ELSEVIER

Mutation Research 517 (2002) 101–111

Genetic Toxicology and Environmental Mutagenesis

www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Arsenic co-exposure potentiates benzo[a]pyrene genotoxicity

Andrew Maier*, Brenda L. Schumann, Xiaoqing Chang, Glenn Talaska, Alvaro Puga

Department of Environmental Health, Center for Environmental Genetics, University of Cincinnati Medical Center, P.O. Box 670056, Cincinnati, OH 45267, USA

Received 12 September 2001; received in revised form 14 January 2002; accepted 7 March 2002

Abstract

Co-exposures to complex mixtures of arsenic and polycyclic aromatic hydrocarbons such as benzo[a]pyrene (BaP) are common in the environment. These two environmental pollutants are carcinogenic, but the nature of their molecular interactions in the induction of cancer is not well understood. Additive or synergistic interactions have been proposed to explain why arsenic, which is not a potent mutagen itself, is comutagenic with a variety of DNA-damaging agents. We have examined the genotoxicity of BaP-arsenic mixtures. We find that exposure of mouse hepatoma Hepa-1 cells to low concentrations of arsenite increases BaP–DNA adduct levels by as much as 18-fold. This effect requires the activation of BaP by cytochrome p450 1A1 (CYP1A1), although arsenite does not alter BaP-inducible CYP1A1 enzymatic activity, suggesting that arsenite acts downstream of metabolic BaP activation. Glutathione homeostasis was important in modulating the potency of arsenite. In cells depleted of reduced glutathione, arsenite increased BaP–DNA adduct formation by an even greater degree than in cells co-treated with BaP and arsenite in control medium. Although arsenic comutagenicity has been attributed to inhibition of DNA repair, arsenite treatment did not alter adduct removal kinetics in BaP-treated cells, suggesting that mechanisms upstream of DNA repair are responsible for increased adduct levels. Concentrations of arsenite and BaP that had no measurable mutagenic effect alone, increased mutation frequency at the *Hprt* locus by eight-fold when given in combination, demonstrating a comutagenic response between BaP and arsenite. These results provide strong support for the positive interaction between arsenic and PAH-induced cancer observed in epidemiology studies, and help to identify additional mechanistic steps likely to be involved in arsenic comutagenesis.

Keywords: Arsenic; Benzo[a]pyrene; DNA adducts; Mixture; Genotoxicity

1. Introduction

Benzo[a]pyrene (BaP) and inorganic arsenic are toxicologically important compounds that are widely distributed in the environment. Important sources of co-exposure to these two compounds include emissions from fossil fuel combustion, cigarette smoke, and migration from hazardous waste sites [1,2]. In addition, occupational exposures to arsenic in nonferrous smelters, pesticide manufacturing, or from consumption of contaminated drinking water, coupled with tobacco use, represents another potential avenue for exposure to BaP and arsenic mixtures.

A primary toxic consequence of concern for both BaP and arsenic exposure is the induction of cancer. BaP is clearly tumorigenic in animal studies by

Abbreviations: BaP, benzo[a]pyrene; BPQ, benzo[a]pyrene-3,6-dione; BPDiol, benzo[a]pyrene-7,8-dihydrodiol; BSO, L-buthionine-*S,R*-sulfoximine; CYP1A1, cytochrome p450 1A1; DMSO, dimethylsulfoxide; GSH, reduce glutathione; HPRT, hypoxanthine-guanine phosphoribosyl transferase; NQO1, NADPH: quinone oxidoreductase; PAH, polycyclic aromatic hydrocarbon

* Corresponding author. Present address: Toxicology Excellence for Risk Assessment, 1757 Chase Avenue, Cincinnati, OH 45223, USA. Tel.: +1-513-542-7475; fax: +1-513-542-7487.
E-mail address: maier@tera.org (A. Maier).

1383-5718/02/$ – see front matter
PII: S1383-5718(02)00057-8

multiple routes of exposure (reviewed in [1]). Evidence for BaP involvement in human cancer is indirect, since environmental and occupational exposures are to mixtures of polycyclic aromatic hydrocarbons (PAH), only one of which is BaP. Nevertheless, the increased lung cancer risk identified in occupational cohorts exposed to PAHs [3–5], or in individuals who smoke tobacco, suggests a causal relationship between BaP exposure and human cancer. The epidemiologic data supporting the human carcinogenicity of arsenic, on the other hand, is very strong (reviewed in [2,6]).

The weight of evidence suggests that estimated human cancer risk based on the carcinogenic potential of either of these chemicals when evaluated singly might underestimate risks resulting from co-exposure. Results from several epidemiology studies suggest more than additive effects on respiratory tract cancer incidence in arsenic-exposed populations when stratified for smoking [7–11], although the available studies do not uniformly support this effect [12–14]. As additional support, tumorigenic responses were significantly enhanced by arsenic and BaP co-treatment for certain tumor types in animal studies [15,16].

The mechanism(s) responsible for interactions between BaP and arsenic carcinogenicity remain unexplained. Arsenic is not a potent mutagen, but it enhances the genotoxicity of DNA damaging agents including ultraviolet radiation [17,18], methyl methanesulfonate [19], and *N*-methyl-*N*-nitrosourea [20]. Inhibition of DNA repair has been suggested as one mechanism for this observed comutagenesis, although the step in the DNA repair process that is most affected is not entirely clear [20–22]. Little work has been done to specifically investigate the potential mechanisms of arsenic and BaP comutagenesis, although arsenic does appear to inhibit the repair of DNA damage induced by BaP [23].

Since BaP initiates genetic damage through the direct interaction of its metabolites with DNA, we hypothesized that this initiation step in BaP mutagenesis could also be a target for modification by arsenic. To test this idea, we examined the ability of the trivalent arsenic compound, sodium arsenite, to enhance BaP–DNA adduct formation in mouse hepatoma, Hepa-1 cells. We find that arsenite strongly potentiates the genotoxicity of BaP.

