



UNIVERSITY OF
NEBRASKA
MEDICAL CENTER

PATHOLOGY AND MICROBIOLOGY

Dr. Angela Nugent, Designated Federal Officer
US EPA Science Advisory Board (1400R)
1200 Pennsylvania Avenue, NW
Washington, DC 20460

Dear Dr. Nugent,

The following comments are concerning the draft ‘Toxicological Review of Inorganic Arsenic’ (2010 IRIS draft) and its review by the Science Advisory Board (SAB). I will also be presenting brief comments to the SAB during the teleconference scheduled for November 22, 2010, which will review the report of the inorganic arsenic working group on its assessment of the sufficiency of US EPA’s responses to the review of the 2005 draft regarding inorganic arsenic, which was published in 2007.

My comments will focus primarily on the issue of mode of action (MOA). In the 2005 draft and in the 2007 SAB report, there was considerable attention paid to the MOA of inorganic arsenic, based on studies not only in animals, but also from human studies and *in vitro* analyses. They concluded that there was strong evidence for a non-linear mode of action, even though a specific mode of action could not be ascertained. This lack of one definitive mode of action has been used by EPA in their IRIS assessment to default to the linear approach to risk assessment. I do not believe that this is in accord with the data, the conclusions of the previous SAB report, nor with the 2005 Cancer Guidelines for EPA.

The evidence strongly indicates that the mode of action for arsenic carcinogenesis is non-linear. In fact, the only possible linear mode of action that can be postulated involves DNA reactivity by inorganic arsenic or one or more of its metabolites. Nesnow and his colleagues at the EPA Laboratories at Research Triangle Park have clearly shown that this does not occur. Indirect effects might occur on DNA, but these all involve non-linear mechanisms. Furthermore, there is extensive literature on animal, *in vitro*, and human studies clearly indicating that the many possible non-DNA reactive modes of action that have been hypothesized are all non-linear.

To default to a linear approach because the definitive mode of action for inorganic arsenic carcinogenicity is not known is not appropriate and not in agreement with the 2005 EPA guidelines for carcinogenicity assessment. Definitive evidence is not called for, but rather, sufficient evidence, and there is certainly sufficient evidence for non-linearity.

In the draft IRIS document, they list possible modes of action and the number of papers that were considered to fit with one or more of these modes of action, out of those papers that were reviewed. This method of counting papers does not address a scientific assessment of the various modes of action that are possible; it merely provides a list. Moreover, a few hundred papers were



not reviewed, about a hundred of which were published before the deadline of 2007. Thus, the mode of action is not adequately addressed in the draft IRIS report.

As I have indicated in my previous comments to the SAB and to the Working Group (my previous comments are attached), there is considerable evidence that the mode of action for arsenic carcinogenicity in animals is cytotoxicity followed by regenerative proliferation. All the modes of action listed by the EPA in their draft IRIS document could readily be a cause or consequence of this cytotoxicity and regenerative proliferation. More importantly, the data from human studies, *in vivo* and *in vitro*, strongly support this mode of action.

This mode of action has been most extensively investigated for the urinary bladder, one of the main target sites for arsenic carcinogenicity and the target organ for which the risk assessment by EPA has been performed based on the Southwest Taiwanese data. In animal studies, there is no question that the mode of action involves cytotoxicity and regeneration (Suzuki et al., *Toxicol Appl Pharmacol*, 244: 99-105, 2010). Recent evidence also suggests that a similar process occurs in humans as was indicated by the high incidence of hematuria (blood in the urine as a consequence of urothelial damage) in approximately one third of workers exposed to high levels of inorganic arsenic in an occupational accident in China. Cytotoxicity of the urothelium usually occurs without evidence of hematuria, since the cytotoxicity usually involves only the superficial layers of the urothelium. Hematuria as seen in the exposed Chinese workers only occurs if there is full-thickness damage of the urothelium with actual ulceration and breaking of the basement membrane.

Furthermore, recent data from the laboratory of Dr. Harvey Clewell (Gentry et al., *Environ Mol Mutagen*, 51:1-14, 2010) utilizing primary urothelial cells from humans clearly indicate that a similar mode of action could occur. What is proving quite interesting is that regardless of the form of arsenic that is administered, whether dimethylarsinic acid (DMA^{V}), arsenite, or arsenate, whether in adult mice (Suzuki, et al, 2010) or transplacentally (Tokar, et al., *Toxicol Sci*, available online), whether it is administered in the diet or drinking water, or whether it is administered to rats or mice, the no effect level for these effects on the urothelium are in the range of one part per million (ppm) of arsenic, either in the drinking water or diet.

We have previously suggested that the cytotoxic effects on the urothelium are due to trivalent arsenicals, such as arsenite, dimethylarsinous acid (DMA^{III}), or methylarsinous acid (MMA^{III}), interacting with critical cellular sulfhydryl groups. Considerable evidence had been accumulating that it was the methylated trivalent arsenicals that were the key components. However, recent evidence from studies in methyltransferase knockout mice clearly indicate that arsenite can also act as the reactive form (Yokohira et al., *Toxicol Appl Pharmacol*, 246:1-7, 2010). Most likely, it is a combination of the trivalent forms that are present in the various tissues that are causing the cytotoxic effects, based on their interaction with critical sulfhydryl groups in the respective tissues. Whether or not a tissue responds to a cytotoxic stimulus from arsenic administration is entirely dependent on achieving a critical concentration of the reactive metabolites in that tissue. Thus, it is a toxicokinetics issue. The 2005 draft document of the SAB clearly indicated that they



believed that interspecies differences and differences in target tissues could readily be explained by toxicokinetic considerations, and considerable progress has been made in delineating the differences between species. This research has come from a variety of laboratories, including Elaina Kenyon at the EPA Laboratories in Research Triangle Park, Dr. Harvey Clewell (The Hamner Institute), Dr. Doug Wolf (EPA), and myself in collaboration with Dr. Chris Le at the University of Alberta.

