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# Effect of dietary treatment with dimethylarsinous acid ($DMA^{III}$) on the urinary bladder epithelium of arsenic (+3 oxidation state) methyltransferase (As3mt) knockout and C57BL/6 wild type female mice

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ABSTRACT

Chronic exposure to inorganic arsenic (iAs) is carcinogenic to the human urinary bladder. It produces urothelial cytotoxicity and proliferation in rats and mice. $DMA^{V}$, a major methylated urinary metabolite of iAs, is a rat bladder carcinogen, but without effects on the mouse urothelium. $DMA^{III}$ was shown to be the likely urinary metabolite of $DMA^{V}$ inducing urothelial changes and is also postulated to be one of the active metabolites of iAs. To evaluate potential $DMA^{III}$-induced urothelial effects, it was administered to As3mt knockout mice which cannot methylate arsenicals. Female C57BL/6 wild type and As3mt knockout mice (10/group) were administered $DMA^{III}$, 77.3 ppm in water for four weeks. Urothelial effects were evaluated by light and scanning electron microscopy (EM) and immunohistochemical detection of bromodeoxyuridine (BrdU) incorporation. EM findings were rated 1–5, with higher rating indicating greater extent of cytotoxicity visualized. $DMA^{III}$ significantly increased the BrdU labeling index, a ratio of BrdU labeled cells to non-labeled cells, in the treated knockout group compared to control and wild type treated groups. $DMA^{III}$ induced simple hyperplasia in more knockout mice (4/10) compared to wild type mice (2/10). All treated knockout mice had more and larger intracytoplasmic granules compared to the treated wild type mice. Changes in EM classification were not significant. In conclusion, $DMA^{III}$ induces urothelial toxicity and regenerative hyperplasia in mice and most likely plays a role in inorganic arsenic-induced urothelial changes. However, $DMA^{V}$ does not induce hyperplasia in mice, suggesting that urinary concentrations of $DMA^{III}$ do not reach cytotoxic levels in $DMA^{V}$-treated mice.



## 1. Introduction

Inorganic arsenic (iAs) is a known human carcinogen producing tumors in urinary bladder, liver, lung, and skin (National Research Council, 1999, 2001). This metalloid occurs mainly in the +5 (arsenate) or +3 (arsenite) oxidation states, and is the 20th most abundant chemical in the earth's crust (IARC, 2004). Because it is present in soils, it can be leached into ground water. As-contaminated ground water is used as drinking water by populations worldwide and constitutes an important source of exposure to this toxicant that is associated with increased incidence of both cancer and non-cancer health effects (Chiou et al., 1995). Although dose–response relations for these adverse health effects are not fully understood (Lamm et al., 2006), it is clear that arsenic exposure contributes to tumor risk (National Research Council, 1999, 2001).

Elucidating the processes underlying the adverse health effects of chronic arsenic exposure is complicated by the extensive metabolism that follows ingestion or inhalation of iAs. Mono-, di-, and trimethylated metabolites are found in tissues and excreta of animals exposed to iAs. The formation of methylated arsenicals is catalyzed in many mammalian species by arsenic (3+ oxidation state) methyltransferase (As3mt) (Thomas et al., 2007). In the Challenger scheme, a series of reactions starting with arsenite produces methylarsonic acid ($MMA^{V}$) which is reduced to methylarsonous acid ($MMA^{III}$) that is converted to dimethylarsinic acid ($DMA^{V}$) and reduced to form dimethylarsinous acid ($DMA^{III}$). In some species, particularly rats and to lesser extent other rodents, $DMA^{III}$ is further methylated to form trimethylarsine oxide ($TMA^{V}O$) (Adair et al., 2007). $TMA^{V}O$ is only formed in humans when there is very high exposure to inorganic arsenic (Aposhian, 1997; Cohen et al., 2006; Thomas, 2007). These methylated metabolites of iAs exert specific