2. Materials and methods

2.1. Cell lines, growth conditions, and chemical treatments

Unless specified otherwise, cells were seeded to 2×10^6 cells per 100 mm tissue culture plate and grown for 24 h prior to treatment in α-minimal essential medium (MEM) (GibcoBRL Life technologies) containing 5% fetal bovine serum and 1% antibiotics. Chemicals were obtained from Sigma unless specified otherwise. For chemical treatments, BaP, benzo[a]pyrene-3,6-dione (BPQ) and benzo[a]pyrene-7,8-dihydrodiol (BPDiol) (both from the NCI Carcinogen Repository) were dissolved in dimethylsulfoxide (DMSO) and stored at 4 °C until use. Sodium arsenite (hereinafter referred to simply as arsenite) was freshly dissolved in sterile deionized water. L-buthionine-*S*,*R*-sulfoximine (BSO) and glutathione ethylester (GSH-ester) (a gift of Howard G. Shertzer) were dissolved in serum free α-MEM and stored at −20 °C until use. Specific treatment regimens are described in the legends to each figure. To assess the dependence of DNA adduct formation on cytochrome p450 1A1 (CYP1A1), we used the *c37* cell line [24], which was derived from Hepa-1 cells, but lacks functional CYP1A1 activity [25]. We also used a second Hepa-1-derived cell line, CX4, that is isogenic with *c37* cells, except that is has been stably transformed with a CYP1A1 expression plasmid [26]. The CX4 cells were grown under similar conditions as Hepa-1 and *c37* cells, except for the addition of 300 μg/ml geneticin to maintain the selection conditions.

2.2. Colony forming efficiency assays

To identify appropriate levels of arsenite to use in subsequent genotoxicity assays, we conducted initial toxicity studies in Hepa-1 cells by measuring changes in cell growth. Hepa-1 cells were seeded at a density of 1000 cells per well in 6-well tissue culture plates and allowed to adhere to the plates for 4 h in untreated medium. The indicated concentrations of arsenite were added to the medium, and following incubation in the presence of arsenite for 24 h, the medium was replaced and the cells were grown until a total of 7 days had elapsed. At that time, the medium was removed,

Table 1
Effect of arsenite treatment on cell proliferation

As (μM)	Colony forming
Control	
1.0	
2.5	
5.0	
7.5	
10	

Hepa-1 cells were seeded at 1000 cells per well and treated with varying concentrations of arsenite for 24 h. The number of colonies is shown as a percent of untreated control cells and each value represents the mean ± S.E. of two independent experiments. The average plating efficiency in untreated controls was 25%. The relative colony formation for the arsenite treatments was compared to controls and statistically significant differences ($P < 0.05$) are denoted with an asterisk (*).

the cells were washed twice with phosphate-buffered saline (PBS), and fixed in a solution of 10% formaldehyde. The colonies were subsequently stained with Giemsa and all visible colonies were counted. Treatments with arsenite over the tested series of concentrations induced from minimal to marked decreases in colony forming (Table 1). Therefore, this range of concentrations was judged as adequate to ensure that varying levels of cytotoxicity were accounted for in the subsequent genotoxicity experiments.

2.3. Genomic DNA purification and DNA adduct analysis

At the end of the treatment period, cells were trypsinized and recovered in PBS. The cells were centrifuged to remove the excess PBS and the cell pellet was stored at −70 °C. Genomic DNA was prepared using a Wizard Genomic DNA Purification Kit (Promega).

The ^{32}P-postlabeling assay was performed as described previously [27] with modifications described in detail elsewhere [28]. Briefly, 2–4 μg of DNA were hydrolyzed with 0.25 units of micrococcal endonuclease and 0.001 units of calf spleen phosphodiesterase for 3 h at 37 °C. The hydrolyzed DNA was digested with 3.5 units of nuclease P1 for 30 min at 37 °C and the adducted nucleotides were subsequently labeled by addition of 50 μCi/sample of [γ-^{32}P]-ATP and 2.8 units of polynucleotide kinase and incubating the reaction mixture for an additional 30 min at 37 °C. The post-labeled mixtures were applied to 20 cm × 20 cm PEI-cellulose plates (Alltech). Chromatography was done using a four solvent system as described previously [29]. The solvents were D1 (0.65 M sodium phosphate, pH 6.8), D3 (3.6 M lithium formate containing 3.5 M urea), D4 (0.8 M LiCl, 0.5 M Tris–HCl containing 3.5 M urea, pH 8.0), and D5 (1.5 M sodium phosphate, pH 6.0). Adducts were visualized by autoradiography, and were quantified by scintillation counting.

2.4. Enzymatic activity assays

CYP1A1 activity was determined by measuring the formation of 3-OH-BaP as described previously [30]. Enzymatic activity was normalized to cellular protein content as determined by the Bradford assay using bovine serum albumin as a standard.

2.5. Determination of glutathione levels

Cells were rinsed twice with PBS and harvested in 750 μl of 0.1 M potassium phosphate buffer (pH 6.8) containing 150 mM potassium chloride and 5 mM diethylenetriaminepentaacetic acid (DPTA). The samples were mixed by vortexing, an aliquot was taken for protein measurement, and an equal volume of a buffer containing 40 mM HCl, 10 mM DPTA, and 10% trichloroacetic acid was added. The sample was centrifuged at 15,000 rpm for 5 min and the deproteinized supernatant was transferred to a fresh tube for subsequent reduced glutathione (GSH) determinations. GSH levels were determined as described elsewhere [31] and were normalized to total protein content as measured using the bicinchoninic acid protein assay (Pierce).

2.6. Hprt mutagenesis assay

The ability of BaP to induce mutations at the *Hprt* locus in Hepa-1 cells has been demonstrated by others [32]. Building on this earlier work, we used a similar approach in conducting the mutagenesis experiments for our studies. Briefly, Hepa-1 cells were seeded to 7.5×10^6 cells per 150 mm plate and grown 24 h prior to treatment. After 24 h of treatment, the cells were trypsinized and live cells were counted by trypan blue exclusion. Cells from each treatment group

were seeded at a density of 1×10^7 live cells per plate in untreated medium. As a minimum, a total of at least 3×10^7 cells were plated for each treatment group. After 24 h, the medium was replaced with medium containing 10 μg/ml 6-thioguanine for selection of cells harboring *Hprt* mutations. At the end of a 2-week selection period, colonies were fixed, stained as described for the cell proliferation assay, and all visible colonies were counted. Mutation frequency was determined as the number of 6-thioguanine resistant colonies per 1×10^6 viable cells plated at the initiation of the selection period.

2.7. Statistical analysis

Group comparisons were made by one- or two-way analysis of variance. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Arsenite co-treatment enhances the formation of stable BaP–DNA adducts

Arsenic is cocarcinogenic with BaP in animals [15,16] and possibly with cigarette smoking in humans [7–11]. We asked whether these observations might be rooted in the ability of arsenic to enhance the interaction of BaP metabolites with DNA. To answer this question, we examined the formation of bulky DNA adducts after treating cells with BaP or BaP plus arsenite. BaP treatment generated a single major adduct that had a migration pattern consistent with that of 7,8-diol-9,10-epoxide-benzo[a]pyrene adducted with guanine [33].

