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Arsenite Targets the Zinc Finger Domains of Tet Proteins and Inhibits Tet-Mediated Oxidation of 5-Methylcytosine

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Abstract

Arsenic toxicity is a serious public health problem worldwide that brings more than 100 million people into the risk of arsenic exposure from groundwater and food contamination. Although there is accumulating evidence linking arsenic exposure with aberrant cytosine methylation in the global genome or at specific genomic loci, very few have investigated the impact of arsenic on the oxidation of 5-methylcytosine (5-mC) mediated by the Ten-eleven translocation (Tet) family of proteins. Owing to the high binding affinity of As(III) toward cysteine residues, we reasoned that the highly conserved C₃H-type zinc fingers situated in Tet proteins may constitute potential targets for arsenic binding. Herein, we found that arsenite could bind directly to the zinc fingers of Tet proteins in vitro and in cells, and this interaction substantially impaired the catalytic efficiency of Tet proteins in oxidizing 5-mC to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-foC), and 5-carboxylcytosine (5-caC). Treatments with arsenite also led to a dose-dependent decrease in the level of 5-hmC, but not 5-mC, in DNA isolated from HEK293T cells overexpressing the catalytic domain of any of the three Tet proteins and from mouse embryonic stem cells. Together, our study unveiled, for the first time, that arsenite could alter epigenetic signaling by targeting the zinc fingers of Tet proteins and perturbing the Tet-mediated oxidation of 5-mC in vitro and in cells. Our results offer important mechanistic understanding of arsenic epigenotoxicity and carcinogenesis in mammalian systems and may lead to novel approaches for the chemoprevention of arsenic toxicity.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

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Additional tables and figures as mentioned in the text (PDF).

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INTRODUCTION

As a naturally occurring metalloid, arsenic constitutes an important and ubiquitous threat to public health due to the prevalence of its contamination in groundwater and the substance's toxic potential.¹ Whereas the World Health Organization (2011) recommends a threshold of 10 µg/L for inorganic arsenic concentration in drinking water, over 100 million individuals in nearly 70 nations are exposed to excessive amounts of arsenic through drinking water and diet.² There is a strong body of evidence linking arsenic exposure with diverse pathophysiological end points in humans, from acute toxicity to chronic diseases including cancer.³ Apart from arsenic-induced oxidative damage in DNA and perturbations of DNA repair,⁴ it becomes increasingly clear that arsenic accumulation also leads to epigenetic dysregulation, including perturbations of DNA methylation in the global genome and at some specific genomic loci.^{5,6} However, it remains unexploited how arsenic exposure influences the enzymatic removal of this pivotal epigenetic mark.

Although 5-methylcytosine (5-mC) is a stable epigenetic mark that mediates long-term gene silencing, mounting evidence indicates that DNA methylation is reversible.⁷ In this vein, the recent discovery of Ten-eleven translocation (Tet) family of Fe²⁺- and α-ketoglutarate-dependent dioxygenases sheds new light on the replication-independent active DNA demethylation in mammals. Tet enzymes can induce the iterative oxidation of 5-mC to yield the 5-hydroxymethyl, 5-formyl, and 5-carboxyl derivatives of cytosine (5-hmC, 5-foC, and 5-caC),^{8,9} and the resultant 5-foC and 5-caC, as well as the deamination product of 5-hmC (i.e., 5-hydroxymethyluracil), can then be removed from DNA by thymine DNA glycosylase.^{10,11} Subsequent repair of the resulting abasic site by the base excision repair machinery restores unmethylated cytosine.

A recent structural analysis of a catalytically active form of human Tet2 revealed three zinc cations in C₃H coordination that are important for the structural integrity, substrate recognition, and catalytic activity of Tet2.¹² In addition, the amino acid residues involved in zinc coordination are highly conserved in Tet proteins, and mutations in these residues are found in a number of human cancers.¹² Furthermore, As(III) was found to be capable of binding to proteins harboring a C₃H- or C₄-type of zinc fingers, e.g. the promyelocytic leukemia protein,¹³ estrogen receptor-α,¹⁴ poly(ADP-ribose) polymerase-1,¹⁵ DNA repair proteins,¹⁶ and some RING-finger histone E3 ubiquitin ligases.¹⁷ Hence, we reason that arsenite may interact with the zinc fingers of Tet proteins, thereby altering their conformation and perturbing Tet-mediated oxidation of 5-mC in mammalian systems.

