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Review

Mechanism of arsenic carcinogenesis: an integrated approach

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Abstract

Epidemiological evidence shows an association between inorganic arsenic in drinking water and increased risk of skin, lung and bladder cancers. The lack of animal models has hindered mechanistic studies of arsenic carcinogenesis in the past, but some promising new models for these cancers are now available. The various forms of arsenic to which humans are exposed, either directly or via metabolism of inorganic arsenic to various methylated forms, further complicate the issue of mechanism, since these compounds can have different effects, both genotoxic and non-genotoxic. This review will try to integrate all of these issues, with a strong bias toward effects that are produced by environmentally relevant arsenic concentrations.
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1. Introduction

Arsenic-related publications have greatly increased in recent years, partly as a result of the enormous disaster in the Bengal region of India and neighboring Bangladesh where millions have been exposed to high levels of arsenic in drinking water. In West Bengal alone, nine districts encompassing an area of 38,000 km^2 and with a population of about 42.7 million are affected [1]. In addition, the recent re-examination by the USEPA of arsenic levels in drinking water was a stimulus to research on the mechanism of arsenic carcinogenesis. In the absence of clear knowledge as to the mechanism of arsenic carcinogenesis, the default assumption (based on the idea that a carcinogen forms DNA adducts, and therefore even a single adduct has a finite chance of causing cancer) was used, leading to a controversial linear extrapolation from observable exposures with no threshold.

Some excellent recent reviews on various aspects of arsenic metabolism, toxicity, and carcinogenicity already exist [2–7]. The genetic and molecular toxicology of arsenic was previously reviewed by this author [8] and more recently Basu et al. [9] has reviewed arsenic's genetic toxicology, giving an extensive compendium of the behavior of arsenic compounds in various genetic toxicology assays. This review will focus on work related to the possible mechanisms of arsenic carcinogenicity, both genotoxic and non-genotoxic, published in peer-reviewed journals during the past 10 years. It will not address the very important issue of arsenic as a chemotherapeutic agent for acute promyelocytic leukemia and other cancers, and the related topic of arsenic-induced apoptosis. These topics have been addressed by others [7,10–12].

Because any event which could lead to carcinogenesis must of necessity allow clonal expansion of altered cells, this review will not contain much

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discussion of in vitro studies using concentrations of arsenic compounds that lead to extensive apoptosis or necrotic cell death. It is becoming increasingly clear that high dose exposure to arsenic compounds differs from low dose exposure with regard to genotoxicity [13], types of reactive species formed [14], signal pathways activated [15] and gene expression [16]. Many "stress proteins" seem to be induced only at high dose [17]. For most human cells treated in clonal culture (<1000 cells/dish) the IC_{50} for arsenite (clonal survival) is between 0.2 and 2 μM and for rodent cells it can be an order of magnitude higher [18]. When cells are treated in subconfluent monolayer cultures, which usually contain at least 5×10^5 cells, IC_{50} values for clonal survival after replating can be 10-fold higher than for direct exposure in clonal culture. Although many studies claim to be using "non-toxic" concentrations of arsenicals, the criteria are often weak, based on assays which are not as stringent as clonal survival or even growth inhibition as a substitute for cells which do not form colonies. We have recently shown that many of these alternate assays are performed too soon after exposure to arsenite, so that dying cells are not scored (Komissarova and Rossman, manuscript in preparation).

2. Arsenic as a human carcinogen

Arsenic is released into the atmosphere from both natural and anthropogenic sources. Global natural emissions of arsenic and arsenic compounds have been estimated to be 8000 t each year, whereas anthropogenic emissions are about three times higher [19]. Chronic arsenic exposure is of concern mainly because of its carcinogenic effects. Inorganic arsenic was one of the earliest identified human carcinogens. Medical treatment of psoriasis with Fowler's solution (1% potassium arsenite) resulted in an excess of skin cancers, a finding that has lead to almost complete elimination of arsenic in human medicine. Further evidence for arsenic as a human carcinogen comes from studies of arsenic ore smelters, pesticide workers, and people exposed to arsenic-containing drinking water. In Taiwan, Chile, Argentina, Bangladesh, and Mexico, people who drink arsenic-containing drinking water develop cancers [20–26]. Although skin cancers are most frequent, strong epidemiological associations exist also between arsenic ingestion and bladder, lung, and perhaps kidney and liver cancers. Arsenic is the most extensively studied of the metals and metalloids found in drinking water. Arsenic contamination of drinking water is a public health issue worldwide, including regions of the West, Midwest and New England in the US [19]. Environmental exposure to arsenic is generally in the form of either arsenite (As^{3+}) or arsenate (As^{5+}). The former is the predominant form in drinking water from deep (anaerobic) wells, while the latter predominates under aerobic conditions. The increase in cancer risk observed in epidemiological studies is attributed mainly to the presence of inorganic trivalent arsenic (arsenite) [27,28]. Any arsenate in the water is rapidly reduced to arsenite once it enters cells (see below).

3. Metabolism

It has been known for some time that arsenite is more toxic than arsenate. This may be due in part to different rates of cellular uptake. At equimolar concentration, arsenite accumulation in many cell types is much faster compared with that of arsenate [29–33]. It has been suggested that since arsenite is uncharged at physiological pH, it can pass through the cell membrane faster than can arsenate which is negatively charged. However, it is now becoming clear that both arsenite and arsenate are actively transported into cells. Arsenite is transported by aquaglycoporins 7 and 9, which transport water and glycerol [34] and arsenate is probably transported by the phosphate transporter [35]. Thus, inorganic arsenic compounds take advantage of transporters whose substrates they resemble. The transport mechanisms of organic arsenicals are unknown but may involve organic ion transporters.

The structures of the arsenic compounds discussed here are shown in Fig. 1. In humans, arsenic compounds are metabolized by methylation followed by excretion in the urine. Methylated arsenic species are excreted much faster than inorganic species [36]. Methylation occurs primarily in the liver with much smaller amounts in other organs such as kidney and lung. The metabolism of arsenic compounds in mammals has recently been reviewed [5,37–39]. Reduction of arsenate to arsenite is necessary before methylation can occur. An enzyme that reduces arsenate to

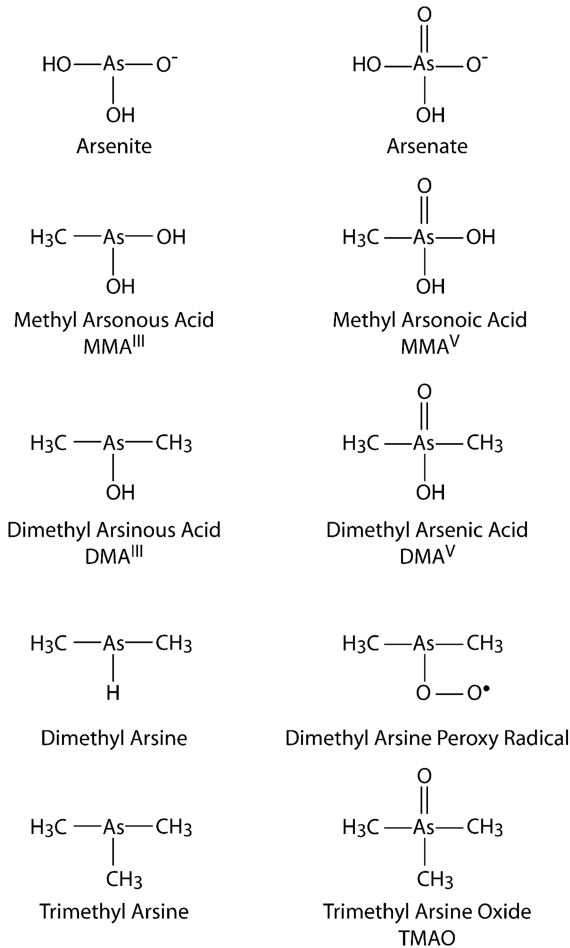

Fig. 1. Structure of arsenic compounds.

arsenite in vitro has been identified as purine nucleoside phosphorylase, with a dithiol, but not glutathione (GSH) as reductant [40,41]. GSH is able to reduce arsenate non-enzymatically [42]. A recent study, however, casts doubt on a role for purine nucleotide phosphorylase in the reduction of arsenate to arsenite in vivo [43].

Arsenite is methylated by enzymatic transfer of the methyl group from *S*-adenosylmethionine (SAM) to arsenite to form monomethylarsonic acid (MMA^V) (structures are shown in Fig. 1). The gene encoding the enzyme responsible for this reaction has been cloned and is identical to the cyt19 gene [44]. The same enzyme or glutathione-*S*-transferase omega class 1-1 (GSTO1-1) [45] then reduces MMA^V to monomethylarsonous acid (MMA^{III}). Cyt19 utilizes thioredoxin and NADPH while GSTO1-1 utilizes GSH as reductant. A second reaction methylates MMA^{III} to dimethlyarsinic acid (DMA^V) [39]. Some DMA^V can then be reduced to DMA^{III} probably also catalyzed by cyt19:

$$\text{arsenite} + \text{SAM} \rightarrow \text{MMA}^{V}$$

$$\text{MMAV} + \text{thiol} \rightarrow \text{MMA}^{III}$$

$$\text{MMA}^{III} + \text{SAM} \rightarrow \text{DMA}^{V}$$

$$\text{DMA}^{V} + \text{thiol} \rightarrow \text{DMA}^{III}$$

Most humans exposed to arsenic excrete 10–30% inorganic arsenic, 10–20% $MMA^{(V+III)}$ and 60–80% $DMA^{(V+III)}$, but some populations differ [38]. Humans are unusual in that they excrete much more MMA than do other species [46]. The pentavalent metabolites MMA^V and DMA^V are less toxic than arsenite or arsenate [36]. The oral LD_{50} for rats for arsenate, arsenite, MMA^V and DMA^V is approximately 100, 41, 961, and 644 mg/kg, respectively [47]. Thus, biomethylation of arsenic has long been considered the major detoxication pathway. However, it is now known that the trivalent methylated arsenic metabolites MMA^{III} and DMA^{III} are more toxic than arsenite both in vitro and in vivo [32,48–50]. Methylated trivalent metabolites are highly reactive and are more potent inhibitors of GSH reductase [48] and thioredoxin reductase [51] compared with arsenite or pentavalent metabolites. Thioredoxin reductase catalyzes the NADPH-dependent reduction of the disulfide bond in oxidized thioredoxin, which is an oxidoreductase with broad biological activity [52]. The carcinogenicity and genetic toxicology of methylated compounds is discussed below.