As expected, DNA adduct formation was dependent on the concentration of BaP. Adduct yields were not distinguishable from background (1.4 adducts per 10^9 nucleotides versus 3.9 adducts per 10^9 nucleotides in the DMSO controls) following treatment with 0.1 μM BaP without arsenite, but were increased by as much as 17-fold over control levels in cells treated with 1.0 μM BaP in the absence of arsenite (Fig. 1).

For each BaP concentration tested, arsenite co-treatment increased the net yield of adducts in a concentration-dependent manner. The potentiating effect of arsenite was greatest at the lowest BaP concentration, in which co-treatment with 10 μM arsenite resulted in an 18-fold increase over the adduct levels observed with 0.1 μM BaP alone (Fig. 1). Co-treatment with 10 μM arsenite resulted in eight- and five-fold higher levels of adducts compared to the corresponding BaP-only treatments of 0.5 and 1 μM BaP, respectively. Although the effects of arsenite were not statistically significant based on pair-wise comparisons ($P > 0.05$), the consistent pattern of increasing adduct yield with increasing arsenite concentration suggests that the results are biologically meaningful.

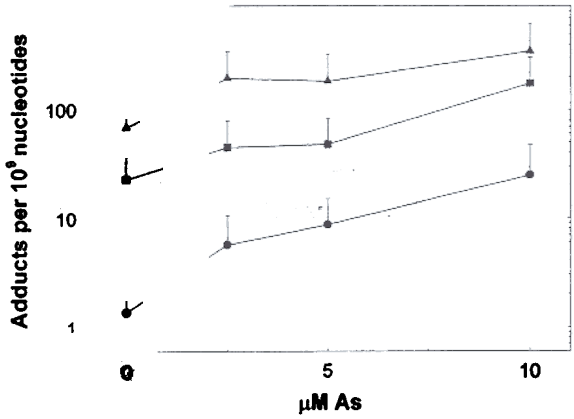

Fig. 1. Formation of BaP–DNA adducts is dose-dependent and is potentiated by arsenite co-treatment. Hepa-1 cells were pre-treated with arsenite for 30 min before addition of BaP to the culture medium, and cells were harvested for adduct determination after 24 h of BaP treatment. Cells were treated with either 0.1 μM (circles), 0.5 μM (squares), or 1.0 μM (triangles) BaP plus the indicated concentration of arsenite. Total adduct level is shown as the number of adducts per 10^9 normal nucleotides. The adduct yield in DMSO controls was 3.9 adducts per 10^9 nucleotides (not shown). Each point represents the mean ± S.E. of two independent experiments. Arsenite treatments were compared to treatments with the same BaP concentration, but without arsenite. None of the increases in adducts with arsenite co-treatments were statistically significant, $P > 0.05$.

3.2. Increased BaP–DNA adduct formation is cytochrome p450 1A1 (CYP1A1) dependent

To verify that the DNA adducts we observed were metabolism dependent, we made use of Hepa-1 derived cells lines that have different (CYP1A1) activity phenotypes. Consistent with our expectations,

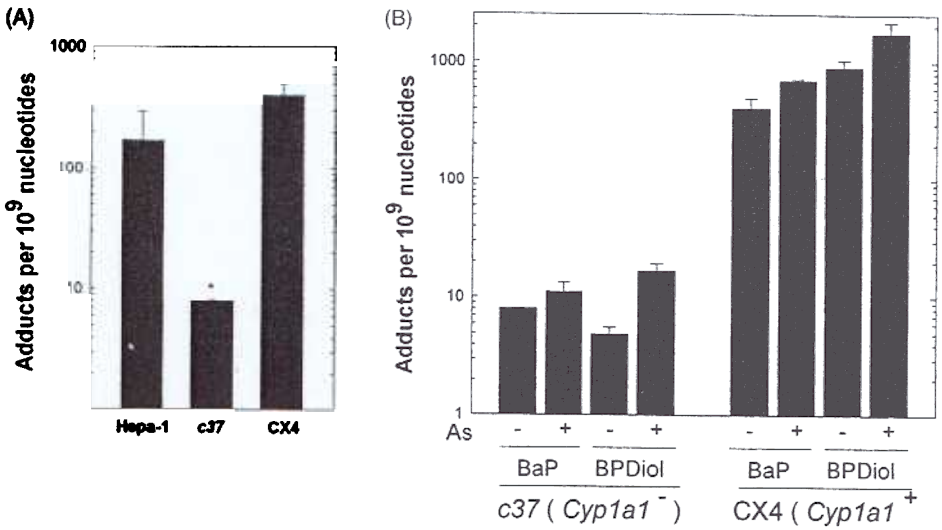

Fig. 2. BaP–DNA adduct formation is CYP1A1-dependent. Panel A: Hepa-1, *c37*, and CX4 cells were pretreated with 10 μM arsenite for 30 min before addition of 0.5 μM BaP to the culture medium, and were harvested for adduct determination after 24 h of BaP treatment. Each bar represents the mean ± S.E. of two independent experiments. Adduct levels in *c37* cells and CX4 cells were compared to Hepa-1 cells. Statistically significant differences are noted with an asterisk (*), $P < 0.05$. Panel B: the same conditions were used as for Panel A, except *c37* and CX4 cells were treated with either 0.5 μM BaP or BPDiol. Adduct levels for different arsenite and BaP or BPDiol treatments for each cell line were compared to the treatment with the PAH without arsenite in the same cell line. None of these comparisons were statistically significant, $P > 0.05$.

formation of bulky adducts was nearly completely dependent on the presence of an active CYP1A1 enzyme, since adduct levels in Hepa-1 cells and CX4 cells, which have functional CYP1A1 activity, were more than an order of magnitude higher than in *c37* cells, which lack CYP1A1 activity (Fig. 2A). In a second experiment, CX4 and *c37* cells were treated with BPDiol as well as BaP to test whether arsenite-induced reactive oxygen species might directly oxidize the dihydrodiol metabolite of BaP, and therefore, increase the formation of DNA reactive metabolites at a step downstream of the initial metabolic activation step by CYP1A1. Although this mechanism has been suggested for adduct enhancement by other sources of oxidative stress [28,34], the absence of a significant number of adducts in *c37* cells co-treated with arsenite and BPDiol argues against direct oxidation of BaP metabolites, and confirms the central role of CYP1A1 in BaP activation.