To test the hypothesis, we examined the interaction between As(III) and zinc finger domains of human Tet proteins in vitro and in mammalian cells with the applications of LC–MS, ultraviolet (UV) absorption spectrophotometry, and streptavidin agarose affinity assay. We also assessed how arsenite exposure affects the Tet-mediated oxidation of 5-mC in vitro and in cultured mammalian cells by determining the catalytic efficiency of Tet1 in sequentially oxidizing 5-mC in a synthetic duplex DNA and by analyzing the levels of 5-hmC in genomic DNA isolated from arsenite-treated cells. The results uncovered a novel molecular mechanism underlying arsenic-induced perturbation in epigenetic signaling, which deepened our understanding on the epigenotoxicity of arsenic.

EXPERIMENTAL PROCEDURES

In Vitro Arsenite Binding Assay

The peptide FPSCRCVERAGHTCEAA, derived from the first zinc finger of human Tet2 with amino acid residues 1130–1137 and 1216–1224, was obtained from Genemed Synthesis Inc. (San Antonio, TX). For mass spectrometric analysis, the lyophilized apo-peptide was prepared at a concentration of 1 mM in 100 mM ammonium acetate (pH 7.2) containing 10 mM tris(2-carboxyethyl)phosphine (TCEP). Aliquots of 100 μ M peptide solution were incubated with 100 μ M NaAsO₂ at room temperature for 1 h. The resultant solution was diluted by 10-fold in 50% methanol and 0.1% formic acid before injection. Electrospray ionization–mass spectrometry (ESI–MS) experiments were performed on an LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Samples were introduced into the inlet at 10 μ L/min in 50% methanol. The voltages for the ESI source, capillary, and tube lens were maintained at 5 kV, 8.6 V, and 41.5 V, respectively. The maximum ion injection time was 50 ms and the temperature for the ion transport tube was held constant at 275 °C throughout the experiment.

UV Spectrophotometric Analysis of As(III)–Peptide Interaction

Aliquots of 200 μ M of the aforementioned apo-peptide in a solution containing 20 mM ammonium acetate (pH 7.2) and 2 mM TCEP were titrated with NaAsO₂ in increments of 20 μ M over a range of 20–220 μ M at room temperature. The UV absorption spectra of the resultant solutions were collected in the wavelength range of 240–370 nm on a Varian Cary 50 UV–visible spectrophotometer (Palo Alto, CA).

Streptavidin Agarose Affinity Assay and Western Blot

The streptavidin agarose affinity assay was conducted with the use of biotin-As as previously described.¹⁷ Briefly, HEK293T cells were transfected with plasmid allowing for expressing the HA-tagged catalytic domain of Tet1 (HA-Tet1CD) for 24 h, followed by treating with 5 μ M biotin-As for 2 h. The cells were then lysed in CellLytic M lysis buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Afterwards, the cell lysates were incubated with streptavidin agarose beads at room temperature for 3 h. Streptavidin agarose beads were subsequently washed with 1 $\times$ PBS containing 1% IGEPAL CA-630 (Sigma-Aldrich) for 3 times and resuspended in SDS-PAGE loading buffer.

The proteins on the beads were resolved by SDS-PAGE and subsequently transferred to a nitrocellulose membrane using a solution containing 10 mM NaHCO₃, 3 mM Na₂CO₃, and 20% methanol. The membranes were blocked with 5% nonfat milk at room temperature for 2 h and then incubated with primary anti-HA antibody (1:10 000 dilution, Sigma-Aldrich) in PBS-T at 4 °C overnight, washed for 5 times in PBS-T (10 min each), and incubated with secondary antirabbit antibody (1:10 000) in PBS-T for 1 h, which was followed with five washes in PBS-T. The secondary antibody was detected by using ECL Advance Western Blotting Detection Kit (GE Healthcare) and visualized with Hyblot CL autoradiography film (Denville Scientific, Inc., Metuchen, NJ). Similar Western blot analysis was carried out for HA-Tet1CD-overexpressing HEK293T cells following a 24-h treatment with 0, 1, 2, or 5 μM NaAsO₂.

Biochemical Assay of Tet1-Mediated Oxidation of 5-mC in DNA

The 5-mC oxidation assay was performed in triplicate by using a 5-mC-containing duplex DNA d-(AGCTC_mCGGTCA)/d(GTGACCGGAGCTG) and purified mouse Tet1 protein included in a 5mC Tet1 Oxidation Kit (Wisegene, IL). The reaction was initiated by incubating 0.15 μL of the Tet1 enzyme with 10 pmol of the duplex DNA at 37 °C in a buffer that contained 50 mM HEPES (pH 8.0), 10 mM NaCl, 2 mM ascorbic acid, 1 mM 2-oxoglutarate, 50 μM (NH₄)₂Fe(SO₄)₂, 1 mM ATP, and different concentrations of NaAsO₂ (i.e., 0, 2, and 5 μM). After reaction for 10 and 30 min, the mixtures were immediately frozen on dry ice.

