpG islands in As_2O_3-treated cells. It has been considered that As_2O_3 exert demethylation effects by a direct inhibition of DNA methyltransferase, and also by a depletion of the methyl donor [Cui et al., 2006; Reichard et al., 2007]. The depletion of the methyl groups would result in an inability to maintain methylated cytosine in DNA, resulting in hypomethylation [Cui et al., 2006; Reichard et al., 2007; Cui et al., 2008].

The biotransformation of As_2O_3 in most mammals such as human, involves a series of reductions and methylations. As_2O_3 is converted to methylated products by AS3MT catalyzes, utilizing S-adenosyl methionine (SAM) an essential co-factor of DNMTs (DNMT1, DNMT3a, DNMT3b) and producing SAH, in order to bring about the DNA hypomethylation [Yi et al., 2000] [Sciandrello et al., 2004]. Therefore, As_2O_3 may therefore play a role in the regulation of tumor suppressor genes by interfering with the DNA methylation patterns. The liver is the most important site for arsenic methylation, but other organs such as the kidney and lungs also show arsenic methylating activity [Healy et al., 1998]. It is reported that As_2O_3 methylates to mono-methylarsenic (MMA) and dimethylarsenic (DMA) in the liver of APL patients [Drobna et al., 2009]. As_2O_3 methylated metabolites is also found in some cancer cell lines such as hepatoma (HepG2) [Chen et al., 2003] and glioblastoma cell lines (U87MG) [Falnoga et al., 2007]; but it is not found in the APL cells (such as NB4) as well as other human leukemia cells such as U937 regardless of the As_2O_3 concentration [Chen et al., 2003]. Whereas, in the present study, we show for the first time that As_2O_3 is metabolized into the methylated metabolites in the NB4 cell, and the formation of these intracellular methylated metabolites may induce DNA hypomethylation and contribute to the therapeutic effect of As_2O_3 in APL.

MATERIALS AND METHODS

CELL LINE AND As_2O_3 TREATMENT

The human APL cell line, NB4, was cultured at a density of 5,000 cell/100 μl/well in RPMI 1640 medium (Invitrogen, Auckland, New Zealand) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Biosera, Ringmer, East Sussex, UK); cells were then incubated in 5% CO_2 at 37 °C. NB4 cells were treated with As_2O_3 at 0.5, 1, and 2 μM that are thought to be the therapeutic range in treating the APL patients. For As_2O_3 treatment, a relevant amounts of stock solution [0.5 mM in RPMI 1640 (Invitrogen)] of As_2O_3 (Sina Darou, Tehran, Iran) was added to a culture medium to attain the desired concentrations. The inhibitory effects of As_2O_3 on the metabolic activity and viability of NB4 cells was assessed by microculture tetrazolium test (MTT, Sigma) and trypan blue exclusion assay as described previously [Hassani et al., 2013].

HPLC STUDY FOR As_2O_3 METABOLITES

The cells were cultured onto 96-well plates. After treatment with As_2O_3 at 1 μM for 4, 24, 48, and 72 h, the cells were separated from medium by centrifugation (12000 rpm × 5 min) and washed three times with PBS solution and lysed with 1% Triton X-100 (Merck, Germany) using an ultrasound instrument then dialyzed by Standard Grade Regenerated Cellulose dialysis membrane in acetonitrile solution. The medium was passed through a 0.2 μm filter, and 5 μl was injected in to the HPLC system. The total arsenic concentration was quantified by using Agilent 1100 Series HPLC. The basic mobile phase consisted of 2 mM phosphate buffer solution (PBS), pH = 11.00 adjusted with NaOH plus 0.2 mM EDTA. The final optimized mobile phase included 10 mM CH_3COONa, 3.0 mM $NaNO_3$, and 1% EtOH [Wang et al., 2004]. The samples were concentrated and injected to HPLC with conventional column (C18 column, Agilent, Santa Clara, CA). Untreated cells were defined as the control group. Peaks separated by HPLC were referred to basically as As_2O_3, MMA, and DMA. The total As_2O_3 level in cultured NB4 cell or in medium was calculated as sum of As_2O_3 + MMA + DMA.

HPLC STUDY FOR SAM AND SAH

For the determination of SAM and SAH, 40 μl of 40% trichloro acetic acid was added to a 200 μl medium or cell extract to precipitate the proteins; it was the mixed well and incubated on ice for 30 min. After a centrifugation for 15 min at 18,000 3 g at 4 °C, supernatants containing SAM and SAH were passed through a 0.2 μm filter, and 5 μl was injected into the HPLC system [Melnyk et al., 2000; Yi et al., 2000].

The separation of SAM and SAH in cell extracts was accomplished by Agilent 1100 Series high performance liquid chromatography (HPLC) and a conventional C18 column (5 mm bead size; 4.6 × 100 mm; MCM) obtained from ESA. The basic mobile phase consisted of methanol and water was performed at an ambient temperature at a flow rate of 1.0 mL/min and a pressure of 100–110 kfg/cm^2 (1500–1800 psi). The standard calibrator for SAM was purchased from New England Biolabs (Beverly, MA); the stock solution with 32 mM was diluted to 16 nM for use as a calibrator compound. SAH was purchased from Sigma–Aldrich (St. Louis, MO) and was diluted to 10 nM and used as the standard.