The dependence on CYP1A1 for the formation of a significant yield of DNA adducts suggested that this enzyme might be a target for arsenite. To test this possibility, we measured CYP1A1 activity following BaP and arsenite co-treatments. CYP1A1 was highly inducible by BaP treatment (Table 2), but arsenite co-treatments had no discernable effect on this activity at any of the BaP concentrations tested, indicating that modulation of CYP1A1 activity by arsenite is not the explanation for the enhancing

Table 2
BaP-inducible CYP1A1 activity with arsenic co-treatment

BaP (μM)	As (μM)	CYP1A1 activity (nmol/min/mg) protein
0.0	0.0	0.2 ± 0.06
0.5	0.0	3.7 ± 0.11
	2.5	4.0 ± 0.25
	10	4.6 ± 0.20
5.0	0.0	6.3 ± 0.96
	2.5	7.6 ± 0.08
	10	7.1 ± 0.16

Hepa-1 cells were treated with varying concentrations of BaP and arsenite for 24 h. Each value represents the mean ± S.E. of two independent samples. Enzymatic activities in the arsenite-treated cells were compared to the activity in cells receiving the same BaP treatment, but without arsenite. None of these comparisons yielded a statistically significant difference ($P > 0.05$).

affect of arsenite on BaP–DNA adduct yields. These data strongly suggest that arsenite potentiates adduct formation at a step downstream of the metabolic activation of BaP, since, although the effect is dependent on CYP1A1 activity, arsenite does not alter this activity.

3.3. Glutathione depletion enhances the potentiating effect of arsenite on adduct formation

Arsenite binds sulfhydryl groups avidly and glutathione has been clearly shown to protect against arsenic-mediated toxicity [35–40]. Glutathione is also important for cellular protection against electrophiles, serving as the primary source of conjugation of BaP-derived epoxide metabolites [41]. Both BaP metabolites and arsenic bind to GSH, suggesting that depletion of GSH by arsenite might serve to decrease BaP metabolite conjugation and, thus, increase the potential for reaction with DNA. To examine the role of cellular thiol status on the potentiation by arsenite of BaP–DNA adduct levels, we modulated GSH levels with exogenous treatments. We used BSO to deplete GSH levels and GSH ethylester to replenish them. Co-treatment with arsenite had no concentration-dependent effect on GSH levels in the cells pretreated in regular medium (Fig. 3, top panel), suggesting that direct competition between arsenite and BaP metabolites was not the cause of increased adduct levels. In cells pretreated with medium containing 20 μM BSO, GSH levels were decreased to between 24 and 65% of cells given the same arsenite treatment, but grown in control medium. The addition of GSH ethylester to the culture medium enhanced the GSH levels to a maximum of 173% over cells grown in control medium.

Changes in cellular GSH status had a noticeable effect on DNA adduct yields. In the cells co-treated with BaP and arsenite in regular medium, adduct yields increased with increasing arsenite concentration to a maximum of 3.5-fold in cells co-treated with 10 μM arsenite (Fig. 3, bottom panel). The potentiation by arsenite was further enhanced in the cells pretreated with BSO, confirming the important role that GSH plays in protection against reactive BaP metabolites. Consistent with this conclusion, treatment of the cells in medium containing GSH ethylester mitigated the potentiating effect of arsenite co-treatment.

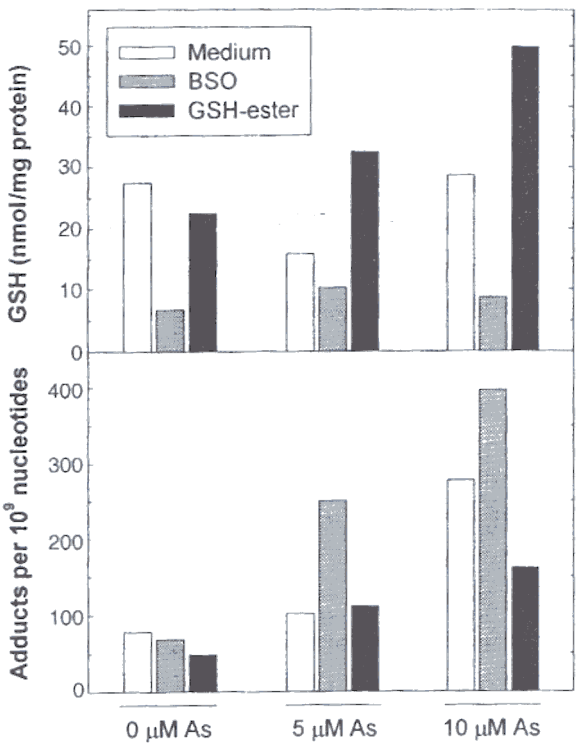

Fig. 3. Depletion of cellular glutathione enhances the ability of arsenite to potentiate BaP–DNA adduct formation. Hepa-1 cells were seeded to 1×10^6 cells per plate and grown 24 h prior to treatment. The cells were then pre-treated for 24 h with 20 μM BSO or for 2 h with 2.5 mM GSH-ethylester. Following these pretreatments, the culture medium was replaced with fresh medium, and the cells were subsequently treated with the indicated concentrations of arsenite for 30 min before addition of 0.5 μM BaP to the culture medium. Cells were harvested after 1.5 h of arsenite and BaP co-treatment for determination of GSH levels or after 24 h for DNA adduct analysis. The GSH level in DMSO controls (not shown) was 19.8 nmol/mg protein.

3.4. Arsenite does not alter the kinetics of adduct removal

To test whether the arsenite-induced increases in net yield of BaP–DNA adducts was the result of an increase in the rate of adduct formation or of a decrease in the rate of adduct removal, we evaluated the rate of adduct removal in the presence of 2.5 μM arsenite (Fig. 4). The presence of arsenite did not