4. Recent animal models for inorganic arsenic carcinogenesis

Until recently, arsenic compounds were the only compounds that IARC considered to have sufficient evidence for human carcinogenicity, but inadequate evidence for animal carcinogenicity [53]. Early tumorigenesis experiments in four species of animals given inorganic arsenic compounds by different routes

of exposure either failed or had serious flaws [27]. There are several reports of lung carcinogenesis using intratracheal instillation of rats and hamsters with inorganic arsenic compounds alone or with other carcinogens [54–57]. However, very toxic (even lethal) doses of arsenic compounds were required for very little tumor induction among survivors.

Because arsenite is not significantly mutagenic at endogenous loci in bacterial or mammalian cells at concentrations giving high levels of survival (see below), it is sometimes considered a tumor promoter. There is little evidence for this view, as arsenite had no promotional activity in skin carcinogenesis using 3-methylcholanthrene as initiator [58] or in H-ras activated transgenic mice [59]. On the other hand, DMA^V does act as a promoter in bladder carcinogenesis [60]. Organic arsenic compounds are discussed below. Inorganic arsenic compounds were also not carcinogenic to animals when tested at reasonable doses as initiators in two-stage carcinogenesis assays [19,27].

A number of new animal models for arsenic carcinogenesis show promise. Transgenic (Tg.AC) mice-containing activated H-ras were exposed to 200 ppm sodium arsenite in drinking water for 4 weeks with or without subsequent skin painting with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Arsenite alone caused no tumors, but arsenite increased the numbers of papillomas induced by TPA, so arsenite could be considered a “co-promoter” in that system [59,61]. Arsenic compounds were assayed in another transgenic mouse, K6/ODC, in which hair follicle keratinocytes (likely targets for skin carcinogens) overexpress ornithine decarboxylase (ODC). ODC is a TPA-inducible enzyme involved in polyamine synthesis (needed in S phase), and its overexpression is sufficient to promote papilloma formation following a subthreshold dose of carcinogen [62]. Ten ppm sodium arsenite or DMA^V (cacodylic acid), given 5 months in drinking water, induced a small number of papillomas [63].

Ultraviolet radiation (UVR) from sunlight is the most prominent carcinogen in our natural environment and the most important cause of skin cancers [64]. We have developed a new mouse model for arsenic carcinogenesis which couples the use of a non-toxic concentration of sodium arsenite in drinking water with a low (non-erythemic) dose of solar UVR [65]. Hairless Skh1 mice given 10 ppm sodium arsenite in drinking water for 26 weeks had a 2.4-fold increase in yield of tumors after 1.7 kJ/m^2 solar UVR three times weekly compared with mice given UVR alone. In a second experiment, with a lower UVR dose (1 kJ/m^2) a maximum enhancement of about five-fold was seen using 5 mg/l arsenite but there was a significant increase even at 1.25 ppm ([66], Burns et al., manuscript submitted). The tumors were mostly squamous cell carcinomas, and those occurring in mice given UVR plus arsenite appeared earlier and were much larger and more highly invasive than those receiving UVR alone [65]. No tumors appeared in any organs in mice given arsenite alone. This is the first demonstration that arsenite can enhance the onset and growth of malignant skin tumors induced by a genotoxic carcinogen in mice.

Sunlight-associated skin cancer is characterized by solar UV-induced genotoxic photoproducts in DNA, mainly cyclobutane dimers and 6-4 photoproducts at dipyrimidine sites, but also some oxidative lesions. The p53 gene is often mutated early in skin carcinogenesis and the mutations are often at sites of dipyrimidines [67]. Subsequent exposures to sunlight are thought to favor the clonal expansion of p53-mutated cells, which have increased resistance to apoptosis [68]. Thus, if arsenite enhanced the mutagenicity of UVR, tumors should arise more quickly and at higher yield.

Several epidemiological studies also implicate arsenic as a co-carcinogen in humans (reviewed in [69]). In Japanese and Taiwanese populations exposed to arsenic in drinking water, associations with increased lung cancer in smokers compared to non-smokers suggest a synergy between the carcinogens [70,71].

An interesting new finding is that arsenite (42.5 and 85 ppm) in drinking water of pregnant C3H mice caused increased tumorigenesis in the offspring [72]. Hepatocellular carcinoma and benign adrenal tumor incidence was increased in male offspring and ovarian and lung tumor incidence was increased in female offspring. It should be pointed out that the C3H mouse has a high background of spontaneous tumors in many organs. It remains to be seen whether this effect would occur in another strain of mice with lower background tumorigenicity, or whether this is also a case of enhancement, but of an endogenous carcinogenic process.

5. Genotoxicity by low level inorganic arsenic

When studying the genetic toxicology of arsenite in cultured cells, it must be kept in mind that some cell lines are able to methylate arsenite. Although hepatocytes are not often used for assessing genotoxicity, rat hepatocytes have a fast rate of methylation compared with human hepatocytes, while the methylation capacities of human keratinocytes and bronchial cells were less than 1% that of human hepatocytes [32]. The methylation rate of normal human keratinocytes did not exceed 0.25 pmol arsenic per 10^6 cells/h, and it was concluded that methylation is not a significant detoxification pathway in keratinocytes [33]. Insignificant amounts or no methylation was seen in Urotsa cells (SV40-transformed human bladder epithelial line) [32], human lymphoblasts (Styblo, personal communication), or various commonly used fibroblast lines such as mouse BALB/3T3 or Chinese hamster V79 [30,73]. Thus, in many cases, with the exception of hepatocytes, the effects of arsenite appear to be caused by arsenite itself, and cannot to attributed to the small amounts (if any) of the methylated metabolites made.

The genetic toxicology of arsenite was previously reviewed [8,9,74]. Unlike many carcinogens, arsenite is not a mutagen in bacteria and acts weakly at high (toxic) concentration at the *hprt* locus in Chinese hamster V79 cells [75]. Because arsenite is such a poor mutagen at endogenous loci such as the X-linked *hprt*, attempts have been made to find genetic markers more likely to be able to detect large deletions, which are often lethal when extended past the *hprt* locus on the (single functioning) X chromosome. In transgenic G12 cells assayed at the *E. coli gpt* locus, which can detect clastogens causing deletions because the single copy of *gpt* is inserted into an autosome [76,77] and in mouse lymphoma cells, which can tolerate deletions at the TK locus due to its autosomal location, weak effects are also seen at toxic doses (15 μM for G12 cells and 10 μM for mouse lymphoma) [78,79]. Analysis of mutants resulting from AS52 transgenic (*gpt*) Chinese hamster ovary cells treated with high concentrations of arsenite (which also gave mutant fractions only twice background levels) showed a higher proportion of deletions than in the spontaneous class [80]. This is also true in A_L cells, which are CHO-K1 cells containing a single copy of human chromosome 11, which can suffer deletions when exposed to toxic concentrations of arsenite [81].

In addition to small numbers of mainly deletion mutations, arsenite also induces chromosome aberrations, aneuploidy, and micronuclei (MN) formation [82]. Chromosome aberrations and endoreduplication were induced by arsenite, but not arsenate, in human fibroblasts and CHO cells at concentrations 1 μM and higher, whereas both arsenite and arsenate cause sister chromatid exchanges (SCE) at concentrations as low as 0.01 μM, but not in a dose-dependent manner [83,84]. An increase in MN frequency after exposure to an agent is evidence of either clastogenesis or aneuploidy induced by that agent [85,86]. Low dose (5 μM) exposure to arsenite for 24 h and high dose (20 μM) arsenite for 4 h resulted in similar levels of arsenic accumulation and toxicity in normal human fibroblasts [13]. Both treatments induced micronuclei, but the low dose protocol resulted mainly in kinetochore positive (K^+) MN, whereas the high dose protocol resulted in mainly MN which were kinetochore negative (K^-). K^+MN are usually derived from whole chromosomes while K^-MN are derived from fragments. X-rays and other clastogens induce high levels of K^-MN [86], whereas agents that induce aneuploidy by interfering with spindle function induce mainly K^+MN [87]. Thus, at low dose, arsenite acts as an aneugen, but at high dose it acts as a clastogen. High concentrations of arsenite may result in its sudden accumulation in cells and may have effects that differ from a slower accumulation, which would allow tolerance mechanisms to come into play [88–91]. Evidence supports arsenite's aneugenic role in other cells such as CHO, Chinese hamster V79, human lymphoblasts, mouse lymphoma, and HeLa [79,84,92–96]. In V79 cells, 10 μM arsenite disrupted mitotic spindles and induced persistent aneuploidy that was maintained even 5 days after it was removed but caused no chromosome aberrations in surviving cells [95,96].