Prior to LC–MS analysis, the enzyme in the reaction mixture was removed by chloroform extraction. The aqueous layer was subjected to LC–MS analyses on an LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) operated in the higher resolution “ultra-zoom-scan” mode, and an Agilent 1200 capillary HPLC (Agilent Technologies, Santa Clara, CA) was used. The separation was carried out on a 0.5 × 250 mm Zorbax SB-C18 column (5 μm particle size, Agilent Technologies, Santa Clara, CA). The flow rate was 8.0 μL/min, and a gradient of 5 min of 0–20% methanol followed by 35 min of 20–40% methanol in 400 mM 1,1,1,3,3,3-hexafluoro-2-propanol (pH adjusted to 7.0 with triethylamine) was employed. The mass spectrometer was operated in the negative-ion mode to monitor the [M – 3H]^{3–} ions of the initially methylated 11-mer DNA and the corresponding products with the 5-mC being oxidized to 5-hmC, 5-foC, and 5-caC. The ESI source, capillary, and tube lens voltages were 4.0 kV, –50 V, and –75 V, respectively, and the temperature for the ion transport tube was 300 °C. Quantification of the oxidation products was conducted based on the intensities of the monoisotopic peak and the M + 1 isotopic peak representing the [M – 3H]^{3–} ions of 5-mC-, 5-hmC-, 5-foC-, and 5-caC-containing 11mer DNA. Because the isotopic peaks of the 5-hmC- and 5-foC-bearing DNA partly overlap, the total intensities of the M + 2 and M + 3 isotopic peaks of the 5-foC-harboring DNA were calculated following its isotopic abundance ratios of M, M + 1, M + 2, and M + 3 (i.e., 73.9:100:76.8:42.9 for an elemental composition of C₁₀₇H₁₃₅N₄₁O₆₅P₁₀) and subtracted from the intensities of the monoisotopic and M + 1 isotopic peaks of the 5-hmC-carrying DNA. The amounts of 5-hmC, 5-foC, and 5-caC produced and 5-mC remained in the reaction mixture were expressed as the percentages of the peak intensity for

each cytosine modification-harboring 11mer DNA over the total peak intensities of DNA containing all four modifications.

Synthesis of [$^{13}\text{C}_5$]-5-methyl-2'-Deoxycytidine ([$^{13}\text{C}_5$]-5-mdC)

5-methyl- N^4 -Benzoylcytosine was synthesized following the established procedures for the preparation of N^4 -benzoylcytosine.¹⁸ Additionally, 3',5'-di-*O*-acetyl-[$^{13}\text{C}_{10}$ - $^{15}\text{N}_2$]-thymidine was synthesized following our previously established procedures.¹⁹ 3',5'-di-*O*-acetyl-[$^{13}\text{C}_{10}$ - $^{15}\text{N}_2$]-thymidine (5.0 mg, 0.015 mmol, Cambridge Isotope Laboratories) and 5-methyl- N^4 -benzoylcytosine (13.3 mg, 0.040 mmol) were added to a 15-mL flask and purged with N_2 gas. Anhydrous acetonitrile (1 mL) was added and, after 10 min of stirring, bis(trimethylsilyl)-acetamide (20 μL , 0.070 mmol, Sigma-Aldrich) was added to the reaction mixture. The solution was then heated to 70 $^\circ\text{C}$. After 15 min, trimethylsilyl triflate (4 μL , 0.020 mmol, Sigma-Aldrich) was subsequently added and the reaction was maintained at 70 $^\circ\text{C}$ for 4 h. The reaction mixture was cooled, removed of solvent, and dried under vacuum. The crude [$^{13}\text{C}_5$]-3',5'-di-*O*-acetyl-5-methyl-2'-deoxycytidine was purged with N_2 gas, and 2 M ammonia in methanol (4 mL, Sigma-Aldrich) was subsequently added. The reaction was heated to 40 $^\circ\text{C}$ and maintained at that temperature overnight. Ammonium hydroxide solution (30%, 4 mL) was added to the reaction mixture, and the solution was stirred at 40 $^\circ\text{C}$ for 72 h. The solvent was removed and [$^{13}\text{C}_5$]-5-methyl-2'-deoxycytidine was purified by HPLC. The identity of [$^{13}\text{C}_5$]-5-methyl-2'-deoxycytidine was confirmed by LC-MS/MS analysis (Supporting Information Figure S2).

