Published in final edited form as:

Toxicol Appl Pharmacol. 2011 November 15; 257(1): 1–13. doi:10.1016/j.taap.2011.08.029.

Interdependent genotoxic mechanisms of monomethylarsonous acid: Role of ROS-induced DNA damage and poly(ADP-ribose) polymerase-1 inhibition in the malignant transformation of urothelial cells

Shawn M. Wnek^a, Christopher L. Kuhlman^a, Jeannie M. Camarillo^a, Matthew K. Medeiros^a, Ke J. Liu^c, Serrine S. Lau^{a,b}, and A.J. Gandolfi^a

^aDepartment of Pharmacology and Toxicology, College of Pharmacy, The University of Arizona, Tucson AZ 85721, United States

^bSouthwest Environmental Health Sciences Center, Department of Pharmacology and Toxicology, The University of Arizona Health Sciences Center, Tucson, AZ 85721, United States

^cDepartment of Pharmaceutical Sciences, College of Pharmacy, The University of New Mexico, Albuquerque NM 87131, United States

Abstract

Exposure of human bladder urothelial cells (UROtsa) to 50 nM of the arsenic metabolite, monomethylarsonous acid (MMA^{III}), for 12 weeks results in irreversible malignant transformation. The ability of continuous, low-level MMA^{III} exposure to cause an increase in genotoxic potential by inhibiting repair processes necessary to maintain genomic stability is unknown. Following genomic insult within cellular systems poly(ADP-ribose) polymerase-1 (PARP-1), a zinc finger protein, is rapidly activated and recruited to sites of DNA strand breaks. When UROtsa cells are continuously exposed to 50 nM MMA^{III}, PARP-1 activity does not increase despite the increase in MMA^{III}-induced DNA single-strand breaks through 12 weeks of exposure. When UROtsa cells are removed from continuous MMA^{III} exposure (2 weeks), PARP-1 activity increases coinciding with a subsequent decrease in DNA damage levels. Paradoxically, PARP-1 mRNA expression and protein levels are elevated in the presence of continuous MMA^{III} indicating a possible mechanism to compensate for the inhibition of PARP-1 activity in the presence of MMA^{III}. The zinc finger domains of PARP-1 contain vicinal sulfhydryl groups which may act as a potential site for MMA^{III} to bind, displace zinc ion, and render PARP-1 inactive. Mass spectrometry analysis demonstrates the ability of MMA^{III} to bind a synthetic peptide representing the zinc-finger domain of PARP-1, and displace zinc from the peptide in a dose-dependent manner. In the presence of continuous MMA^{III} exposure, continuous 4-week zinc supplementation restored PARP-1 activity levels and reduced the genotoxicity associated with MMA^{III}. Zinc supplementation did not produce an overall increase in PARP-1 protein levels, decrease the levels of MMA^{III}-induced reactive oxygen species, or alter Cu-Zn superoxide dismutase levels. Overall, these results present two potential interdependent mechanisms in which

© 2011 Elsevier Inc. All rights reserved.

Correspondence should be addressed to: A. Jay Gandolfi, Ph.D., College of Pharmacy, 1703 E. Mabel, P.O. Box 210207, Tucson, Arizona 85721, Tel: 520-626-7317, Fax: 520-626-2466.

The authors declare no Conflict of Interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

MMA^{III} may increase the susceptibility of UROtsa cells to genotoxic insult and/or malignant transformation: elevated levels of MMA^{III}-induced DNA damage through the production of reactive oxygen species, and the direct MMA^{III}-induced inhibition of PARP-1.

Keywords

Arsenic; Monomethylarsonous acid; Bladder cancer; UROtsa; Genotoxicity; Poly(ADP-ribose) polymerase-1

Introduction

A major issue regarding the discovery of arsenic's mechanism of action following low-level exposures is the multitude of biological systems in which arsenic can elicit toxic effects. A key research concern regarding arsenic toxicity is the potential mechanisms involved in arsenic-induced carcinogenesis. The human urinary bladder is an organ associated with arsenic-induced cancer due in part to the elimination of arsenic via the urinary tract, and the bioconcentration of inorganic arsenic and the multiple methylated metabolites within the urine (Chen et al., 1988; Calderon et al., 1999; Aposhian et al., 2000a, 2000b). Of particular interest is the arsenic metabolite, monomethylarsonous acid (MMA^{III}), which is 20 times more cytotoxic in mammalian cells than inorganic arsenite (As^{III}) (Styblo et al., 2002; Bredfeldt et al., 2006). MMA^{III} conjures particular interest because this metabolite may be responsible for the toxic effects and maladies associated with the human urinary bladder as human produce and excrete a large percentage of MMA^{III} relative to other mammalian species (Vahter, 1994).

To investigate the particular mechanisms involved in arsenical-induced carcinogenesis of the bladder, the immortalized non-tumorigenic human urothelial cell line, UROtsa, has been established as an *in vitro* model to study the molecular changes induced by chronic arsenical exposure (Rossi et al., 2001; Eblin et al., 2008a). Derived from normal urothelium, UROtsa cells are phenotypically similar to the transitional epithelium of the urinary bladder (Rossi et al., 2001). Thus, UROtsa cells provide a useful *in vitro* model to investigate the mechanisms driving arsenical-induced malignant transformation from an initial immortalized state. Recent research has demonstrated the acquisition of initial characteristics of malignant transformation in UROtsa cells following 12 weeks of exposure to 50 nM MMA^{III}, providing a revised timeline to mechanistically evaluate the specific biological changes caused by MMA^{III} leading to malignant transformation (Wnek et al., 2010).

As the specific mechanisms by which arsenic acts as a carcinogen are unknown, multiple mechanisms have been suggested (Ludwig et al., 1998; Kitchin, 2001; Kligerman et al., 2003; Eblin et al., 2006; Kitchin and Wallace, 2008; Jensen et al., 2008). Of these mechanisms, the potential of arsenicals to generate DNA damaging reactive oxygen species (ROS) and inhibit DNA repair processes may play an important underlying role in the development of cancer. DNA damage is recognized as a potential toxicity associated with arsenic exposure (Ding et al., 2005; Klein et al., 2007). Furthermore, research demonstrates that the methylated trivalent arsenicals MMA^{III} and dimethylarsinous acid, DMA^{III}, produce elevated levels of DNA damage when compared to inorganic arsenic (Mass et al., 2001; Amhad et al., 2002; Nesnow et al., 2002; Kligerman et al., 2003). The use of both ROS scavengers and Cu-Zn superoxide dismutase knockout mice suggest the potential role of arsenical-induced ROS in damaging DNA following exposure to either MMA^{III} or DMA^{III} (Wnek et al., 2009; Tennant and Kligerman, 2010). Previous studies in our laboratory have demonstrated that continuous exposure of UROtsa cells to 50 nM MMA^{III} through 52 weeks results in a time-dependent increase in the levels of DNA single-strand breaks, which remain

persistent after a 2-week removal of previous chronic MMA^{III} exposure (Wnek et al., 2009). DNA strand break formation induced by the generation of ROS following exogenous exposure to chronic arsenicals may pose a major threat to genomic integrity. In the presence of a continuous genotoxic insult, base excision repair is a key mechanism involved in the repair of DNA single-strand breaks, and is an essential component in the maintenance of genomic stability.

There are multiple DNA repair enzymes involved in the repair of damaged DNA; however, poly(ADP-ribose) polymerase-1 (PARP-1) is a critical enzyme involved in the initiation of base excision repair following oxidative DNA damage, such as DNA single-strand breaks (Gradwohl et al., 1990; Caldecott, 2007). Despite the existence of 17 different members of PARP, PARP-1 constitutes 90% of the overall cellular poly(ADP-ribosylation) activity (Burkle et al., 2005; Schreiber et al., 2006; Qin et al., 2008). PARP-1 specifically binds to DNA strand breaks in a zinc-dependent manner. The coordination of zinc ion binding between vicinal sulfhydryl groups within the N-terminal zinc-finger domains of PARP-1 is necessary for normal enzyme function (Gradwohl et al., 1990). Following the generation of DNA strand breaks, PARP-1 rapidly binds to the site of damage and undergoes automodification using NAD⁺ as a cofactor. Activation of PARP-1 results in the formation of branched chains of poly(ADP-ribose) polymers (PAR) that bind to PARP-1 and multiple nuclear proteins to facilitate the initiation and recruitment of DNA repair processes (Herceg and Wang, 2001). However, evidence suggests that trivalent arsenicals may inhibit DNA repair enzymes activated in the presence of ROS-induced DNA damage. As the affinity of trivalent arsenicals to bind vicinal sulfhydryl containing peptides and proteins is well established, PARP-1 is a plausible target following MMA^{III} exposure (Kitchin and Wallace, 2008).

The available research examining the ability of continuous, low-level MMA^{III} to elicit genotoxicity and inhibit the DNA repair enzyme, PARP-1, is limited as the majority of studies have focused primarily on biological alterations following acute exposures. In the current study, the revised model of MMA^{III}-induced malignant transformation in UROtsa cells following 12-week exposure to 50 nM MMA^{III} was utilized to establish potential mechanisms leading to the acquisition of a malignant phenotype (Wnek et al., 2010). MMA^{III}-exposed UROtsa cells were used to investigate the generation of DNA single-strand breaks, the induction of ROS, and the inhibition of PARP-1 following continuous low-level MMA^{III} exposure through 12 weeks. The ability of trivalent arsenicals to bind vicinal sulfhydryl groups and displace zinc from the zinc finger domains of PARP-1 is a suggested event causing the arsenical-induced inhibition of PARP-1 (Kitchin and Wallace, 2005; Kitchin and Wallace, 2008). Mass spectrometry analysis reveals the ability of MMA^{III} to displace zinc from a synthetic peptide representing the zinc finger domains of PARP-1. As MMA^{III} may target PARP-1 and compete with zinc for the same binding site within the zinc-finger domain, zinc supplementation was used to restore PARP-1 activity in the presence of continuous MMA^{III} exposure. The results herein, support an interdependent mechanism by which MMA^{III} may contribute to the malignant transformation of UROtsa cells through enhanced genotoxicity as a result of ROS-induced DNA damage and the direct MMA^{III}-induced inhibition of PARP-1.