Micronuclei are induced in vivo in the bone marrow of mice treated with arsenite [28], and are detected in exfoliated bladder cells, buccal cells, sputum cells, and lymphocytes from arsenic exposed humans [95–98]. Some studies report increased chromosome aberrations in the lymphocytes in humans exposed to arsenic in drinking water [9,99–102] The bladder cell MN assay has been proposed as the most appropriate biological marker of in vivo arsenic genotoxicity

[103]. However, in an arsenic-exposed population in the West Bengal region of India, MN frequencies in peripheral blood lymphocytes seemed to be a more sensitive indicator of arsenic exposure compared with MN in exfoliated bladder epithelial cells or in buccal cells [98]. The majority of studies where the populations had long-term exposure to 400 ppb arsenic via drinking water gave clear evidence of increased chromosome aberrations in peripheral blood lymphocytes and increased MN formation in lymphocytes, exfoliated oral mucosa cells, and exfoliated urinary bladder epithelial cells [95–100].

There is significant evidence demonstrating heterogeneity in the in vitro response to arsenite by cells from different individuals. An evaluation of arsenite-induced aneuploidy in peripheral blood lymphocytes from human donors shows quite large inter-individual variation [92]. Donors who were most sensitive to arsenite-induced aneuploidy were also most sensitive to arsenite-induced mitotic arrest and chromosome aberrations. Large interindividual variations were obtained by other laboratories studying arsenite-induced chromosome aberrations [104], DNA strand breaks in the single cell gel electrophoresis (Comet) assay [105], SCE [106,107], and inhibition of lymphoblast proliferation [108].

6. Oxidant production and oxidative DNA damage induced by arsenite

Although arsenite does not react directly with DNA, cells treated with arsenite show evidence of oxidative DNA damage. The concept that arsenite increases oxidant levels is supported by studies demonstrating protection against arsenite genotoxicity by GSH elevation and antioxidants Vitamin E, catalase, superoxide dismutase (SOD), and squalene [81,89,109–113]. H_2O_2-resistant CHO cells are cross-resistant to arsenite [114]. Mutagenicity of A_L cells by arsenite was blocked by dimethyl sulfoxide, a free radical scavenger [81]. SOD and catalase reduce arsenite-induced production of oxyradicals and 8-oxo-2′-deoxyguanine (8-oxo-dG) production in DNA in these cells [113,115]. The X-ray sensitive CHO variant XRS5, which is deficient in catalase and GSH peroxidase, is also hypersensitive to micronucleus induction by arsenite [116].

It is of interest that increased levels of serum lipid peroxides and decreased levels of non-protein sulfhydryls were found in a Chinese population chronically exposed to arsenic in drinking water [117]. Lipid peroxidation is also seen in various tissues after exposure of rats to arsenite [118], and this effect is independent of GSH levels.

In cell culture, depletion of GSH increases the toxic and clastogenic effects of arsenite [119], and some of the oxidative effects of arsenite may be caused by arsenite-induced GSH depletion. Arsenite readily reacts with GSH [42]. Depletion of GSH may be a high dose effect, since cells normally contain mM concentrations of GSH. Low arsenite concentrations actually cause increased GSH levels [90]. On the other hand, it is not clear what the long-term affects would be of small changes in redox status caused by reduction of GSH and/or reduced thioredoxin levels. Both GSH reductase and thioredoxin reductase can be inhibited by arsenite and its trivalent methylated metabolites [48,51].

Arsenite induces proteins which are induced by and protect against oxidative stress. Induction of metallothionein (MT) is seen in cells and mice after treatment with arsenite [120–122]. MT overexpression gives some protection against arsenite toxicity even though MT does not have a high affinity for arsenite itself [123] and MT-I/II null mice are hypersensitive to the toxic effects of inorganic arsenicals [122]. Most likely, the MT blocks oxidant stress rather than reacting with arsenite. Arsenite treatment results in induction of the DNA damage-inducible protein GADD153 and this is suppressed by the antioxidant *N*-acetyl cysteine [124]. Levels of GSH, ferritin, and heme oxygenase (HO) are also increased after treatment of human cells with arsenite [89,90,125–127].

Induction by arsenite of HO warrants further discussion, as it has been suggested as a response biomarker for arsenite exposure [128]. It is induced by treatment with low concentrations of arsenite in human keratinocytes [129], in human embryonic kidney cells [130], and in rat liver and kidney [47] where it was related to inorganic arsenic levels. HO induction is an indicator of oxidant stress [131–133]. HO cleaves the heme tetrapyrrole ring in cellular hemoproteins involved in redox reactions, thus causing cellular redox potential to shift towards reduction. The enzymatic product of HO, biliverdin, is then converted to

bilirubin which is also an antioxidant [132,133]. Induction of HO is responsive to cellular levels of GSH [132,133], and its induction by arsenite was blocked by antioxidants [89]. An arsenite-resistant variant of a human lung adenocarcinoma cell line has elevated levels of HO, and arsenite-resistance in these cells could be blocked by tin-protoporphyrin, an inhibitor of HO [134]. Tin-protoporphyrin also enhances arsenite-induced DNA strand breaks and MN [135]. Wild type cells, but not the arsenite-resistant variant, showed increased oxidant content after exposure to arsenite for 24 h, thus supporting the role of oxidants in arsenite toxicity and the protective role of HO.

Oxidative DNA damage and DNA–protein crosslinks may be the major DNA lesions induced by arsenite [136]. Skin samples from individuals with arsenic-related lesions and arsenic-unrelated lesions were assayed for 8-oxo-dG by immunohistochemistry. Only 1/11 of the arsenic-unexposed group showed staining for 8-oxo-dG, whereas staining was seen in 22/28 of the arsenic-exposed group [137]. Mice given arsenite or higher doses of arsenate show evidence of free radicals in the liver detected by a spin trap agent [122]. Results such as these suggest that DNA damage induced by arsenite are mediated by reactive oxygen species (ROS).

The single cell electrophoresis (Comet) assay has been used to measure DNA strand breaks (DSB) in cells treated with genotoxicants. However, detection of DSB in the standard Comet assay or by alkaline elution is inadequate for arsenic compounds. The small number of DSB detectable probably result from excision repair of lesions. In cells exposed to low concentrations of arsenite, there are increased DSB when the DNA is treated with enzymes [138]. These include the DNA repair enzymes formamidopyrimidine-DNA glycosylase (Fpg) and endonuclease III (endo III) as well as proteinase K (PK). Fpg and endo III catalyze the excision of oxidized bases [139]. PK would release DNA which is bound in DNA–protein crosslinks [140], and data suggested that the DSB were produced secondarily [141]. A more sensitive Comet assay has been developed in which the slides are digested sequentially with endo III, Fpg and PK to increase the number of DSB, allowing detection of DNA damage by 0.1 μM arsenite in leukemia cells [138]. The relative increase in strand breaks after arsenite treatment was endo III $>$ Fpg $>$ PK. Endo III cleaves oxidized pyrimidines such as thymine glycol, 5,6-dihydrothymine, 5-hydroxydihydrothymine, 5-hydroxycytosine, 5-hydroxyuracil and uracil glycol [139,142]. Fpg cleaves mainly oxidized purines such as 8-oxoguanine, 2,6, diamino-4-hydroxy-5-*N*-methylformamidopyrimidine, and 4,6-diamino-5-formamidopyrimidine, but also can cleave the modified pyrimidines 5-hydroxycytosine and 5-hydroxyuracil [142]. While PK does not increase the number of strand breaks in all cell types, Fpg always does [136]. The increased DSB produced by Fpg in human lymphocytes treated with arsenite showed similar repair kinetics to those in H_2O_2-treated cells [143].

ROS and nitric oxide (NO) have been detected in some cells treated with arsenite [80,111]. What is the source of the oxidants seen after arsenite treatment? One possibility is the activation of NAD(P)H oxidase found on the plasma membrane of vascular epithelial cells and other cells [144–147]. NAD(P)H oxidases catalyze the one-electron reduction of O_2 to superoxide and its activation leads to increases in superoxide and other ROS. Arsenite-induced superoxide production was blocked by transfection of antisense oligonucleotides directed against an essential component of NAD(P)H oxidase [145]. In addition, H_2O_2 can react with Cl^- (catalyzed by myeloperoxidase) to produce hypochlorous acid which also causes DNA damage. Myeloperoxidase inhibitors reduce arsenite-induced DSB [136]. Even in the absence of overt evidence of increased oxidant stress, it is possible that oxidant-related cell signaling can be affected. Low arsenite concentrations (0.5–5 μM) increased oxidant signaling and oxidant-dependent activation of NFκB in the absence of oxidant stress in porcine endothelial cells [14]. The increased oxidants appear to result from activation of membrane-bound NAD(P)H oxidase.