Cell Culture, Transfection, and NaAsO₂ Treatment

The HEK293T human embryonic kidney cells were cultured in Dulbecco's Modified Eagle Medium (ATCC) supplemented with 10% fetal bovine serum and 100 IU/mL penicillin. The mouse embryonic stem cells were cultured in High Glucose Dulbecco's Modified Eagle Medium (Life Technologies), supplemented with 10% ES-qualified fetal bovine serum (Atlanta Biologicals), 100 IU/mL penicillin-streptomycin, MEM Non-Essential Amino Acids (Life Technologies), 0.1 mM β -mercaptoethanol (Sigma), and 60 000 units of Leukemia Inhibitory Factor (Stemgent). Culture vessels were pretreated with 0.1% gelatin (STEMCELL Technologies) for 5–10 min prior to culture removal and cell plating. All cells were maintained in a humidified atmosphere with 5% CO_2 at 37 $^\circ\text{C}$ and medium was renewed 2–3 times a week according to cell densities. For plasmid transfection, cells were transferred to the same media as described above without the addition of penicillin.

HEK293T cells, seeded in 6-well plates at a density of $\sim 3 \times 10^5$ cells per well, were transfected individually with 1.5 μg expression vectors carrying the catalytic domains of human Tet1 (amino acids 1418–2136), human Tet2 (amino acids 1129–2002), or mouse Tet3 (amino acids 697–1669) using Lipofectamine 2000 (Invitrogen), as described previously.²⁰ The media were discarded at 6-h following transfection, and fresh media containing NaAsO₂ at final concentrations of 1, 2, and 5 μM were subsequently added. The cells were cultured for 24 h and harvested for DNA extraction. Following the similar procedures, mESCs were seeded in 6-well plates, treated with 0, 1, 2, and 5 μM NaAsO₂ for 24 h, and harvested for DNA extraction.

DNA Extraction and Enzymatic Digestion

Total genomic DNA was isolated from treated cells using a previously published high-salt method.²¹ The resulting DNA sample (1 µg) was treated with nuclease P1 (0.5 U) and phosphodiesterase 2 (0.00025 U) at 37 °C for 12 h in a digestion buffer (30 mM sodium acetate, 1 mM zinc acetate, 1 mM EHNA, pH 5.6), followed by the addition of alkaline phosphatase (0.05 U) and phosphodiesterase 1 (0.0005 U) in 50 mM Tris-HCl (pH 8.9) for 2 h. After being neutralized by formic acid, the enzymatic digestion mixture of 50–300 ng of genomic DNA was mixed with 48 fmol of [1,3-¹⁵N₂-2'-D]-5-hmdC, 600 fmol of [¹³C₅]-5-mdC, and 16.2 pmol of [¹⁵N₅]-2'-deoxyguanosine ([¹⁵N₅]-dG). The samples were subsequently extracted with chloroform and the aqueous layer was subjected to LC–tandem MS analysis.

LC–Tandem MS Quantification of 5-mdC, dG, and 5-hmdC

Measurements of 5-mdC, dG, and 5-hmdC were conducted on an LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an Agilent 1200 capillary HPLC (Agilent Technologies, Santa Clara, CA). The mass spectrometer was operated in the positive-ion mode, where the MS/MS for the [M + H]⁺ ions of 5-mdC (*m/z* 242) and [¹³C₅]-5-mdC (*m/z* 247) were acquired, and the MS/MS/MS arising from the further cleavages of the [M + H]⁺ ions of the nucleobase portions of dG (*m/z* 152), [¹⁵N₅]-dG (*m/z* 157), 5-hmdC (*m/z* 142), and [1,3-¹⁵N₂, 2'-D]-5-hmdC (*m/z* 144) were recorded (Figures S3–S5). The HPLC separation was carried out on a 0.5 × 250 mm Zorbax SB-C18 column (5 µm particle size, Agilent Technologies, Santa Clara, CA) at a flow rate of 8.0 µL/min. A solution of 2 mM ammonium bicarbonate (pH 7.0, solution A) and methanol (solution B) were used as mobile phases, and a gradient of 5 min 0–20% B followed by 25 min 20–70% B was employed for the separation. MS settings were as follows: electrospray voltage, 5 kV; capillary temperature, 275 °C; capillary voltage, 38 V; tube lens voltage, 60 V; sheath gas flow rate, 15 arbitrary units. The calibration curve for 5-hmdC was constructed previously,²² and the calibration curves for 5-mdC and dG were generated by spiking 600 fmol and 16.2 pmol of [¹³C₅]-5mdC and [¹⁵N₅]-dG with different amounts of 5-mdC and dG, respectively (Figure S6). The numbers of moles of 5-mdC, dG, and 5-hmdC in each sample were calculated from the peak area ratios of the unlabeled analytes to the corresponding stable isotope-labeled standards, the calibration curves, and the amounts of stable isotope-labeled standards added. The levels of 5-hmdC and 5-mdC were expressed as the percentages of dG.

