The Role of Biomethylation in Toxicity and Carcinogenicity of Arsenic: A Research Update

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Recent research of the metabolism and biological effects of arsenic has profoundly changed our understanding of the role of metabolism in modulation of toxicity and carcinogenicity of this metalloid. Historically, the enzymatic conversion of inorganic arsenic to mono- and dimethylated species has been considered a major mechanism for detoxification of inorganic arsenic. However, compelling experimental evidence obtained from several laboratories suggests that biomethylation, particularly the production of methylated metabolites that contain trivalent arsenic, is a process that activates arsenic as a toxin and a carcinogen. This article summarizes this evidence and provides new data on (a) the toxicity of methylated trivalent arsenicals in mammalian cells, (b) the effect of methylated trivalent arsenicals on gene transcription, and (c) the mechanisms involved in arsenic methylation in animal and human tissues. Key words: AP-1, arsenic, cancer, inhibition, methylated arsenic, methylation, methytransferase, toxicity, transcription control. Environ Health Perspect 110(suppl 5):767–771 (2002). http://ehpnet1.niehs.nih.gov/docs/2002/suppl-5/767-771styblo/abstract.html

The metabolism of inorganic arsenic (iAs) in humans involves two types of chemical reactions, the reduction of pentavalent arsenicals to trivalency and the oxidative methylation of trivalent arsenicals to yield methylated pentavalent metabolites (1) (Figure 1). Glutathione (GSH) has been shown to reduce pentavalent arsenicals (arsenate iAs V and methylarsonic acid (MAs V), and dimethylarsonic acid (DMA s V)) in aqueous solutions (2,3). As V reductases may enzymatically reduce these arsenicals in mammalian tissues (4,5). Methylation of trivalent arsenicals [arsenite (iAs III) and methylarsonous acid (MAs III)] is catalyzed by As III-methyltransferases that use S-adenosylmethionine (AdoMet) as the methyl group donor (6,7). Because MAs V and DMA s V are not toxic in acute lethality assays, methylation of iAs has long been considered a detoxification mechanism. However, methylated arsenicals that are chemically consistent with trivalent methylated metabolites, MAs IIII O and dimethylarsonous acid (DMA s IIII O), have been shown to be more potent enzyme inhibitors and cytotoxins than either iAs V or MAs IIII O (8). Diiodomethylarsine (MA s IIII 2) and methylarsine oxide (MA s IIII O) are potent inhibitors of glutathione disulfide (GSSG) reductase (9), pyruvate dehydrogenase (10), and especially thioredoxin reductase (11). MAs IIII O and MA s IIII 2 are also far more toxic than iAs III for various types of mammalian cells (12–14). DMA s IIII derivatives [iodomethylarsine (DMA s IIII I) and dimethylarsinous-glutathione (DMA s IIII GS)] are at least as cytotoxic as iAs III for most cell types examined. Notably, exposures to low concentrations of either MAs IIII O or DMA s IIII I induce cell proliferation and production of growth-promoting cytokines in normal human keratinocytes (NHEK) (15). Unlike iAs III and iAs V, MA s IIII O and DMA s IIII react directly with DNA, nicking naked DNA in vitro and damaging nuclear DNA in intact human leukocytes (16). Evidence for the formation of methylated trivalent arsenicals in the course of the metabolism of iAs in humans has been obtained using optimized analytical techniques (17,18). MAs III and DMA s IIII have been detected in urine of individuals chronically exposed to iAs in drinking water (5,18–20) and in cultured human hepatic cells exposed to various concentrations of iAs III (18). Studies are currently under way in several laboratories to elucidate the role of methylated trivalent metabolites in the systemic toxicity and carcinogenicity of iAs. This report summarizes some recent work linking the metabolism of arsenic to its biological effects.

Toxicity of Methylated Trivalent Arsenicals in Mammalian Cells

Cytotoxic effects of trivalent and pentavalent arsenicals have previously been examined in several cell types, including primary human hepatocytes, primary human bronchial epithelial cells (HBEC), NHEK, SV-40-immortalized human bladder epithelial (UROtsa) cells, HeLa cells (12,13), and Chang liver cells (14). Pentavalent arsenicals were significantly less cytotoxic than their trivalent counterparts (12–14). Among trivalent arsenicals, MA s IIII O and MA s IIII 2 were the most cytotoxic species, followed by DMA s IIII, DMA s IIII GS, and iAs III.