Materials and methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), antibiotic-antimycotic (100×), 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) and 1× trypsin-ethylenediaminetetraacetic acid (EDTA) (0.25%) were acquired from Gibco Invitrogen/Molecular Probes Corporation (Carlsbad,

CA). Diiodomethylarsine (MMA^{III} iodide, CH_3AsI_2) was prepared by the Synthetic Chemistry Facility Core (Southwest Environmental Health Sciences Center, Tucson AZ) using the technique of Millar et al., 1960. Hydrogen peroxide solution was purchased from Sigma Aldrich Chemical Company (St. Louis, MO). Etoposide was purchased from Trevigen, Inc. (Gaithersburg, MD). Water used in studies was distilled and de-ionized.

Dosing solutions

Dosing solutions and procedures were derived from Bredfeldt et al., 2006. Pure MMA^{III} was stored in ampules at 4 °C. Fresh stock solutions of 25 mM MMA^{III} were made and diluted to a final concentration of 5 μM prior to dosing (1 to 100 dilution) to obtain a final concentration of 0.05 μM (50 nM) MMA^{III} . All dosing solutions were sterile filtered with a 0.2 μm acrodisc and stored in sealed, sterile tubes at 4 °C that were opened only for dosing in a sterile cell culture hood. MMA^{III} solutions in distilled, de-ionized water were stable for approximately 4 months at 4 °C with no degradation observed when monitored using high performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICPMS) (Gong et al., 2001).

Cell culture

UROtsa cells were a generous gift from Drs. Donald and Maryann Sens (University of North Dakota, ND). All cells were cultured in DMEM (Gibco Invitrogen/Molecular Probes Corporation, Carlsbad, CA) supplemented with 5% vol/vol FBS (Gibco Invitrogen/Molecular Probes Corporation, Carlsbad, CA) and 1% vol/vol antibiotic-antimycotic (100 $\times$) (Catalogue # 15240-062, Gibco Invitrogen/Molecular Probes Corporation, Carlsbad, CA) at 37°C with 5% CO_2 as described by Bredfeldt et al. 2004. The MMA^{III} -exposed UROtsa variant cells (URO-MSc and URO-MSc(-) cells) were created in our laboratory as previously reported (Wnek et al., 2010). Cell culture conditions were derived from those previously described by Rossi et al., 2001 and Bredfeldt et al., 2004. All UROtsa and URO-MSc cells used in this study tested negative for the presence of mycoplasma contamination. Cell line DNA fingerprinting was conducted on UROtsa and URO-MSc cells to verify their identity. Parallel cultures of UROtsa cells were maintained in MMA^{III} -free medium and served as passage-matched controls. Duration of MMA^{III} in culture after dosing was determined in previous studies (Wnek et al., 2009). The URO-MSc cells used in the following studies were exposed to continuous 50 nM MMA^{III} exposure (dosed every other day) through 4, 8, and 12 weeks. URO-MSc cells were analyzed either in the presence of continuous MMA^{III} exposure following 4, 8, and 12 weeks [URO-MSc cells: URO-MSc4, URO-MSc8, and URO-MSc12] to investigate cellular alterations in the presence of MMA^{III} , or after the 2-week removal of MMA^{III} following previous 4, 8, or 12 week exposure to 50 nM MMA^{III} [URO-MSc(-) cells: URO-MSc4(-), URO-MSc8(-), URO-MSc12(-)] to examine biological alterations following the removal of continuous MMA^{III} exposure. The 2-week removal of MMA^{III} and cell culture splitting upon cell confluence was to ensure that MMA^{III} was no longer present within URO-MSc(-) cells prior to experimentation.

Alkaline comet assay

The comet assay was performed to measure DNA single-strand breaks. URO-MSc4, URO-MSc8, and URO-MSc12 were utilized and cultured in 100 $\times$ 20 mm tissue culture dishes (BD Falcon, Franklin Lakes, NJ) at 5 $\times$ 10⁵ cells per plate and allowed to reach 90% confluence. Adherent cells were removed via trypsin-EDTA (0.25%), quenched with DMEM containing 5% v/v FBS and 1% antibiotic-antimycotic, and centrifuged at 400 rpm for 5 min. Cells were resuspended in 1 $\times$ PBS (Ca^{2+} and Mg^{2+} free) and counted via trypan blue exclusion assay (Bredfeldt et al., 2006). Cells were washed in ice cold 1 $\times$ PBS, resuspended at 1 $\times$ 10⁵ cell/ml in ice cold 1 $\times$ PBS, and were combined with a low melting

point agarose at a ratio of 1 to 10. Cells in agar were allowed to solidify onto a comet slide (Trevigen Inc., Gaithersburg, MD) and placed in prechilled lysis solution overnight. Comet slide was placed in alkali solution (1 h) followed by horizontal electrophoresis. After electrophoresis, slides were rinsed in distilled water and immersed in 70% ethanol for 5 min and allowed to dry. Prior to fluorescence microscopy, samples were stained with SYBR green (Trevigen Inc., Gaithersburg, MD).

Image analysis

Comet images were viewed via epifluorescence microscopy at excitation and emissions of 494/521 nm. The images were captured using an Olympus IMT-2 inverted fluorescence microscope, using a 20× objective. The camera used was a Hamamatsu ORCA-100 CCD camera (chip resolution 1280 × 1024) in bin2 mode, with the actual image resolution set at 640 × 512 using the SimplePCI version 6.5 (Hamamatsu) instrument control software. At least 75 randomly selected cells were analyzed per *n*-value. An image analysis system based on the Matlab Computer Program (Mathworks, Natick, MA) developed by Dr. Joceline Lega (Department of Mathematics, University of Arizona) was used to quantitate average comet moment.

Analysis of single stranded DNA damage via flow cytometry

Single-stranded DNA in 4, 8, and 12 week MMA^{III}-exposed UROtsa cells and after the 2-week removal of previous MMA^{III} exposure were analyzed via flow cytometry. Cell samples were cultured in 75 cm² tissue culture flasks from Sarstedt (Newton, NC) until confluence was reached. Cells were removed via trypsin-EDTA (0.25%), and resuspended in DMEM supplemented with 5% vol/vol FBS (Gibco Invitrogen/Molecular Probes Corporation, Carlsbad, CA) and 1% vol/vol antibiotic-antimycotic (100×) (Catalogue # 15240-062, Gibco Invitrogen/Molecular Probes Corporation, Carlsbad, CA). Cell count was performed using the trypan blue exclusion assay (Bredfeldt et al., 2006). URO-MSC and URO-MSC(-) cells following 4-, 8-, and 12-week MMA^{III} exposure and UROtsa control cells (1×10^6) were transferred into individual 5 ml polystyrene round bottom tubes (BD Falcon, Franklin Lakes, NJ) and fixed using 1 ml fixative solution (6:1 methanol/PBS solution) for 48 h. Samples were denatured using 1× denaturing buffer, ≥ 99.5% formamide (Sigma Co., St. Louis, MO), for 10 min at 80 °C. Samples were then blocked and stained using a monoclonal antibody (F7-26) (Cell Technology, Mountain View, CA), which recognizes deoxycytidine of single-stranded DNA of at least 25–30 bases in length at a concentration of 10 µl of undiluted primary antibody per 0.5×10^6 cells. Following incubation with the primary antibody, 0.5×10^6 cells were incubated with 10 µl the secondary anti-mouse IgM fluorescein isothiocyanate-labeled antibody (1:10 dilution) (Cell Technology, Mountain View, CA). Samples were analyzed at the Arizona Cancer Center Cytometry Core Facility using a FACScan cytometer (BD Biosciences, San Jose CA) equipped with a 15 mW Argon laser. Cells were analyzed via excitation/emission wavelengths at 488/530 nm.

ROS analysis

MMA^{III}-exposed UROtsa cell variants were cultured in a 96-well plate at 3.5×10^4 cells per well and grown to 80% confluence. Adherent cells were washed with 1× PBS (37 °C) and loaded with 25 µM CM-H₂DCFDA (Molecular Probes/Invitrogen, Carlsbad, CA) suspended in DMEM containing 0.5% v/v FBS for 30 min at 37 °C. Cells were washed with 1× PBS (37 °C) twice and resuspended in DMEM containing no phenol red. As a positive control, adherent UROtsa control cells were exposed to either 100 µM tert-butylhydroperoxide (tBOOH) or 100 µM hydrogen peroxide (1 h) prior to addition of CMH₂DCFDA. Fluorescence emission was measured at 530 nm on a SpectraMax Gemini XS multi-well plate reader (Molecular Devices Co., Sunnyvale, CA) with excitation filter set at 485 nm.

Temperature was maintained at 37 °C and readings were performed every min for a total of 1 h (Wang and Joseph, 1999).

PARP-1 activity analysis

PARP-1 activity in MMA^{III}-exposed UROtsa cells was determined using a semi-quantitative ELISA in a 96-well format. Relative PARP-1 activity was determined through detection of poly (ADP-ribose) polymer, PAR, a polymer produced by PARP-1 upon enzyme activation. PARP-1 activity was assessed in URO-MSC4, URO-MSC8, and URO-MSC12 cells or following the 2-week removal of previous, chronic MMA^{III} exposure [URO-MSC4(-), URO-MSC8(-), and UROMSC12(-) cells]. UROtsa or MMA^{III}-exposed variant cells were grown in culture, lysed, and quantified for protein levels via the bicinchoninic acid assay. PARP-1 activity analysis was performed similar to the methods described previously (Wnek et al., 2009).

Zinc Studies

UROtsa control cells, URO-MSC8 and URO-MSC12 cells were incubated in the continuous presence of 10 or 50 μ M zinc chloride for 4 weeks. The addition of zinc chloride in the presence of MMA^{III} did not decrease the concentration of arsenic in solution following either short-term or long-term incubations. Zinc concentrations used were derived from previous studies (Qin et al., 2008). Zinc chloride supplementation in URO-MSC8 and URO-MSC12 cells was in conjunction with continuous exposure to 50 nM MMA^{III}. As a positive control, UROtsa cells were continuously cultured in a medium enriched with 1 μ M cadmium chloride, a known DNA damaging agent. Cadmium concentrations used were derived from those previously described (Sens et al., 2004). Zinc chloride supplementation (10 or 50 μ M) was in conjunction with continuous exposure to 1 μ M cadmium chloride.