RESULTS

As(III) Binds to the Zinc Finger Domains of Tet Proteins in Vitro and in Cells

To test whether NaAsO₂ can bind directly to the zinc finger domains of Tet proteins, we first performed a mass spectrometry (MS)-based in vitro binding assay using a synthetic peptide derived from the first zinc finger motif of human Tet2 protein, which carries a.a. 1130–1137 and a.a. 1216–1224. Positive-ion electrospray ionization–mass spectrometry (ESI–MS) revealed the [M + 2H]²⁺ ion of the apo-peptide at *m/z* 919 (Figure 1a). Incubation of the apo-peptide with As(III) leads to a + 36 *m/z* shift (Figure 1a), which corresponds to the binding with one As(III) after the release of three protons from the Cys residues in the Tet2

peptide. In accordance with the mass increase for coordination of As(III) with other C₃H-type zinc finger proteins,^{23,24} the MS result revealed that arsenite can bind to the peptide derived from the first zinc binding site of Tet2. The coordination of As(III) with thiol was also confirmed by UV spectrophotometric analysis. In the UV absorption spectra, titrations of arsenite into Tet2 peptide-containing solution led to increased absorbance in the wavelength range of 240–370 nm (Figure 1b), indicating the new charge-transfer electronic transitions occurring in the near UV range upon As–S bond formation.²⁵

To further exploit the potential binding of arsenite to Tet proteins in mammalian cells, we treated HEK293T cells with a *p*-aminophenylarsenoxide-conjugated biotin probe,^{17,26} and assessed its interaction with ectopically expressed Tet1 by streptavidin agarose affinity assay. The Western blot result showed that biotin-As probe pulled down Tet1 protein that was overexpressed in mammalian cells (Figure 1c), confirming Tet1 as an As(III)-binding protein. In a control experiment, we failed to pull down Tet1 without the addition of the biotin-As probe (Figure 1c).

Arsenite Suppresses the Sequential Oxidation of 5-mC in a Synthetic Duplex DNA

Given that mammalian Tet proteins could induce sequential oxidation of 5-mC to yield 5-hmC, 5-foC, and 5-caC,^{8–10} we next assessed how binding to arsenite affects the catalytic efficiency of Tet1 in oxidizing 5-mC in duplex DNA. To this end, we conducted an in vitro Tet reaction in the presence of different concentrations of arsenite, where a duplex DNA d(AGCTCXGGTCA)/d-(GTGACCGGAGCTG) (X = 5-mC) was employed as the substrate. The compositions of the resulting reaction mixture were characterized and quantified by LC–ESI–MS analysis (Figures 2 and S1). In the absence of arsenite, the MS results revealed a rapid loss of approximately 83.2% 5-mC in 10 min and an additional decrease of 8.6% in 30 min (Figure 3 and Table S1), suggesting the high activity of Tet1 toward oxidizing 5-mC in duplex DNA. Consistently, we observed rapid formation of the three oxidized 5-mC derivatives. At 10 min, the levels of duplex DNA carrying 5-hmC, 5-foC, and 5-caC were increased by 42%, 26%, and 15%, respectively; and at the later time point (30 min), an accumulation of 57% 5-caC was observed, which was accompanied by drops in the levels of 5-hmC and 5-foC by 2.7- and 1.4-fold, respectively (Figure 3 and Table S1). In contrast to the control reactions, the addition of 2 μ M arsenite led to an inhibition of Tet1 activity, as reflected by a nearly 50% reduction in the production of 5-caC at 30 min (Figure 3 and Table S1). Such a retardation in Tet1-mediated oxidation of 5-mC to 5-caC was exacerbated when a higher concentration of arsenite (5 μ M) was present in the reaction mixture, where 5-caC was produced at a level that was about one-third of its yield in the control reaction (Figure 3 and Table S1). Together, the MS data indicated that NaAsO₂ exerted significant inhibitory effects on the oxidation of 5-mC in duplex DNA by Tet enzymes.