We have recently examined cytotoxicity of arsenicals in several other mammalian cell types, including human hepatocellular carcinoma (HepG2) cells, human bladder transient carcinoma (T24) cells, human acute promyelocytic leukemia (NB4) cells, human monoblastoid (U937) cells, human osteosarcoma (HOS) cells, human neuroblastoma (SK-N-SH) cells, mouse 3T3 adipocytes, primary guinea pig hepatocytes, and Chinese hamster lung (V79-4) cells (Table 1). Regardless of the cell type, trivalent monomethylated arsenicals, MAs IIII O and MA s IIII 2, were the most potent cytoxins, with LC 50 values ranging from 0.4 to 5.5 µM. DMA s IIII derivatives were as cytotoxic as MAs IIII species and more cytotoxic than iAs III in most cell types.

The thiazolyl blue (MTT) assay that monitors the activity of mitochondrial dehydrogenases in viable cells has been used to examine cytotoxicity of arsenicals in all these cell types. The neutral red assay that measures the uptake of the dye by viable cells has also been used in some experiments. Because the cell viability values determined by the neutral red assay were lower than those obtained by the MTT assay, the results of MTT assay were used for these comparisons. It is important to realize however, that the neutral red assay measures cell viability but does not provide information about cell structure and function, such as metabolic activity.

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assay (12,15), it is possible that the latter assay underestimates cytotoxic effects of arsenicals in cultured cells. Figure 2 shows an example of the concentration-dependent effects of trivalent arsenicals on cell viability in human leukemia NB4 and U937 cell lines. Increased cell viability values found after 24-hr exposures to low concentrations of arsenicals were associated with increased cell proliferation rates. The induction of cell proliferation by low concentrations of trivalent arsenicals has previously been reported in several cell types (e.g., NHEK (19)). Notably, among cell types examined, NB4 cells were most sensitive to cytotoxic effects of trivalent arsenicals.

As shown in Table 1, there was no apparent correlation between the capacity of cells to methylate iAs and their sensitivity to the cytotoxic effects of trivalent arsenicals, indicating that the capacity to methylate has little to do with the resistance of cells to acute toxicity of AsIII. In some cases, iAsIII was more toxic in cells with a high methylation capacity (e.g., rat hepatocytes) than in cells that do not methylate this arsenical (e.g., guinea pig hepatocytes). Consequently, mechanisms other than methylation (e.g., transport of arsenicals across the cell membrane or protein binding) may play a critical role in the detoxification of trivalent arsenicals under acute exposure conditions. These results suggest that production and accumulation of MAIII and/or DMAIII, the most cytotoxic species among biologically relevant arsenicals, may be directly linked to adverse effects associated with in vivo exposures to iAs. We have previously shown that HepG2 cells exposed to iAsIII produced both MAIII and DMAIII. In addition, both MAIII and DMAIII synthesized in HepG2 cells were released into culture medium (18). Hence, MAIII and DMAIII may be translocated from methylating cells to tissues and cells that cannot methylate iAs. Notably, production of MAIII and DMAIII by HepG2 cells increased with increasing concentrations of iAsIII in the culture. Similarly, epidemiologic studies have shown that urinary levels of MAIII and DMAIII in individuals exposed to iAsIII in drinking water are positively correlated with exposure levels (5,18). These results suggest that individuals exposed to higher levels of iAs may be at greater risk associated with the production of these toxic methylated metabolites.

**Effects of Methylated Trivalent Arsenicals on Gene Transcription**

Various hypotheses have been proposed to explain the carcinogenicity of iAs (28). Nevertheless, molecular mechanisms by which this arsenical induces cancer are still poorly understood. Results of previous studies indicated that iAs does not act through classic genotoxic and mutagenic mechanisms, but rather may be a tumor promoter that modifies signal transduction pathways involved in cell growth and proliferation (29). iAsIII has been shown to modulate expression and/or DNA-binding activities of several key transcription factors, including nuclear factor kappa B (30), tumor suppressor p53 (p53) (31), and activating protein-1 (AP-1) (32–34). Mechanisms of AP-1 activation by iAsIII include stimulation of the mitogen-activated protein kinase (MAPK) cascade with a consequent increase in the expression and/or phosphorylation of the two major AP-1 constituents, c-Jun and c-Fos (29). The modulation of AP-1–dependent gene transcription by iAsIII may contribute to the induction of cell proliferation in cultured cells exposed to this arsenical. However, there are no data on the effects of methylated trivalent arsenicals on AP-1 composition and DNA-binding activity.

Recently, we have examined c-Jun and c-Fos expression and AP-1 DNA-binding activity.

![Figure 1](image1.png)

**Figure 1. Scheme of the metabolic conversions of iAs in humans.** AdoHcy, S-adenosylhomocysteine; R, AsIII reductase; M, AsIII methyltransferase.