GSH ASSAY

The intracellular GSH level in As_2O_3 treated cells grown in microplates was assessed using glutathione reductase as described previously. Briefly, the cells (1×10^7) were harvested, washed and then lysed in 0.9 ml of 100 mM Tris-HCl by freeze-thaw cycling. Cell lysates were centrifuged in 15,000 g for 10 min, 0.5 ml of the supernatants were transferred to test tubes containing 1 ml of 4% Sulfosalicyclic acid and incubated on ice for 60 min. Cell extracts were centrifuged for 5 min at 3000 rpm. Supernatants were transferred to new tubes containing 0.2 mM NADPH, 0.52 mM DTNB, and 0.15 mM EDTA in 165 μl of 0.1 M sodium phosphate buffer (pH 7.5). After an incubation at 37 °C for 15 min, the plates were read spectrophotometrically. GSH-dependent glutathione reductase 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was converted to 5-thionitrobenzoic acid (TNB) and its colored product was read at 412 nm [Davison et al., 2003].

mRNA EXPRESSION OF AS3MT AND DNMTs

Total RNA from NB4 cultured cells isolated by FastPure RNA kit (Takara Bio Inc., Otsu, Japan). The amount of RNA samples was assessed spectrophotometrically using Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). Alterations in mRNA expression of desired genes were surveyed by real-time PCR on ABI PRISM 7500 Sequence Detection System (PE Applied Biosystems, Foster City, CA). A total of 1 μg RNAs were reverse transcribed into cDNA by PrimeScriptRT reagent kit (Takara Bio Inc.) according to the manufacture's specifications. PCR assay was performed in a final volume of 20 μl containing 10 μl of SYBR Premix Ex Taq technology (Takara Bio Inc.) master mix, 2 μl of cDNA samples, 0.5 μl of each forward and reverse primers (10 pmol), and 7 μl of nuclease-free water (Qiagen, Hilden, Germany). Thermal cycling conditions involved an initial activation step for 30 s at 95 °C followed by 45 cycles including a denaturation step for 5 s at 95 °C and a combined annealing/extension step for 20 s at 60 °C. Melting curve analysis was applied to validate whether all primers yielded a single PCR product (Table I). Hypoxanthine phosphoribosyltransferase1 (HPRT1) was amplified as a normalizer and fold change in expression of each target mRNA relative to HPRT1 was calculated based on $2^{-\Delta\Delta Ct}$ relative expression formula [Momeny et al., 2010].

RESULTS

CONVERSION OF As_2O_3 TO METHYLATED METABOLITES IN NB4 CELL AND MEDIUM

APL-derived NB4 cells were cultured under standard conditions. Prior to study the As_2O_3 metabolism, MTT and trypan blue exclusion assays were performed to investigate the suppressive effect of As_2O_3 on metabolic activity and viability of NB4 cells after 24, 48, and 72 h treatments at concentrations of 0.5, 1, and 2 μM. As_2O_3 inhibited metabolic activity and viability of NB4 cell in concentration- and time-dependent manners. As_2O_3 treatment at 0.5, 1, and 2 μM reduced the metabolic activity of NB4 cells by 17.7%, 37.0%, 55.9% after 24 h; 25.4%, 55.9%, and 83.6% after 48 h; and 31.8%, 73.7%, and 94.0% after 72 h, respectively ($P < 0.05$).

HPLC technique was used to determine the conversion of As_2O_3 to the methylated metabolites in APL cells. NB4 cells were exposed to 1 μM concentration of As_2O_3 for 4, 24, 48, and 72 h, the level of As_2O_3 and its metabolites was detected in the supernatant of cell lysate and also in the cell culture medium. Figure 1 shows the presence of methylated species in NB4 cells after exposure to 1 μM As_2O_3; indicating that methylation of As_2O_3 does occur in the APL cell. After 24 h exposure, the peaks related to the MMA and DMA metabolites were appeared in the cell lysate supernatant; and after 48 and 72 h their levels were significantly increased relative to the As_2O_3 level. The peaks related to the MMA compound was the predominant form of intracellular methylated metabolites. However, the peaks related to the DMA compound was the major methylated metabolite in the medium. As shown in Figure 1B, at each exposure time, the ratios of MMA and DMA yields were calculated relative to the total As_2O_3 level (sum of $As_2O_3 + MMA + DMA$). The result shows that the combined methylated metabolites ($MMA + DMA$) yield was significantly increased, while unmethylated As_2O_3 level was decreased almost linearly in NB4 cell exposed to 1 μM As_2O_3 for up to 72 h (Fig. 1B).

ALTERATION IN SAM AND SAH LEVELS AFTER TREATMENT WITH As_2O_3

The NB4 cell was treated with 1 μM As_2O_3 for 24–72 h, and the presence and increase of SAM and SAH levels were determined by an HPLC technique. The size of the eluted fractions was compared to SAM and SAH calibration standards elution profiles (Fig. 2A and B). In the cell extracts, an evident increase of SAH level was found after 24–72 h of exposure to 1 μM arsenic, while the alteration in SAM peak was negligible (Fig. 2C and D).

As_2O_3 INCREASES GSH LEVEL IN NB4 CELLS

We investigated the effect of As_2O_3 on GSH levels. The level of intracellular GSH was measured in the NB4 cell line during the As_2O_3 treatment, and the levels were adjusted to the untreated control. Data presented in Figure 3 shows that the treatment of the cells with 0.5, 1, and 2 μM As_2O_3 for 24, 48, and 72 h increased the GSH level in NB4 cells in both time course (Fig. 3A) and dose response manners (Fig. 3B). The levels of GSH were slightly decreased during the 24 h

TABLE I. Nucleotide Sequences of the Primers Used for Real-Time RT-PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
HPRT	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTTA
AS3MT	CGTCTATACGAGCCTTGAAC	AACGACAGTCACCGATAA
DNMT1	GATCGAATTCATGCCGGCGCGTACCGCCCCAG	ATGGTGGTTTGCCTGGTGC
DNMT3a	GGGGACGTCCGCAGCGTCACAC	CAGGGTTGGACTCGAGAAATCGC
DNMT3b	CCTGCTGAATTACTCACGCCCC	GTCTGTGTAGTGCACAGGAAAGCC