In CHO cells, arsenite-induced MN production can be suppressed by SOD and inhibitors of NO synthase, suggesting the involvement of NO and/or peroxynitrite in arsenite genotoxicity [146–148]. At least three mechanisms have been proposed: formation of *N*-nitroso compounds; deamination of bases (G $\rightarrow$ X, A $\rightarrow$ HX; C $\rightarrow$ U; 5MeC $\rightarrow$ T); and oxidation after conversion to peroxynitrite and/or hydroxyl radical [149]. Peroxynitrite treatment of a plasmid which is then transfected into mammalian cells causes point mutations consistent with deamination or oxidation at GC sites, as well as deletions [150]. Treatment

with arsenite increases NO synthase production and NO levels in bovine endothelial cells [151]. In these and some other cells, arsenite-induced DSB after Fpg treatment can be greatly decreased by NO synthase inhibitors, superoxide scavengers and peroxynitrite scavengers, suggesting that 8-oxo-G (or other lesions susceptible to cleavage by Fpg) might result from arsenite-induced NO production. However, some cells do not seem responsive to NO modulators, but only to ROS modulators [136]. Also, the increases in NO may only occur at higher arsenite concentrations. NO was not produced by porcine endothelial cells exposed to 5 μM or less arsenite, even though there is increased levels of superoxide and H_2O_2 which are capable of activating transcription factor NFkB [14]. In contrast, 0.5 μM arsenite induced peroxynitrite production as well as cyclooxygenase-2 in bovine aortic endothelial cells [152]. NO, peroxynitrite and H_2O_2 are all capable of producing DNA damage cleavable by Fpg [151]. Further details concerning the role of NO in arsenic carcinogenesis appear in another review in this issue [153].

Recent studies show that in some cells both intracellular Ca and peroxynitrite are involved in the increased oxidant stress seen after arsenite treatment. A 4 h treatment of CHO cells with arsenite above 5 μM caused a dose-dependent increase in peroxides (measured by DCF fluorescence), nitric oxide, and Ca levels [148]. The increase in peroxide level was blocked by NOS inhibitors and Ca chelators, as was oxidative DNA damage and DNA–protein crosslinks [154]. MN induction by arsenite can also be blocked by NOS inhibitors, Ca chelators, SOD and uric acid, suggesting that peroxynitrite mediates arsenite-induced MN in these cells in a Ca-dependent fashion.

Besides inducing oxidant DNA damage in cells, it has been suggested that arsenite may also inhibit the repair of endogenously-produced lesions. A large portion of spontaneous mutagenesis appears to be caused by endogenous oxidants [155]. However, if arsenite inhibited repair of spontaneous lesions, one might expect to see an increase in mutation rate in cells growing in arsenite. Instead, we find no change in the mutation rate until more than 20 generations of growth in arsenite concentrations that do not inhibit growth [156], indicating that any increased DNA damage seen in cells growing in low concentrations of arsenite is not converted into mutations.

7. In vitro transformation by arsenicals

In contrast to its weak mutagenicity, arsenite induces cell transformation of various cells to a more malignant phenotype. Some early studies found a lack of mutagenesis in the same cells that were transformed by arsenite suggesting a non-mutagenic mode of transformation. For example, no mutagenicity was seen at two loci in Syrian hamster embryo (SHE) cells, where arsenite caused cell transformation and cytogenetic damage [157]. These cells did, however, undergo gene amplification at the *dhfr* locus [158]. In $10T^{1/2}$ mouse embryo fibroblasts, where arsenite induced morphological transformation and increased steady-state levels of *c-myc* transcripts, no mutagenesis was induced either [159].

A number of new cell transformation systems have recently been developed to study arsenic compounds. We have reported that human osteogenic sarcoma (HOS) cells can be transformed to anchorage-independence by exposure to extremely low concentrations of arsenite [156]. The dose-dependent transformation by arsenite of HOS cells was accompanied by a similar dose-dependent apparent mutagenesis at the *hprt* locus (see below), but the mutagenesis showed a delay of more than 20 generations, while transformation showed a delay of more than 30 generations. MMA^{III} induced neither mutation nor transformation in this system, although it was more toxic than arsenite [156].

Arsenite has been shown to transform mouse epidermal JB6 C141 cells to anchorage-independence. Transformation was blocked when the cells were transfected with a dominant negative ERK2 gene. In contrast, transfection of a dominant negative JNK1 gene increased arsenite-induced transformation. These results demonstrate that arsenite-induced activation of ERKs is transforming, but activation of JNKs is not [15]. Activation of JNKs appears to be required for arsenite-induced apoptosis.

Arsenite also induces transformation (to tumorigenicity after injection into nude mice) of RWPE-1 cells, a line derived from normal human prostate epithelium immortalized with human papillomavirus [160]. Transformation occurred after 29 weeks of exposure to 5 μM arsenite and was concomitant with increased expression of matrix metalloproteinase-9 secretion, a marker for malignant prostate cancer.

Transformation to anchorage-independence by arsenite of rat liver-derived TRL 1215 cells was associated with global DNA hypomethylation, depletion of SAM pools, decreased DNA methyltransferase activity and activation (overexpression) of the proto-oncogene c-myc [161]. In a follow up, the overexpression of c-myc was confirmed at the transcription level and found to be increased in a dose-dependent fashion during the transformation process. Expression of c-myc was highly correlated with increased cell proliferation, genomic hypomethylation, and overexpression of proliferating-cell nuclear antigen PCNA and cyclin D1 [162]. Although the methylation status of the c-myc promoter was not examined, c-myc overexpression is consistent with either gene amplification or hypomethylation.

Hypomethylation is also important in arsenite-induced transformation of SHE cells. Using long-term culture after a 48 h treatment, hypomethylation of the 5′-CCGG sequences of c-myc and c-Ha-ras oncogenes was seen [163]. The arsenite-exposed cells needed between 17 and 113 passages before acquiring anchorage-independence.

8. DNA methylation, gene amplification, and genomic instability induced by arsenite

It has become increasingly apparent that aberrant promoter methylation at CpG sites alters gene function, which may give a selective advantage to neoplastic cells in the same way that mutations do [164]. The first report of arsenite inducing methylation changes was the increased cytosine methylation in the p53 promoter in human adenocarcinoma A549 cells [165]. Later it was found that there was both hypo- and hypermethylation (of different genes) in human kidney UOK cells treated with arsenite [166]. As mentioned above, when SHE cells were transformed by arsenite, specific oncogenes were more highly expressed due to hypomethylation [163]. In the case of TRL1215 cells, there was global DNA hypomethylation and evidence of decreased DNA methyltransferase activity [161]. In mice given a methyl-deficient diet, arsenite in drinking water for 130 days caused a dose-related DNA hypomethylation in the liver [167]. Folate-deficient mice also show increased MN formation when given arsenite [168]. These findings are consistent with the usual DNA methylation changes observed in cancer, in which global methylation is reduced but some gene-specific promoter methylation is increased [169]. Arsenite does not appear to cause methylation and silencing of the transgenic *E. coli gpt* gene in Chinese hamster G12 cells, in contrast to nickel compounds which do [170]. However, there are probably multiple pathways by which genes can become hypermethylated, and sequence context may be important.

Arsenite induced gene amplification (a sign of genomic instability) at the *dhfr* locus in human and rodent cells, but failed to cause amplification of SV40 sequences in SV40-transformed human keratinocytes or Chinese hamster cells [156,158,171,172]. This suggests that arsenite treatment does not result in signaling typical of DNA-damaging agents (which induce SV40 amplification in these systems), but rather appears to feed into checkpoint pathways common to those involving p53, whose disruption lead to cellular gene amplification [173]. The effects of arsenite on p53 signaling are discussed below.

Recently, we found that extremely low concentrations of arsenite (but not MMA^{III}) can induce a robust delayed mutagenesis at the *hprt* locus in human osteosarcoma (HOS) cells, as well as transformation to anchorage-independence and gene amplification [156]. The delayed mutagenesis occurred after more than 20 generations of growth in the arsenite and transformation required more than 30 generations. However, as the $hprt^-$ variants have not yet been characterized, hypermethylation (gene silencing) rather than mutation, cannot be ruled out. This effect suggests a progressive genomic instability which is manifest as increased mutagenesis (or hypermethylation) after many generations.

Besides changes in DNA methylation or other causes of aberrant gene expression, genomic instability can result from telomerase inhibition. Arsenic trioxide is a trivalent arsenic compound that is less soluble at physiological pH and thus less toxic when injected, compared with arsenite. It has been shown to be effective as chemotherapy for acute myelogenous leukemia [10]. In human leukemia cells, arsenic trioxide (0.75–1 μM, 2 weeks) induces chromosomal end fusions that correlate with the inhibition of telomerase activity [174]. Telomerase is a ribonucleoprotein that

maintains terminal chromosome telomere sequences (TTAGGG)n by de novo synthesis of telomere DNA. The reverse transcriptase subunit of telomerase is encoded by the hTERT gene. In normal human somatic cells, telomerase activity is negligible and cell senescence is believed to occur via progressive telomere shortening [175]. However, loss of telomeres can lead to genomic instability and carcinogenesis [176,177]. Telomerase knock-out mice show a four to six-fold increase in spontaneous tumor incidence and genomic instability [178]. The tumors arise in tissues with high proliferation rate including skin, a major site for arsenic carcinogenesis. In addition, the skin of these mice show epidermal hyperplasia and hyperkeratosis, also characteristic of arsenic exposure. Cells in these mice lack telomere repeats detectable by fluorescent in situ hybridization (FISH), and show end-to-end chromosome fusions. Chromosome ends that lack a FISH signal most often form end-to-end fusions, supporting the concept that telomere repeats suppress end joining (i.e., chromosome ends can be distinguished from breaks). End-to-end fusion is non-homologous end joining resulting in loss of all telomeric and some non-telomeric sequences. Tumors in telomerase knock-out mice show increased chromosome fusion and aneuploidy compared with wild type.