![Figure 2](image2.png)

**Figure 2. Effects of trivalent arsenicals on cell viability in (A) NB4 and (B) U937 cultures.** Cell viability was determined by the MTT assay after 24-hr exposures to iAsIII (circles), MAIII (squares), or DMAIII (triangles). Each symbol and error bar represents mean and SD for n = 4. Asterisk (*) indicates cell viability in treated cultures is significantly different (p < 0.05) from that in untreated cultures as determined by analysis of variance with the Dunnett multiple comparison posttest.

| Cell type                  | iAsIII | MAIII | DMAIII | DMAIII S | DMAIII G | References |
|----------------------------|--------|-------|--------|----------|----------|------------|
| Primary rat hepatocytes    | 10–20  | 2.8   | 1.8    | 14.5     | 2.7      | 19         |
| Primary human hepatocytes  | 20     | 5.5   | >20    |          |          | 3.3        |
| NHK                        | 10–20  | 2.6   | 6.8    | 14.2     | >20      | ND (12,13) |
| HBEC                       | 3.2    | 2.7   | 0.8    |          | 0.25     | (12,13)    |
| UROtsa                     | 1.8    | 2.8   | >20    |          |          | (12,13)    |
| HepG2                      | >50    | 4.0   |        | 8.0      | 0.5      | (21)       |
| T24                        | >10    | 2.5   |        | 10       |          | ND (21)    |
| NB4                        | 1.0    | 0.4   |        | 0.4      |          | ND (22)    |
| U937                       | 5.0    | 1.0   |        | 1.0      |          | ND (22)    |
| HeLa                       | 25     | 1.6   |        | 7.0      | 0.25     | (22)       |
| Hep360                      | 40     | 2.7   |        | 2.0      |          | (24)       |
| Primary guinea pig hepatocytes | 100   | 5.0   |        | 6.0      | 0.3      | (29)       |
| Primary guinea 50 3T3adipocytes | 50   | 3.2   |        | 5.0      |          | (20)       |
| V79                         | 4.5    | 0.5   |        | 0.4      |          | ND (27)    |

Abbreviations: ND, not determined. *LC50* is defined as the concentration of an arsenical that resulted in a 50% decrease in cell viability over a 24-hr incubation period. The MTT assay was used to examine cell viability in all cell types.

- Methylation activity not determined.
- Methylation activity not determined.
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activity in several human cell lines, including UROtsa, T24, HepG2, and primary human hepatocytes exposed to trivalent or pentavalent inorganic or methylated arsenicals. Short-time exposures to trivalent, but not to pentavalent, arsenicals increased AP-1 DNA-binding activity in all these cell types. Most profound effects were found in UROtsa and T24 cells. In these cell lines, exposures to MAIII or DMAIII significantly increased the levels of nuclear phospho-c-Jun (p-c-Jun) but had no effects on either c-Jun or c-Fos levels (35). Importantly, MAIII and DMAIII were considerably more potent inducers of c-Jun phosphorylation and AP-1 activation than was iAsIII. Neither iAsV nor methylated pentavalent arsenicals, MAIV or DMAIV, modified c-Jun phosphorylation. Figure 3 shows nuclear levels of p-c-Jun in UROtsa cells exposed for 1 hr to iAsIII, MAIII, or DMAIII (0.5, 1, or 5 µM). MAIII was the most potent inducer of p-c-Jun, followed by DMAIII. In contrast, exposures to iAsIII suppressed p-c-Jun levels in this cell line. The AP-1 DNA-binding activity was induced in UROtsa cells exposed to as little as 0.1 µM MAIII (35), a concentration that is well below the LC50 value for these cells (Table 1).

The potencies of MAIII and DMAIII to stimulate the AP-1–dependent gene transcription have further been demonstrated using UROtsa and T24 cells transiently transfected with an AP-1–dependent promoter-reporter construct (35). Somewhat different AP-1 activation patterns were observed in primary human hepatocytes (Figure 4). Among trivalent arsenicals examined, MAIII was the most potent inducer of c-Jun phosphorylation in these cells. However, only a weak induction of p-c-Jun was observed in human hepatocytes exposed to iAsIII or DMAIII. A significant induction of the AP-1 DNA-binding activity was detected by the electrophoretic mobility shift assay (EMSA) only in cells exposed to 5 µM MAIII (Figure 5). Under these exposure conditions, p-c-Jun, but not c-Fos, was the major constituent of the AP-1 DNA-binding complex. Based on these results, the AP-1 DNA-binding activity appears to be less sensitive to induction by trivalent arsenicals in primary human hepatocytes than in either UROtsa or T24 cell lines that are derived from human urinary bladder. Accordingly, trivalent arsenicals, particularly MAIII, are likely to induce the AP-1–dependent gene transcription in human bladder to a greater extent than in the liver. Notably, both hepatic and urinary bladder cancers have been associated with chronic exposures to iAs in drinking water. However, the incidence of bladder cancer exceeds that of hepatic cancer (36–38). Thus, trivalent methylated arsenicals that are chemically consistent with trivalent methylated metabolites of iAs are more potent than iAs, inducing the DNA-binding activity of AP-1, a key transcription factor that is involved in regulation of cell proliferation and death (29).