Nucleic acid isolation

UROtsa cells or MMA^{III}-exposed UROtsa variants were plate in 6-well plates (Greiner Bio One, Monroe, NC) at a density of 6×10^5 cells per well and grown as previously described (Eblin et al., 2008b). Nucleic acids were isolated as previously described (Oshiro et al., 2005). Total RNA was isolated from all cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). Samples were quantified using absorbance at 260 nm on the NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, DE).

Real-time reverse transcription-PCR

mRNA expression was measured using quantitative real-time reverse transcription (RT)-PCR (Jensen et al., 2008). Total RNA (250 ng) was reverse transcribed to complementary DNA (cDNA) (Applied Biosystems, Foster City, CA). Converted cDNA was added to Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and specific PARP-1 Taqman Primer/Probe (Applied Biosystems, Foster City, CA) and subjected to real-time PCR analysis using Roche Universal Probe technology (Roche, Branchburg, NJ) using the ABI 7500 Real-Time Detection System (Applied Biosystems, Foster City, CA). Results were calculated using the delta, delta *Ct* method normalized to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) expression for each sample. ABI Assay ID numbers: Hs00242302_m1.

Western blot analysis

UROtsa, MMA^{III}-exposed variants, or zinc supplemented cells were plated in 6-well plates (Greiner Bio One, Monroe, NC) at a density of 5×10^5 cells per well. Plated cells were directly lysed on 6-well plates using radioimmunoprecipitation (RIPA) lysis buffer containing 50 mM Tris-HCl (pH 8.6), 1% NP-40, 0.25% C₂₄H₃₉NaO₄, 150 mM NaCl, 1mM

PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM NaF, 1 mM Na₃VO₄, 1 mM EDTA, and 10 µg/ml protease inhibitor cocktail. The cell lysates were sonicated and centrifuged at 14,000 rpm for 10 min at 4 °C. Supernatant protein concentrations were determined by the bicinchoninic acid assay for protein determination (Sigma-Aldrich, St. Louis, MO). Thirty micrograms of each sample was loaded onto 8% SDS/polyacrylamide gels. Samples were separated via SDS-PAGE with Mini-Protein II (Bio-Rad) and transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Inc./GE Healthcare, Piscataway, NJ) and blocked for 1 h at 4 °C with 5% nonfat dry milk in Tris-buffered saline Tween-20. Blots were incubated overnight at 4 °C with primary antibodies at manufacturer's recommended dilutions: anti-poly (ADP-ribose) polymerase-1 (PARP-1), anti-Cu/Zn superoxide dismutase 1 (SOD1) (Cell Signaling Technology, Danvers, MA), or anti-PAR Affinity Purified Polyclonal Antibody (Trevigen Inc., Gaithersburg, MD) with anti- α -Tubulin (Cell Signaling Technology, Danvers, MA) utilized as a loading control. The appropriate secondary antibody linked to horseradish peroxidase was used for detection of primary antibody. Images were scanned with a Scanjet 5370C (Hewlett Packard, Palo, Alto, CA) at maximum resolution and prepared in Adobe Photoshop 3.0 (San Jose, CA).

Mass spectrometry analysis: MMA^{III} peptide binding

The zinc finger PARP-1 peptide (PARPzf) was commercially synthesized by Genemed Synthesis Inc., (San Antonio, TX) and is comprised of the following amino acid sequence (GRASCKKCSIESIPKDKVPHWYHFSCFWKV). The lyophilized PARPzf peptide was resuspended at a concentration of 1 mM in 10 mM Tris buffer, pH 7.4 containing 0.1 mM of the known reducing agent, Tris(2-carboxyethyl)phosphine (TCEP), to allow the cysteine amino acid residues to remain in the reduced state prior to incubations. Stock concentrations of MMA^{III} were prepared at a concentration of 50 mM and diluted to appropriate working concentrations in 10 mM Tris, pH 7.4 containing 0.1 mM TCEP. Aliquots of 200 µM PARPzf peptide were incubated with Zn(II) (as ZnCl₂) (4 mM, final concentration) for 30 min at 37 °C alone or for competition studies with MMA^{III} (200 nM through 2 µM, final concentrations) for 30 min at 37 °C. Following the total incubation period, samples were diluted to final concentrations in 10 mg/ml α -cyano-4-hydroxycinnamic acid in a 1:1 (v/v) acetonitrile/water solution, and analyzed in duplicate. Samples were dried and analyzed via matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. MALDI-TOF spectra were recorded on an Applied Biosystems Voyager DE-STR instrument with a 2 m flight path, operating in positive ion mode. The instrument was equipped with a nitrogen laser operating at 337 nm. Whole peptide spectra were acquired in reflectron mode over the mass range of 3440 – 3600 dalton (Da) with a laser intensity of 1000 and a low mass gate of 500 Da. Final mass spectra were the summation of 3 sub-spectra, each acquired with 100 laser pulses.

Statistics

Graphs were generated in Microsoft Office Excel (Microsoft Corp., Redmond, WA), and the data within each graph are represented as the means with the error bars in each column representative of the $\pm$ SEM unless noted otherwise in the Figure Legends. The particular statistical analysis used and *p*-value is noted in the corresponding Figure Legends for each experiment. The alkaline comet assay data included an *n* = 4 for each experimental value with a minimum of 75 cells assessed per each *n*-value. Detection of single-strand DNA damage via flow cytometry, ROS analysis, and PARP-1 activity analysis was expressed as the average of four experiments. Data from PARP-1 mRNA expression analysis represent an *n* = 3 for each experimental value. Western blot data for PARP-1, PAR and SOD1 represent a total of four experiments.

Results

DNA strand break formation following continuous low-level MMA^{III} exposure

The formation of DNA strand breaks are a common indicator of genotoxic insult. Previous research in our laboratory has demonstrated an increase in DNA strand breaks within the UROtsa cell model following continuous MMA^{III} (50 nM) exposure through 52 weeks and persistent DNA damage following a 2-week removal of previous chronic MMA^{III} exposure (Wnek et al., 2009). Having resolved an earlier timeline in which continuous MMA^{III} exposure results in the irreversible malignant transformation of UROtsa cells (Wnek et al., 2010), mechanistic studies are focused on assessing the genotoxic potential of continuous low-level MMA^{III} exposure through 12 weeks.

In the present study, UROtsa cells exposed to continuous MMA^{III} (50 nM) through 12 weeks of exposure were examined using the alkaline comet assay and flow cytometry. Using the alkaline comet assay, representative images of single cells exposed to continuous MMA^{III} exposure following 4, 8, and 12 weeks [URO-MSC4, URO-MSC8, and URO-MSC12, respectively] results in a significant increase in DNA single-strand breaks (Fig. 1A). Quantitative data based on the average comet moment (defined as the product of the comet tail length and the fraction of DNA within the comet tail) demonstrate a time-dependent increase in the mean comet moment through 12 weeks of exposure (Fig. 1B). To validate the levels of DNA single-strand breaks determined using the alkaline comet assay, flow cytometry was completed using a monoclonal antibody against single-stranded DNA. Using flow cytometry, comparable results were obtained with a time-dependent increase in DNA single-strand breaks through 4, 8, and 12 weeks MMA^{III} exposure (Fig. 1C). Acute incubation of UROtsa cells with 100 μ M etoposide was used as positive control for the induction of DNA strand breaks (Fig. 1A, 1B, and 1C). These results indicate that within 12 weeks of continuous exposure, low-level MMA^{III} induces a genotoxic insult. The greatest levels of DNA damage occurring at 8 and 12 weeks exposure are consistent with previous findings of UROtsa cells obtaining initial characteristics of malignant transformation.

Elevated levels of ROS following continuous, low-level MMA^{III} exposure

The majority of studies demonstrating arsenical-induced oxidative stress are the result of acute exposures (Kitchin, 2001; Nesnow et al., 2002; Eblin et al., 2006, 2008b). Previous research in our laboratory has demonstrated that chronic 52 week exposure to 50 nM MMA^{III} results in a time-dependent increase in ROS levels and an increase in endogenous levels of superoxide following 52 weeks MMA^{III} exposure (Eblin et al., 2009; Wnek et al., 2009). However, levels of ROS have not been investigated following continuous MMA^{III} exposure prior to 12 weeks.

To determine when initial levels in ROS are elevated through 12 weeks of MMA^{III} exposure UROtsa cells were examined following 4, 8, and 12 weeks 50 nM MMA^{III} exposure. Utilizing the dye, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) spectrofluorometric analysis of ROS levels in UROtsa cells following continuous MMA^{III} exposure resulted in a time-dependent increase in relative fluorescent units; however, statistical significance was only reached after 12 weeks of MMA^{III} exposure (Fig. 2). The relative levels of ROS following 12 weeks of exposure are comparable to the acute exposure of two known ROS-inducing agents tert-butyl hydroperoxide and hydrogen peroxide. The following results demonstrate that in the presence of MMA^{III}, the elevated ROS level at 12 weeks of exposure coincide with increased levels of DNA damage in URO-MSC12 cells (Fig 1B, 1C).

Effect of MMA^{III} on PARP-1 activity, PARP-1 mRNA expression, and PARP-1 protein levels in UROtsa cells

UROtsa cells were exposed continuously to 50 nM MMA^{III} for 4, 8, and 12 weeks in the continuous presence of MMA^{III} [URO-MSC4, URO-MSC8, and URO-MSC12, respectively] or after a 2-week removal of MMA^{III} following previous continuous exposure [URO-MSC4(-), URO-MSC8(-), and URO-MSC12(-), respectively] prior to protein isolation. Following protein isolation, total levels of intracellular poly(ADP-ribose) polymers were analyzed to evaluate overall PARP-1 activity. In the presence of MMA^{III}, PARP-1 activity in URO-MSC4, URO-MSC8, and URO-MSC12 cells are comparable to the levels of PARP-1 activity demonstrated in UROtsa cells (Fig. 3A). Upon a 2-week removal of MMA^{III} following previous exposure, PARP-1 activity is significantly increased in URO-MSC8(-) and URO-MSC12(-) cells (Fig. 3A). Upon assessment of DNA single-strand breaks using flow cytometry, DNA damage in URO-MSC4, URO-MSC8, and URO-MSC12 cells increases in a time-dependent manner through 12 weeks of exposure, consistent with previous results (Fig. 1A, 1B); however, the extent of DNA damage in URO-MSC4(-), URO-MSC8(-), and URO-MSC12(-) cells decreased significantly, similar to the basal levels demonstrated in UROtsa control cells (Fig. 3B). The following results demonstrate that in the presence of MMA^{III} PARP-1 activity do not increase relative to the increase in DNA single-strand breaks; however, following the 2-week removal of MMA^{III} exposure, PARP-1 activity increases coinciding with a decrease in DNA single-strand breaks.