Arsenite Inhibits Tet-Mediated Hydroxylation of 5-mdC in Mammalian Cells

Having demonstrated that arsenite suppresses the enzymatic activity of Tet proteins toward 5-mdC oxidation in vitro, we next examined how NaAsO₂ exposure perturbs the levels of 5-mdC and its oxidation product 5-hmdC in HEK293T cells individually overexpressing the catalytic domains of the three Tet proteins, namely Tet1CD, Tet2CD, and Tet3CD. In this vein, we did not assess if arsenite exposure alters the levels of 5-fodC and 5-cadC in cellular

DNA because of the facile cleavage of these two modified nucleosides from DNA by thymine DNA glycosylase.^{10,27} After arsenic treatments, we isolated genomic DNA from the cells, digested the DNA with enzymes, and subjected the resulting nucleoside mixture to LC–MS/MS for the simultaneous quantification of 5-methyl-2'-deoxycytidine (5-mdC), 2'-deoxyguanosine (dG), and 5-hydroxymethyl-2'-deoxycytidine (5-hmdC). The levels of 5-mdC and 5-hmdC were expressed as the percentages of dG. The identical elution time and similar tandem mass spectra of the analytes and their respective stable isotope-labeled standards facilitated the unambiguous identification and reliable quantification of these nucleosides in the digestion mixture of cellular DNA (Figures S3–S6). Our quantification results revealed that HEK293T cells with ectopic expression of Tet1CD displayed a high level of 5-hmdC (~0.33% dG), whereas 24-h treatments with 1, 2, and 5 μ M NaAsO₂ diminished the levels of 5-hmdC by 1.3-, 1.4-, and 2.1-fold, respectively (Figure 4a and Table S2). Moreover, we found that the arsenite-induced dose-dependent decrease in the yield of 5-hmdC was accompanied by an elevated level of 5-mdC in genomic DNA (up to a 28% increase at the highest dose) in HEK293T cells ectopically expressing Tet1CD (Figure 4b and Table S2), suggesting that arsenite inhibited the conversion of 5-mdC to 5-hmdC in cells. Likewise, exposure to arsenite resulted in dose-dependent reductions in the levels of 5-hmdC, but not 5-mdC, in HEK293T cells ectopically expressing the catalytic domains of Tet2 and Tet3 (Figure 4 and Table S2). Additionally, Western blot result showed that arsenite treatments did not alter significantly the expression level of Tet1CD in HEK293T cells (Figure S7). Hence, we inferred that the reduced 5-hmdC formation is attributed to impaired catalytic function of Tet enzymes, rather than decreased expression at the protein levels.

We next asked whether similar findings can be extended to mammalian cells without ectopic expression of Tet proteins. It turned out that arsenite exposure did not alter substantially the oxidation of 5-mC to 5-hmC in HEK293T cells without the overexpression of any Tet proteins (Figure S8). We reason that mouse embryonic stem cells (mESCs) with relatively high levels of Tet-induced oxidation products²⁸ could be a unique system for studying the impact of arsenic on Tet-mediated oxidation of 5-mdC. Indeed, the MS-based quantification data revealed substantial decreases in the levels of 5-hmdC in mESCs treated with NaAsO₂, where the levels of 5-hmdC were 0.092, 0.096, 0.070, and 0.063 modifications per 100 dG in cells treated with NaAsO₂ at concentrations of 0, 1, 2, and 5 μ M, respectively (Figure 5a and Table S3). The drop in the levels of 5-hmdC, particularly in mESCs exposed to 2–5 μ M arsenite, was in line with the inhibitory effect of arsenite on 5-hmdC generation in cells ectopically expressing Tet proteins (Figure 4a). We also found that the same treatments did not result in statistically significant decreases in the level of 5-mdC (Figure 5b and Table S3), indicating that 5-hmdC diminution was induced by the suppressed enzymatic activity of Tet proteins, but not the reduced level of 5-mC.