Early in carcinogenesis a small percentage of cells bypass senescence by acquiring a “critical threshold” of genetic changes during the “massive” genomic instability that arises during cellular crisis [179]. One of these advantageous changes is the activation of telomerase, which may be low or barely detectable soon after crisis, but becomes more pronounced later in tumorigenesis [180]. Still later, telomere dysfunction can lead to further genomic instability characterized by telomeric associations and anaphase bridges that arise by terminal fusions of chromosomes without functional telomeres. Telomerase activity and concurrent stabilization or lengthening of telomeres is detectable in germ cells, stem cells, and in most immortalized and tumor cells. In immortalized human cells telomere maintenance may also occur by a telomerase-independent recombination-mediated mechanism called ALT (alternative lengthening of telomeres) (reviewed in [181,182]), usually when telomerase is inactivated. Chromosomal end-to-end fusions are characteristic of the ALT mechanism, as evidenced by the presence of dicentric chromosomes, and by microscopic FISH visualization of interstitial telomere sequences [183]. However, ALT is not universally viewed as a telomere maintenance mechanism, but rather may signal telomere dysfunction associated with chromosomal instability that leads to tumor progression [183]. Indeed, telomere dysfunction has been reported to accompany increases in mutation rate and genomic instability in telomerase null mice [183]. An interesting fact to note here is that unlike humans, mice have long telomeres, “promiscuous” telomerase expression [184] and do not exhibit telomere-based crisis that seems to correlate with early human cancer cell immortality (reviewed in [185]). This may be important in light of the well-known paradox that inorganic arsenic is a documented human carcinogen, but by itself (without a co-carcinongen) is not a demonstrable rodent carcinogen except perhaps transplacentally.

9. Enhancing effects of arsenite

One of the most serious threats to genome stability is replication of DNA with unrepaired or badly repaired damage. Low concentrations of arsenite which are not mutagenic nevertheless can effect the mutagenicity of other carcinogens, probably by interfering with DNA repair. Arsenite enhances the mutagenicity of UV in *E. coli* [186] and the mutagenicity and/or clastogenicity of UV, *N*-methyl-*N*-nitrosourea (MNU), diepoxybutane, X-rays, and methylmethane sulfonate in mammalian cells [75,78,104,187–191]. Arsenite enhances the mutagenicity of UVC [78], which causes DNA lesions repairable by nucleotide excision repair (NER), as well as mutations induced by MNU [75], which causes DNA adducts repairable by base excision repair (BER). When the effects of arsenite on NER in UV-irradiated repair-proficient fibroblasts and repair-deficient XPC cells were studied, the incision step of repair was inhibited by a low dose of 2.5 μM arsenite, whereas the ligation step of repair was inhibited at much higher cytotoxic doses [192]. The data suggests that both global and transcription-coupled repair processes can be altered by arsenite.

Since V79 cells lack 0^6-methylguanine DNA methyltransferase, pre-mutagenic MNU adducts in those cells would be predominantly subject to BER. A

nick-translation assay for DNA strand breaks or gaps showed that in V79 cells treated with MNU + arsenite, breaks remained open 3 h after MNU treatment, whereas in the absence of arsenite, the breaks had closed by that time [78]. This suggested that either the polymerase or the ligase step of base excision repair had been blocked by arsenite. In subsequent experiments, nuclear extracts of cells treated with arsenite were found to have decreased total ligase activity, and in particular the enzyme now called DNA ligase III (previously called DNA ligase II) [193]. The alkylating agent MNU induced a robust activation of DNA ligase III activity after 3 h. This activation was blocked by cotreatment of cells with arsenite, and, in fact, a 50% inhibition of ligase activity was seen even cells treated with arsenite alone. When the effect of arsenite on DNA ligase III activity in nuclear preparations was examined, inhibition was seen only at concentrations of arsenite 1000-fold higher than that seen after cellular exposure to arsenite, indicating that arsenite does not directly inhibit the ligase enzyme [193]. More recently, similar results were obtained using purified DNA ligase III [194]. In the Comet assay, arsenite was found to inhibit DNA strand break rejoining. This was also attributed primarily to inhibition of DNA ligase III by using a ligase III-specific substrate [195].

Although arsenite inhibits DNA repair in cells, the effect does not appear to be via enzyme inhibition by arsenite. In the case of NER, arsenite-induced inhibition of pyrimidine dimer excision may be mediated by NO production [136]. The activities of DNA repair proteins Fpg and XPA were not inhibited by arsenite up to 1 mM [196]. DNA polymerases also are not sensitive to inhibition by arsenite. DNA polymerase β (polβ) in fact is stimulated by arsenite concentrations up to at least 12 mM [171,194]. Overexpression of polβ is associated with a mutator phenotype, microsatellite instability and increased tumorigenesis [197–199]. The replication fidelity of polβ is lower than that of some other polymerases and causes many base pair deletions in mononucleotide repeats [200]. Some of the comutagenic effects of arsenite might be mediated by increased polβ activity. It is possible that arsenite may also affect DNA repair by down-regulating the synthesis of DNA repair enzymes [201].

Besides repair enzymes, many other accessory proteins may be targets of arsenite inhibition. A decreased amount of poly(ADP-ribose) in human T-cell lymphoma-derived Molt-3 cells was observed after treatment with toxic arsenite concentrations [202]. More recently, an extremely low concentration (10 nM) of arsenite was found to block poly(ADP-ribosyl)ation in HeLa cells [203]. The mechanism by which arsenite inhibits poly(ADP-ribosyl)ation in cells is not known. The enzyme poly(ADP-ribosyl) polymerase-1 (PARP-1) accounts for at least 75% of the cell's activity, but three other PARPs (PARP-2, -3, -4) have been identified in mammalian cells (reviewed in [204]). PARP-1, the best studied PARP, is constitutively expressed in most tissues, but its activity is stimulated 500-fold by DNA with single-strand or double-strand breaks. PARP functions as a "nick sensor". Upon DNA damage by ionizing radiation, alkylating agents or oxidants, there is an immediate transfer of ADP-ribosyl moieties from NAD^+ to PARP itself ("automodification") and to other nuclear proteins. Subsequent sequential attachments of ADP-ribosyl moieties to ribose (on ADP-ribosyl moieties) create long chains up to 200 U with multiple branching points. A poly(ADP-ribose) binding sequence motif of 20 amino acids was found on several proteins important for genomic stability, including DNA methyltransferase 1, p53, p21, XPA, MSH6, DNA ligase III, XRCC1, DNA polymerase-ε, DNA-PK_{cs}, Ku70, NFκB and telomerase [205].

Mice carrying mutations in PARP-1 are viable, perhaps because the PARP homologs compensate. However, these mice have a number of abnormalities including chromosome instability, defects in DNA repair, and immune deficiencies. PARP-1 null mice exhibit increased susceptibility to nitrosamine carcinogenesis [206]. Studies on cells in which PARP activity has been abrogated through use of low MW competitive inhibitors, expression of dominant negative mutants, or antisense RNA expression all firmly establish a role for PARP-1 in cellular recovery from DNA damage, particularly damage repaired by BER [207]. During BER, PARP binds to nicks in DNA along with other components of the BER complex. This binding activates PARP catalytic activity. PARP induces decondensation of chromatin by poly(ADP-ribosyl)ating histones in nucleosomes. It also accelerates ongoing transcription while inhibiting de novo transcription, thus enabling DNA repair to take place [208]. Inhibition or depletion of PARP

by chemical inhibitors or by expression of dominant negative mutants or antisense RNA results in genomic instability (DNA strand breakage, and increases in recombination, gene amplification, aneuploidy, MN formation, and SCE) after exposure to genotoxicants [209].

Two new members of the PARP family, tankyrase (TRF-1 interacting ankyrin-related ADP-ribose polymerase) 1 and 2, catalyze a similar reaction. (The genes are now called ADPRT 1–4 and TNKS 1–2.) TRF-1 (telomere repeat binding factor) is a negative regulator of telomere length. Tankyrase poly(ADP-ribosyl)ates TRF-1 bound to telomeres (reviewed in [210]). This prevents TRF-1 from binding to DNA and allows telomerase to add DNA repeats to chromosome ends, promoting telomere elongation [211]. Thus, inhibition of tankyrases by arsenite, should it occur, is expected to have the same effect as inhibiting telomerase.

10. Effects of arsenite on DNA damage response and cell cycle control

Many different DNA lesions can trigger common signaling pathways that collectively are referred to as the DNA damage response [212]. An important feature of this pathway is the slowing or arrest of the cell cycle which is thought to be necessary to allow efficient DNA repair to take place prior to DNA replication. If damaged DNA is replicated, it may be mutated or lost due to chromosome breaks. Cell cycle checkpoints are signal transduction pathways that prevent late events from being initiated until earlier events are completed. These checkpoints can halt the cell cycle at various points if DNA is damaged. DNA damage results in an accumulation of P53 protein, mainly via post-translational stabilization [213]. P53 transactivation of downstream genes such as $p21^{waf-1/cif-1}$ (hereafter referred to as p21) helps to block cell cycle progression, allowing time for DNA repair before replication [214] or else causes apoptosis (via induction of BAX and down-regulation of bcl-2) in heavily damaged cells [215].