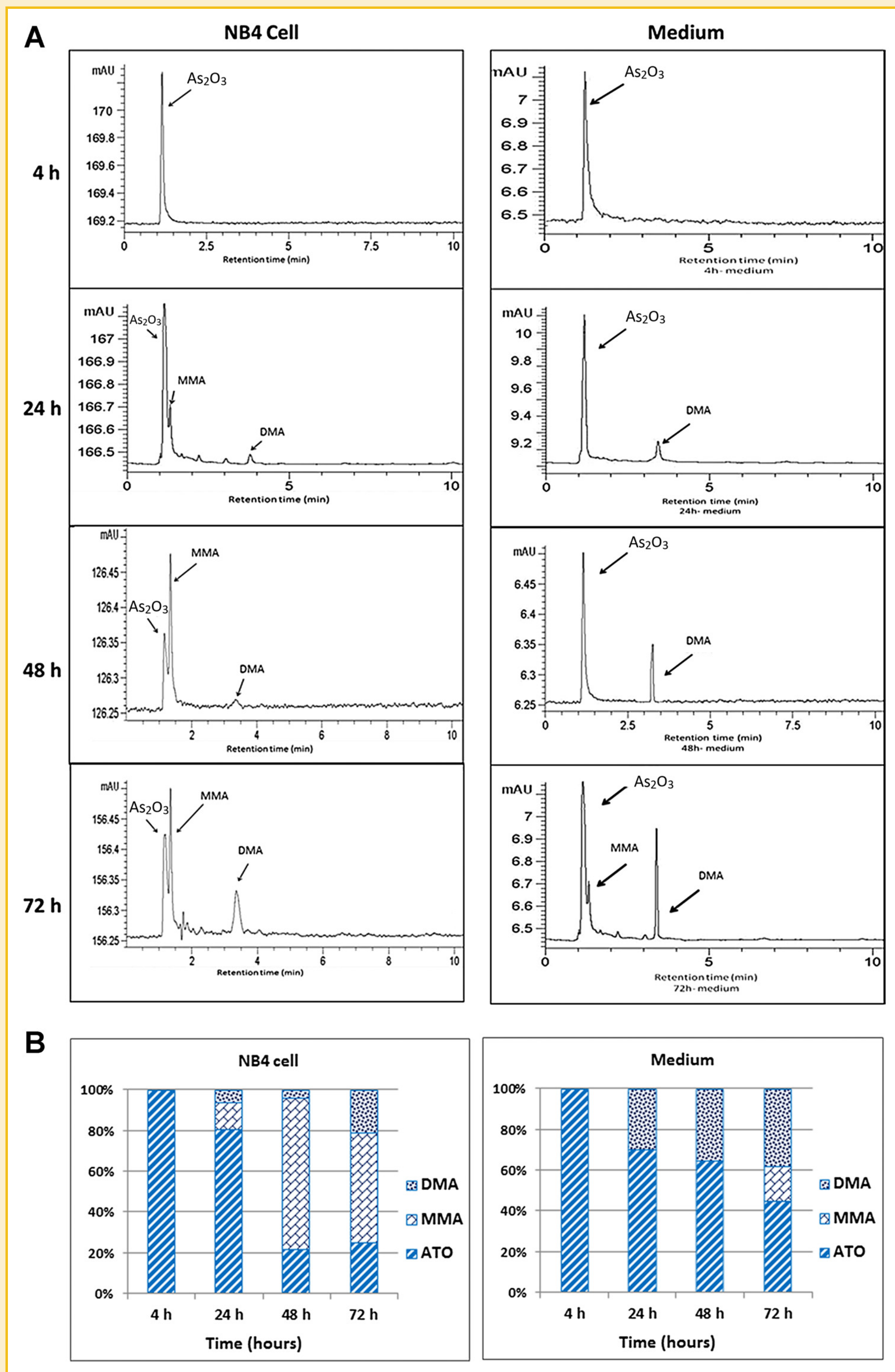

Fig. 1. Biomethylation of As_2O_3 to methylated metabolites. A: Anion-exchange chromatogram (HPLC) of As_2O_3 and its methylated metabolites in intracellular (cell lysate) and medium of NB4 cell exposed to 1 μM As_2O_3 for 4, 24, 48, and 72 h. The peaks related to the methylated metabolites (MMA and DMA) were appear in the cell lysate supernatants, indicating that methylation of As_2O_3 does occur in APL cell as a result of biomethylation of As_2O_3. B: Relative amounts of As_2O_3 and its methylated metabolites in intracellular and medium of NB4 cell. Relative amounts of As_2O_3, MMA, and DMA are expressed as a percentage of total in each assay. Although not shown, SD values did not exceed 10% of the corresponding mean value.