All of the studies, *in vitro* and *in vivo*, clearly demonstrate that a critical, threshold level of these reactive metabolites has to be reached before cytotoxicity or other deleterious effects can occur in a tissue. Low level exposure to inorganic arsenic in the drinking water will not produce such critical levels. In fact, in populations exposed to low levels of inorganic arsenic where arsenical concentrations have been measured, the trivalent arsenicals are not excreted in the urine at detectable levels (detection limits of approximately less than 10 nM or lower). It is only when higher exposures occur, usually above 100 ppb, that detectable levels of these reactive metabolites are achieved. Based on *in vitro* studies, utilizing a variety of epithelial cell types, it is clear that at least 100 nM is necessary for cytotoxicity to occur.

Recently, various thio reactive metabolites have been detected in animal species and in humans. It has become clear that some of these can also be highly reactive with cells *in vitro*, sometimes at concentrations approaching the cytotoxic levels seen with the trivalent oxygen containing analogs (Suzuki et al., 2010). However, the reactivity of these thio metabolites, which are pentavalent, occurs primarily because of their rapid uptake into the cells and rapid conversion to reactive trivalent oxygen-containing metabolites, such as DMA^{III}.

Clearly, the greatest evidence for the mode of action of cytotoxicity and regenerative proliferation has been documented for the urinary bladder, based on animal, human, and *in vitro* studies involving animal and human cell lines. However, there is considerable evidence that a similar process occurs in the two other main target tissues of arsenic carcinogenesis, the skin and the lung.

In vitro, cytotoxicity occurs with human keratinocytes at concentrations that are approximately the same as seen for human urothelial cells for the various trivalent reactive metabolites. Furthermore, the histology of arseniasis clearly supports a toxicity and regenerative proliferative process. The key characteristic findings of arseniasis are referred to as actinic keratosis. This consists of a hyperkeratotic epidermis, with increased epidermal proliferation and underlying chronic inflammatory infiltrates. Furthermore, keratinocytes are a logical target for arsenic carcinogenesis because of their enormous content of protein thiol groups. Differences between species could readily explain the species differences, as humans appear to be much more susceptible to arsenic skin carcinogenesis than are mice or rats. The distribution of free thiol groups in the skin within the various keratins differs significantly between species, and furthermore, other critical proteins in the skin could vary considerably with respect to their free thiol groups and thus susceptibility to targeting by trivalent arsenicals.



The one tissue for which there has been limited data regarding toxicity and regeneration as a mode of action has been the lung. However, we have recently demonstrated that *in vitro*, human bronchial epithelial cells behave exactly the same in response to exposure to arsenicals as human keratinocytes and human urothelial cells *in vitro* (see attached manuscript). The concentrations required for cytotoxicity in all three cell types (keratinocytes, urothelial cells, bronchial epithelial cells) are approximately the same for the different trivalents. It should be noted that for the methylated pentavalent arsenicals cytotoxicity occurs in the millimolar range, in contrast to the submicromolar concentrations required for the trivalent arsenicals. Since oxidative damage has been considered as one possible contributor to the toxicity of arsenicals, the lung is a target for arsenic carcinogenicity based on the high oxygen concentration that is present related to respiration, as well as the possible distribution of thiol groups within specific proteins.

Carcinogenicity in the lung in mouse and rat models has been difficult if not impossible to demonstrate for arsenicals. However, this is not too surprising given the fact that the rodent lung is anatomically different from the human, the aerodynamics are different, and the entire carcinogenic process within these tissues contrast significantly with that seen in humans. Cigarette smoking has not been shown to be carcinogenic to the mouse or rat lung, either, further illustrating the difficulties of utilizing these rodent models for the evaluation of human carcinogenesis of the lung. In the mouse model, there is a sequence of broncho-alveolar hyperplasia leading to adenomas leading to carcinomas. This does not occur in humans, rather, most human lung cancers arise from the bronchial epithelium and do not go through an adenoma precursor lesion. Furthermore, a majority of human lung cancers are squamous cell or small cell undifferentiated (oat cell) carcinomas, although adenocarcinomas can occur. It is interesting that recent studies from Taiwan have suggested that squamous cell and small cell carcinomas are the more common types seen in individuals exposed to high levels of arsenic, raising the strong possibility that these are associated with cigarette smoking more than with arsenic since these two cell types are the most commonly seen in cigarette smokers.

In summary, it is my belief that the 2005 EPA Guidelines for assessing carcinogenicity do not require definitive proof of a clear mechanism, but rely on an evaluation of the sufficiency of the data. There is strong evidence that arsenic does not act as a carcinogen by DNA reactivity, which is the only possible linear mode of action. The various modes of action that are being considered for arsenic carcinogenesis can readily be related to cytotoxicity and regeneration, either as cause or effect, and all of the aspects of these findings rely on a non-linear dose response. Furthermore, the toxicity and carcinogenicity of arsenic is reliant on the formation of trivalent species, rather than pentavalent species, and these need to be present at critical concentrations to have a toxic effect on cells. This critical value appears to be approximately 100 nM or higher, which is a concentration that is not attainable with exposure to inorganic arsenic in the drinking water at levels of 10 ppb or below.

We have recently completed a survey of the dermatologists in the state of Nebraska, population approximately 1.8 million, with drinking water inorganic arsenic levels between 1-50 ppb, but occasionally higher. Only one case of arseniasis has been seen by these dermatologists, and that



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was in an individual administered Gay's solution, an arsenic solution. Given the population and arsenic exposure, more than zero cases of arseniasis would be expected.

I strongly encourage the SAB to take this information into consideration as they deliberate further regarding the draft IRIS report from EPA. The SAB 2005 Science Panel, in their report, which was published in 2007, made a clear recommendation to EPA to do an analysis of a risk assessment based on non-linear modes of action. It is my impression that this has yet to be done. Rather, the EPA has defaulted to a linear risk assessment despite all of the scientific evidence pointing to a non-linear mode of action.

I am appending a manuscript which we have submitted to Toxicological Sciences describing the recent findings regarding toxicity of arsenicals for human bronchiole epithelial cells. I would be happy to provide any specific information that the SAB believes would be helpful in their deliberations.

Sincerely yours,

Samuel M. Cohen, M.D., Ph.D.