Conflicting data exist on the effects of arsenite on p53 abundance. Exposure of human cells to increasing concentrations (0.1–100 μM) of arsenite for 24 h resulted in a dose-dependent increase in the level of P53 protein expression, especially in cells with wild type p53 genes [216]. In another study [217], exposure to 5 μM arsenite-induced DSB in human fibroblasts accompanied by phosphorylation and accumulation of P53 protein and increases in P53 target genes, including p21 and MDM2. In these cells, arsenite failed to increase p53 accumulation in the presence of Wortmannin or in Ataxia telangiectasia fibroblasts, implicating activation via ATM kinase, which has been shown to phosphorylate P53 in response to ionizing radiation [218]. In contrast, exposure of immortalized human keratinocyte HaCaT cells to low levels of arsenite (0.01–1 μM) caused a time- and dose-dependent decrease in p53 protein levels [219]. The interpretation of these results is problematic because HaCaT cells have mutations in both p53 alleles and normally overexpress P53 protein [220]. Arsenite had no effect on P53-dependent transcription activity in p53 promoter-transfected JB6 Cl41 mouse cells treated with 12.5–200 μM arsenite [15].

This laboratory has recently shown that long-term (14 days) low dose (0.1 μM) arsenite caused a modest increase in P53 protein levels in WI38 normal human fibroblasts, while only toxic (50 μM) concentrations increased P53 levels after short-term (18 h) exposure [16]. When these cells were irradiated with ionizing radiation (6 Gy), P53 and P21 protein concentrations were increased after 4 h, as expected. Arsenite (long-term, low dose and short-term, high dose) caused a slight reduction in radiation-induced increases in P53 levels, but greatly suppressed the increase in P21 abundance. P21 binds to and inhibits cyclinE-Cdk-2 phosphorylation of pRb, causing G1 arrest [221], and also binds to proliferating-cell nuclear antigen (PCNA), impairing DNA replication. Blocking P21 increases after DNA damage would allow cell cycle progression and DNA replication of a damaged template. In addition, long-term, low dose (but not short-term, high dose) exposure to arsenite resulted in increased expression of cyclin D1. This is expected to have the same effect as blocking P21 increases, leading to faulty DNA replication/repair and comutagenesis. Others have also found that low (non-toxic) exposure to arsenite enhances positive growth signaling [14,59,162,222,223]. The absence of normal p53 functioning along with increased positive growth signaling in the presence of DNA damage may both contribute to defective DNA repair and account

for the comutagenic effects of arsenite. In support of this hypothesis, it was recently found that arsenite treatment prevented the S phase arrest in human lung tumor cells that were irradiated with UVC [224].

Inactivation of the p53 pathway favors genomic instability. When p53 activity is inactivated by the E6 protein of HPV16, UV-induced mutations are elevated about two-fold and a large increase in deletions is seen at the human *hprt* locus [225,226]. One possible explanation for the increased deletion mutagenesis is that deletion-prone intermediates, such as strand breaks or gaps, accumulate during faulty repair. Arsenite also increases UV-mutagenesis about two-fold [78]. Loss of p53 activity is also associated with other types of genomic instability such as gene amplification, chromosome aberrations, and aneuploidy [173,227,228]. As discussed above, arsenite can also induce all of these effects.

The mechanism by which arsenite induces gene amplification may be via alteration of p53 activity or abundance. Regulation of P53 activity is complex, involving covalent modifications such as phosphorylation, cysteine oxidation, acetylation, and sumoylation as well as protein–protein interactions. The transactivating activity of P53 is inhibited in thioredoxin reductase null yeast, and this effect was ascribed to the presence of oxidized thioredoxin [229]. Since both arsenite and (even better) trivalent methylated arsenicals, can inhibit thioredoxin reductase [51] it is possible that the accumulation of oxidized thioredoxin in mammalian cells may be responsible for p53 malfunction. Because GSH is present in mM concentrations in the cell, its redox state is often considered representative of the redox state of the cell. Although trivalent arsenicals all inhibit GSH reductase [48], oxidation of GSH was found to be neither necessary nor sufficient for p53 inhibition [229].

11. Carcinogenicity of methylated arsenic species

DMA^V (cacodylic acid) is widely used in herbicides. Human exposure occurs during production and use of these herbicides as well as from food contaminated with them and through ingestion of some seaweed in which it occurs naturally [19]. As discussed above, DMA^V is the main urinary metabolite of inorganic arsenic. It is also the major metabolite of the arsenosugars that occur naturally in seaweed [230]. When rodents and human ingest DMA^V, most of it is rapidly excreted unchanged, but approximately 5% is converted to TMAO (see Fig. 1) [36,231]. Only trace amounts of TMAO are seen after ingestion of high doses of inorganic arsenic.

The carcinogenicity of DMA^V has been reviewed [3,232]. After treatment with five carcinogens, DMA^V administration to rats acted as a promoter for bladder (strongest response), kidney, liver and thyroid tumors [60]. It also increased numbers of premalignant renal and hepatic foci, and ODC activity. In the bladder, promotion is seen at 10 ppm in drinking water (for papilloma; 25 ppm for carcinoma) and it was suggested that this was due to increased cell proliferation [233]. Urothelial hyperplasia is seen in rats given >40 ppm DMA^V in food [234], and there is evidence of necrosis at even lower concentrations, suggesting a scenario of cytotoxicity followed by regenerative hyperplasia. The abilities of various arsenicals were tested as promoters in the rat bladder induced with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. Arsenite (17.3 ppm) was not active. The most active promoting compound was DMA^V (184 ppm), but MMA^V (187 ppm) and TMAO (182 ppm) were also promoters [233].

DMA^V administration causes single strand breaks in mouse lung, but not in liver or kidney [47,235,236]. DMA^V increased ODC activity in liver and kidney [60,233,237]. In mice, but not in rats, DMA^V caused progression of 4-NQO-induced benign lung tumor nodules [238]. The same group also saw enhanced UVB-induced (2 kJ/m^2, twice weekly) skin tumorigenesis with 1000 ppm (but not with 400 ppm) DMA^V in drinking water, but only during weeks 13–19. The total tumor yield was not increased [239]. In A/J mice, which are normally very susceptible to lung tumors, 400 ppm DMA^V in drinking water increased the tumor yield [240]. 8-oxo-dG was detected in skin, lung, liver, and bladder, as well as in urine of mice given DMA^V [241].

DMA^V was then shown to act as a complete carcinogen in the rat bladder, causing transitional cell carcinoma at 50 ppm in water and higher in a 2-year exposure protocol [242]. No tumors were seen in any other organs. 8-oxo-dG and COX-2 levels were increased in the bladders of rats receiving 200 ppm DMA^V for 2 weeks [243].

Table 1
Increased tumorigenesis by arsenicals in drinking water (effective concentrations)

Animal and strain	Compound and effective concentrations	Effects	References
Tg.AC mice (activated H-ras)	200 ppm sodium arsenite, 4 weeks	Increased TPA-induced papillomas	[61]
K6/ODC mice (ODC expressed in skin)	100 ppm sodium arsenite or DMA^{V}, 5 months	Induced a small number of papillomas	[63]
Skh1 mice (hairless)	1.25–10 ppm sodium arsenite, 26 weeks	Increased UVR-induced squamous cell carcinomas	[65,66]
C3H mice	42.5 and 85 ppm sodium arsenite, 10 days	Transplacental carcinogenesis, various sites	[72]
F344/DuCrj rat	50 ppm DMA^{V} (for bladder) or 100–400 ppm (for other organs), 30 weeks	Promotion of various tumors after initiation with five carcinogens	[60]
F344 rat	10 ppm DMA^{V} (for total tumor) and 25 ppm (for carcinoma)	Promotion of nitrosamine-induced bladder papilloma and carcinoma	[233]
F344 rat	50 and 200 ppm DMA^{V}, 2 years	Complete bladder carcinogen	[242]
ddY mice	400 ppm DMA^{V}, 25 weeks	Increased yield of 4NQO-induced lung tumors	[238]
A/J mice	400 ppm DMA^{V}, 50 weeks	Increased lung tumor yield over a high background level	[240]
Hos:HR-1 mice (hairless)	1000 ppm DMA^{V}, 25 weeks	Increased yield of UVR-induced skin tumors	[239]
C57BL/6J mice, wild type and $p53^{+/-}$	50 ppm DMA^{V}, 80 weeks (wild type) or 200 ppm DMA^{V}, 80 weeks ($p53^{+/-}$)	Increased total tumor incidence (mainly lymphomas and sarcomas)	[299]

Table 1 summarizes the effects of inorganic and organic arsenicals on tumorigenesis in animal models. The minimal drinking water concentration of DMA^{V} needed for promotion (bladder papillomas) is 10 ppm and for complete bladder carcinogenesis is 50 ppm. Compare this to 1.25 ppm arsenite needed for co-carcinogenesis in mouse skin ([65,66], Burns et al., manuscript submitted). This argues that arsenite, and not its methylated metabolites (or at least not DMA^{V} or further metabolites), may be the carcinogenic species for skin. Keratinocytes have very slow rates of arsenic methylation, and only MMAs were produced [33]. It is of interest that, in contrast to other cells, keratinocytes are more sensitive to the toxic effects of arsenite compared with MMA^{III} and DMA^{III} [33]. Perhaps arsenite targets the skin, while its metabolites may target other organs. The case for bladder may be different because the metabolites concentrate in urine, although the toxicity of inorganic arsenic compounds would limit the ability to accumulate such high concentrations of methylated metabolites. Even assuming 100% conversion, in order to generate the equivalent of 50 ppm DMA^{V} (0.36 mM), one would need 46.8 ppm sodium arsenite in drinking water. In a mouse that drinks about 6 ml per day water (Uddin, unpublished), this comes to 280 mg per day. Assuming a weight of 28 g for an adult mouse [65], this would come to 10,000 mg/kg per day sodium arsenite in drinking water. The 96 h LD_{50} for arsenic trioxide is in the range of 25–40 mg/kg for mice, and sodium arsenite is reported to be 3–10 times more toxic than arsenic trioxide [244]. Therefore, it is not possible to generate a carcinogenic dose of DMA^{V} from inorganic arsenic.