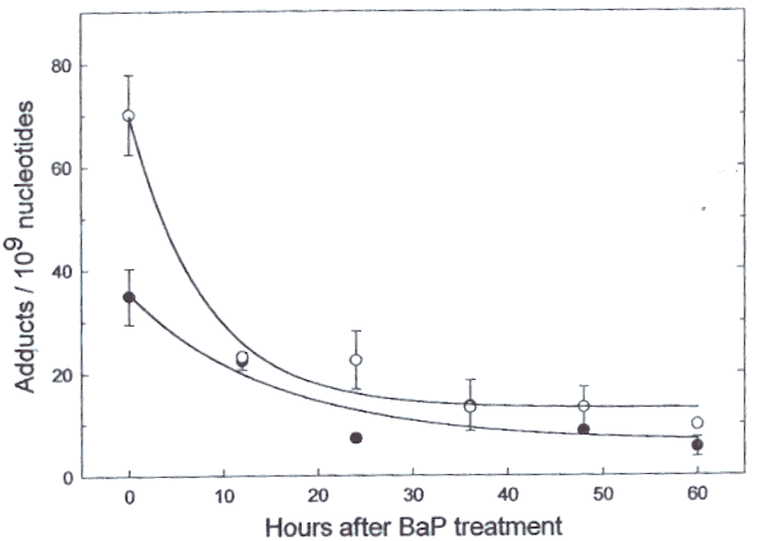

Fig. 4. Arsenite does not disrupt BaP–DNA adduct removal. Hepa-1 cells were seeded to 1×10^6 cells per plate and grown 24 h prior to treatment. Two treatment groups were followed kinetically. One group (open circles) was pre-treated for 24 h with 0.5 μM BaP and 2.5 μM arsenite, and then at time 0 h was switched to medium containing 2.5 μM arsenite. The second group (filled circles) was pre-treated 24 h with BaP only, and then at time 0 h was switched to control medium. Each point represents the mean ± S.E. of at least two independent experiments. Three parameter regression curves are shown for the samples with or without 2.5 μM arsenite during the incubation period after BaP treatment.

disrupt the rapid removal of adducts, showing that DNA adduct excision was not substantially affected by arsenite treatment, and suggesting that arsenite affects the genotoxicity of DNA reactive compounds by mechanisms upstream of DNA repair.

3.5. *Arsenite synergizes with BaP mutagenicity*

To determine whether the enhancement in BaP–DNA adduct levels induced by arsenite co-treatment is converted into an increased mutagenic response, we measured mutation frequencies in Hepa-1 cells co-exposed or singly exposed to 0.5 μM BaP and 2.5 μM arsenite. We followed the appearance of mutant colonies at the *Hprt* locus by selecting cells for their ability to grow in the presence of 6-thioguanine. We found that the low concentrations of BaP and arsenite that we used had no significant effect on mutation frequencies relative to vehicle-treated control cells; however, the combined exposure resulted in a seven-fold increase in the number of mutant colonies (Fig. 5), indicating that the effect of arsenite on BaP–DNA adduct levels has a profound consequence on the mutagenicity and genotoxicity of BaP.

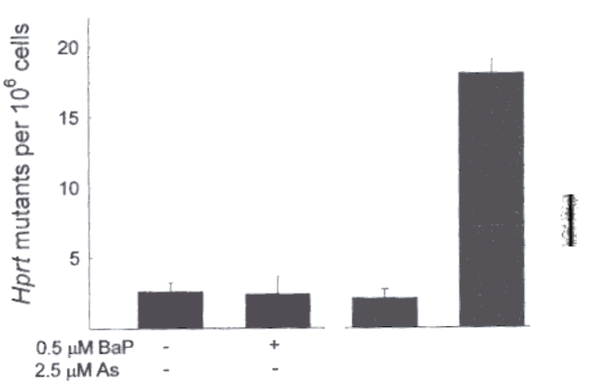

Fig. 5. Arsenic and BaP are comutagenic. Hepa-1 cells were seeded to 7.5×10^6 cells per 150 mm plate and grown 24 h prior to treatment. For the co-treatment group, cells were treated with 2.5 μM arsenite for 30 min prior to the addition of 0.5 μM BaP. The number of *Hprt* mutant colonies was normalized to the number of cells surviving the initial BaP and arsenite treatments as shown in the figure. The average number of colonies counted per plate were 27 for DMSO controls, 24 for BaP alone, 17 for arsenite alone, and 183 for the combined BaP and arsenite treatment. Each bar represents the mean ± S.E. from three independent samples. Treated groups were compared to DMSO controls and statistically significant differences ($P < 0.05$) are denoted with an asterisk (*).

4. Discussion

We report here that arsenite co-treatment potentiates the formation of bulky DNA adducts induced by BaP, and that this effect is a major factor in the mutagenicity of mixtures of these compounds. We tested several hypotheses that addressed potential mechanisms for the enhanced genotoxicity of BaP-arsenite mixtures. BaP–DNA adduct formation required functional CYP1A1 activity, but arsenite did not directly enhance the activity of this enzyme, suggesting that the target for the biological activity of arsenite resides downstream of the metabolic activation of BaP. This absence of an arsenite effect on CYP1A1 activity is inconsistent with other recent reports using chick embryo hepatocytes [42] or human hepatocytes [43], but is consistent with our earlier studies in Hepa-1 cells [44]. One potential explanation for the difference may relate to species-specificity of arsenite-induced changes. If this were the case, it would be of significant interest to test whether arsenite also potentiates BaP–DNA adduct formation in human cells that represent targets for arsenic and BaP toxicity, including cardiovascular, lung, and skin cells.

Although we did not observe an effect of arsenite on phase I metabolism of BaP, arsenite might affect the fate of DNA reactive metabolites of BaP by altering phase II conjugation reactions. Arsenic is an inducer of phase II genes, such as glutathione-*S*-transferases (*Gsta1*) [45–47] as well as genes that regulate GSH synthesis [44]. The data are mixed as to whether *GSTM1* null phenotype may be an important determinant of DNA adduct formation by BaP [48–50], but *GSTP1* over-expression in tissue culture cells appears to block the formation of adducts [51]. Our results suggest that GSH is an important modulatory agent in BaP–DNA adduct formation, since arsenite was much more potent in enhancing BaP–DNA adduct yields in GSH-depleted cells, and this effect was blocked by excess GSH. While our results show an important role of GSH homeostasis in arsenite comutagenesis, they do not suggest that arsenite increases BaP–DNA adducts through direct competition with BaP metabolites for the cellular GSH pool. Our data are most consistent with a model in which depletion of GSH levels increases unbound arsenite in the cell, which can then act to enhance adduct formation through an as of yet unidentified mechanism(s).

Arsenite also modulates other conjugation reactions important for BaP metabolism, for example by regulating the expression of NADPH:quinone oxidoreductase (*Nqo1*) [52]. DNA adduct formation in vitro has been reported for 3,6-BPQ [53], and 7,8-BPQ [54–57], which are substrates for NQO1. We did not find evidence that stable quinone adducts were formed in our experiments following treatment with up to 5 μM 3,6-BPQ (data not shown). It has been suggested that metabolites of BaP can form depurinating adducts [58]. Therefore, it is possible that the formation of depurinating adducts or other adducts not detected by our chromatography system could also contribute to arsenite comutagenesis.