Cytotoxicity of Arsenicals on Human Bronchial Epithelial Cells

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CYTOTOXICITY OF ARSENICALS ON HUMAN BRONCHIAL EPITHELIAL CELLS

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ABSTRACT

Inorganic arsenic induces tumors of the skin, urinary bladder, and lung in individuals exposed to high levels, primarily through drinking water. Lung cancer is the most common cause of mortality associated with arsenic exposure. We have shown that the mode of action for the increased incidence of urothelial tumors is cell death followed by regenerative cell proliferation, ultimately inducing cancer. We postulated that during metabolism of arsenic highly toxic metabolites are formed, including arsenite (InAs^{III}), dimethylarsinous acid (DMA^{III}) and monomethylarsenous acid (MMA^{III}), which are cytotoxic to the bladder urothelium. We hypothesized that this mechanism could also be involved in arsenic-induced lung carcinogenesis. To evaluate this hypothesis, we investigated the cytotoxicity of inorganic and organic arsenicals on human bronchial epithelial (HBE) cells *in vitro* and compared their toxicity to that on human urothelial 1T1 cells. In HBE cells, MMA^{III} and DMA^{III} were considerably more cytotoxic than the corresponding pentavalent arsenicals, MMA^{V} and DMA^{V} . InAs^{III} was more cytotoxic on HBE cells than InAs^{V} . In both HBE cells and 1T1 urothelial cells, the LC_{50} values of MMA^{V} and DMA^{V} were in the millimolar range, whereas the LC_{50} values of InAs^{III} , InAs^{V} , MMA^{III} and DMA^{III} were in the micromolar range. In urothelial 1T1 cells, dimethylmonothioarsinic acid (DMMTA^{V}), although a pentavalent arsenical, showed cytotoxicity similar to that of the trivalent arsenicals. Our results support the hypothesis that in lung, like in the bladder, reactive arsenic metabolites induce cytotoxicity, producing cell death and stimulating a regenerative cell proliferative response, ultimately leading to lung cancer formation.

INTRODUCTION

Inorganic arsenic¹ is a known human carcinogen, inducing tumors of the skin, urinary bladder, and lung in individuals exposed to high levels, primarily through drinking water containing arsenic at concentrations above 150 µg/L (NRC, 1999; 2001; Chiou *et al.*, 1995; Lamm *et al.*, 2006). Exposure occurs also through the diet and by inhalation in certain occupational exposure scenarios, such as mining (NRC, 1999) and coal burning (Bencko, 2009). Smith *et al.* (1998) reported that lung cancer is the most common cause of mortality associated with arsenic exposure from drinking water in a region of northern Chile. In a systematic review of nine ecological studies conducted in southern Taiwan, Japan (Niigata), and northern Chile, Celik *et al.* (2008) observed a consistently markedly higher risk of mortality and incidence of lung cancer in groups exposed to high concentrations of arsenic in drinking water (100 µg/L or higher) than in groups exposed to low arsenic concentrations (0-10 µg/L).

The mechanism by which inorganic arsenic induces tumors is being actively investigated. During the past decade, animal models have been developed which have contributed to a better understanding of the mechanisms of arsenic-induced carcinogenesis of the urinary bladder. Inorganic arsenic and its metabolites do not react with DNA (Nesnow *et al.*, 2002), rather, a proliferative

¹ Inorganic arsenic occurs mainly in two oxidation states +5 and +3. The state -3 also occurs. The inorganic pentavalent arsenic (InAs⁺⁵, or InAs^V) occurs as arsenic pentoxide, arsenic acid (H₃AsO₄) and its salts, arsenates (V); The inorganic trivalent arsenic (InAs⁺³, or InAs^{III}) occurs as arsenic trioxide, arsenous acid (HAsO₂ or H₃AsO₃) and its salts, arsenites, or arsenates(III). In this paper, we will use the terms “arsenate” or “InAs^V” to denote inorganic compounds of pentavalent arsenic. The term “arsenite” or “InAs^{III}” is used to denote inorganic compounds of trivalent arsenic. As^V symbolizes any compound of pentavalent arsenic. As^{III} symbolizes any compound of trivalent arsenic.

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3 mechanism is involved (Cohen and Ellwein, 1990; 1991). Treatment with InAs^{V} or InAs^{III} causes a
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6 hyperplastic response in the urinary bladder epithelium of rat and mouse (Simeonova *et al.*, 2000;
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9 Suzuki *et al.*, 2008). Treatment of rats with 100 $\mu\text{g}/\text{mL}$ of arsenic as sodium arsenate (Na_3AsO_4) or
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12 sodium arsenite (NaAsO_2) in the drinking water or 100 $\mu\text{g}/\text{g}$ of arsenic as NaAsO_2 in the diet induced
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15 cytotoxicity and increased cell proliferation in the bladder epithelium of rats and mice (Suzuki *et al.*,
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18 2008).