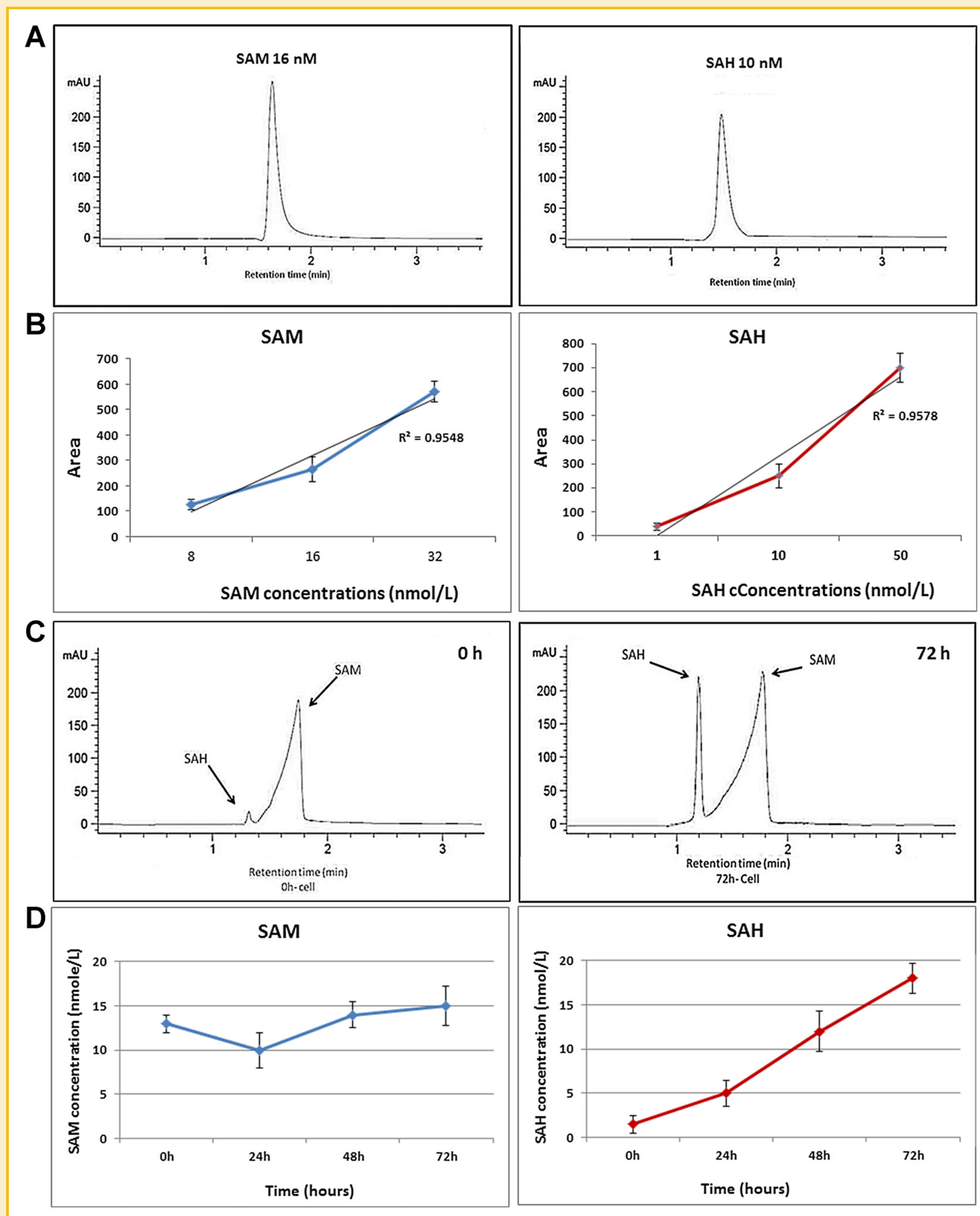

Fig. 2. Chromatograms showing elution profiles for SAM and SAH in As_2O_3 treated NB4 cells. The effect of As_2O_3 on the SAM and SAH levels was determined by HPLC technique. A,B: SAM and SAH elution profiles in calibrator standard solutions and plots of mean ± SD of three independent experiments. C: SAM and SAH elution profiles in untreated and in 1 μM As_2O_3 treated NB4 cell for 72 h. D: plots of SAM and SAH levels in NB4 cell treated with 1 μM As_2O_3 for 0, 24, 48, and 72 h. Values are given as mean ± SD of three independent experiments.

treatment, while after that, the GSH levels were increased in dose-dependent manners at 48 h ($P < 0.05$) and 72 h ($P < 0.01$).

As_2O_3 INHIBITS mRNA EXPRESSION LEVELS OF DNMTs AND AS3MT IN NB4 CELLS

Three DNMTs (DNMT1, DNMT3a, and DNMT3b) are required for the establishment and maintenance of DNA methylation patterns. DNMT3a and DNMT3b seem to mediate the establishment of new or de novo DNA methylation patterns, while DNMT1 appears to be responsible for the maintenance of established patterns of DNA methylation [Jin et al., 2011]. Arsenic methyltransferase (AS3MT) is a key enzyme that catalyzes the conversion of inorganic arsenic to methylated products. To investigate the mechanism of As_2O_3-induced DNA hypomethylation, we measured DNMTs and AS3MT mRNA levels in As_2O_3-treated NB4 cells line by quantitative real-time PCR. As shown in Figure 4A, As_2O_3 inhibited DNMT1, DNMT3a,

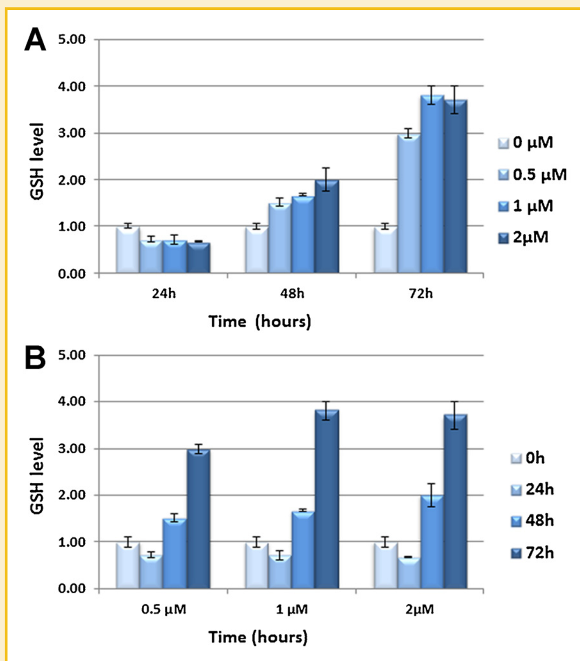

Fig. 3. The effect of As_2O_3 on the intracellular GSH level in NB4 cell. NB4 cells were treated with varying concentrations of As_2O_3 for 24, 48, and 72 h, assessed for the intracellular GSH level, and the levels were adjusted to the untreated control. As_2O_3 induced the generation of GSH levels in NB4 cells in a dose- and time-dependent manner (A and B, respectively). Values are given as mean ± SD of three independent experiments ($P < 0.05$ at 48 h; $P < 0.01$ at 72 h).

and DNMT3b mRNA levels in NB4 cells in a dose- ($P < 0.01$) and a time-dependent manner (data not shown). The expression of AS3MT was slightly increased after the treatment which it was not statistically significant.