To determine the extent of PARP-1 inhibition following low-level MMA^{III} exposure, PARP-1 inhibitory capacity was examined following acute exposure to increasing concentrations of MMA^{III}. To determine if a dose-response relationship exists between PARP-1 inhibitory capacity and concentration of MMA^{III}, UROtsa cells were treated with increasing, non-cytotoxic concentrations of MMA^{III} (0.1–1000 nM) for 18 h followed by the addition of a known DNA damaging agent, H₂O₂ (100 μ M for 5 min), to stimulate PARP-1 activation. Bredfeldt et al. (2006) demonstrated that only concentrations of MMA^{III} greater than 5 μ M demonstrated cytotoxicity in UROtsa cells. As elevated levels of DNA damage have been demonstrated only at concentrations higher than 5 μ M in UROtsa cells following acute exposures, H₂O₂ was used as a DNA damaging agent to stimulate PARP-1 activity in the presence of noncytotoxic concentrations of MMA^{III}. Increasing concentrations of MMA^{III} strongly affected the extent of H₂O₂-induced PARP-1 activity (Fig. 3C). Concentrations as low as 0.1 nM MMA^{III} led to a significant reduction in the extent of PARP-1 activity. Quantitative assessment demonstrated a time-dependent decrease in PARP-1 activation with increasing concentrations of MMA^{III}. Furthermore, incubation with 1 μ M MMA^{III} caused a 30% decrease in PARP-1 activity. The following results demonstrate the inhibitory capacity of MMA^{III} on PARP-1 activity following acute exposure in the presence of a known DNA damage agent (H₂O₂) and validate the PARP-1 inhibitory capacity of MMA^{III} in UROtsa cells following continuous, low-level exposure.

Interestingly, when PARP-1 mRNA expression was analyzed, UROtsa cells in the presence of continuous MMA^{III}, [URO-MSC4, URO-MSC8, and URO-MSC12, respectively] demonstrated a roughly 2.5 fold increase in PARP-1 mRNA expression levels when compared to control UROtsa cells (Fig. 4A). Meanwhile cells following a 2-week removal of MMA^{III} [URO-MSC4(-), URO-MSC8(-), and URO-MSC12(-), respectively], demonstrated levels of PARP-1 mRNA expression similar to that of UROtsa control (Fig. 4A). Analysis of PARP-1 protein levels at these same time points demonstrated an increase in PARP-1 protein levels in URO-MSC4, URO-MSC8, and URO-MSC12 cells, while PARP-1 protein levels in URO-MSC4(-), URO-MSC8(-), and URO-MSC12(-) cells were similar to that of UROtsa control cells correlating with the changes demonstrated in the PARP-1 transcript levels (Fig. 4B) demonstrating a possible means to compensate for the inhibition of PARP-1 activity in the presence of MMA^{III}.

Molecular interaction of MMA^{III} with zinc finger domain of PARP-1

The two zinc finger domains located in the N-terminal region of PARP-1 may act as a potential site for MMA^{III} to bind, displace zinc ion, and render PARP-1 inactive. The ability of MMA^{III} to bind the zinc finger domain of PARP-1 was investigated using a synthetic peptide representing the zinc-finger domains of PARP-1 (PARPzf) using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. Incubation of the PARPzf peptide (3458.4 Da) (Fig. 5A) with Zn(II) [4 mM] leads to the formation of a new molecule (Zn(II)PARPzf) at (3521.4 Da) (Fig. 5B). The 63-Da shift following incubation of the PARPzf peptide with Zn(II) suggests the PARPzf peptide must release 2 hydrogens before binding to Zn(II). Incubation of the PARPzf peptide (3458.4 Da) with MMA^{III} [500 nM] leads to a 88-Da shift corresponding with the binding of MMA^{III} (MMAPARPzf, 3546.4 Da) to the PARPzf peptide after the release of two hydrogens from the cysteine residues of the representative C3H1 PARPzf peptide (Fig. 5C). These results demonstrate that MMA^{III} can bind the zinc finger domain of PARP-1 with a far greater affinity when compared to Zn(II).

To demonstrate the ability of MMA^{III} to displace Zn(II) from the zinc finger domain of PARP-1, the PARPzf peptide was preincubated with Zn(II) followed by incubation with increasing concentrations of MMA^{III}. Incubation of the PARPzf peptide (3458.4 Da) with Zn(II) [4 mM] resulted in the formation of a new Zn(II)PARPzf molecule (3521.4 Da) demonstrating the PARPzf binding of Zn(II). Following preincubation of the PARPzf peptide with Zn(II), incubation with increasing concentrations of MMA^{III} [200 nM – 2 μ M] caused a dose-dependent decrease in the Zn(II)PARPzf signal (3521.4 Da) while increasing the MMAPARPzf signal intensity (3546.4 Da) (Fig. 6A–F). Overall, these data suggest a competition between Zn(II) and MMA^{III} for the same binding site on the PARPzf peptide and suggest the possibility that MMA^{III} can act to displace Zn(II) from the zinc finger domain of PARP-1 rendering the enzyme inactive in the presence of MMA^{III}-induced DNA damage.

Effects of zinc supplementation on PARP-1 activity and reversal of MMA^{III}-induced DNA damage

Research indicates that PARP-1 is rapidly activated by the generation of DNA strand breaks, and the repair activity of PARP-1 is dependent on binding zinc ion via two zinc-finger domains located within the N-terminal DNA-binding domain of PARP-1 (Gradwohl et al., 1990; Ikejima et al., 1990; Caldecott, 2007). Because trivalent arsenicals may compete with zinc ion at the site of binding, the resulting zinc displacement may inhibit protein function; therefore, zinc supplementation may restore PARP-1 activity in the presence of MMA^{III}-induced DNA damage. Zinc supplementation was used to restore PARP-1 activity in the presence of continuous MMA^{III} exposure, URO-MS8 and URO-MS12 cells were incubated in the presence of zinc chloride (10 or 50 μ M) for 4 weeks (dosed every other day) in conjunction with continuous exposure to 50 nM MMA^{III}. As initially demonstrated, in the presence of continuous MMA^{III}, URO-MS8, and URO-MS12 cells demonstrate PARP-1 activity levels similar to UROtsa control despite having elevated levels of DNA damage. Zinc supplementation increased PARP-1 activity in both URO-MS8 and URO-MS12 cells following continuous 50 μ M supplementation (Fig. 7A). As expected, zinc supplementation alone did not increase PARP-1 activity in UROtsa cells following 4-week supplementation, as UROtsa control cells demonstrate negligible levels of endogenous DNA damage.

To validate the increase in PARP-1 activity, total levels of poly(ADP-ribosylation) of proteins following zinc supplementation (10 or 50 μ M) in URO-MS12 cells were measured via Western blot analysis using a poly(ADP-ribose) (PAR) specific antibody. An

increase in PAR antibody binding to bands around 116 kD (mass of PARP-1) was demonstrated following continuous, 4-week 10 μ M or 50 μ M zinc supplementation in 12-week MMA^{III}-exposed UROtsa cells (URO-MSC12) [Fig. 7B]. To determine whether the effects of zinc supplementation on increased PARP-1 activity were dependent upon an induction of protein, total PARP-1 protein levels were analyzed via Western blot analysis. There were no significant changes in PARP-1 protein levels following supplementation of URO-MSC12 cells with 10 μ M or 50 μ M zinc supplementation (Fig. 7C). Continuous zinc supplementation increased PARP-1 activity in the presence of continuous 50 nM MMA^{III} exposure; the increased PARP-1 activity following zinc supplementation in the presence of MMA^{III} was not a result of PARP-1 protein induction.

The role of PARP-1 enzyme in base excision repair following oxidative DNA damage, including DNA strand breaks, has been suggested as a critical component for the detection and initiation of DNA repair as a result of ROS-induced DNA damage. 4-week zinc supplementation was able to increase PARP-1 activity in the presence of continuous MMA^{III} exposure (Fig. 6A, 6B). Having demonstrated the ability of continuous, 50 nM MMA^{III} exposure to induce a time-dependent increase in DNA single-strand breaks (Fig. 1), it was necessary to investigate whether zinc supplementation would lead to a decrease in DNA damage in URO-MSC cells cultured in the continuous presence of MMA^{III}. URO-MSC8 and URO-MSC12 cells incubated with 10 μ M or 50 μ M zinc supplementation every other day for 4 weeks (in conjunction with continuous 50 nM MMA^{III} exposure) resulted in a significant decrease in the levels of DNA single-strand breaks as measured through the alkaline comet assay (Fig. 7D). Zinc supplementation alone did not increase DNA single-strand break levels in UROtsa control cells. As a positive control, UROtsa cells were exposed to 4-week Cd exposure (1 μ M); as Cd has been demonstrated to elicit genotoxicity (Misra et al., 1998; Mourón et al., 2001; Rozgaj et al., 2002; Mourón et al., 2004), and bind sulfhydryl groups resulting in the displacement of zinc from zinc-finger domains (Predki et al., 1992; Hanas et al., 1996). UROtsa cells exposed to 1 μ M Cd demonstrated an increase in DNA single-strand breaks; following 4-week Cd exposure in conjunction with 10 μ M or 50 μ M zinc supplementation (4 weeks), DNA damage levels were partially decreased (Fig. 7D). Overall, these results suggest that the increase in PARP-1 activity following zinc supplementation plays a role in the abrogation of DNA single-strand breaks following continuous MMA^{III} exposure.

ROS levels following zinc supplementation

Because it has been demonstrated that chronic MMA^{III} exposure in UROtsa cells can induce DNA damage via the generation of ROS (Wnek et al., 2009), it is feasible that continuous zinc supplementation could decrease DNA damage levels via an antioxidant response resulting in a reduction of MMA^{III}-induced ROS levels. Research has suggested a functional role of zinc as an antioxidant; however the exact mechanisms by which zinc can function as an antioxidant are unclear (Bray and Bettger, 1990; Frassinetti et al., 2006). Following continuous, 4-week 10 μ M or 50 μ M zinc supplementation in conjunction with 12-week 50 nM MMA^{III}-exposed UROtsa cells (URO-MSC12), zinc supplementation does not result in a significant decrease in the production of ROS in the presence of continuous MMA^{III} exposure (Fig. 8A). These results validate the elevated levels of ROS previously demonstrated following continuous, 12-week 50 nM MMA^{III} (URO-MSC12) [Fig. 2]. As zinc supplementation did not decrease MMA^{III}-induced ROS levels, it is unlikely that an antioxidant process was responsible for the decrease in MMA^{III}-induced DNA damage.