We found that arsenite co-treatment did little to change the rapid removal of DNA adducts. Most of the existing data describing the inhibitory role of arsenite in DNA repair suggests that inhibition of repair occurs at the ligation step that joins the newly repaired DNA to the pre-existing strand. Inhibition of ligation would contribute to DNA damage and mutagenesis, but would not necessarily play a role in DNA adduct removal. Although less well studied, inhibition of other aspects of DNA repair, including the incision process has been reported [59]. While our data are not inconsistent with a contribution of DNA repair to the increase in mutagenesis that we observe, our results identify a step preceding DNA repair as a major contributor to the enhanced genotoxicity of BaP in the presence of arsenite.

There are other effects of arsenite that may be important in regulating the efficiency by which reactive BaP metabolites interact with DNA. For example, gene methylation changes induced by arsenic may alter nuclear architecture and increase the number of targets for BaP metabolite–DNA interactions. Arsenic has been reported to generate global DNA hypomethylation [60] and global hypomethylation promotes a more open chromatin structure [61,62], perhaps allowing for the enhancement of BaP metabolite–DNA interactions. Arsenic has also been shown to cause DNA hypermethylation [63–65], which may also have an impact on the formation of DNA adducts since methylated CpG islands have been shown to favor BaP–DNA adduct formation [66]. Hence, DNA methylation changes induced by arsenite exposure might be important elements in the ability of arsenic to potentiate BaP metabolite–DNA adduct formation.

Uncovering the mechanisms involved in arsenic co-mutagenesis has important public health implications. Our results suggest that co-exposure to arsenite and BaP leads to synergistic toxic responses that differ considerably from those that would be predicted based on the assumption of response-additivity, currently used for quantitative mixtures risk assessment for carcinogens. Further development of these data would allow for direct testing of the appropriateness of this additivity assumption for co-exposures to metals and other PAHs and could contribute to the development of quantitative risk assessment methods for mixtures that more accurately estimate human health risk under environmental exposure conditions.

Acknowledgements

We thank H. Shertzer for a gift of GSH ethylester, A. Senft for assistance with the GSH assays, and K. Dixon for critical discussions on BaP-induced mutagenesis and DNA repair. This work was funded by NIEHS grants ES10807 and ES06096 to Alvaro Puga, and an USEPA STAR predoctoral fellowship to Andrew Maier.

References

[1] ATSDR. Toxicological Profile for Polycyclic Aromatic Hydrocarbons (PAHs), Agency for Toxic Substances Disease Registry, Atlanta, 1995.
[2] ATSDR. Toxicological Profile for Arsenic, Agency for Toxic Substances Disease Registry, Atlanta, 1999.
[3] J.W. Lloyd, Long-term mortality study of steel workers: V. Respiratory cancer in coke plant workers, J. Occup. Med. 13 (1971) 53–68.
[4] S. Mazumdar, C.K. Redmond, W. Sollecito, N. Sussman, An epidemiological study of exposure to coal tar pitch volatiles among coke oven workers, J. Air Pollut. Control Assoc. 25 (1975) 382–389.
[5] E. Redmond, B. Strobino, R. Cypress, Cancer experience among coke by-product workers, Ann. New York Acad. Sci. 271 (1976) 96–107.
[6] C.O. Abernathy, Y.P. Liu, D. Longfellow, H.V. Aposhian, B. Beck, B. Fowler, R. Goyer, R. Menzer, T. Rossman, C. Thompson, M. Waalkes, Arsenic: health effects, mechanisms of actions, and research issues, Environ. Health Perspect. 107 (1999) 593–597.
[7] G. Pershagen, S. Wall, A. Taube, L. Linnman, On the interaction between occupational arsenic exposure and smoking and its relationship to lung cancer, Scand. J. Work Environ. Health 7 (1981) 302–309.
[8] L. Jarup, G. Pershagen, Arsenic exposure, smoking, and lung cancer in smelter workers—a case-control study (published erratum appears in Am. J. Epidemiol. 136 (9) (1992) 1174), Am. J. Epidemiol. 134 (1991) 545–551.
[9] I. Hertz-Picciotto, A.H. Smith, D. Holtzman, M. Lipsett, G. Alexeeff, Synergism between occupational arsenic exposure and smoking in the induction of lung cancer (see comments), Epidemiology 3 (1992) 23–31.
[10] I. Hertz-Picciotto, A.H. Smith, Observations on the dose–response curve for arsenic exposure and lung cancer, Scand. J. Work Environ. Health 19 (1993) 217–226.
[11] T. Tsuda, A. Babazono, E. Yamamoto, N. Kurumatani, Y. Mino, T. Ogawa, Y. Kishi, H. Aoyama, Ingested arsenic and internal cancer: a historical cohort study followed for 33 years, Am. J. Epidemiol. 141 (1995) 198–209.
[12] A.C. Rencher, M.W. Carter, D.W. McKee, A retrospective epidemiological study of mortality at a large western copper smelter, J. Occup. Med. 19 (1977) 754–758.
[13] K. Welch, I. Higgins, M. Oh, C. Burchfiel, Arsenic exposure, smoking, and respiratory cancer in copper smelter workers, Arch. Environ. Health 37 (1982) 325–335.
[14] P.R. Taylor, Y.L. Qiao, A. Schatzkin, S.X. Yao, J. Lubin, B.L. Mao, J.Y. Rao, M. McAdams, X.Z. Xuan, J.Y. Li, Relation of arsenic exposure to lung cancer among tin miners in Yunnan Province, China, Br. J. Ind. Med. 46 (1989) 881–886.
[15] N. Ishinishi, Y. Kodama, K. Nobutomo, A. Hisanaga, Preliminary experimental study on carcinogenicity of arsenic trioxide in rat lung, Environ. Health Perspect. 19 (1977) 191–196.
[16] G. Pershagen, G. Nordberg, N.E. Bjorklund, Carcinomas of the respiratory tract in hamsters given arsenic trioxide and/or benzo[a]pyrene by the pulmonary route, Environ. Res. 34 (1984) 227–241.
[17] J.H. Li, T.G. Rossman, Comutagenesis of sodium arsenite with ultraviolet radiation in Chinese hamster V79 cells, Biol. Met. 4 (1991) 197–200.
[18] J.K. Wiencke, J.W. Yager, A. Varkonyi, M. Hultner, L.H. Lutze, Study of arsenic mutagenesis using the plasmid shuttle vector pZ189 propagated in DNA repair proficient human cells, Mutat. Res. 386 (1997) 335–344.
[19] S.F. Lee-Chen, J.R. Gurr, I.B. Lin, K.Y. Jan, Arsenite enhances DNA double-strand breaks and cell killing of methyl methanesulfonate-treated cells by inhibiting the excision of alkali-labile sites, Mutat. Res. 294 (1993) 21–28.
[20] J.H. Li, T.G. Rossman, Mechanism of comutagenesis of sodium arsenite with *n*-methyl-*n*-nitrosourea, Biol. Trace Elem. Res. 21 (1989) 373–381.
[21] S. Lynn, H.T. Lai, J.R. Gurr, K.Y. Jan, Arsenite retards DNA break rejoining by inhibiting DNA ligation, Mutagenesis 12 (1997) 353–358.
[22] Y. Hu, L. Su, E.T. Snow, Arsenic toxicity is enzyme specific and its affects on ligation are not caused by the direct inhibition of DNA repair enzymes, Mutat. Res. 408 (1998) 203–218.
[23] A. Hartmann, G. Speit, Effect of arsenic and cadmium on the persistence of mutagen-induced DNA lesions in human cells, Environ. Mol. Mutagen. 27 (1996) 98–104.