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21 In humans and in most experimental animals, inorganic arsenic undergoes sequential reductions
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24 and oxidative methylations to form organic pentavalent and trivalent arsenicals (Aposhian *et al.*, 2000;
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27 Le *et al.*, 2000; Thomas *et al.*, 2001; Cohen *et al.*, 2002; Wang *et al.*, 2004; Cohen *et al.*, 2006). Only a
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30 small amount of inorganic arsenic is excreted in the urine (Vahter, 1994; Valenzuela *et al.*, 2005; Suzuki
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33 *et al.*, 2010). Once InAs^{V} enters the gastrointestinal tract, or is absorbed via another route, it is rapidly
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36 reduced to InAs^{III} , which is oxidatively methylated to monomethylarsonic acid (MMA^{V} , the
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39 monomethyl form of pentavalent arsenic) by arsenic methyltransferase. MMA^{V} is then reduced to
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42 monomethylarsenous acid (MMA^{III} , the monomethyl form of trivalent arsenic), which is methylated to
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45 dimethylarsinic acid (DMA^{V} , the dimethyl form of pentavalent arsenic). In certain species, DMA^{V} is
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48 subsequently reduced to dimethylarsinous acid (DMA^{III} , the dimethyl form of trivalent arsenic). We
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51 previously reported that the methylated trivalent arsenic intermediates (MMA^{III} and DMA^{III}) are highly
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54 reactive and markedly more cytotoxic than the corresponding pentavalent forms. These highly toxic
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57 trivalent arsenicals likely cause the observed effects of urothelial cytotoxicity that are followed by
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3 regenerative cell proliferation, ultimately leading to bladder cancer (Cohen *et al.*, 2002; Suzuki *et al.*,
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6 2008). Symptoms of workers, including hematuria, that were exposed to high levels of inorganic arsenic
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9 in a recent occupational incident in China (Xu *et al.*, 2008) suggest that high exposures are cytotoxic to
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12 the human urothelium, consistent with cytotoxicity and proliferation, which is the proposed mode of
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15 action for induction of bladder cancer in humans (Suzuki *et al.*, 2008).
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19 In addition to the methylated metabolites of arsenic, a thio-metabolite, dimethylmonothioarsinic
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21 acid (DMMTA^V) was recently identified in the urine of human and experimental animals
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24 (Naranmandura *et al.*, 2007; Raml *et al.*, 2007; Suzuki *et al.*, 2010). Suzuki *et al.* (2010) reported that
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27 DMMTA^V has cytotoxic effects on urothelial cells from rat (MYP3) and human (1T1) that are similar to
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30 the effects caused by MMA^{III} and DMA^{III}. Although it is a pentavalent form of arsenic, it is rapidly
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33 taken up by the cells and converted to the reactive DMA^{III}. Cytotoxicities of the various arsenicals,
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36 similar in potency to those observed using bladder urothelial cells, have been demonstrated in squamous
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39 keratinocytes treated *in vitro* (Klimecki *et al.*, 1997). Cytotoxicity and regeneration are consistent with
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42 the occurrence of hyperkeratosis and inflammation in the skin of humans in cases of arseniasis, the
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45 precursor lesion to skin cancer induced by inorganic arsenic (Tollestrup *et al.*, 2005).
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50 Less information is available concerning the effects of arsenicals on the lung, the other major
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52 target organ of cancer in humans exposed to high levels of arsenic (NRC, 1999). In the present study, we
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55 tested the hypothesis that the mode of action of arsenic-induced carcinogenesis in lung is also
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58 cytotoxicity followed by increased cell proliferation, similar to the mode of action in bladder and skin.
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3 We investigated the cytotoxic effects of inorganic arsenic and its metabolites on human bronchial
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6 epithelial (HBE) cells *in vitro*, and compared the cytotoxicities on HBE cells with the effects of the same
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9 compounds on human 1T1 urothelial cells.
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11 12 13 14 15 **MATERIALS AND METHODS**

16 17 18 *Test Substances:*

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21 Sodium arsenite (NaAsO_2) of 99% purity, sodium arsenate (Na_3AsO_4) of 99.7% purity, and
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24 sodium azide of >99.5% purity were purchased from Sigma (St. Louis, MO) and used as received,
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27 without additional analysis. Monomethylarsonic acid (MMA^{V}) of 99.5% purity and dimethylarsinic acid
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30 (DMA^{V}) of 99.5% purity were provided by Luxembourg Industries Ltd. (Tel Aviv, Israel).
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33 Diiodide- MMA^{III} and monoiodide- DMA^{III} were synthesized by Dr. William Cullen (University of
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36 British Columbia, Vancouver, Canada). The identity of both chemicals was confirmed by NMR analysis
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39 at the University of British Columbia, and their purity was confirmed to be at least 99%. DMMTA^{V} was
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42 synthesized by Dr. Hua Naranmandura from the laboratory of Dr. X.C. Le (University of Alberta,
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45 Edmonton, Alberta, Canada). ICP-MS analysis showed that the purity of the DMMTA^{V} was 96% with
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48 the remaining 4% identified as DMA^{V} , NaAsO_2 , Na_3AsO_4 , MMA^{V} , DMA^{V} and sodium azide (used as
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51 the positive control) were stored desiccated at room temperature. MMA^{III} and DMA^{III} were stored in the
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54 dark at approximately 4°C. DMMTA^{V} was stored at -80°C.
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Cell Line

Primary cultures of human bronchial epithelial (HBE) cells were obtained from endobronchial biopsies from an ongoing project conducted by Dr. Stephen I Rennard. The epithelial cells were and separated from fibroblasts by selective use of medium as previously described (Wang *et al.*, 2001).. Only one lot of HBE cells was used in the present study. HBE cells were cultured in 60-mm dishes (Advanced Biomatrix, San Diego, CA) coated with 30 µg/mL PureCol® purified bovine collagen solution in a 1.5:1 mixture of LHC-9 medium and RPMI-1640 medium (both from Gibco, Grand Island, NY) supplemented with 0.5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 50 U/ml penicillin, and 50 µg/ml streptomycin (supplied as a penicillin and streptomycin mixture from Gibco). HBE cells were grown in an atmosphere of 95% air and 5% CO₂ at 37°C. The HBE cells showed approximately 50% survival in 0.001% sodium azide, which was used as a positive control for toxicity testing of each arsenical (data not shown).

Determination of arsenic toxicity to human bronchial epithelial cells

The HBE cells were seeded on 24-well microplates (2500 cells/well). One day after seeding the cells, the medium was removed and 3-day treatment with various arsenicals was started. Cell viability was determined by staining with trypan blue and counting the area of four squares in a hemocytometer. The survival was determined by calculating the ratio between cell number in the arsenical-treated cell culture and that in the negative control culture. The concentration of arsenicals that was lethal to 50% of

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3 cultured cells (LC₅₀) was calculated by linear regression analysis (Cohen *et al.*, 2002; Suzuki *et al.*,
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6 2010).
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10 11 12 **RESULTS** 13