To further investigate the potential role of antioxidant activities in decreasing the levels of MMA^{III}-induced DNA damage levels following zinc supplementation, Cu-Zn superoxide dismutase (SOD1) levels were analyzed. SOD1 is an essential enzyme in the protection of cells from oxidative stress including one of the main ROS species, superoxide (Kumagai et

al., 1994; Pi et al., 2002). Because SOD1 binds copper and zinc ions, changes in SOD1 protein levels were investigated in UROtsa cells continuously exposed to 50 nM MMA^{III} following 4-week zinc supplementation (10 μ M or 50 μ M). SOD1 protein expression does not see a significant change following 12-week exposure to 50 nM MMA^{III} (URO-MS12), or in URO-MS12 cells following 4-week zinc supplementation when compared to UROtsa control cells (Fig. 8B).

Discussion

The exact mechanism in which arsenicals participate in the development of cancer remains unknown; however, mechanistic evidence suggests the ability of trivalent arsenicals to induce the formation of ROS leading to DNA damage, and causing the direct arsenical-induced inhibition of DNA repair systems vital for the repair of oxidative DNA damage (Petrick et al., 2001; Hartwig et al., 2003; Kligerman et al., 2003). The majority of mechanistic evidence regarding arsenic-induced indirect genotoxicity is limited to the biological alterations following acute exposures; conversely, the vast majority of human exposures are to chronic, low-level concentrations of arsenic. Thus, the UROtsa cell line and continuous, low-level MMA^{III}-exposed UROtsa variants were used to investigate the mechanistic role in which MMA^{III}-induced DNA damage may contribute to malignant transformation of UROtsa cells through the inhibition of the DNA repair enzyme, PARP-1.

Inorganic arsenic and methylated arsenical-induced oxidative stress is a plausible theory in the generation of arsenical-induced carcinogenesis (Kitchin and Wallace, 2008). Experimental evidence demonstrates that both inorganic arsenic and trivalent methylated arsenicals can generate DNA damaging ROS; however, the methylated trivalent arsenicals are associated with higher levels of cytotoxicity and genotoxicity in comparison to inorganic arsenicals following acute exposures (Styblo et al., 2000; Mass et al., 2001; Petrick et al., 2001; Schwerdtle et al., 2003). Since irreversible malignant transformation has been demonstrated in UROtsa cells following 12-week exposure to 50 nM MMA^{III} (Wnek et al., 2010), the current study investigated the ability of MMA^{III} to elicit a genotoxic insult through 12 weeks of exposure. Continuous exposure to 50 nM MMA^{III} through 12-weeks resulted in a time-dependent increase in DNA single-strand breaks. The genotoxicity in UROtsa cells following MMA^{III} exposure, support findings of Kligerman et al., (2003) in which trivalent methylated arsenicals are potent inducers of DNA damage. Elevated levels of ROS at 12 weeks of MMA^{III} exposure coincide with the exposure length in which DNA damage levels are the greatest. The role of ROS in generating DNA damage has been previously demonstrated in UROtsa cells. When 12-week MMA^{III} exposed UROtsa cells were incubated separately with the ROS scavengers, potassium iodide, superoxide dismutase, or catalase each resulted in a significant decrease in the levels of DNA damage measured, supporting the role of MMA^{III}-induced ROS in eliciting a genotoxic insult (Wnek et al., 2009). The results in the current study demonstrate the ability of continuous 50 nM MMA^{III} to generate an increase in genotoxicity in UROtsa cells as early as 8 weeks (URO-MS8), which increases through 12 weeks of exposure (URO-MS12), consistent with characteristics of hyperproliferation, colony growth in soft agar, and tumor growth in immunocompromised mice at 12 weeks of MMA^{III} exposure (Wnek et al., 2010).

The ability of trivalent arsenicals to interfere with DNA repair processes necessary for the repair of oxidative DNA damage may exacerbate the severity of an ROS-induced genotoxic insult. PARP-1 is a DNA-repair protein rapidly activated upon formation of DNA single-strand breaks and is a critical component in the initiation of downstream DNA repair (Gradwohl et al., 1990; Caldecott, 2007). Multiple studies have demonstrated the acute effects of low-level, trivalent arsenical exposure in the inhibition of PARP-1 activity in the presence of an exogenous ROS-inducing agent (Hartwig et al., 2003; Walter et al., 2007;

Qin et al., 2008). Our model evaluates PARP-1 activity through 12 weeks of exposure to 50 nM MMA^{III} in the continuous presence of MMA^{III} [URO-MSC4, URO-MSC8, and URO-MSC12, respectively] or following a 2-week removal of MMA^{III} exposure from 4-, 8-, and 12-week exposed UROtsa cells [URO-MSC4(-), URO-MSC8(-), and URO-MSC12(-), respectively]. Research indicates that upon formation of DNA strand breaks PARP-1 activity normally increases (Hartwig et al., 2003). Despite the increase in MMA^{III}-induced DNA strand breaks following continuous MMA^{III} exposure, PARP-1 activity was similar to that of UROtsa control. After a 2-week removal of MMA^{III}, URO-MSC8(-) and URO-MSC12(-) cells demonstrated a significant increase in PARP-1 activity that coincide with a significant decrease DNA single-strand breaks. Previous research demonstrated no changes in PARP-1 activity in URO-MSC12 cells post 2-wk MMA^{III} removal (Wnek *et al.*, 2009); however, the use of high-passage control UROtsa cells may have obscured the actual increase in PARP-1 activity in URO-MSC8(-) and URO-MSC12(-) cells demonstrated in the current study. Moreover, increasing concentrations of MMA^{III} following acute exposure diminished the extent of H₂O₂-induced PARP-1 activity, further suggesting the ability of MMA^{III} to alter PARP-1 activity. These results suggest the inhibition of PARP-1 in the presence MMA^{III}, which may have a direct impact on the severity of ROS-induced DNA damage. These results suggest the inhibition of PARP-1 in the presence MMA^{III}, which may have a direct impact on the severity of ROS-induced DNA damage as a result of continuous MMA^{III} exposure.

Contrary to the decrease in PARP-1 activity in the continuous presence of MMA^{III}, PARP-1 mRNA expression and protein levels were increased following 4, 8, and 12 weeks MMA^{III} exposure [URO-MSC4, URO-MSC8, and URO-MSC12, respectively]. Following the 2-week removal of MMA^{III}, these levels were similar to that of control. These results were similar to those demonstrated by Komissarova and Rossman (2010) in which 14-day exposure to 0.1 μ M arsenite in HaCaT caused a decrease in total protein poly(ADP-ribosylation) and an increase in total PARP-1 protein levels. It has been suggested that PARP-1 gene transcription is self-regulated via the auto-poly(ADP-ribosylation) of PARP-1 protein. In support, inducible PARP-1 has been reported to inhibit PARP-1 promoter activity via PARP-1 auto-poly(ADP-ribosylation) (Schweiger et al., 1992; Soldatenkov et al., 2002); thus, the inhibition of PARP-1 activity in the presence of MMA^{III} and resulting lack of PARP-1 auto-poly(ADP-ribosylation) may result in the increase in PARP-1 mRNA and protein levels.

PARP-1 is comprised of two zinc-fingers located in the N-terminal domain. The binding of zinc within each of these zinc-fingers is critical for the ability of PARP-1 to bind to DNA single-strand breaks and initiate downstream base excision repair. A plausible explanation for the decrease in PARP-1 activity following continuous exposure to 50 nM MMA^{III} may be the direct interaction of MMA^{III} with PARP-1 through binding of the cysteine amino acid residues located within the zinc-finger domains of PARP-1. Based on the ability of trivalent arsenicals to bind to vicinal sulfhydryl groups, it has been postulated that MMA^{III} may render DNA repair enzymes, such as PARP-1, inactive through the displacement of zinc from within the zinc-finger domains of PARP-1 (Kitchin and Wallace, 2005; Kitchin and Wallace, 2008). Data herein, provides evidence of a direct interaction of MMA^{III} with a PARP-1 zinc finger peptide representative of the first zinc finger within the N-terminal DNA binding domain of human PARP-1. Following incubation of the PARPzf peptide with Zn(II), incubation with MMA^{III} [200 nM – 2 μ M] reduces the interaction of Zn(II) within the PARPzf (Zn(II)PARPzf) and an increases the signal intensity of the MMAPARPzf corresponding to the binding of MMA^{III} to the PARPzf. Recently, Ding *et al.*, 2009 demonstrated via mass spectrometry that arsenite interacts with the synthetic PARPzf peptide representative of the PARP-1 zinc-finger domain, and that increasing concentrations of arsenite diminished the ability of zinc to bind the PARP-1 peptide. However, these

findings demonstrate the binding of MMA^{III} to the PARPzf peptide and subsequent zinc displacement. The binding of MMA^{III} to the PARPzf peptide occurs at concentrations demonstrated in the urine of arsenic exposed populations and are within the range of those used to malignantly transform UROtsa cells (Aposhian et al., 2000; Mandal et al., 2001). The decrease in the Zn(II)PARPzf as a function of increasing MMA^{III} concentration implies a potential competition between Zn(II) and MMA^{III} for the same bind site on the PARPzf peptide at the expense of Zn(II). These results exemplify the high reactivity of MMA^{III} for vicinal sulfhydryl groups, in particular those present in the cysteine amino acids of the PARP-1 zinc finger domain. Recent research has demonstrated the ability of arsenite to binds zinc-finger peptides containing three or four cysteine amino acid residues, but not to zinc-finger peptides containing only two cysteines; these results demonstrate the binding selectivity of arsenite (Zhou et al., 2011). Furthermore, results present PARP-1 as a potential molecular target for low concentrations of MMA^{III} to selectively displace Zn(II) from the zinc finger domain and render the enzyme inactive, contributing to the genotoxicity associated with MMA^{III} exposure.