[24] O. Hankinson, R.D. Andersen, B.W. Birren, F. Sander, M. Negishi, D.W. Nebert, Mutations affecting the regulation of transcription of the cytochrome P1-450 gene in the mouse Hepa-1 cell line, J. Biol. Chem. 260 (1985) 1790–1795.

[25] S. Kimura, H.H. Smith, O. Hankinson, D.W. Nebert, Analysis of two benzo[a]pyrene-resistant mutants of the mouse hepatoma Hepa-1 P1-450 gene via cDNA expression in yeast, Embo. J. 6 (1987) 1929–1933.

[26] A. Puga, B. Raychaudhuri, K. Salata, Y.H. Zhang, D.W. Nebert, Stable expression of mouse Cyp1a1 and human CYP1A2 cDNAs transfected into mouse hepatoma cells lacking detectable p450 enzyme activity, DNA Cell Biol. 9 (1990) 425–436.

[27] M.V. Reddy, K. Randerath, Nuclease P1-mediated enhancement of sensitivity of 32P-postlabeling test for structurally diverse DNA adducts, Carcinogenesis 7 (1986) 1543–1551.

[28] J. Cheu, G. Talaska, M. Miller, C. Rice, D. Warshawsky, Benzo[a]pyrene coated ferric oxide and aluminum oxide particles: uptake, metabolism and DNA binding in hamster pulmonary alveolar macrophages and tracheal epithelial cells in vitro (published erratum appears in Carcinogenesis 18 (5) (1997) 1123), Carcinogenesis 18 (1997) 167–175.

[29] G. Talaska, K.L. Dooley, F.F. Kadlubar, Detection and characterization of carcinogen–DNA adducts in exfoliated urothelial cells from 4-aminobiphenyl-treated dogs by 32P-postlabelling and subsequent thin-layer and high-pressure liquid chromatography, Carcinogenesis 11 (1990) 639–646.

[30] D.W. Nebert, H.V. Gelboin, Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme, J. Biol. Chem. 243 (1968) 6242–6249.

[31] A.P. Senft, T.P. Dalton, H.G. Shertzer, Determining glutathione and glutathione disulfide using the fluorescence probe *o*-phthalaldehyde, Anal. Biochem. 280 (2000) 80–86.

[32] O. Hankinson, Evidence that benzo[a]pyrene-resistant, aryl hydrocarbon hydroxylase-deficient variants of mouse hepatoma line, Hepa-1, are mutational in origin, Somatic Cell Genet. 7 (1981) 373–388.

[33] G. Talaska, M. Jaeger, R. Reilman, T. Collins, D. Warshawsky, Chronic, topical exposure to benzo[a]pyrene induces relatively high steady-state levels of DNA adducts in target tissues and alters kinetics of adduct loss, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 7789–7793.

[34] P.J. Borm, A.M. Knaapen, R.P. Schins, R.W. Godschalk, F.J. Schooten, Neutrophils amplify the formation of DNA adducts by benzo[a]pyrene in lung target cells, Environ. Health Perspect. 105 (Suppl. 5) (1997) 1089–1093.

[35] R.D. Snyder, P.J. Lachmann, Thiol involvement in the inhibition of DNA repair by metals in mammalian cells, Mol. Toxicol. 2 (1989) 117–128.

[36] H. Huang, C.F. Huang, D.R. Wu, C.M. Jinn, K.Y. Jan, Glutathione as a cellular defence against arsenite toxicity in cultured Chinese hamster ovary cells, Toxicology 79 (1993) 195–204.

[37] T.S. Wang, Y.F. Shu, Y.C. Liu, K.Y. Jan, H. Huang, Glutathione peroxidase and catalase modulate the genotoxicity of arsenite, Toxicology 121 (1997) 229–237.

[38] D. Quig, Cysteine metabolism and metal toxicity, Altern. Med. Rev. 3 (1998) 262–270.

[39] M. Shimizu, J.F. Hochadel, B.A. Fulmer, M.P. Waalkes, Effect of glutathione depletion and metallothionein gene expression on arsenic-induced cytotoxicity and c-myc expression in vitro, Toxicol. Sci. 45 (1998) 204–211.

[40] C.H. Yang, M.L. Kuo, J.C. Chen, Y.C. Chen, Arsenic trioxide sensitivity is associated with low level of glutathione in cancer cells, Br. J. Cancer 81 (1999) 796–799.

[41] P.L. Grover, Pathways involved in the metabolism and activation of polycyclic hydrocarbons, Xenobiotica 16 (1986) 915–931.

[42] J. Jacobs, R. Roussel, M. Roberts, D. Marek, S. Wood, H. Walton, B. Dwyer, P. Sinclair, J. Sinclair, Effect of arsenite on induction of CYP1A and CYP2H in primary cultures of chick hepatocytes, Toxicol. Appl. Pharmacol. 150 (1998) 376–382.

[43] D.D. Vakharia, N. Liu, R. Pause, M. Fasco, E. Bessette, Q.Y. Zhang, L.S. Kaminsky, Effect of metals on polycyclic aromatic hydrocarbon induction of CYP1A1 and CYP1A2 in human hepatocyte cultures, Toxicol. Appl. Pharmacol. 170 (2001) 93–103.