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15 The cytotoxicity of arsenicals to HBE cells was investigated by assessing the survival of HBE
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18 cells treated with various compounds and comparing the results with the cytotoxicity for human 1T1
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21 urothelial cells, expressed as LC₅₀ values (Cohen *et al.*, 2002; Suzuki *et al.*, 2010). The LC₅₀ values of
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24 the various arsenicals to HBE cells and to human 1T1 urothelial cells are reported in Table 1. The
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27 trivalent methylated arsenicals were 3-4 orders of magnitude more cytotoxic to the HBE cells than the
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30 corresponding pentavalent arsenicals, similar to the toxicities of arsenicals to human 1T1 urothelial cells
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33 previously reported (Cohen *et al.*, 2002; Suzuki *et al.*, 2010). The difference observed between the
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36 toxicity of InAs^V and the more cytotoxic InAs^{III} was not as striking. The LC₅₀ values of MMA^V and
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39 DMA^V to both HBE cells and 1T1 cells were in the millimolar range, whereas the LC₅₀ values of InAs^{III},
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42 InAs^V, MMA^{III} and DMA^{III} were in the micromolar range. Dimethylmonothioarsinic acid (DMMTA^V),
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45 although a pentavalent arsenical, showed cytotoxicity to HBE cells in the micromolar range, similar to
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48 that of the trivalent arsenicals.
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52 The LC₅₀ values of MMA^{III}, DMA^{III} and InAs^{III} to HBE cells were similar to the LC₅₀ values of
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54
55 the same compounds to human 1T1 urothelial cells (Cohen *et al.*, 2002; Suzuki *et al.*, 2010). The LC₅₀
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58 values of MMA^V, DMA^V, DMMTA^V and InAs^V were higher for HBE cells than the LC₅₀ values of the
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3 same compounds to human 1T1 urothelial cells. The LC_{50} value for $InAs^V$ was higher than that of $InAs^{III}$
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6 to both HBE cells and human 1T1 urothelial cells (Table 1).
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10 11 12 **DISCUSSION**