As evidence suggests the ability of MMA^{III} to displace Zn(II) from the zinc finger domain of PARP-1 and inhibit the increase in PARP-1 activity following MMA^{III}-induced DNA strand breaks, supplementation of zinc in MMA^{III}-exposed UROtsa cells is a plausible treatment to re-establish PARP-1 activity. In the presence of continuous MMA^{III} exposure through 8 and 12 weeks, continuous zinc supplementation (10 μ M or 50 μ M) for 4 weeks significantly increased PARP-1 activity levels. To assess the functional role of increased PARP-1 activity following zinc supplementation in the repair of MMA^{III}-induced DNA damage, the levels of DNA strand breaks were assessed. Zinc supplementation in conjunction with continuous MMA^{III} exposure significantly decreased the levels of DNA single-strand breaks compared to UROtsa cells without zinc supplementation exposed to 50 nM MMA^{III} for 8 and 12 weeks. Zinc supplementation did not increase the overall PARP-1 protein levels; therefore, the increase in PARP-1 activity is not a direct result of PARP-1 protein induction following zinc supplementation. These results support a mechanistic role of continuous, low-level MMA^{III} in the inhibition PARP-1, and maintain previous findings of the importance of PARP-1 activity in the repair of DNA strand breaks. The ability of zinc supplementation in the presence of continuous low-level MMA^{III} to restore PARP-1 activity supports previous research which has demonstrated the ability of acute zinc supplementation to increase relative PARP-1 activity and reverse oxidative damage (8-hydroxyl-2'-deoxyguanosine, 8-OHdG levels) induced by arsenite in human keratinocytes (Qin et al., 2008; Ding et al., 2009).

The mechanism in which zinc supplementation restores PARP-1 activity in the presence of MMA^{III} is unknown. Zinc has been shown to have an antioxidant role in defined chemical systems (Bray and Bettger, 1990). However, following 4-week zinc supplementation (10 μ M or 50 μ M) in the presence of 12-week MMA^{III} exposure, ROS levels were elevated; these ROS levels were similar to the elevated ROS levels demonstrated in 12-week MMA^{III}-exposed UROtsa cells (URO-MSC12). Zinc supplementation did not increase overall protein levels of the antioxidant enzyme, Cu-Zn superoxide dismutase (SOD1). As zinc supplementation did not directly decrease the total levels of MMA^{III}-induced ROS, these data strengthen the possibility that zinc supplementation decreased DNA strand breaks levels through a mechanism other than an antioxidant process.

These results suggest two interdependent mechanisms in which MMA^{III} can elicit indirect genotoxicity: the ability of continuous, low-level exposure to elicit a genotoxic response in the form of DNA single-strand breaks through MMA^{III}-induced ROS generation, and the inhibition of PARP-1, a critical repair enzyme involved with the initiation of DNA strand break repair (Fig. 9). Restoration of PARP-1 activity following zinc supplementation

suggests a biochemical interaction of MMA^{III} with dithiol groups within PARP-1 zinc fingers, which may result in the displacement of zinc ion, rendering PARP-1 inactive in the presence of MMA^{III}-induced DNA damage. However, further studies are necessary to elucidate the exact biochemical role MMA^{III} may play in the displacement of zinc from PARP-1 and determine the significance of MMA^{III}-induced PARP-1 inhibition in the malignant transformation of UROtsa cells. Overall, these data present a plausible mechanism in which MMA^{III}-induced DNA damage can contribute to the acquisition of malignant characteristics in UROtsa cells following 12-weeks of low-level MMA^{III} exposure through the inhibition of PARP-1.

Acknowledgments

The authors would like to thank Drs. Donald and Mary Ann Sens and Dr. Scott Garret for the UROtsa cells and assistance with culturing conditions, Dr. Joceline Lega (Department of Mathematics, University of Arizona) for the comet analysis software, Doug Cromey (SWEHS Imaging Core Facility) for his help with image analysis, Mike Kopplin for ICP-MS analysis (UA SRBP), and the Synthetic Chemistry Facility Core (Southwest Environmental Health Sciences Center, Tucson, AZ) for the Diiodomethylarsine (MMA^{III}). The authors would also like to thank Daniel Troup and Tam Mihn le for performing the MMA^{III}/zinc solubility studies. The research herein was made possible by the NIEHS Superfund Basic Research Program (ES 04940) and the Southwest Environmental Health Sciences Center (ES 06694). SMW is funded by the NIEHS SRP UA Training Core and NIEHS Training Grant (ES 07091).

Supported by NIEHS grants ES04940, ES06694, ES07091

References

- Ahmad S, Kitchin KT, Cullen WR. Plasmid DNA damage caused by methylated arsenicals, ascorbic acid and human liver ferritin. *Toxicol Lett.* 2002; 133:47–57. [PubMed: 12076509]
- Aposhian HV, Gurzau ES, Le XC, Gurzau A, Healy SM, Lu X, Ma M, Yip Li, Zakharyan RA, Maiorino RM, Dart RC, Tircus MG, Gonzalez-Ramirez D, Morgan DL, Avram D, Aposhian MM. Occurrence of monomethylarsonous acid in urine of humans exposed to inorganic arsenic. *Chem Res Toxicol.* 2000a; 13:693–697. [PubMed: 10956055]
- Aposhian HV, Zheng B, Aposhian MM, Le XC, Cebrian ME, Cullen W, Zakharyan RA, Ma M, Dart RC, Cheng Z, Andrewes P, Yip Li, O'Malley GF, Maiorino RM, Van Voorhies W, Healy SM, Titcomb A. DMPA-arsenic challenge test. II Modulation of arsenic species, including monomethylarsonous acid (MMAIII), excreted in human urine. *Toxicol Appl Pharmacol.* 2000b; 165:74–83. [PubMed: 10814555]
- Caldecott KW. Mammalian single-strand break repair: mechanisms and links with chromatin. *DNA Rep.* 2007; 6:443–453.
- Calderon RL, Hudgens E, Le XC, Schreinemachers D, Thomas DJ. Excretion of arsenic in urine as a function of exposure to arsenic in drinking water. *Environ Health Perspect.* 1999; 107:663–667. [PubMed: 10417365]
- Chen CJ, Kuo TL, Wu MM. Arsenic and cancers. *Lancet.* 1988; 1:414–415. [PubMed: 2893213]
- Bredfeldt TG, Kopplin MJ, Gandolfi AJ. Effects of arsenite on UROtsa cells: low-level arsenite causes accumulation of ubiquitinated proteins that is enhanced by reduction in cellular glutathione levels. *Toxicol Appl Pharmacol.* 2004; 198 (3):412–418. [PubMed: 15276421]
- Bredfeldt TG, Jagadish B, Eblin KE, Mash EA, Gandolfi AJ. Monomethylarsonous acid induced transformation of human bladder cells. *Toxicol Appl Pharmacol.* 2006; 216:69–79. [PubMed: 16806342]
- Bray TM, Bettger WJ. The physiological role of zinc as an antioxidant. *Free Radic Biol Med.* 1990; 8 (3):281–291. [PubMed: 2187766]
- Burkle A, Diefenbach J, Brabeck C, Beneke S. Ageing and PARP. *Pharmacol Res.* 2005; 52:93–99. [PubMed: 15911337]
- Ding W, Hudson LG, Liu KJ. Inorganic arsenic compounds cause oxidative damage to DNA and protein by inducing ROS and RNS generation in human keratinocytes. *Mol Cell Biochem.* 2005; 279 (1–2):105–112. [PubMed: 16283519]

- Ding W, Liu W, Cooper KL, Qin XJ, de Souza Bergo PL, Hudson LG, Liu KJ. Inhibition of poly(ADP-ribose) polymerase-1 by arsenite interferes with repair of oxidative DNA damage. *J Biol Chem.* 2009; 284 (11):6809–6817. [PubMed: 19056730]
- Eblin KE, Bowen ME, Crome DW, Bredfeldt TG, Mash EA, Lau SS, Gandolfi AJ. Arsenite and monomethylarsonous acid generate oxidative stress response in human bladder cell line. *Toxicol Appl Pharmacol.* 2006; 217 (1):7–14. [PubMed: 16930658]
- Eblin KE, Bredfeldt TG, Gandolfi AJ. Immortalized human urothelial cells as a model of arsenic-induced bladder cancer. *Toxicology.* 2008a; 248:67–76. [PubMed: 18456381]
- Eblin KE, Hau AM, Jensen TJ, Futscher BW, Gandolfi AJ. The role of reactive oxygen species in arsenite and monomethylarsonous acid-induced signal transduction in human bladder cells: Acute studies. *Toxicol.* 2008b; 250 (1):47–54.
- Eblin KE, Jensen TJ, Wnek SM, Buffington SE, Futscher BW, Gandolfi AJ. Reactive Oxygen Species regulate properties of transformation in UROtsa cells exposed to monomethylarsonous acid by modulating MAPK signaling. *Toxicol.* 2009; 255 (1–2):107–114.
- Frassinetti S, Bronzett G, Caltavuturo L, Cini M, Croce CD. The role of zinc in life: a review. *J Environ Pathol Toxicol Oncol.* 2006; 25 (3):597–610. [PubMed: 17073562]
- Gong Z, Lu X, Cullen WR, Le C. Unstable Trivalent Arsenic Metabolites, Monomethylarsonous Acid and Dimethylarsinous Acid. *J Anal At Spectrom.* 2001; 16:1409–1413.
- Gradwohl G, Menissier De Murcia J, Molinete M, Simonin F, Koken M, Hoeijmakers JHJ, De Murcia G. The second zinc-finger domain of poly(ADP-ribose) polymerase determines specificity for single-stranded breaks in DNA. *Proc Natl Acad Sci USA.* 1990; 87 (8):2990–2994. [PubMed: 2109322]
- Hanas JS, Gunn CG. Inhibition of transcription factor IIIA-DNA interactions by xenobiotic metal ions. *Nucleic Acids Res.* 1996; 24:924–930. [PubMed: 8600461]
- Hartwig A, Blessing H, Schwerdtle T, Walter I. Modulation of DNA repair processes by arsenic and selenium compounds. *Toxicology.* 2003; 193 (1–2):161–169. [PubMed: 14599775]
- Herceg Z, Wang ZQ. Functions of poly(ADP-ribose) polymerase (PARP-1) in DNA repair, genomic integrity and cell death. *Mutat Res.* 2001; 477 (1–2):97–110. [PubMed: 11376691]
- Ikejima M, Noguchi S, Yamashita R, Ogura T, Sugimura T, Gill DM, Miwa M. The zinc fingers of human poly(ADP-ribose) polymerase are differentially required for the recognition of DNA breaks and nicks and the consequent enzyme activation. Other structures recognize intact DNA. *J Biol Chem.* 1990; 265 (35):21907–21913. [PubMed: 2123876]
- Jensen TJ, Novak P, Eblin KE, Gandolfi AJ, Futscher BW. Epigenetic remodeling during arsenical-induced malignant transformation. *Carcinogenesis.* 2008; 29 (8):1500–1508. [PubMed: 18448484]
- Kitchin KT. Recent advances in arsenic carcinogenesis: modes of action, animal model systems, and methylated arsenic metabolites. *Toxicol Appl Pharmacol.* 2001; 172 (3):249–261. [PubMed: 11312654]
- Kitchin KT, Wallace K. Arsenite binding to synthetic peptides based on the Zn finger region and the estrogen binding region of the human estrogen receptor-alpha. *Toxicol Appl Pharmacol.* 2005; 206 (1):66–72. [PubMed: 15963345]
- Kitchin KT, Wallace K. The role of protein binding of trivalent arsenicals in arsenic carcinogenesis and toxicity. *J Inorg Biochem.* 2008; 102 (3):532–539. [PubMed: 18164070]
- Klein CB, Leszczynska J, Hickey C, Rossman TG. Further evidence against a direct genotoxic mode of action for arsenic-induced cancer. *Toxicol Appl Pharmacol.* 2007; 222:289–297. [PubMed: 17316729]
- Kligerman AD, Doerr CL, Tennant AH, Harrington-Brock K, Allen JW, Allen JW, Winkfield E, Poorman-Allen P, Kundu B, Funasaka K, Roop BC, Mass MJ, DeMarini DM. Methylated trivalent arsenicals as candidate ultimate genotoxic forms of arsenic: induction of chromosomal mutations but not gene mutations. *Environ Mol Mutagen.* 2003; 42 (3):192–205. [PubMed: 14556226]
- Komissarova EV, Rossman TG. Arsenite induced poly(ADP-ribosylation) of tumor suppressor P53 in human skin keratinocytes as a possible mechanism for carcinogenesis associated with arsenic exposure. *Toxicol Appl Pharmacol.* 2010; 243:399–404. [PubMed: 20036271]