12. Genotoxicity of methylated metabolites of arsenic

Various pathways of metabolism have been proposed to explain the effects of DMA^{V}. These include: (1) reduction of DMA^{V} to DMA^{III}, which is more toxic and genotoxic (discussed below); (2) formation of TMAO; (3) reduction of DMA^{V} to yield dimethyl arsine, which forms the peroxyradical (see Fig. 1), hydroxyl radical, and superoxide [245,246]. To determine whether DMA^{III} mediated the bladder hyperplasia in rats, 2,3-dimercaptopropane-1-sulfonic acid (DMPS), a chelator of trivalent arsenicals, was coadministered with DMA^{V}. DMPS blocked the

cytotoxicity and regenerative hyperplasia in the bladder epithelium [247]. DMPS also blocked the conversion of DMA^{V} to TMAO, suggesting that DMA^{III} is an intermediate.

Earlier experiments on the genotoxic effects of methylated arsenic compounds concentrated on the pentavalent species. Very high concentrations (>10,000 μg/ml) of DMA^{V} induced mutations in mouse lymphoma L5 178Y/TK$^{+/-}$ cells [79]. The mutant colonies were of the small colony type, suggesting clastogenic effects. MMA^{V} was active at half that concentration, and arsenite was active at 1–2 μg/ml (but none of these compounds was highly mutagenic). The clastogenic effects of a number of arsenicals were assayed in human fibroblasts. The order of potency as clastogens based on concentration needed was: arsenite > arsenate > DMA^{V} > MMA^{V} > TMAO. In fact, >7 mM DMA^{V} is required, whereas only 0.8 μM arsenite was clastogenic. DMA^{V} at >7 mM was considered very potent and caused chromosome pulverizations in most metaphases [119]. Depletion of GSH increased the clastogenicity of all compounds except DMA^{V}, where clastogenicity was suppressed by GSH depletion, suggesting that GSH is required for the clastogenicity induced by DMA^{V}, but not by arsenite, arsenate, MMA^{V} or TMAO.

DMA^{V} at 10 mM induced DSB and DNA-protein crosslinks in human alveolar type II cells, possibly via formation of a DMA peroxy radical (with dimethyl arsine as an intermediate) [245]. Oral administration of DMA^{V} to rats causes the same lesions to form in lung DNA, but not in other tissues [47,234]. The DMA peroxyradical can cause DNA strand breakage in vitro [241]. It has been suggested that the carcinogenicity of DMA^{V} is via the DMA peroxy radical. If so, one would expect to see evidence of oxidative damage to DNA in target organs. Oral administration of DMA^{V} to mice increased 8-oxo-dG formation in skin, lung, liver, and urinary bladder, whereas arsenite did not [241]. Similar results were seen in rat kidney [248]. Administration of DMA^{V} to mice also induced aneuploidy in bone marrow [249]. However, there is no evidence that the DMA peroxy radical can form during metabolism of inorganic arsenic. DMA^{V} (5 μM) was found to induce more repair polymerization than MMA^{V} or arsenite [250]. This was interpreted as evidence of greater genotoxicity. However, as discussed above, arsenite was found to stimulate pol β activity. If DMA^{V} is more active than arsenite in this regard, the increased "repair polymerization" might be explained.

DMA^{V} (10.6 mg/kg for 5 days) was injected i.p. into MutaMouse, and mutations at the lacZ transgene as well as the endogenous cII gene were assayed [251]. Only a weak (1.3X) increase in lacZ mutations were detected in lung, and no increase was seen in liver or bone marrow. Only "marginal" effects were seen at the cII locus in lung. Arsenic trioxide (7.6 mg/kg) induced no mutagenesis.

Recently, attention has turned to the trivalent methylated metabolites, after it was found that they can nick DNA in vitro and can cause DSB in plasmids and alkali-labile sites in the Comet assay using human lymphocytes [252]. However, concentrations of 30 and 150 μM of MMA^{III} and DMA^{III}, respectively, were needed for the plasmid nicking reaction and highly toxic concentrations were also needed in the lymphocyte assay. Arsenite was hardly effective even at 1 mM. The plasmid nicking by trivalent metabolites was inhibited by the ROS inhibitors Tiron, melatonin and Trolox. A radical species was detected by ES spectroscopy with a spin trap agent [253]. Some of the genotoxic effects of methylated metabolites might be mediated by ROS from Fenton reactions involving iron [254]. Both DMA^{V} and DMA^{III} (10 mM) have been shown to cause the release of iron from ferritin and the ability of DMA^{III} to nick plasmid DNA can be blocked by iron chelation. Excess free iron induces HO [132,133]. Yet, arsenite is a good HO inducer but DMA^{V} and MMA^{V} are not [47]. Ahmad et al. [254] point out that the enzymatic action of HO releases free iron into the cell.

The induction of DSB in lymphocytes, analyzed by the Comet assay, showed the following order of activity: DMA^{III} > MMA^{III} ≫ arsenite = arsenate > MMA^{V} > DMA^{V}. Different results (and at much lower concentrations) were obtained using the sequential enzyme digestion in the Comet assay [138]. In the absence of the sequential digestion, the order of DNA strand break activity in HL60 cells was: MMA^{III} > arsenite > MMA^{V} > DMA^{V}. But with sequential digestion, the order became: arsenite > MMA^{III} > MMA^{V} > DMA^{V}. Also, the relative increase in strand breaks induced by the different enzymes differed in the different arsenicals. For arsenite, it is: endoIII > Fpg > PK. For MMA^{III} and MMA^{V}, it is: PK > Fpg > endoIII. Thus, the organic compounds induce

more DNA–protein crosslinks, whereas arsenite induces more oxidized pyrimidines. The genotoxicity of arsenite in leukocytes from various species does not correlate with their abilities to methylate arsenite [255], nor is there any correlation between sensitivity to arsenite toxicity and ability to methylate arsenite [32].

MMA^{III} has been detected in the bile of rats exposed to inorganic arsenic [256] and in the urine of humans drinking arsenic-contaminated water [257,258]. In arsenic-exposed individuals from West Bengal, India, 48% had MMA^{III} in their urine and 72% had DMA^{III} [258].

An important difference between DMA^{V} and arsenite concerns their interactions with GSH. As discussed above, GSH is protective against arsenite toxicity and genotoxicity. In contrast, DMA^{V} actually requires GSH to induce apoptosis and becomes less toxic after GSH depletion [259]. Because inhibitors of GSH-*S*-transferase are also able to block DMA^{V} toxicity, it is suggested that DMA^{V} conjugated to GSH is the toxic species.

13. Enhanced cell proliferation by arsenicals

Cell proliferation that results from mitogenic stimuli or from regeneration after cytotoxicity can enhance carcinogenesis [260]. Proliferation of mammalian cells is regulated by a number of mitogens including growth factors, mitogenic lipids, inflammatory cytokines and hormones, modified by integrin-mediated adhesion [261]. A considerable amount of evidence suggests that arsenite (and perhaps some of its metabolites) act as co-carcinogens in part by activating signal transduction pathways which enhance cell proliferation, reduce antiproliferative signaling, and override checkpoints controlling cell division after genotoxic insult. In animals treated with arsenite, hyperplasia is seen in the urinary bladder epithelium and in skin ([59,222,262], Burns et al., manuscript submitted). DMA^{V} induces increased cell proliferation in rat bladder and kidney [233,234]. Mice given 100 ppm sodium arsenite for 4 weeks show bladder epithelial hyperplasia with no microscopic evidence of inflammation or necrosis [262]. Although the urine contained almost exclusively DMA in those experiments, the bladder epithelium contained mostly arsenite.

In cell culture studies, arsenite increases cell proliferation in human keratinocytes [2,263] and in other cell lines [14,223]. Low levels of arsenite ($<5\,\mu M$) caused increased proliferation of porcine aortic endothelial cells, which was associated with increased H_2O_2 and superoxide levels as well as c-src activation and NFκB transcription [14,264]. Tumor growth factor α (TGFα) and granulocyte macrophage colony stimulating factor (GM-CSF) is upregulated in human keratinocytes exposed to arsenite [222]. Arsenite can also enhance the mitogenic effect of sub-optimal serum concentrations on quiescent $C3H10T^{1/2}$ cells [265]. All trivalent arsenicals (arsenite, MMA^{III} and DMA^{III}) increased thymidine incorporation (a surrogate for cell proliferation) in normal human epidermal keratinocytes at very low concentration [33]. No stimulation was seen with any pentavalent arsenical.

In addition to upregulating proliferative signaling, arsenite also down-regulates antiproliferative signaling. Murine fibroblasts chronically exposed to low concentrations of arsenite show increased proliferative response to epidermal growth factor (EGF) and increased expression of c-myc and E2F-1 (positive growth regulators) [223]. At the same time, there was a decrease in expression of the negative growth regulators MAP kinase phosphatase (MKP1) and $p27^{kip1}$. MKP1 functions as a negative regulator by removing the phosphate from activated ERK1 and 2 (discussed below). EGF is unable to induce MKP1 expression in the presence of arsenite, thus prolonging the activation of mitogenic signaling. Similar effects were seen in human fibroblasts where $0.1\,\mu M$ arsenite increased cyclin D1 (positive growth regulator) abundance and decreased expression of p21 (negative growth regulator) after genotoxic insult [16].