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15 Previous studies have demonstrated that the mode of action of arsenical-induced rat bladder
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18 tumors involves bladder epithelial cell death followed by regenerative hyperplasia, due to the presence
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21 of high urinary concentrations of cytotoxic metabolites (Cohen *et al.*, 2002; Suzuki *et al.*, 2008). It is
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24 also known that compounds of As^{III} , the trivalent forms of arsenic (inorganic and organic) are
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27 significantly more cytotoxic than compounds of As^V (their respective pentavalent forms) to human (1T1)
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30 and rat (MYP3) urothelial cells (Cohen *et al.*, 2002; Suzuki *et al.*, 2010). Several studies have shown
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33 that inorganic arsenic compounds ($InAs^{III}$ and $InAs^V$) are more toxic than the pentavalent methylated
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36 metabolites (Klimecki *et al.*, 1997; Styblo *et al.*, 2000; Cohen *et al.*, 2002; Suzuki *et al.*, 2010).
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39 $DMMTA^V$, although a pentavalent arsenical, has a LC_{50} value for 1T1 cells in the micromolar range like
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42 the trivalent arsenicals (MMA^{III} , DMA^{III} and $InAs^{III}$) (Suzuki *et al.*, 2010). This is apparently due to its
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45 rapid uptake by the cells and conversion to the highly toxic DMA^{III} (Suzuki *et al.*, 2010).
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49 In the present study, we investigated the possibility that arsenic-induced lung carcinogenesis
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52 could also involve cytotoxicity followed by increased cell proliferation. We evaluated the cytotoxicities
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55 of various arsenicals that are known to be involved with urothelial cell toxicities, on human bronchial
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58 epithelial (HBE) cells *in vitro*. Similar to urothelial cells, the LC_{50} values of MMA^{III} and DMA^{III} to HBE
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3 cells were in the micromolar range and 3-4 orders of magnitude more cytotoxic than MMA^V and DMA^V.
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6 InAs^V was less cytotoxic than InAs^{III}, although the difference between the cytotoxicities of the inorganic
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9 arsenicals (InAs^{III} and InAs^V) was much less than the difference between the LC₅₀ values of organic As^{III}
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12 and organic As^V. The higher toxicity of the trivalent forms may be due to the more efficient cellular
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15 uptake of these forms than that of the pentavalent forms (Hirano *et al.*, 2003). Similar to 1T1 urothelial
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18 cells, the LC₅₀ values of the inorganic arsenicals As^{III} and As^V and the organic arsenicals MMA^{III} and
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21 DMA^{III} were in the micromolar range whereas the LC₅₀ values of MMA^V and DMA^V to HBE cells were
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24 in the millimolar range. DMMTA^V had cytotoxic potential similar to MMA^{III} and DMA^{III}, rather than to
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27 the pentavalent methylated metabolites. Since DMMTA^V is nearly completely converted to DMA^V in the
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30 cells, presumably through the toxic DMA^{III} intermediate (Naranmandura *et al.*, 2008; Naranmandura *et*
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33 *al.*, 2009), this pentavalent arsenical has a potent cytotoxic effect on HBE cells and IT1 urothelial cells.
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36 The LC₅₀ values of the pentavalent arsenic compounds for both types of cells were less than 10 mM and
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39 less than 10 uM for DMMTA^V and the trivalent arsenic compounds, although HBE cells were more
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42 resistant to the cytotoxic effects of MMA^V, DMA^V, and DMMTA^V than the human urothelial 1T1 cells.
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45 Our results indicate that in human epithelial cells, the cytotoxicities of inorganic arsenic and its
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48 metabolites are similar for bladder urothelial cells and for lung bronchial epithelial cells.
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52 Most arsenic metabolites are efficiently excreted in the urine, however, some of these
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55 metabolites may be deposited in tissues such as the lung, liver, kidney, and skin, including hair, and nails
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58 (Chiou *et al.*, 1995). Both MMA^{III} and DMA^{III} are excreted in the urine of humans (Aposhian *et al.*,
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3 2000; Le *et al.*, 2000), suggesting that these cytotoxic metabolites are formed *in vivo* and possibly
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6 transported through the target tissues. Kenyon *et al.* (2005) reported the tissue distribution of inorganic
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9 arsenic, MMA, and DMA (including both As^{III} and As^V) after a single oral dose of inorganic arsenic in
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12 mice. Due to technical limitations, these researchers could not distinguish between pentavalent and
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15 trivalent arsenic compounds. The maximum concentration of arsenic in lung occurred in the form of
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18 DMA, and the concentration of total DMA (including DMA^{III} and DMA^V) was greater than inorganic
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21 arsenic and MMA in lung. The levels of InAs^V and InAs^{III} in the human blood and urine are lower than
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23
24 those of the methylated metabolites (Vahter, 1994; Hall *et al.*, 2007; Li *et al.*, 2009). The specific levels
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26
27 of the reactive methylated trivalent arsenic metabolites in human blood have not been measured. The
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29
30 mechanism of preferential accumulation of DMA in mouse lung and the relevance of mouse tissue
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33 distribution to humans are not clear. However, considering that the lung is exposed to the total amount of
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36 blood circulating through the organism which contains high levels of methylated metabolites (Vahter,
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39 1994; Hall *et al.*, 2007; Li *et al.*, 2009), it is likely that the human lung absorbs high amounts of DMA
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41
42 from the blood. El-Masri and Kenyon (2008) published a model to estimate levels of arsenic and its
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45 metabolites in human tissues and urine after oral exposure to either InAs^V or InAs^{III}. According to that
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48 model, a considerable, high concentration of DMA is predicted in human lung. The possible
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51 accumulation of DMA in the form of the highly toxic DMA^{III} in the human lung may be the major
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54 contributor to arsenic-induced effects on the lung in humans and may cause cytotoxic effects followed
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56
57 by increased cell proliferation and eventually tumor formation in the lung.
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3 The lung may be particularly susceptible to arsenic-induced tumorigenesis since lungs are
4
5 exposed to the highest oxygen concentrations in the body, and oxidative damage has been suggested as
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7 contributing to the carcinogenicity of arsenic (Kitchin, 2001; Kitchin and Conolly, 2010).
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11 Evidence has already been presented that increased proliferation might be involved with
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13 arsenic-induced lung carcinogenesis in humans. Andrew *et al.* (2009) suggested that the
14
15 arsenic-activated epidermal growth factor receptor (EGFR) pathway and cyclin D expression may be
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17 associated with arsenic-induced lung cancer. Following exposure to InAs^{III}, they observed increased
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19 levels of EGFR protein expression, EGFR phosphorylation, and the expression of its pro-ligand, heparin
20
21 binding-EGF in human bronchial epithelial cells (Beas-2B). InAs^{III} upregulated cyclin D expression,
22
23 which promotes cell cycle progression through the EGFR pathway. These researchers found higher
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25 levels of EGFR protein and EGFR phosphorylation in the lung tumor tissues of arsenic-exposed patients
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27 than in lungs of non-exposed patients.
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40 Animal models of arsenic-induced lung cancer have given inconsistent results, although several
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42 studies in the literature have shown lung-specific toxicity of inorganic arsenic and/or the organic
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44 metabolites which might be involved in arsenic-induced carcinogenesis (Yamanaka *et al.*, 1996; Hayashi
45
46 *et al.*, 1998). Long term exposure to high doses of inorganic arsenic, as InAs^{III} (up to 250 mg/L) or
47
48 sodium InAs^V (up to 400 mg/L), did not cause tumors in rats, mice, beagles, or cynomolgus monkeys,
49
50 indicating that arsenic by itself is not a lung carcinogen in animals (Kitchin, 2001). MMA^V was not
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52 carcinogenic to either rats or mice in 2-year bioassays (Arnold *et al.*, 2003). Relatively high doses of
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3 DMA^V administered through the diet or drinking water for two years, caused an increased incidence of
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6 bladder tumors in F344 rats (Wei *et al.*, 2002; Arnold *et al.*, 2006), but did not induce any tumors at any
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8
9 sites in B6C3F1 mice after treatment for 2 years (Arnold *et al.*, 2006). Although no increase in incidence
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11
12 of lung tumors was observed with DMA^V treatment in ddY mice, DMA^V significantly increased the
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14
15 multiplicity of lung tumors induced by a DNA reactive carcinogen, 4-nitroquinoline 1-oxide (Yamanaka
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17
18 *et al.*, 1996). DMA^V increased the multiplicity of lung tumors in A/J mice, a highly susceptible strain to
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20
21 the induction of lung tumors by chemical carcinogens, however no dose-related trends were observed in
22
23
24 the incidence, total number of tumors, or size of tumors (Hayashi *et al.*, 1998). The relevance of the
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26
27 results reported by Yamanaka *et al.* (1996) and Hayashi *et al.* (1998) to lung carcinogenesis in humans is
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29
30 questionable because of the study design in which a DNA reactive carcinogen was used to initiate lung
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33 tumors in a strain of mice highly susceptible to lung tumorigenesis. In addition, there are marked
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36 differences between the anatomy of the mouse respiratory tract and the pathogenesis of lung tumors in
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39 mice and in humans. In mice, lung tumors arise through bronchoalveolar hyperplasia, adenomas, then
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42 carcinomas, whereas in humans most lung cancer is bronchogenic in origin and there is no sequence
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45 involving bronchoalveolar hyperplasia and adenoma formation. Because of these reasons, mice are
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47
48 probably not an acceptable model for investigations of the mode of action of arsenic lung carcinogenesis
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51 in humans.
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55 In summary, we showed that the sensitivity of the human bronchial epithelial cells to the
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58 cytotoxicity of inorganic arsenic and its trivalent and pentavalent metabolites is similar to the sensitivity
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3 of human urothelial cells *in vitro*. The results of our study support the hypothesis that reactive
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5
6 arsenic-induced cytotoxicity produces cell death, which stimulates a regenerative cell proliferative
7
8
9 response, ultimately leading to lung cancer formation.
10

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17
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19
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30 manuscript.
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TABLE 1

Comparison of values obtained in this study for the LC₅₀ of various metabolites of arsenic to human bronchial epithelial cells *in vitro*, with LC₅₀ values from previous studies of the same compounds to human urothelial cells (ITI)^a.

	InAs ^{III}	InAs ^V	MMA ^{III}	MMA ^V	DMA ^{III}	DMA ^V	DMMTA ^V
1T1 ^b	4.8	31.3	1.0	1700	0.8	500	----- ^c
1T1 ^d	8.3	34.6	0.9	2700	1.0	230	1.4
HBE cells	5.8	46.5	1.0	6100	1.4	960	5.5

^a LC₅₀ values are expressed as μM.