DISCUSSION

Role of Zinc Fingers in the Toxicity of Arsenic and Other Metal Ions

Because As(III) can selectively bind to C₃H- and C₄-type zinc fingers with high affinity,^{29–31} the interaction between arsenic and zinc finger proteins plays a crucial role in

the toxicity and carcinogenesis of the metalloid. Binding of As(III) to the zinc finger domain of poly(ADP-ribose) polymerase 1 (PARP-1) and xeroderma pigmentosum complementation group A (XPA) proteins is known to interfere with DNA repair.^{32,33} In addition, substitution of Zn(II) with As(III) in the RING finger motif of RNF20–RNF40 heterodimer, a RING finger E3 ubiquitin ligase, was found to impair the ubiquitination of lysine 120 in histone H2B, which gives rise to a chromatin environment that is less biochemically accessible and inhibits DNA double strand break repair.¹⁷ Our present study demonstrated that arsenite can bind to the zinc finger motifs of Tet proteins. In this vein, zinc fingers of Tet2 are highly conserved and are essential for the structural integrity, DNA interaction, and catalytic activity of the three Tet proteins.¹² Therefore, our discovery of diminished Tet activities arising from arsenic binding is closely associated with the disruption of the coordination sphere of zinc in the zinc finger environment and consequent perturbation of the zinc finger function. The results also supported that the zinc fingers of Tet proteins serve an essential and critical function in oxidizing 5-mC. Apart from arsenic, other metals and metalloids with high affinities to thiolates can also compromise zinc fingers by displacing zinc and disrupting the conformations of the zinc finger proteins. For instance, cadmium, cobalt, nickel, and antimony ions were found to cause zinc ion release from the zinc finger motif of XPA protein.^{34–36} Similarly, the displacement of zinc from zinc finger structures by cadmium and mercury also resulted in the inactivation of bacterial formamidopyrimidine-DNA glycosylase (Fpg), a DNA repair enzyme involved in base excision repair.³⁷ In this vein, owing to the metal binding properties of zinc fingers,¹⁶ it can be envisaged that Tet proteins may also be susceptible to inhibition by a variety of carcinogenic metal ions.

Disturbance to DNA Methylation Fidelity by Arsenic

As a milestone in epigenetic research, the recent discovery of Tet family proteins revealed a plausible DNA demethylation process that can be initiated through Tet-mediated successive oxidation of 5-mC to 5-hmC, 5-foC, and 5-caC.^{8,9} The functions of Tet proteins necessitate their binding with metal ions (i.e., Zn(II) and Fe(II)) and cosubstrate (i.e., α -ketoglutarate). It has been demonstrated that some cancer-associated mutations in isocitrate dehydrogenases 1 and 2 (IDH1/2) and the resulting aberrant accumulation of (*R*)-2-hydroxyglutarate could lead to inhibition of Tet enzymes by occupying the α -ketoglutarate binding site, which contributes to diminished levels of 5-hmC and global genome hypermethylation in some cancer cells.^{38,39} It was also observed that the catalytic functions of Tet proteins could be stimulated by vitamin C.^{40–42} Here, we found that arsenite could bind to the zinc finger domain of Tet proteins and inhibit the formation of the three oxidized derivatives of 5-mC in vitro, and consistently, it compromised the catalytic efficiency of Tet enzymes in cells and led to a substantial loss of 5-hmC in the global genome of cultured mammalian cells. 5-hmC has now been widely accepted as the sixth base owing to its ubiquitous presence in mammalian DNA.^{43,44} Aside from being an intermediate in active DNA demethylation, 5-hmC can be recognized by specific cellular proteins,^{45,46} and thus acts as an epigenetic mark on its own right and regulates gene transcription.⁴⁷ Additionally, changes in the levels of 5-hmC are often accompanied by human diseases, especially cancer.^{47,48} Therefore, the arsenite-induced alteration of this modification provides a novel mechanism pertaining to carcinogenic effects of arsenic exposure. It is also noteworthy that failure in efficiently

oxidizing 5-mC to 5-hmC may lead to an enrichment of 5-mC in the genome. In this regard, DNA hypermethylation has been observed in the global genome in various human cell lines and human peripheral blood leukocytes after arsenic exposure, and targeted hypermethylation was also found in the promoter regions of some tumor suppressor genes.⁴⁹ Hence, arsenite-induced impairment of the functions of Tet proteins could confer multiple risks on the control of DNA methylation fidelity and exacerbate its perturbation of the epigenome.