- Kumagai Y, Shinyashiki M, Sun GF, Shimojo N, Sagai M. An efficient method for purification of cuprozinc superoxide dismutase from bovine erythrocytes. *Experientia*. 1994; 50:673–676. [PubMed: 8033975]
- Ludwig S, Hoffmeyer A, Goebeler M, Kilian K, Hafner H, Neufeld B, Han J, Rapp U. The stress inducer arsenite activates mitogen-activated protein kinases extracellular signal-regulated kinases 1 and 2 via MAPK kinase 6/p38-dependent pathway. *J Biol Chem*. 1998; 273:1917–1922. [PubMed: 9442025]
- Mass MJ, Tennant A, Roop BC, Cullen WR, Styblo M, Thomas DJ, Kligerman AD. Methylated trivalent arsenic species are genotoxic. *Chem Res Toxicol*. 2001; 14 (4):355–361. [PubMed: 11304123]
- Millar IT, Heany H, Heinehey DM, Fernelius WC. Methyliodoarsine. *Inorg Synth*. 1960; 6:113–115.
- Misra R, Smith G, Waalkes M. Evaluation of the direct genotoxic potential of cadmium in four different rodent cell lines. *Toxicology*. 1998; 126:103–114. [PubMed: 9620542]
- Mourón SA, Golijow CD, Dulout FN. DNA damage by cadmium and arsenic salts assessed by the single cell gel electrophoresis assay. *Mutat Res*. 2001; 498:47–55. [PubMed: 11673070]
- Mourón SA, Grillo CA, Dulout FN, Golijow CD. A comparative investigation of DNA strand breaks, sister chromatid exchanges and K-ras gene mutations induced by cadmium salts in cultured human cells. *Mutat Res*. 2004; 568 (2):221–231. [PubMed: 15542109]
- Nesnow S, Roop BC, Lambert G, Kadiiska M, Mason RP, Cullen WR, Mass MJ. DNA damage induced by methylated trivalent arsenicals is mediated by reactive oxygen species. *Chem Res Toxicol*. 2002; 15 (12):1627–1634. [PubMed: 12482246]
- Oshiro MM, Kim CJ, Wozniak RJ, Junk DJ, Munoz-Rodrigues JL, Burr JA, Fitzgerald M, Pawar S, Cress AE, Domann FE, et al. Epigenetic silencing of DSC3 is a common event in human breast cancer. *Breast Cancer*. 2005; 7:669–680.
- Petrick JS, Jagadish B, Mash EA, Aposhian HV. Monomethylarsonous acid (MMAIII) and arsenite: LD(50) in hamsters and in vitro inhibition of pyruvate dehydrogenase. *Chem Res Toxicol*. 2001; 14 (6):651–656. [PubMed: 11409934]
- Pi J, Yamauchi H, Kumagai Y, Sun G, Yoshida T, Aikawa H, Hopenhayn-Rich C, Shimojo N. Evidence for induction of oxidative stress caused by chronic exposure of Chinese residents to arsenic contained in drinking water. *Environ Health Perspect*. 2002; 110 (4):331–336. [PubMed: 11940449]
- Predki PF, Sarkar B. Effect of replacement of ‘zinc finger’ zinc on estrogen receptor DNA interactions. *J Biol Chem*. 1992; 267:5842–5846. [PubMed: 1556100]
- Qin XJ, Hudson LG, Liu W, Ding W, Cooper KL, Liu KJ. Dual actions involved in arsenite-induced oxidative DNA damage. *Chem Res Toxicol*. 2008; 21 (9):1806–1813. [PubMed: 18707137]
- Rossi MR, Masters JR, Park S, Todd JH, Garrett SH, Sens MA, Somji S, Nath J, Sens DA. The immortalized UROtsa cell line as a potential cell culture model of human urothelium. *Environ Health Perspect*. 2001; 109:801–808. [PubMed: 11564615]
- Rozgaj R, Kašuba V, Fučić A. Genotoxicity of cadmium chloride in human lymphocytes evaluated by the comet assay and cytogenetic test. *J Trace Elem Med Biol*. 2002; 16:187–192. [PubMed: 12437157]
- Sens DA, Park S, Gurel V, Sens MA, Garrett SH, Somji S. Inorganic cadmium- and arsenite-induced malignant transformation of human bladder urothelial cells. *Toxicol Sci*. 2004; 79:56–63. [PubMed: 14976345]
- Schreiber V, Dantzer F, Ame JC, de Murcia G. Poly(ADP-ribose): novel functions for an old molecule. *Nat Rev Mol Cell Biol*. 2006; 7:517–528. [PubMed: 16829982]
- Schweiger, M.; Auer, B.; Herzog, H.; Hirsch-Kauffmann, M.; Kaizer, P.; Flick, K.; Nagl, U.; Schneider, R. ADP-Ribosylation reactions. Springer-Verlag; New York: 1992. p. 20-30.
- Schwerdtle T, Walter I, Mackiw I, Hartwig A. Induction of oxidative DNA damage by arsenite and its trivalent and pentavalent methylated metabolites in cultured human cells and isolated DNA. *Carcinogenesis*. 2003; 24 (5):967–974. [PubMed: 12771042]
- Soldatenkov VA, Chasovskikh S, Potaman VN, Trofimova I, Smulson ME, Dritschilo A. Transcriptional repression by binding of poly(ADP-ribose) polymerase to promoter sequences. *J Biol Chem*. 2002; 277 (1):665–670. [PubMed: 11684688]

- Styblo M, Del Razo LM, Vega L, Germolec DR, LeCluyse EL, Hamilton GA, Reed W, Wang C, Cullen WR, Thomas DJ. Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. *Arch Toxicol*. 2000; 74 (6):289–299. [PubMed: 11005674]
- Styblo M, Drobná Z, Jaspers I, Lin S, Thomas DJ. The role of biomethylation in toxicity and carcinogenicity of arsenic: a research update. *Environ Health Perspect*. 2002; 110:767–771. [PubMed: 12426129]
- Tennant AH, Kligerman AD. Superoxide dismutase protects cells from DNA damage induced by trivalent methylated arsenicals. *Environ Mol Mutagen*. 2010;10.1002/em.20609
- Vahter M. Species differences in the metabolism of arsenic compounds. *Appl Organomet Chem*. 1994; 8:175–182.
- Walter I, Schwerdtle T, Thuy C, Parsons JL, Dianov GL, Hartwig A. Impact of arsenite and its methylated metabolites on PARP-1 activity, PARP-1 gene expression and poly(ADP-ribosyl)ation in cultured human cells. *DNA Repair (Amst)*. 2007; 6 (1):61–70. [PubMed: 17011244]
- Wang H, Joseph JA. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Rad Biol Med*. 1999; 27:612–616. [PubMed: 10490282]
- Wnek SM, Medeiros MK, Eblin KE, Gandolfi AJ. Persistence of DNA damage following exposure of human bladder cells to chronic monomethylarsonous acid. *Toxicol Appl Pharmacol*. 2009; 241 (2):202–209. [PubMed: 19699219]
- Wnek SM, Jensen TJ, Severson PL, Futscher BW, Gandolfi AJ. Monomethylarsonous acid produces irreversible events resulting in malignant transformation of a human bladder cell line following 12 weeks of low-level exposure. *Toxicol Sci*. 2010; 116 (1):44–57. [PubMed: 20375083]
- Zhou X, Sun X, Cooper KL, Wang F, Liu KJ, Hudson LG. Arsenite interacts selectively with zinc finger proteins containing C3H1 or C4 motifs. *J Biol Chem*. 2011; 286 (26):22855–22863. [PubMed: 21550982]

Highlights

- Time-dependent increase in DNA strand breaks in UROtsa cells following 4-, 8-, and 12-wk 50 nM MMA^{III} exposure.
- ROS levels are elevated above UROtsa control cells following 12-wk 50 nM MMA^{III} exposure.
- In the presence of MMA^{III} PARP-1 activity is inhibited despite the increase in DNA strand breaks.
- MMA^{III} can directly displace zinc from PARP-1 zinc-finger domain rendering enzyme inactive.
- Zinc supplementation was able to restore PARP-1 activity in the presence of continuous MMA^{III} exposure.