^b Cohen *et al.*, 2002

^c Not performed.

^d Suzuki *et al.*, 2010



UNIVERSITY OF
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PATHOLOGY AND MICROBIOLOGY

March 24, 2010

Hon. Lisa Jackson
Administrator
US Environmental Protection Agency
Ariel Rios Building
1200 Pennsylvania Ave., N.W.
Washington, D.C. 20460

Dear Administrator Jackson:

I am writing regarding NCEA's Science Advisory Board meeting concerning the IRIS toxicological review of inorganic arsenic for cancer that is scheduled for April 6-7, 2010. I have a long standing history of research in chemical carcinogenesis with extensive experience regarding the urinary bladder. Because I am also trained as a medical doctor specializing in surgical pathology, I have been involved with research in humans as well as in animal models. I began working in the field of arsenic toxicology and carcinogenesis approximately 15 years ago, and have published extensively in this research area. In 1997, I served on a Science Advisory Panel for EPA regarding arsenic.

Originally, my group's research focused on dimethylarsinic acid (DMA^V), including establishment of the mode of action (MOA) for its effects on the urinary bladder in rats. Our data were used extensively by the EPA for its review on DMA, and formed the basis of many of the conclusions by the previous SAB Panel (2005) that was charged with questions regarding DMA and arsenic. Much of this work has been corroborated and extended by other investigators, including several at EPA's NHERL laboratory. During the past five years, my laboratory has increasingly focused on the issue of inorganic arsenic rather than organic arsenicals.

I have reviewed the draft IRIS document, Toxicological Review of Inorganic Arsenic, which became publically available in February, 2010. I have also reviewed the recently released charge questions to the arsenic panel that is to be convened April 6-7.

I would like to make a few comments regarding some specific issues raised in parts of the IRIS document. There is an enormous amount of material that is covered in the document, with considerable effort made in obtaining the necessary literature through the year of 2007; nevertheless, there are major publications which are not included in the reference list, including studies *in vitro* and *in vivo* as well as interactions with specific cellular molecules. Many of these papers contain information that conflicts with the mode of action and hypotheses conveyed in the IRIS document or provide alternative explanations.



The charge questions appear to be primarily focused on the interpretation of epidemiology studies, but I strongly believe that to properly interpret these questions the overall knowledge concerning mode of action for inorganic arsenic-induced cancer should be considered. The question of mode of action was addressed by the SAB 2005 Panel, with the conclusion that the specific mode of action for inorganic arsenic was not known, and therefore the agency should default to the linear extrapolation of the dose response data in humans (primarily based on the southwest Taiwan population). However, the Panel concluded that “[t]his issue is an extremely important area for research attention, and it is an issue that should be evaluated in EPA’s continuing risk assessment for iAs.” (p. 6)

Although the specific mode of action for inorganic arsenic-induced carcinogenesis is not known, the 2005 SAB clearly indicated in its conclusions as well as in its discussion that all of the possible modes of action involve not only a non-linear dose response but most likely involve a threshold.

It has been demonstrated conclusively that arsenicals will not react directly with DNA. Much of this work was performed at the NHERL laboratories of the EPA. Rather than default to a linear extrapolation, I believe that it is appropriate and sufficiently conservative to utilize a non-linear, threshold consideration for the dose response, taking into account what we know from animal studies and from studies in humans.

As indicated in the IRIS document, there are considerable differences between species in the carcinogenic potencies of inorganic arsenic. It would appear that the human is more susceptible than rats or mice. These differences in susceptibility appear to be primarily related to differences in toxicokinetics between species rather than in cellular responses to exposures to the arsenicals. The kinetic basis for the interspecies differences is stated in the IRIS document (page 75).

In all species, as indicated in the IRIS document, it is quite clear that of the various arsenicals it is the trivalent forms of arsenic are the critical molecules causing the toxicity and carcinogenicity. Following exposures to inorganic arsenic there are three trivalent forms present in the body. These are: inorganic arsenic itself, in the form of arsenite, and the monomethyl (MMA^{III}) and dimethyl (DMA^{III}) arsenicals. In humans, trimethyl arsenicals do not occur except at exceedingly high exposure levels, and it does not appear that the trivalent form of trimethyl arsenic is produced in humans.

Although we do not know the specific biological consequences that are critical to arsenic-induced carcinogenesis, the overall mode of action actually is already discernible at the present time. This involves exposure to arsenic, leading to generation of trivalent metabolites. These trivalent metabolites react with critical cellular sulfhydryl groups (most likely specific proteins), leading to consequent biological responses. It is quite likely that multiple biological effects by these trivalent arsenicals are essential for the eventual induction of tumors.



These three trivalent forms of arsenic that occur in humans are highly reactive chemicals. The reactivity is with thiol groups existing either in small molecules, such as glutathione, or more commonly, in proteins as free sulfhydryl groups. The reactivity of the three trivalent forms of arsenicals with these various thiol groups differs, and there is variation between species. The interspecies differences in reactivity are primarily due to differences in the amino acid sequences of specific proteins, with free sulfhydryl groups sometimes being present in the corresponding protein in one species but not in others. This is demonstrated by hemoglobin. In the rat hemoglobin, there is an additional free sulfhydryl group to which DMA^{III} binds, acting as a “storage sink” for arsenic in the rat. This is the primary reason for the unique toxicokinetics of all arsenicals in rats compared to other species, including humans. In the mouse, the accumulation of arsenic appears to pool in the urothelium of the lower urinary tract, including the urinary bladder, where it is stored in the form of arsenite within the mitochondria. In humans, there does not appear to be a storage or accumulation site for arsenic, and that explains part of the difference in susceptibility between humans and rodents.

The specific mode of action for arsenic in the various tissues of different species is dependent on the toxicokinetics of the trivalent forms of arsenic, and also importantly, is dependent on the reactivity of these trivalent arsenicals with specific proteins in the target tissues. It is not surprising that the primary cancer tissues for arsenic are the skin, lung, and urinary bladder, since these tissues contain significant levels of sulfhydryl groups with which trivalent arsenicals can react.