[44] A. Maier, T.P. Dalton, A. Puga, Disruption of dioxin-inducible phase I and phase II gene expression patterns by cadmium, chromium, and arsenic, Mol. Carcinog. 28 (2000) 225–235.

[45] T. Prestera, W.D. Holtzclaw, Y. Zhang, P. Talalay, Chemical and molecular regulation of enzymes that detoxify carcinogens, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 2965–2969.

[46] T. Prestera, P. Talalay, Electrophile and antioxidant regulation of enzymes that detoxify carcinogens, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 8965–8969.

[47] P. Talalay, J.W. Fahey, W.D. Holtzclaw, T. Prestera, Y. Zhang, Chemoprotection against cancer by phase 2 enzyme induction, Toxicol. Lett. 82/83 (1995) 173–179.

[48] K. Hemminki, C. Dickey, S. Karlsson, D. Bell, Y. Hsu, W.Y. Tsai, L.A. Mooney, K. Savela, F.P. Perera, Aromatic DNA adducts in foundry workers in relation to exposure, life style and CYP1A1 and glutathione transferase M1 genotype, Carcinogenesis 18 (1997) 345–350.

[49] R. Pastorelli, M. Guanci, A. Cerri, E. Negri, C. La Vecchia, F. Fumagalli, M. Mezzetti, R. Cappelli, T. Panigalli, R. Fanelli, L. Airoldi, Impact of inherited polymorphisms in glutathione *S*-transferase M1, microsomal epoxide hydrolase, cytochrome p450 enzymes on DNA, and blood protein adducts of benzo[a]pyrene-diolepoxide, Cancer Epidemiol. Biomarkers Prev. 7 (1998) 703–709.

[50] M. Rojas, K. Alexandrov, I. Cascorbi, J. Brockmoller, A. Likhachev, K. Pozharisski, G. Bouvier, G. Auburtin, L. Mayer, A. Kopp-Schneider, I. Roots, H. Bartsch, High benzo[a]pyrene diol-epoxide DNA adduct levels in lung and blood cells from individuals with combined CYP1A1 MspI/Msp-GSTM1*0/*0 genotypes, Pharmacogenetics 8 (1998) 109–118.

[51] W.R. Fields, C.S. Morrow, A.J. Doss, K. Sundberg, B. Jernstrom, A.J. Townsend, Overexpression of stably transfected human glutathione *S*-transferase P1-1 protects against DNA damage by benzo[a]pyrene diol-epoxide in human T47D cells, Mol. Pharmacol. 54 (1998) 298–304.

[52] K.C. Falkner, G.P. McCallum, J.R. Bend, Effects of arsenite treatment on NAD(P)H:quinone acceptor oxidoreductase

activity in liver, lung, kidney, and heart of the rat. Comparison to induction by the polyaromatic hydrocarbon, beta-naphthoflavone, Drug Metab. Dispos. 21 (1993) 334–337.
[53] P. Joseph, A.K. Jaiswal, NAD(P)H:quinone oxidoreductase1 (DT diaphorase) specifically prevents the formation of benzo [a]pyrene quinone–DNA adducts generated by cytochrome p4501A1 and p450 reductase, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 8413–8417.
[54] M. Shou, R.G. Harvey, T.M. Penning, Reactivity of benzo[a]pyrene-7, 8-dione with DNA Evidence for the formation of deoxyguanosine adducts, Carcinogenesis 14 (1993) 475–482.
[55] L. Flowers-Geary, R.G. Harvey, T.M. Penning, Identification of benzo[a]pyrene-7,8-dione as an authentic metabolite of (+/−)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene in isolated rat hepatocytes, Carcinogenesis 16 (1995) 2707–2715.
[56] K.D. McCoull, D. Rindgen, I.A. Blair, T.M. Penning, Synthesis and characterization of polycyclic aromatic hydrocarbon *o*-quinone depurinating N7-guanine adducts, Chem. Res. Toxicol. 12 (1999) 237–246.
[57] T.M. Penning, M.E. Burczynski, C.F. Hung, K.D. McCoull, N.T. Palackal, L.S. Tsuruda, Dihydrodiol dehydrogenases and polycyclic aromatic hydrocarbon activation: generation of reactive and redox active *o*-quinones, Chem. Res. Toxicol. 12 (1999) 1–18.
[58] E.L. Cavalieri, E.G. Rogan, P.D. Devanesan, P. Cremonesi, R.L. Cerny, M.L. Gross, W.J. Bodell, Binding of benzo [a]pyrene to DNA by cytochrome p450 catalyzed one-electron oxidation in rat liver microsomes and nuclei, Biochemistry 29 (1990) 4820–4827.
[59] A. Hartwig, U.D. Groblinghoff, D. Beyersmann, A.T. Natarajan, R. Filon, L.H. Mullenders, Interaction of arsenic (III) with nucleotide excision repair in UV-irradiated human fibroblasts, Carcinogenesis 18 (1997) 399–405.
[60] C.Q. Zhao, M.R. Young, B.A. Diwan, T.P. Coogan, M.P. Waalkes, Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 10907–10912.
[61] A.P. Bird, A.P. Wolffe, Methylation-induced repression—belts, braces, and chromatin, Cell 99 (1999) 451–454.
[62] K.D. Robertson, P.A. Jones, DNA methylation: past, present and future directions, Carcinogenesis 21 (2000) 461–467.
[63] M.J. Mass, L. Wang, Arsenic alters cytosine methylation patterns of the promoter of the tumor suppressor gene p53 in human lung cells: a model for a mechanism of carcinogenesis, Mutat. Res. 386 (1997) 263–277.
[64] H.K. Hamadeh, M. Vargas, E. Lee, D.B. Menzel, Arsenic disrupts cellular levels of p53 and mdm2: a potential mechanism of carcinogenesis, Biochem. Biophys. Res. Commun. 263 (1999) 446–449.
[65] W. Boonchai, M. Walsh, M. Cummings, G. Chenevix-Trench, Expression of p53 in arsenic-related and sporadic basal cell carcinoma, Arch. Dermatol. 136 (2000) 195–198.
[66] M.S. Tang, J.B. Zheng, M.F. Denissenko, G.P. Pfeifer, Y. Zheng, Use of UvrABC nuclease to quantify benzo[a]pyrene diol epoxide–DNA adduct formation at methylated versus unmethylated CpG sites in the p53 gene, Carcinogenesis 20 (1999) 1085–1089.