Implications in Environmental Exposure to Arsenic

Although the currently permitted concentration of arsenic in water is 10 µg/L, millions of people worldwide are exposed to arsenic at concentrations exceeding this limit. For instance, arsenic concentrations of up to 800–7550 µg/L have been detected in drinking water in some areas in Argentina, Bangladesh, Chile, China, India, Mexico, and the United States.³ Even when the levels of arsenic are low and considered safe by the EPA, the impact of long-term cumulative arsenic exposure on public health is substantial.³ For instance, Zierold et al.⁵⁰ reported that individuals who drink well water with low concentrations of arsenic (2–10 µg/L) for more than 20 years were significantly more likely to exhibit depressive symptoms than those drinking water with less than 2 µg/L arsenic. On the basis of these studies, although some low-concentration treatments in our work did not result in statistically significant changes in the level of 5-hmdC within 24 h, it cannot be ruled out that chronic or excessive arsenic exposure may have a pronounced effect in inhibiting the activities of mammalian Tet proteins.

From our cellular experiment, the notably dose-dependent accumulation of 5-mC observed in Tet1CD-, but not Tet2CD- and Tet3CD-overexpressing cells can be explained by the expression of a relatively high level of Tet1CD. This result indicates a potentially high correlation between Tet expression and arsenic toxicity. Viewing that 5-hmC is relatively abundant in the brain and that its presence is Tet protein-dependent, arsenic may have a greater impact on epigenetic regulation in this organ. Whereas exposure to arsenic has been shown to induce neurotoxicity in children and adults,⁵¹ our study suggests a link between arsenite-induced aberrant cytosine methylation/hydroxymethylation and cognitive/mental disorders. In addition, because Tet enzymes and 5-hmC are present in various embryonic and adult cell types, and play crucial roles in pluripotency as well as embryonic and adult development,⁵² the present work sets the stage for future studies on the roles of arsenic interaction with Tet proteins in perturbing the methylation patterns during development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Binding behavior between $NaAsO_2$ and human Tet proteins. (a) Higher-resolution “ultra-zoom-scan” ESI-MS showing the $[M + 2H]^{2+}$ ions of a synthetic peptide derived from human Tet2 in the absence or presence of As(III). (b) UV absorption spectra of the synthetic Tet2 peptide titrated with increasing concentrations of arsenite. (c) Streptavidin agarose affinity pull-down assay using biotin-As as a probe revealed the binding between As(III) and Tet1 in cells.

(a) 5-mC-containing 11mer (0 min)

(b) 0 μM NaAsO_2 (30 min) (c) 2 μM NaAsO_2 (30 min) (d) 5 μM NaAsO_2 (30 min) **Figure 2.**

LC-ESI-MS for monitoring the Tet1-mediated oxidation of 5-mC in a duplex DNA, d(AGCTC^mCGGTCA)/d(GTGACCGGAGCTG), at 0 and 30 min. Shown are the higher resolution “ultra-zoom-scan” ESI-MS results for monitoring the $[M - 3H]^{3-}$ ions of the initial 5-mC-bearing 11mer DNA (a) and the corresponding reaction mixtures at 30 min after initiation of the Tet1 reaction in the presence of 0 μM (b), 2 μM (c), and 5 μM (d) of NaAsO_2 .

Figure 3.

Time- and dose-dependent alterations of 5-mC (a) and its oxidation products, i.e. 5-hmC (b), 5-foC (c), and 5-caC (d), in duplex DNA, d(AGCTC_mCGGTCA)/d(GTGACCGGAGCTG). The peak intensity for each modification-carrying DNA was quantified by LC-MS (Figures 2 and S1) and expressed as a percentage of the total peak intensities for DNA harboring all four types of modifications ($n = 3$). The p values were calculated using unpaired two-tailed Student's t -test, and the asterisks designate significant differences between arsenite treatments and untreated controls at the same time point (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 4.

NaAsO₂ inhibits the formation of 5-hmdC in HEK293T cells individually overexpressing the catalytic domains of Tet1, Tet2, and Tet3 (Tet1CD, Tet2CD, and Tet3CD). Shown are the levels of 5-hmdC (a) and 5-mdC (b) in cells treated with 0, 1, 2, and 5 μM of NaAsO₂ (*n* = 3). The *p* values were calculated using unpaired two-tailed Student's *t*-test, and the asterisks designate significant differences between arsenite treatments and untreated controls (*, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001).

Figure 5.

NaAsO₂ suppresses the formation of 5-hmdC in mouse embryonic stem cells. Displayed are the levels of 5-hmdC (a) and 5-mdC (b) in cells treated with 0, 1, 2, and 5 μM of NaAsO₂ ($n = 3$). The p values were calculated using unpaired two-tailed t -test, and the asterisks designate significant differences between arsenite treatments and untreated controls (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).