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toxicities in animal models. For example, $DMA^V$ produces urinary bladder tumors in rats but is not carcinogenic in mice (Arnold et al., 2006; Wei et al., 1999, 2002). $DMA^{III}$ is thought to be the reactive metabolite that produces changes in the urothelium associated with exposure to $DMA^V$. Development of urinary bladder tumors in $DMA^V$-exposed rats is possibly related to regenerative proliferation caused by the cytotoxic effect of the reactive metabolite $DMA^{III}$ (Cohen et al., 2006, 2007; Waalkes et al., 2006). In contrast, $MMA^V$ is negative as a carcinogen in rat and mouse bioassays (Arnold et al., 2006) but is a transplacental carcinogen in the mouse (Tokar et al., 2012). Thus, because the metabolism of iAs forms methylated metabolites that are more reactive and toxic than the parent compound (Cohen et al., 2002; Petrick et al., 2000; Styblo et al., 2000), it is likely that the methylated arsenicals contribute to the spectrum of adverse health effects associated with chronic exposure to iAs (Cohen et al., 2006).

Given the central role of arsenic methylation in the formation of metabolites that mediate the toxic and carcinogenic effects associated with chronic exposure to iAs, manipulation of the capacity to methylate arsenic is one approach to understanding underlying molecular processes. Studies in As3mt knockout mice have shown that this enzyme plays a central role in the control of the disposition and retention of arsenic in arsenate-treated mice (Drobna et al., 2009; Hughes et al., 2010). Studies of the effects of arsenite exposure in wild-type and As3mt knockout mice have shown that changes in the urothelium are qualitatively similar but more pronounced in knockout mice than in wild-type mice (Chen et al., 2011; Yokohira et al., 2010, 2011). The genotypic differences in the effects of arsenite exposure on the urothelium can largely be explained by the high retention of iAs in the urinary bladders of As3mt knockout mice.

The present study examines effects on urothelial structure after repeated exposure of wild-type and As3mt knockout mice to $DMA^{III}$, the putative active metabolite of $DMA^V$. Here, the As3mt genotype affected morphological changes in the urothelium with a greater response in As3mt knockout mice than in wild-type mice. Evidence of regenerative proliferation in mice of either As3mt genotype suggests that cytotoxicity and cell regeneration could plausibly underlie urinary bladder tumorigenesis in the mouse.

## 2. Materials and methods

### 2.1. Chemicals

Dimethylarsinous iodide ($DMA^{III}$) was obtained from Eurolabs Ltd. (Cheshire, UK) and stored desiccated at 2–8 °C. The purity of the chemical was greater than 95%. Bromodeoxyuridine (BrdU) was purchased from (Sigma–Aldrich, St. Louis, MO). Nembutal (Lundbeck, Deerfield, IL) was obtained from the Nebraska Medical Center pharmacy.

### 2.2. Animals

#### 2.2.1. As3mt knockout mice

Four female and two male mice homozygous for the disrupted As3mt gene (Drobna et al., 2009) were obtained from Dr. David Thomas (U.S. Environmental Protection Agency, Research Triangle Park, NC). Exons 3 through 5 were deleted by homologous recombination to generate the As3mt KO homozygous mice. The altered gene was introduced and maintained in strain 129S6 mice before being introduced into strain C57BL/6 mice by marker-assisted accelerated backcrossing performed by Charles River Laboratories (Wilmington, MA) to produce homozygous $As3mt^{-/-}$ mice. The mice were fertile, so brother/sister matings were used to maintain the homozygous As3mt KO genotype. Twenty As3mt KO mice from the F6 generation, approximately 11 weeks old, were transferred from the breeding colony at the University of Nebraska Medical Center, Omaha, NE.

#### 2.2.2. Wild type mice

Twenty female C57BL/6 mice (Charles River Breeding Laboratories, Kingston, NY) approximately 11 weeks old at the time of arrival were used in the study.