In addition, we used a comparative C_t analysis to compare the basal expression level of these genes in the untreated NB4 cell line (Fig. 4B). The expression level of DNMT3b was very low in NB4 cell. The relative basal expression levels of DNMT3a and particularly DNMT1 were highly elevated in the NB4 cell line as compared to that in the DNMT3b (>3 and >4 logs, respectively).

DISCUSSION

Recent studies show that As_2O_3 treatment induces the up-regulation of several tumor suppressor genes in various cancer cells by demethylation effect [Tong and Lin, 2002]. It was shown that treatment with low dose of As_2O_3 induced CpG island demethylation of tumor suppressor genes p16INK4a, RASSF1A, E-cadherin, and GSTP1 by inhibition of DNA methyltransferase and it reactivated these partially/fully silenced genes in HepG2 and Huh-7 liver cancer cell lines [Cui et al., 2006]. A growing body of evidence indicates that the DNA demethylation effect induced by As_2O_3 is associated with the As_2O_3 unique metabolism [Cui et al., 2006; Zhou et al., 2008]. The liver is the main site of the As_2O_3 metabolism and for the production of methylated metabolites. Analysis of patient's urine samples, collected from intravenous injections of arsenic revealed the presence of the intermediate methyl arsenic metabolites, monomethylarsonous acid (MMA^{III}), and dimethylarsinous acid (DMA^{III}) [Wang et al., 2004]. In an in vitro study, As_2O_3 methylated metabolites have been shown in hepatoma cell line, HepG2 [Chen et al., 2003], Also, traces of methylated products (mainly monomethylarsenic acid) were detected in cell lysates of glioblastoma cell line (U87MG) [Falnoga et al., 2007]. In an experiment conducted by Chen et al. [2003] no methylated metabolites were detected in the APL cell culture (NB4) as well as other human leukemia cells such as U937 regardless of As_2O_3 concentration. In another experiment using a combined culture of NB4 and HepG2, they concluded that the APL cells do not methylate As_2O_3; however, the MMA and DMA metabolites formed and released from the liver cells of patients treated with As_2O_3 are preferentially taken up by the APL cells, and these methylated As_2O_3 metabolites may then contribute to the therapeutic effects of As_2O_3 in APL patients [Chen et al., 2003]. However, in the present study, we show that APL cell is capable of metabolizing As_2O_3. After entry of As_2O_3 into the NB4 cell, it was metabolized and converted into the methylated metabolites. The MMA was the predominant form of intracellular methylated metabolites in the NB4 cell exposed to 1 µM As_2O_3. The intermediate products of arsenic methylation are known to be more toxic than inorganic arsenicals [Styblo et al., 2000; Sakurai et al., 2006]; among them, MMA^{III} is the most toxic arsenic species [Ren et al., 2011]. Thus, formation of methylated species should enhance the As_2O_3 cytotoxicity and potentiate its anticancer effects as this probably occurs in the As_2O_3-treated APL cells. When we examined the culture medium, DMA was the major methylated metabolite in the culture medium. These findings are consistent with a previous report by Drobna et al. [2010] who found that MMA was the major methylated metabolite retained in the cell, and DMA was the major methylated metabolite in the medium of hepatocytes cell culture exposure to low levels of As_2O_3. They suggested that the export of methylated metabolites to the medium may be associated with activities of the basolateral membrane transporters that regulate the entry, retention, and removal of arsenicals in primary cultures of human hepatocytes. This may also play an important role in the efflux of As_2O_3 metabolites formed in the NB4 cell into the medium.

Two pathways for the metabolism of inorganic arsenic by AS3MT have been postulated: oxidative and reductive methylation pathways. The oxidative methylation pathway proposed by Challenger [1951], which involves oxidative addition of a methyl group to a trivalent arsenical to yield a methylated product containing pentavalent arsenic. Recently, the reductive methylation pathway was proposed by Hayakawa et al. [2005] in that iAs^{III} (As_2O_3) reacts with GSH (or other thiol-containing proteins) and becomes ATG, and ATG is methylated by AS3MT by transfer of a methyl group from SAM, becomes MADG. MADG is further methylated by AS3MT to DMAG, or becomes MMA^{III}. DMAG also becomes DMA^{III} (see Fig. 5 for postulated As_2O_3 methylation pathway and also for abbreviations). This new pathway agrees perfectly with our experimental finding. Our experiment revealed that As_2O_3 was metabolized and converted into the methylated MMA and DMA in the APL cell. Since

