

September 1, 2005

USEPA SAB Arsenic Review Panel

Dear Panel Members,

Thank you for the opportunity to present to the EPA Science Advisory Board Committee that has been formed to again review arsenic. We have been involved with research on arsenic for the past 10 years, arising out of our laboratory's long standing interest in bladder carcinogenesis in both animal models and in humans. Over the past year, we have sent all of our raw data on experiments related to dimethylarsinic acid ( $\text{DMA}^{\text{V}}$ ) to the EPA at their request. It is gratifying to see that a significant amount of our work was incorporated into the mode of action analysis by the EPA and also into the quantitative assessment for dose response. As you are well aware, for scientists, having our research utilized for practical, real world issues is rewarding.

Two primary issues need to be addressed by the SAB:

- 1) an assessment of the potential carcinogenic risk of  $\text{DMA}^{\text{V}}$  at low exposure levels in humans based on the animal data since relatively little information is available for the organic arsenicals in humans; and
- 2) low dose extrapolation for determining a relatively safe level of inorganic arsenic in the drinking water.

It is our expectation that the best science available be brought to bear on these issues. Two broad issues are fundamental to the evaluation of cancer risk from inorganic or organic arsenic in humans: a) mode of action analysis; and b) dose response relationships (especially linearity versus non-linearity). However, because the metabolism and kinetics of  $\text{MMA}^{\text{V}}$  and  $\text{DMA}^{\text{V}}$  produced endogenously during the metabolism of inorganic arsenic differs from the metabolism and kinetics of  $\text{MMA}^{\text{V}}$  and  $\text{DMA}^{\text{V}}$  from exogenous exposure, these two issues must be assessed separately for inorganic and organic arsenic.

### **DMA<sup>V</sup>**

#### **a) Mode of Action Analysis**

On the first of these issues, mode of action analysis, we have published extensively regarding the animal model of  $\text{DMA}^{\text{V}}$  in rat bladder carcinogenesis. Our work was summarized extensively in the attached manuscript, which has been accepted for publication in *Critical Reviews in Toxicology*. In addition, we have published several articles on this topic based on the research we have performed in our laboratory, some of which was done in collaboration with Dr. X. Chris Le at the University of Alberta. Much of this work was also summarized in the document prepared by the EPA entitled, "Science Issue Paper: Mode of Carcinogenic Action for Cacodylic Acid (Dimethylarsinic Acid,  $\text{DMA}^{\text{V}}$ ) and Recommendations for Dose Response Extrapolation."

Based on our research and that from several other laboratories, it is clear that the mode of action for DMA<sup>V</sup>-induced bladder carcinogenesis in the rat is cytotoxicity with consequent persistent regenerative cell proliferation, ultimately leading to a relatively low incidence of bladder tumors at the end of a two-year bioassay. In a two-year bioassay feeding DMA<sup>V</sup> in the diet, there was a clear dose response, with tumors significantly increased following exposure to 100 ppm DMA<sup>V</sup> in the diet and hyperplasia present in the groups fed with 40 ppm or higher DMA<sup>V</sup> in the diet with the response greater in females than in males. The laboratory of Dr. Shoji Fukushima at Osaka City University saw approximately the same dose response when they administered DMA<sup>V</sup> in the drinking water to male rats with tumors induced at 50 and 200 ppm but not at 12.5 ppm. Based on short-term studies, there is a clear NOEL for cell proliferation of 2 ppm in the diet with marginal effects at 10 ppm of the diet. No tumors or preneoplastic changes were observed at significant incidences in a two-year bioassay in mice, and no preneoplastic changes were seen in the urinary tract of hamster in a short-term (10-week) experiment. The doses used in these experiments are exceedingly high compared to human exposures. There are serious questions concerning the validity of other studies reporting that treatment with DMA<sup>V</sup> was tumorigenic in other tissues including the fact that the findings in these studies have not been repeated. Other concerns we have with these studies are detailed in our *Critical Reviews in Toxicology* manuscript.

Most important in looking at the mode of action, is the generation of cytotoxic metabolites in the urine, namely DMA<sup>III</sup>. We have shown that there is a clear dose response to DMA<sup>III</sup> formation in the urine which correlates well with the biological effects following oral administration of DMA<sup>V</sup>. At low concentrations, particularly 2 ppm DMA<sup>V</sup> in the diet, the concentration of DMA<sup>III</sup> in the urine is well below a concentration that would be expected to produce cytotoxicity.