In addition to hemoglobin, the ability of trivalent arsenicals to react with proteins has been extensively evaluated for several specific proteins, including metalloproteinases, estrogen receptors and related peptides. Much of this research has been performed in the laboratories of Dr. Chris Le at the University of Alberta and Dr. Kirk Kitchin at EPA’s NHERL.

Depending on the cellular proteins that are affected by trivalent arsenicals, a potential different mode of action and very different biological consequences could occur. For example, there is considerable evidence that a common consequence of this reaction is cytotoxicity followed by regenerative proliferation. Other possibilities include indirect genotoxic effects, direct mitogenesis, or apoptosis. Several of these biological consequences are listed as key events in the IRIS document.

Although there are several theoretical possibilities for the carcinogenic MOA of arsenic, there is considerable information *in vivo* in animals as well as in humans as to which of these is more relevant. Although important information can be garnered from *in vitro* studies, such results are fraught with considerable uncertainty in extrapolating back to the intact organism. For example, it is essential that appropriate cell types be assessed in *in vitro* studies. Specifically, in animal models as well as in humans, carcinogenicity occurs in epithelial tissues. Therefore, *in vitro* studies should involve epithelial cells from the bladder, skin, or lung, and possibly some other target sites for the arsenicals. Evaluation of arsenicals in various *in vitro* systems involving hematopoietic or mesenchymal cells is not relevant to the critical biological effects..



Oxidative damage is also mentioned in the IRIS document as a possible key event. However, the data supporting this effect is unclear since much of it was derived from *in vitro* studies at cytotoxic concentrations. As the IRIS document points out, *in vitro* studies with trivalent arsenicals at concentrations above 10 μM probably involve cytotoxicity and cell lethality. In fact, trivalent arsenicals are lethal *in vitro* with epithelial cell systems at concentrations greater than 1.0 μM .

The information concerning oxidative damage is unclear regarding cause or effect, as is much of the information concerning indirect genotoxicity. Apoptosis is an unlikely key event *in vivo*, as it is very uncommon in urothelium or lung, and there is no evidence for it in human skin lesions secondary to arsenic exposure. Thus, the most likely modes of action are cytotoxicity and regeneration and/or direct mitogenesis.

In identifying the response in humans, it thus becomes essential to identify the concentrations of the trivalent arsenicals that must be produced for the reaction with critical cellular sulfhydryl groups to occur, leading to a biological consequence, such as cytotoxicity, cell proliferation, or indirect DNA damage. Based on *in vitro* studies with epithelial cell systems, the critical concentrations were shown to be 0.01-1.0 μM . In animal models of the urothelial effects, the concentration appears to be somewhere between 0.1-1.0 μM . As quoted in the IRIS document (page 88), the concentration of trivalent arsenicals present in the tissues of humans and other species exposed to high doses *in vivo* are concentrations that produce biological effects *in vitro*.

The key question is what is the minimal concentration of arsenic required to produce the biological consequences. Or, in other words, what is the level of arsenic to which humans must be exposed to generate these levels of trivalent arsenicals in the target tissues. In populations exposed to high levels of inorganic arsenic (usually greater than 400 ppb) trivalent arsenicals were detected in the urine at levels that correspond with the critical *in vitro* concentrations. Direct measurements of samples of individuals exposed to much lower levels of arsenic (<10 ppb) should be able to ascertain critical levels of trivalent arsenicals necessary to produce toxicological consequences. Unfortunately, levels of the trivalent arsenic compounds in the urine or other body fluids or tissues have not yet been measured in populations with lower exposures. Until such measurements are made, some of the PBPK modeling that has been performed by Dr. Elaina Kenyon at the EPA laboratories is helpful in this regard. Also, animal studies provide indication that exposures to inorganic arsenic by humans need to be in excess of the current standard for inorganic arsenic in the drinking water (i.e., 10 ppb) to generate biologically relevant concentrations of trivalent arsenicals to cause effects. The minimum level of detection for these trivalent species is less than 0.05 μM , which is well below the concentration necessary to produce a biological response in tissue culture or in organisms.

Thus, although the exact details of the mechanism of arsenic-induced carcinogenesis are not known, sufficient information is available to ascertain the overall mode of action for inorganic arsenic in animal models as well as in humans, involving the key events that I described above.



The currently available information is sufficient for enabling us to quantitatively evaluate a threshold response to environmental inorganic arsenic in humans.

At the bottom of page 99, the mode of action that we have put forth regarding DMA induction of bladder cancer in rats is described. We and others have demonstrated that a similar mode of action is likely to be occurring in the urinary bladder of mice and rats in response to exposure to inorganic arsenic. The question, of course, is whether this mode of action is relevant to humans. There is considerable evidence that cytotoxicity and regeneration occurs in response to high exposures of arsenicals in humans. The preneoplastic lesions for arsenic-induced skin cancer involve a specific type of pigmentation change and a proliferative response referred to as actinic keratosis. This keratosis involves a chronic inflammatory infiltrate and related increased epidermal proliferation and keratin production. Regarding bladder cancer, a recent occupational accident in China resulted in exposures to extremely high levels of inorganic arsenic, with nearly one third of the affected individuals developing hematuria (blood in the urine). Hematuria is a sign of severe toxicity of the urothelium. Urothelial toxicity could also occur without evidence of hematuria if the toxicity does not penetrate the full thickness of the urothelium. This is similar to scraping the skin versus cutting the skin. If the scrape is superficial, there is no bleeding, but there is still cell death with consequent regeneration. If a cut penetrates the full thickness of the epidermis, it leads to bleeding as well as consequent regeneration.

In summary, it is my belief that the current understanding of the mode of action of inorganic arsenic-induced cancer is sufficiently known so that we can perform a risk assessment without defaulting to a linear extrapolation. This should be taken into consideration in evaluating the epidemiology data that are being presented to the SAB as well as responding to the charge questions. I would be happy to provide more detailed information regarding these statements as well as appropriate references. I also plan to present at the meeting on April 6 or 7, 2010.

Sincerely yours,

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