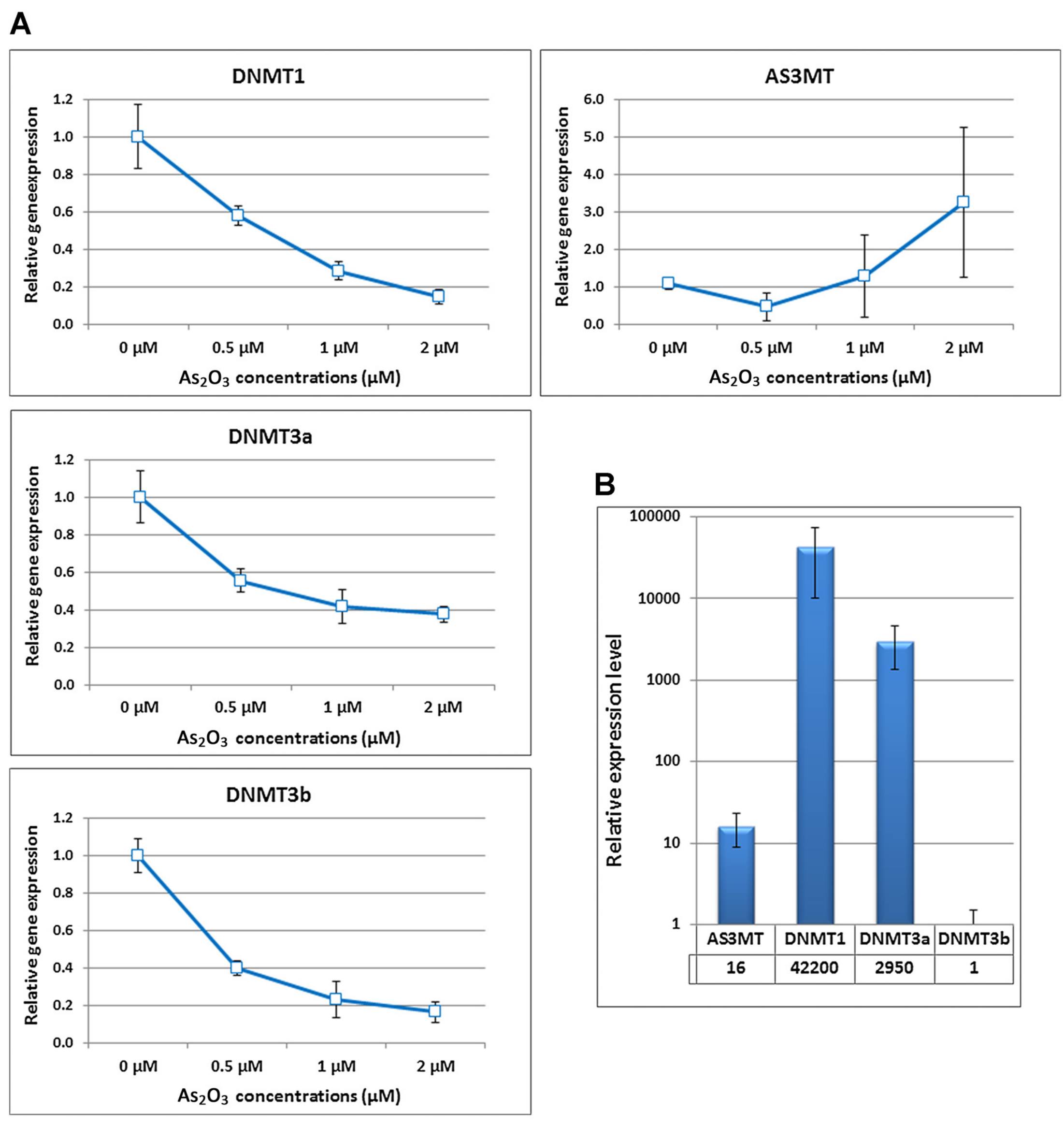

Fig. 4. A: The effect of As_2O_3 on transcriptional levels of DNMT1, DNMT3a, DNMT3b, and AS3MT in NB4 cell. Data are shown as fold change in relative expression levels of transcripts determined by quantitative real-time PCR. Values are normalized using the expression of the housekeeping HPRT1 on the basis of comparative Ct ($2^{-\Delta\Delta Ct}$) method. Values are given as mean $\pm$ SD of three independent experiments. For AS3MT, values are given as mean $\pm$ SD of 10 independent sets of experiments. B: Comparison of relative basal transcriptional levels of DNMT1, DNMT3a, and DNMT3b and AS3MT in untreated NB4 cell lines. The expression of DNMT1 and then DNMT3a were highly elevated in the NB4 cell line compared to the DNMT3b. Values are given as mean $\pm$ SD of three independent experiments.

both MADG and DMAG are unstable in solution and hydrolyzed and oxidized to MMA and DMA, respectively [Kobayashi et al., 2005], thus this could be the reason that their presence were undetectable in the present study. Our data shows that a combined MMA and DMA yield was significantly increased almost linearly in NB4 cell relative to the total As_2O_3 for up to 72 h (Fig. 1). Relative ratio of metabolites to the As_2O_3 level in cell lysate may indicate that the methylation rate of As_2O_3 was significant in the NB4 cells.

In the present study, treatment of NB4 cells with chemotherapeutically achievable concentrations of As_2O_3 (0.5–2 µM) during the period of 48–72 h resulted in a dose-dependent increase in GSH level. During first 24 h of treatment, the level of GSH was slightly decreased; it may indicate the addition of GSH to the reaction and probably stimulated the overall rate of arsenic methylation reaction. While our results show significant dose- and time-dependent increases in GSH levels in the As_2O_3-treatment cells, several investigators have reported downregulation of GSH levels in hepatocyte cells [Thomas et al., 2001] and in in vivo exposures to high dose of arsenic [Liu et al., 2000; Maiti and Chatterjee, 2000; Santra et al., 2000]. However, our study is in agreement with previous studies using human keratinocytes, fibroblasts, and Chinese hamster ovary cells, showing that an exposure to As_2O_3