Signal transduction changes leading to altered transcription after arsenite treatment have been reviewed recently [7,266,267]. In addition, other articles in this issue also address this subject [12,268]. Therefore, I will only touch on this briefly. The immediate target of arsenite with regard to cell proliferation might be one of the receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR). Receptor protein tyrosine kinases are frequently found overexpressed in tumors. EGFR normally phosphorylates itself after being activated to a protein tyrosine kinase by binding EGF. Arsenite induced phosphorylation of EGFR in rat PC12 cells [269]. In UROtsa cells (immortalized

human urothelium), arsenite increases thymidine incorporation along with EGFR activation which is independent of EGF but dependent on c-src [270]. C-src is also upregulated by arsenite in endothelial cells [14]. The same pathway was stimulated in urinary bladder of mice exposed to 50 ppm arsenite in water for 8 weeks, and may account for the proliferation and persistant AP-1 activation in these mice [270].

Growth factor receptors use multiple cytoplasmic signaling pathways to regulate G_1 phase cyclins and their associated cyclin-dependent kinases (cdks). In mammalian cells, the decision to start DNA synthesis or to stop cell proliferation is made at the G_1/S boundary [271]. This transition is regulated by the retinoblastoma (Rb) protein, whose activity is controlled by its phosphorylation by cyclin D1/cdk4. Phosphorylation of Rb relieves the arrest of cells in G_1 phase by releasing transcription factor E2F1 in an active form and allowing transactivation of E2F1-controlled genes [272]. Virtually all human cancers have alterations in the Rb pathway, either through inactivation of Rb itself or through overexpression of cyclin D1 and cdk4 or inactivation of cdk inhibitors [273].

Like other cyclins, cyclin D1 has a very short half life and is tightly regulated in normal cells by a number of signaling pathways that are activated by arsenite, including extracellular signal-related kinases (ERKs), phosphotidylinositol 3-kinase (PI3K) and NFκB [261,272]. Cyclin D1 expression generally requires the sequential activation of Ras, Raf1, mitogen-activated protein kinase (MEK1/2) and sustained activation of ERKs (discussed below), but there are circumstances under which ERKs activation may be dispensable for cyclin D1 induction [274]. Following serum stimulation, PI3K and downstream Akt (a cell survival pathway) play a role in the induction of cyclin D1 in quiescent NIH 3T3 fibroblasts [275]. Activated Akt promotes cell survival by phosphorylating BAD and caspase-9, and can over-ride a G2/M cell cycle checkpoint induced by DNA damage [276]. Thus, activation of this pathway may enhance survival of cells with DNA damage. In human HaCaT cells, keratinocytes and fibroblasts, the PI3K/AKT pathway was activated by arsenite treatment ([127,277]; Huang, personal communication). The relative importance of PI3K, ERK and other signaling in the induction of cyclin D1 remains to be determined.

Activating protein 1 (AP-1) transcription factor regulates expression of a number of proteins important in proliferation, such as cyclin D1, as well as p53, p21, $p19^{ARF}$, and p16 [278]. Low concentrations of arsenite activate AP-1 and induce immediate early genes and ornithine decarboxylase, whose products stimulate cell proliferation [2,237,279]. AP-1 transactivation is increased in the bladders of mice exposed to at least 20 mg/l arsenite [280]. AP-1 is composed of homodimers or heterodimers of the jun and fos families. The arsenite-induced urinary bladder epithelial hyperplasia is accompanied by activation of AP-1, specifically c-Jun/c-Fos [280]. In rabbit renal cortex slices treated with arsenite, increased AP-1 DNA binding was associated with increased c-fos but not c-jun expression [126]. Prolonged expression of c-jun has been shown to prevent the growth inhibitory effect of p53 by down-regulating p21 transcription [278]. This is a possible mechanism for the down-regulation of p21 by arsenite [16,223].

The activation of AP-1 by arsenite appears to be mediated by PKC and mitogen-activated protein (MAP) kinase family members [281]. MAPKs are major mediators of signal transduction pathways controlling cell proliferation, differentiation, and death (reviewed in [282]). The three major classes of MAPKs are the ERKs, c-Jun N-terminal kinases (JNKs) and p38 kinases. Most reports show that these three classes are differentially activated by arsenite (reviewed in [7]). ERKs are phosphorylated in JB6 cells after treatment with 0.2 μM arsenite whereas concentrations >50 μM are needed to activate JNKs [15]. ERKs are important in the proliferative response and transmit signals from growth factors. The best defined signaling pathways leading to ERKs activation is via receptor tyrosine kinases such as EGFR. Src can also activate ERKs directly [270]. ERKs can phosphorylate AP-1 (c-Jun + c-Fos) and ATF-2 transcription factors, leading to a proliferative response. In contrast, activation of JNKs is often associated with apoptosis. Thus, ERKs activation would favor carcinogenesis, while JNKs activation would tend to block it.

NFκB is a rapidly induced stress responsive transcription factor for genes controlling cytokines, growth factors and acute response proteins. MAPK signaling activates NFκB via degradation of its inhibitor IκB. It tends to be antiapoptotic when it consists of RelA-containing complexes which it does

most commonly [266]. Both arsenite and arsenate induce activation of NFκB in mouse epidermal JB6 Cl41 cells, but not in 30.7b cells, which are known to have low levels of ERKs, suggesting that ERKs activation is involved in NFκB activation by arsenite [281]. NFκB binding to the cyclin D1 gene promoter is critical for the regulation of cyclin D1 expression [274,275]. Low concentrations of arsenite tend to activate NFκB in endothelial cells, whereas higher concentrations prevent its activation [283]. Antioxidants prevented the activation in this system.

Most of the genes controlled by the E2F1 transcription factor (which controls entry into S phase) are also regulated by c-myc. The c-myc gene is a protooncogene, often activated during carcinogenesis, and associated with DNA hypomethylation [284]. Upregulation of c-myc was observed after arsenite treatment [59,126,159,285]. In the case of chronic exposure of rat liver TRL 1215 cells, upregulation of c-myc takes 18 weeks of exposure to 0.5 μM arsenite, and correlates with malignant transformation [286]. Activation of AP-1 and NFκB transcription factors may account for arsenite-induced c-myc expression.

Treatment of primary human keratinocytes in culture with arsenite induced a unique cytokine profile which included transforming growth factor α (TGFα), tumor necrosis factor α (TNFα) and granulocyte-macrophage colony stimulating factor (GM-CSF) [59]. GM-CSF is a regulator of keratinocyte growth and differentiation whose expression is induced via AP-1 by interleukin-1 (IL-1) released by epithelial cells [287]. These same cytokines were seen in arsenite-treated Ha-ras transgenic mice, where arsenite enhanced TPA promotion [59,61,222]. In addition to arsenite, MMA^{III} and DMA^{III}-GSH conjugate induced secretion of GM-CSF and TNFα at extremely low doses [33]. Activation of NFkB leads to increased TNFα and IL-1α and other growth-promoting cytokines. Arsenite increases IL-1α in murine keratinocytes, but not in some other cells (reviewed in [267]).

14. Additional considerations

Tumorigenesis is often accompanied by mechanisms that override cellular programs for terminal differentiation. Arsenite prevents terminal differentiation of pre-adipocytes stimulated by insulin + dexamethasone [288]. In this system, arsenite blocked the upregulation of c/EBPα and p21 that normally accompanies differentiation [289]. It also suppresses differentiation of a human keratinocyte line [290], an affect mediated by AP-1 [291]. Perhaps the arsenite-induced activation of c-jun blocks the p21 induction in that system as well.

Low levels of arsenite (0.5–10 μM) cause an increase in ubiquitinated proteins [292]. This may be a result of arsenite-induced protein damage and the cell's attempt to digest the abnormal proteins by the ubiquitin-dependent proteosome. Inhibition of the 20S proteosome required 10 μM arsenite. Possibly related to this may be the finding that overexpression of a protein with a ubiquitin-like domain, Fau, confers arsenite resistance [293]. Recently we found that expression of the ubiquitin-like domain alone actually sensitizes cells to arsenite and may be oncogenic [294], perhaps by blocking arsenite-induced ubiquitination.

Arsenite has also been shown to modulate glucocorticoid receptor function [295]. Glucocorticoids suppress tumor promotion in two-stage carcinogenesis, and a progressive loss of hormone responsiveness was seen in later stages of skin cancer [296].

15. Conclusion

Both arsenite and its metabolites can have a variety of genotoxic effects, which may be mediated by oxidants or free radical species. All of these species also have effects on signaling pathways leading to proliferative responses. There are interesting differences in the activities of inorganic and organic species both in terms of target organ carcinogenicity and genotoxic and toxic mechanisms. Animal experiments show that following chronic exposure, arsenite accumulates in the skin and hair [297]. In mice which were made hepatic methyl donor deficient through a choline-deficient diet, there was a shift in arsenite-induced genotoxicity from liver and bladder (in normal mice) to skin (in methyl-deficient mice) [298]. This supports the idea that the skin may be targeted by arsenite. It is possible that the methylated metabolites may play a role in bladder and perhaps some other cancers. Even in the bladder, lower concentrations of DMA were required for promotion (Table 1). Thus, the question

still remains: Does arsenic require a carcinogenic partner?

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