### 2.3. Animal experiment

All animals were placed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility and quarantined for 7 days prior to treatment. The level of care provided met or exceeded the basic requirements outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). The animals were housed in polycarbonate cages (5 per cage) with micro-isolator tops and dry corncob bedding. Nestlets (Ancare, Bellmore, NY) were placed inside the cages for environmental enrichment. Animals were maintained at approximately 22 °C and 50% relative humidity with a 12-h light/dark schedule, and were provided with pelleted Purina 5002 diet (Dyets Inc., Bethlehem, PA) and water (hyperchlorinated, RO) ad libitum throughout the study.

Animals were randomized into two groups per genotype. One group in each genotype served as the control and the other group was administered 77.3 ppm dimethylarsinous iodide (equivalent to 25 ppm elemental arsenic that previously produced urothelial hyperplastic changes in these mice after 4 weeks of treatment (Yokohira et al., 2010, 2011)) in drinking water by injecting into water bags (Hydropac® bags, Lab Products, Seaford, DE). Freshly prepared $DMA^{III}$ was injected into bags just before replacement. The water bags in both the control and $DMA^{III}$-treated groups were replaced once every two days.

The animals were treated for four weeks. One hour before sacrifice the mice were injected with BrdU, 100 mg/kg intraperitonitally. At sacrifice the urinary bladders were inflated in situ with Bouin's fixative, removed and placed in Bouin's fixative along with a small section of intestine. After fixation, bladders were weighed, observed macroscopically and divided in half longitudinally. One half of the bladder was cut into longitudinal strips, embedded into paraffin with a slice of intestine, sectioned and stained with hematoxylin and eosin for histopathological examination. A diagnosis of mild hyperplasia was made when there were four to five cell layers in the bladder epithelium and moderate hyperplasia when six to eight layers were present. Histopathological diagnosis is made without knowledge of treatment group. The other half of each bladder was processed for examination by scanning electron microscopy (EM) and classified in one of five categories as previously described (Cohen et al., 1990). The categories have the following characteristics: class 1 bladders contain polygonal superficial urothelial cells; class 2 bladders have occasional small foci of superficial urothelial necrosis, especially in the dome of the bladder; class 3 bladders have numerous small foci of superficial urothelial necrosis; class 4 bladders have extensive superficial urothelial necrosis, especially in the dome of the bladder; class 5 bladders have necrosis and piling up (hyperplasia) of rounded urothelial cells. Normal animals show class 1 or 2, or occasionally class 3. Kidneys were removed, fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin and examined histopathologically.

Unstained bladder and intestinal tissue slides were processed for immunohistochemical detection of BrdU (Cohen et al., 2007). Intestinal tissue served as a positive control. Anti-BrdU (Millipore Corporation, Temecula, CA) was used at a dilution of 1:200. The BrdU-labeled cells in at least 3000 urothelial cells were counted to determine a labeling index. Microscopic examination and counting of BrdU-labeled cells was done on blinded slides.

### 2.4. Statistics

Comparison of all data collected on body weights, food and water consumption, bladder weights, and the labeling index was performed by the SAS general linear models procedure and Duncan's multiple range test. Selected intra-genotype and inter-genotype comparisons for control and treated groups were performed. All means are accompanied by calculation of standard errors. Histology results were analyzed using Fisher's Exact test (2-tail).

## 3. Results

### 3.1. General findings

All animals including treated KO mice showed no adverse signs, and none died during the experimental period. There was no significant difference in body weight or food and water intake between respective groups. There was a statistically significant but small increase of the relative kidney weight in treated compared to the control knockout mice.

### 3.2. Histopathology

Simple hyperplasia was observed in 4 of the 10 $DMA^{III}$-treated knockout mice and only 2 of the 10 treated wild-type mice, with none in the control knockout or wild-type mice (Fig. 1C, F and Table 1). The superficial layer of the urothelium contained eosinophilic intracytoplasmic granules (Suzuki et al., 2008)