Figure 1.

Analysis of single-stranded DNA damage levels in UROtsa cells continuously exposed to 50 nM MMA^{III} through 12 weeks. **(A)** Representative fluorescent images of UROtsa cells continuously exposed to 50 nM MMA^{III} for 4, 8, and 12 weeks [URO-MSC4, URO-MSC8, and URO-MSC12, respectively]. **(B)** Alkaline comet assay analysis of the average comet moment. (*) Marks statistically significant difference ($p \leq 0.05$) in DNA single-strand break levels when compared to UROtsa control cells using Student's *t*-test. **(C)** Flow cytometric analysis of single-stranded DNA damage in UROtsa cells continuously exposed to 50 nM MMA^{III} for 4, 8, and 12 weeks ($n = 4$). (*) Marks statistically significant difference ($p \leq 0.05$) in DNA single-strand break levels when compared to UROtsa control cells using Student's *t*-test.

Figure 2.

Detection of ROS generation in UROtsa cells continuously exposed to 50 nM MMA^{III} through 12 weeks. UROtsa cells continuously exposed to 50 nM MMA^{III} for 4, 8, and 12 weeks [URO-MSC4, URO-MSC8, and URO-MSC12, respectively]. (*) Marks a statistically significant difference ($p \leq 0.05$) in ROS levels when compared to UROtsa control cells using Student's *t*-test; $n = 4$.

Figure 3.

Comparison of relative PARP-1 activity and single-stranded DNA damage levels in UROtsa cells in the presence of MMA^{III} (URO-MSC cells) or following removal of previous, chronic MMA^{III} exposure (URO-MSC(-) cells). **(A)** Relative PARP-1 activity in the presence of continuous 50 nM MMA^{III} exposure through 4, 8, and 12 weeks [URO-MSC4, URO-MSC8, and URO-MSC12, respectively] or following the 2-week removal of 50 nM MMA^{III} exposure following previous MMA^{III} exposure through 4, 8, and 12 weeks [URO-MSC4(-), URO-MSC8(-), and URO-MSC12(-), respectively]; $n = 4$. (*) Marks a statistically significant difference ($p \leq 0.05$) in PARP-1 activity between URO-MSC8(-) and URO-MSC12(-) cells compared to UROtsa control cells identified using Student's t -test. (†) Marks a statistically significant difference ($p \leq 0.05$) between URO-MSC8/URO-MSC8(-) and URO-MSC12/URO-MSC12(-) determined using Student's t -test. **(B)** Analysis of single-stranded DNA damage in the presence of continuous 50 nM MMA^{III} exposure for 4, 8, and 12 weeks or following the 2-week removal of 50 nM MMA^{III} exposure following previous, chronic MMA^{III} exposure through 4, 8, and 12 weeks; $n = 4$. (*) Marks statistically significant difference ($p \leq 0.05$) in DNA single-strand break levels when compared to UROtsa control cells using Student's t -test. (†) Marks a statistically significant difference ($p \leq 0.05$) between URO-MSC8/URO-MSC8(-) cells and URO-MSC12/URO-MSC12(-) cells determined using Student's t -test. **(C)** Inhibition of H₂O₂-induced PARP activation by acute, low-level MMA^{III}. Graph demonstrates relative PARP-1 activity in UROtsa cells following 18 h pre-incubation with increasing concentrations of MMA^{III} (0.1–1000 nM) followed by acute exposure (5 min) to 100 μ M H₂O₂; $n = 4$. (*) Marks a statistically significant difference ($p \leq 0.05$) compared to UROtsa control cells identified using Student's t -test.

Figure 4.

Assessment of PARP-1 mRNA and protein expression levels in UROtsa cells in the presence of MMA^{III} (URO-MSC cells) or following removal of previous, chronic MMA^{III} exposure (URO-MSC(-) cells). **(A)** Quantitative real-time RT-PCR expression of PARP-1 mRNA in the presence of continuous 50 nM MMA^{III} exposure for 4, 8, and 12 weeks [URO-MSC4, URO-MSC8, and URO-MSC12, respectively], or following the 2-week removal of 50 nM MMA^{III} exposure following previous MMA^{III} exposure through 4, 8, and 12 weeks [URO-MSC4(-), URO-MSC8(-), and URO-MSC12(-), respectively]. (*) Marks

statistically significant increase in transcript levels when compared with control UROtsa ($p \leq 0.05$) using Student's t-test; $n = 3$. **(B)** PARP-1 protein expression of parental UROtsa and MMA^{III}-exposed UROtsa variants (URO-MSC/URO-MSC(-) cells). (*) Marks statistically significant increase in PARP-1 protein levels when compared with UROtsa control cells ($p \leq 0.05$) using Student's t-test; $n = 4$.

Figure 5.

Analysis of Zn(II) and/or MMA^{III} binding to PARPzf peptide via MALDI-TOF-MS. **(A)** Initial PARPzf peptide was detected using MALDI-TOF-MS with a mass of 3458.4 Da. **(B)** Zn(II) in the form of zinc chloride [4 mM] was incubated with PARPzf peptide for 30 min at 37°C. Incubation of PARPzf peptide with Zn(II) leads to the formation of Zn(II)PARPzf at 3521.4 Da peak resulting from a 63-Da shift corresponding to Zn(II) binding after the release of 2 hydrogens. **(C)** MMA^{III} [500 nM] was incubated with PARPzf peptide for 30 min at 37°C. Incubation of MMA^{III} with PARPzf peptide leads to the formation of MMAPARPzf at 3546.4 Da peak resulting from the 88-Da shift corresponding to MMA^{III} binding after the release of 2 hydrogens.

Figure 6. Analysis of MMA^{III}-induced Zn(II) displacement from PARPzf peptide via MALDI-TOF-MS. (A) Initial PARPzf peptide was detected using MALDI-TOF-MS with a mass of 3458.4 Da. (B) Incubation of PARPzf peptide with Zn(II) leads to the formation of Zn(II)PARPzf at 3521.4 Da peak resulting from a 63-Da shift corresponding to Zn(II) binding after the release of 2 hydrogens. (C-E) MMA^{III} binding to the PARPzf peptide following increasing concentrations of MMA^{III} [200 nm – 2 μM]. MMA^{III} binding is indicated by a 88-Da shift and increase in the MMAIII-PARPzf (3546.4 Da) peak from the original PARPzf peptide (3458.4 Da) peak and the subsequent reduction of the 3521.4 Da peak representative of Zn(II) binding to the PARPzf peptide (Zn(II)PARPzf).

C.

D.

Figure 7.

Relative PARP-1 activity of MMA^{III}-exposed UROtsa and levels of MMA^{III}-induced DNA damage cells following zinc supplementation. (A) Relative PARP-1 activity in URO-MSC8 and URO-MSC12 cells in conjunction with 4-week exposure to 10 µM or 50 µM zinc supplementation. (*) Marks a statistically significant difference ($p \leq 0.05$) in PARP-1 activity compared to UROtsa control identified using Student's *t*-test. (†) Marks a statistically significant difference ($p \leq 0.05$) between URO-MSC8 cells supplemented with

50 μ M zinc and URO-MSC8 cells determined using Student's *t*-test. ($\dagger\dagger$) Marks a statistically significant difference ($p \leq 0.05$) between URO-MSC12 cells supplemented with 50 μ M zinc and URO-MSC12 cells determined using Student's *t*-test; $n = 4$. **(B)** Validation of increase in poly(ADP-ribosyl)ation of proteins in URO-MSC12 cells following zinc supplementation (10 or 50 μ M). (*) Marks statistically significant increase in poly(ADP-ribosyl)ated protein levels when compared with UROtsa control ($p \leq 0.05$) using Student's *t*-test; $n = 4$. **(C)** PARP-1 protein expression of parental UROtsa, URO-MSC12 and URO-MSC12 cells following 4-week zinc supplementation (10 μ M or 50 μ M); $n = 4$ for all test groups. **(D)** The graph demonstrates the average comet moment of URO-MSC8 and URO-MSC12 cells in conjunction with 4-week zinc supplementation (10 μ M or 50 μ M). (*) Marks a statistically significant difference ($p \leq 0.05$) in DNA single-strand break levels when compared to UROtsa control cells using Student's *t*-test. ($\dagger$) Marks a statistically significant difference ($p \leq 0.05$) compared to URO-MSC8 determined using Student's *t*-test. (#) Marks a statistically significant difference ($p \leq 0.05$) compared to URO-MSC12 determined using Student's *t*-test. (\$) Marks a statistically significant difference ($p \leq 0.05$) compared to 4-week Cd-exposed UROtsa cells determined using Student's *t*-test ($n = 4$).

Figure 8. Effect of zinc supplementation on the levels of MMA^{III}-induced ROS in 12-week MMA^{III}-exposed UROtsa cells. **(A)** Relative fluorescent units (RFU) of UROtsa cells exposed to continuous 50 nM MMA^{III} exposure for 12 weeks (URO-MSC12) and URO-MSC12 cells in conjunction with 4-week zinc supplementation (10 μM or 50 μM). (*) Marks statistically significant difference ($p \leq 0.05$) in ROS levels when compared to UROtsa control cells using Student's *t*-test; $n = 4$. **(B)** SOD1 protein expression of parental UROtsa, 12-week MMA^{III}-exposed UROtsa cells (URO-MSC12) and URO-MSC12 cells following 4-week zinc supplementation (10 μM or 50 μM). No significant changes in SOD1 protein levels were determined following zinc supplementation using the Student's *t*-test; $n = 4$.

Figure 9.

Diagram demonstrating the indirect roles of MMA^{III} in eliciting genotoxicity following chronic, low-level exposures. Continuous, low-level MMA^{III} elicits a genotoxic response in the form of DNA single-strand breaks through ROS generation and demonstrates the potential to inhibit PARP-1, a critical repair enzyme involved in the initiation of DNA strand break repair. The ability of trivalent arsenicals such as MMA^{III} to bind sulfhydryl groups may play a role in repair enzyme inhibition as zinc supplementation as been shown to increase PARP-1 activity and diminish MMA^{III}-induced DNA damage.