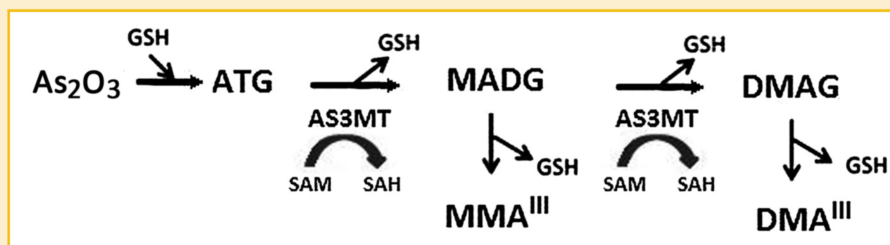

Fig. 5. Postulated reductive biomethylation pathway for arsenic trioxide by AS3MT in NB4 cell line. The reductive methylation pathway proposed for inorganic arsenic trioxide in that As_2O_3 reacts with GSH and become ATG, and ATG methylated by AS3MT by transfer of the methyl group from SAM, becomes MADG. MADG further methylated by AS3MT to DMAG, or became MMA^{III}. DMAG also become DMA^{III} [Hayakawa et al., 2005]. As_2O_3, arsenic trioxide; ATG, arsenic glutathione; MADG, monomethylarsenic diglutathione; DMAG, dimethylarsinic glutathione; MMA^{III}, monomethylarsonous acid; DMA^{III}, dimethylarsinous acid; GSH, reduced glutathione; SAM, S-adenosyl-L-methionine; SAH, S-adenosylhomocysteine; AS3MT, Arsenic methyltransferase.

causes a significant increase in GSH levels in all cell types [Lee and Chong, 1995; Ochi, 1997; Schuliga et al., 2002]. The increase in GSH levels by As_2O_3 in APL cell are probably related to the increase in the methylated metabolites production, and it may be attributed to the decomposition of the GSH arsenical complex (Fig. 5), and it also shows the correct AS3MT enzyme function. Its increase could also be part of an adaptive cytoprotective response against the acute cytotoxic effects of arsenic and its metabolites, to counteract the As_2O_3 induced oxidative stress. Moreover, there are ample evidences that the effects of arsenic are quite tissue specific and also dose dependent. A high dose for a short-term exposure to arsenic has quite different effect compared to a long-term chronic exposure to subtoxic arsenic [Schuliga et al., 2002].

Our results show that DNMT1 was highly overexpressed in APL cell, followed by DNMT3a expression, >4 logs and >3 logs respectively as compared to DNMT3b (Fig. 4B). Overexpression of DNMT1 and DNMT3a genes was previously reported to be an early event in some cancers [Saito et al., 2001], and an increased DNMT1 protein level was reported to be a poor prognostic marker in HCC [Saito et al., 2003]. Previous research showed that As_2O_3 suppressed DNMT1 mRNA level specifically in HepG2 cells, while the expression of DNMT3a and DNMT3b was remained unchanged [Cui et al., 2006]. However, our results showed that As_2O_3 significantly suppressed DNMT1, DNMT3a, and DNMT3b mRNA levels in dose- and time-dependent manners in APL cell. It has been suggested that inhibition of DNMTs, along with the inhibition of the histone deacetylase, may prevent the hypermethylation and silencing of these key genes, and this inhibition may contribute to the treatment of cancer and/or prevention of carcinogenesis. In recent years, considerable initial research efforts have been made in this direction [Saunders et al., 1999; Bovenzi and Momparler, 2001; Yoshida et al., 2001; Zhou et al., 2002; Aparicio et al., 2003; Shaker et al., 2003; Shi et al., 2003].

Arsenic methyltransferase (AS3MT) plays a central role in arsenic metabolism, catalyzing the biotransformation of As_2O_3 to the methylated products. Exposure to As_2O_3 did not produce any significant change in expression of the gene that encodes AS3MT (Fig. 4A). This contradicts our expectations regarding a previous report on the induction of AS3MT in PBMN cells due to the demethylation status of AS3MT promoter in individuals exposed to arsenic [Gribble et al., 2013]. However, a recent array-based study, evaluating arsenic exposure levels and methylation status of AS3MT in populations from Bangladesh and Argentina, found no association with CpG sites at 5′AS3MT [Engstrom et al., 2013]. Moreover, in a recent study, the effect of shRNA silencing of AS3MT expression on the capacity of cell to methylate arsenic was examined. In a stable clonal HepG2 cell line, AS3MT mRNA and protein levels were reduced by 83% and 88%, respectively; however, the methylation capacity was decreased only 70% [Drobna et al., 2006]. This suggests that an AS3MT-independent pathway might exist and contribute partly to the arsenic methylation. Also Drobna et al. produced AS3MT knockout mice to investigate the in vivo roles of AS3MT in the metabolism of arsenic. The results showed that the knockout of AS3MT does not completely abolish the methylation of arsenic, suggesting that there are alternative pathways for arsenic methylation in these animals [Drobna et al., 2009]. Recently, N-6 adenine-specific DNA methyltransferase (N6AMT1) was identified as a novel arsenic methyltransferase [Ren et al., 2011]. Collectively, although AS3MT is the main enzyme in the As_2O_3 methylation pathway; however, factors other than AS3MT expression or polymorphism play important roles in determining the capacity of cell to methylate As_2O_3, such as the intracellular cofactors that support AS3MT activity and the transport systems that regulate the kinetics of the methylation reactions inside the cells by regulating uptake, efflux, or cellular retention of As_2O_3 and/or its metabolites [Drobna et al., 2010].