**Mechanism of iAs Methylation**

The enzymatic reactions involved in the reduction and methylation of arsenicals have
transferases that catalyze methylation of iAsIII and of MAsV to MAsIII (4,39). Both these enzymes require thiols (e.g., GSH) for reducing activity. The MAsV reductase ($K_m = 2.6$ mM) has recently been identified as GSH-S-transferase omega (40). Methyltransferases that catalyze methylation of iAsIII and MAsIII have also been identified. A rabbit liver enzyme that converts iAsIII to MAs and MAsIII to DMAs has been purified and partially characterized (6). This cytosolic protein has a molecular weight of about 60 kDa and requires both AdoMet and a thiol for activity. Consistent with the metabolic scheme in Figure 1, the purified enzyme has a greater affinity for MAsIII than for MAsV.

A novel AsIII methyltransferase ($M = 41$ kDa) has recently been purified by Lin and co-workers (7) from rat liver. This enzyme methylates iAsIII in a two-step reaction, in which MAs is an intermediate and DMAs is the final product. The two-step kinetics of this reaction is consistent with kinetic patterns of iAsIII methylation reported in in vitro studies using tissue extracts (41,42). AdoMet is the essential methyl group donor for both methylation steps (Table 2). MAsIII is also a substrate for this enzyme in a methylation reaction yielding DMAs. A kinetic analysis of this reaction showed a low $K_m$ of 250 nM MAsIII. Thus, this enzyme can effectively methylate at very low concentrations of MAsIII in tissues. However, high concentrations of MAsIII ($≥ 5$ µM) inhibit DMAs synthesis. The rat AsIII methyltransferase requires a dithiol for its activity. Dithiothreitol (DTT) has been used as an enzyme co-factor in in vitro assays with purified rat AsIII methyltransferase. Protein and cDNA sequences for the rat AsIII methyltransferase have been obtained. Sequence analyses have revealed a high degree of homology with a putative human methyltransferase CYT19, indicating that CYT19 is the human AsIII methyltransferase. Using reverse-transcription polymerase chain reaction, mRNA for AsIII methyltransferase has been detected in rat tissues (heart, liver, lung, kidney, adrenal, bladder, and brain) and also in human hepatoma (HePG2) cells that are known to methylate iAsIII (18). In contrast, mRNA for this enzyme has not been found in UROtsa cells, human urinary bladder cells that do not produce methylated metabolites when exposed to iAsIII in culture (12,13).

Based on the results of the in vitro studies, the presence of a dithiol is an essential requirement for the rat AsIII methyltransferase activity. Thioredoxin (TRx), a small (12 kDa) protein with a pair of redox-active cysteine residues ($9.5$ (active from pH 7 to 11)

Table 2. Properties of AsIII methyltransferase from rat liver.

| Molecular mass | 42,000 Da (determined by PAGE); 41,056 Da (calculated) |
| Primary structure | 369 amino acid residues; cysteine-rich protein (12 cysteine residues); common methyltransferase motifs, including the AdoMet binding motifs |
| Kinetic characteristics | $K_m =$ 250 nM, $V_{max} = 68$ pmol/mg protein/min (with MAsIII as a substrate); pH optimum = 9.5 (active from pH 7 to 11) |
| Essential co-factors | Dithiol (e.g., DTT, TRx; AdoMet (the methyl group donor) |
| Known inhibitor | MAsIII ($≥ 5$ µM) inhibits DMAs formation in the second methylation reaction |

*Data from Lin et al. (17) and Lin and Thomas (43).

Figure 6. Hypothetical mechanism of the methylation of iAs by AsIII methyltransferase: the role of Trx and TR. AsV-R, AsV reductase; AsIII-MT, AsIII methyltransferase.

Conclusions

The results of previous studies and new experimental data presented here suggest that exposures to methylated trivalent arsenicals are associated with a variety of adverse effects that have a profound impact on cell viability or proliferation. The known effects include a) inhibition of several key enzymes, b) damage to DNA structure, and c) activation of AP–1–dependent gene transcription. Notably, trivalent methylated arsenicals, MAsIII and/or DMAsIII derivatives, are more potent than iAsIII in producing these effects. These findings are consistent with the concept of biomethyla-
tion being a process that potentiates toxicity and carcinogenicity of iAs.

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