Both, the DNMT-mediated DNA methylation and the AS3MT-mediated As_2O_3 methylation may use SAM as the methyl donor and produces SAH (Fig. 6). Thus, SAM and SAH are important metabolic indicators of the cellular methylation status [Yi et al., 2000]. Glutathione (GSH) serves as a reducing agent [Schuliga et al., 2002; Nemeti and Gregus, 2004]. It is considered that demethylation of the CpG islands in As_2O_3-treated cells might be induced not only by a direct inhibition of DNMT1, but also through a depletion of the methyl donor SAM [Cui et al., 2006]. In this study, we used an HPLC method for the detection of SAH & SAM levels in NB4 Cell lysate. The SAH level was found to increase linearly with time of exposure to As_2O_3; however, The SAM level was not affected. Similar observations regarding the lack of correlation between SAM and SAH have been reported and it may suggest that SAM is not a limiting factor for the DNA methyltransferase, at least within physiologic ranges. An increase in SAH, with or without a decrease in SAM, is the more important variable in predicting DNMTs

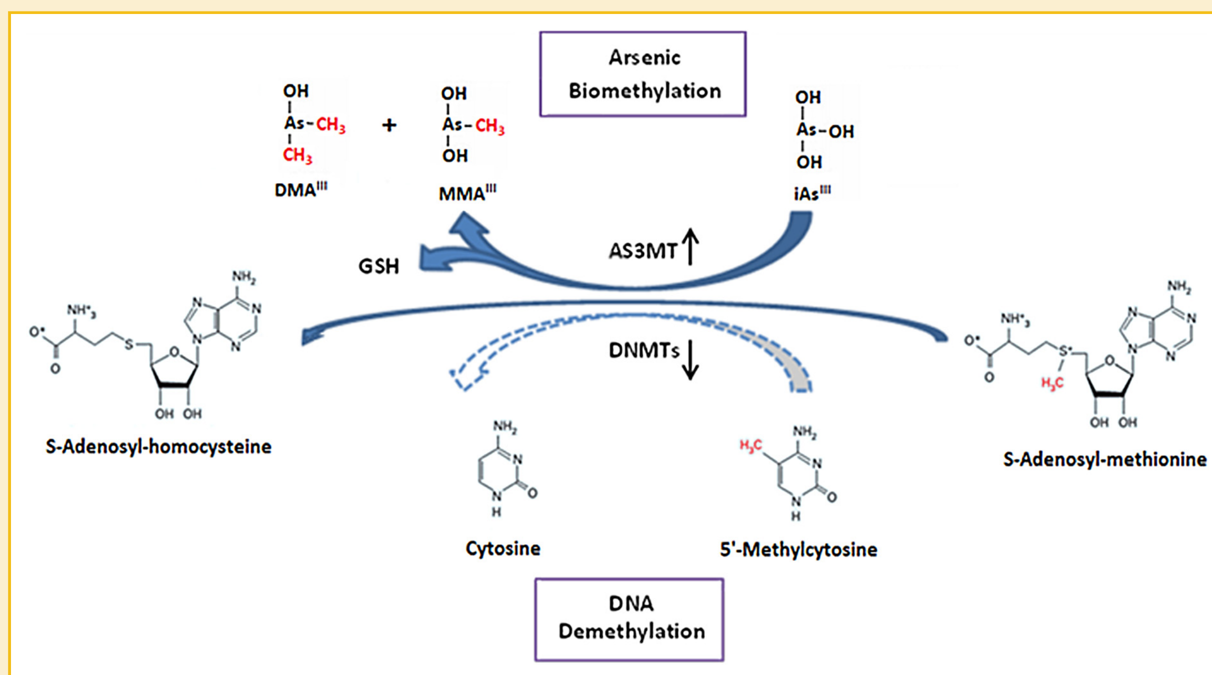

Fig. 6. Simplified scheme of As_2O_3 methylation and DNA demethylation. Both DNMT-mediated DNA methylation and AS3MT-mediated As_2O_3 methylation probably use SAM as the methyl donor. Arsenic exposure leads to a dose-dependent reduction of mRNA levels of DNMT1, DNMT3a, and DNMT3b and can directly interact with DNMTs and inhibit their activities. Also, during the methylation of As_2O_3 and its metabolites, the formation of SAH would be markedly increased. SAH is known an effective feedback inhibitor of DNMTs activities; therefore, the elevated intracellular levels of SAH as a result of the As_2O_3 methylation may increasingly cause a significant inhibition of the DNMT-mediated DNA methylation in a fashion related to the extent of DNA demethylation.

inhibition and an increase in cellular hypomethylation [Yi et al., 2000]. Furthermore, there is substantial evidences that SAH is a very potent inhibitor of DNMT activity [Sciandrello et al., 2004]. Enzyme kinetic analyses indicated that when a fixed concentration of SAM is present, increasing the concentrations of SAH resulted in a continuous decrease in the V_{MAX} values of the DNMT1-mediated DNA methylation (the KM values not altered), indicating that SAH is a noncompetitive inhibitor with respect to the formation of the methylated DNA products [James et al., 2002; Lee et al., 2005]. Thus, the elevated intracellular levels of SAH as a result of the As_2O_3 methylation might increasingly cause a significant inhibition in DNMTs enzymes activity and a consequent reduction in the DNMTs-mediated DNA methylation.

In summary, the anticancer mechanisms of As_2O_3 on APL are not only from direct or indirect influences on the genetic levels, but are also closely correlated with the unique arsenic metabolism. In this study, we have shown for the first time that APL cell is capable of metabolizing As_2O_3. After entry of As_2O_3 into the cell, it is metabolized and converted into the methylated metabolites. As_2O_3 exposure led to a dose- and time-dependent reduction in the mRNA levels of all three DNMTs (DNMT1, DNMT3a, and DNMT3b). The levels of GSH and SAH were increased in the As_2O_3-treated cells; however, the level of SAM was not affected. Thus, direct inhibition of the DNMTs expression plus increase of the SAH level (a potent inhibitor of DNMTs activity) by arsenic biotransformation presumably would affect the DNMTs-methylated DNA methylation in a fashion related to the extent of DNA hypomethylation.

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