There has been considerable discussion in the literature regarding the actual mechanism related to the carcinogenicity of DMA<sup>V</sup>. Most investigators accept that DMA<sup>V</sup> is not directly genotoxic (not DNA reactive). While DMA<sup>III</sup> may be indirectly genotoxic under some circumstances, genotoxicity does not appear to be the driving factor in the mode of action. Rather, DMA<sup>V</sup>-induced cytotoxicity (most likely through DMA<sup>III</sup>) appears to be the integral factor in the mode of action, although the cytotoxic mechanism is unknown. Multiple cytotoxicity mechanisms have been suggested for DMA<sup>V</sup>. The two dominant ones are: 1) oxidative damage; or 2) interaction of trivalent arsenicals with sulfhydryl groups of critical cellular proteins. We have begun to examine both of these. In our publication last year, we showed the effects of a number of antioxidants on urothelial cytotoxicity both *in vitro* and *in vivo*. What has become clear is that some antioxidants have an effect whereas most do not. We are interpreting these results to indicate that oxidative damage might be involved, but is clearly not the only aspect of the mechanism. This is particularly true if one is looking at oxidative damage to DNA rather than to other cellular constituents. Cytotoxicity is evident morphologically within six hours of administration of DMA<sup>V</sup> to the rats, indicating that there must be cytotoxicity occurring earlier, although we have not looked at earlier time points. Increased cell proliferation is evident by 7 days post exposure. Keeping in mind that the urothelium proliferates extremely slowly under normal circumstances (turnover time of approximately 200 days), oxidative damage to DNA as the cause of cytotoxicity or regenerative proliferation is highly unlikely.

If oxidative damage due to the trivalent metabolite DMA<sup>III</sup> is ultimately shown to be involved in DMA<sup>V</sup>-induced rat bladder carcinogenesis, this would also appear to have a non-linear dose response, based on *in vitro* studies and a few *in vivo* studies. Most notably, the *in vitro* studies

have involved the use of concentrations of methylated trivalent arsenicals that actually are cytotoxic when indirect genotoxicity is detected. In fact, in most instances, the concentration required to produce cytotoxicity is less than that required to produce detectable levels of oxidative damage. This certainly raises the possibility that the oxidative damage is a consequence of the cytotoxicity, and thus, would clearly imply a non-linear (and even likely a threshold) dose response.

The alternative hypothesis is interaction with sulfhydryl groups of critical cellular proteins. Dr. Chris Le has clearly demonstrated quantitative interactions with hemoglobin and with metallothionein, which would be expected given the propensity of trivalent arsenicals to react with free sulfhydryl groups. For the urothelium, uroplakins are logical target proteins, as they are the most plentiful proteins in the urothelial cell membrane on the luminal surface facing the urine, and have sulfhydryl groups available to interact with chemicals that are present in the urine. Dr. Le's work interestingly shows that much of the interaction appears to be between DMA<sup>III</sup> and the cellular proteins, rather than MMA<sup>III</sup> or arsenite.

#### **b) Dose-Response Relationship for DMA<sup>V</sup>**

The second major issue regarding DMA risk assessment, of course, is the issue of low-dose extrapolation. Clearly, based on the animal experiments, and to a large extent *in vitro* experiments, there is a non-linear dose response for DMA<sup>V</sup>-induced rat bladder carcinogenesis. If cytotoxicity is indeed the mode of action, this would also imply a threshold process and a margin of exposure approach to risk assessment.

The key for interpreting the effects of DMA<sup>V</sup> for humans is that it is a high-dose phenomenon and the mode of action is based on cytotoxicity with regenerative, increased cell proliferation. This cytotoxicity is likely caused by generation of a reactive metabolite, DMA<sup>III</sup>, which at exposures relevant to humans, is barely formed because of limited cellular uptake and metabolism of DMA<sup>V</sup>. Like other cytotoxic processes, such as chloroform in the liver and kidney, this would be expected to have a non-linear, threshold dose response. To extrapolate to humans, assuming that they have a similar mode of action, a margin of exposure approach would be appropriate, rather than a linear extrapolation. Importantly, in taking into account interspecies extrapolation, it strongly appears that the rat is much more susceptible to the effects of DMA<sup>V</sup> administration than are other species, including most likely humans. Thus, it is inappropriate to use an uncertainty factor of 10 for the interspecies extrapolation.

### **Inorganic Arsenic**

#### **a) Mode of Action**

Although epidemiological data shows that exposure to high levels of inorganic arsenic causes increased incidences of cancer of the skin, bladder, and lung the exact mode of action or mechanism has not been identified mainly due to difficulty in developing a good animal model. Treatment with high doses of inorganic arsenic in standard 2-year bioassays has not been tumorigenic in experimental animals. Two recently developed mouse models require additional investigation. One model indicated that inorganic arsenic might be a transplacental carcinogen but subsequent investigation suggested that the tumorigenic effects are possibly due to alterations in estrogen regulation and not directly related to treatment with inorganic arsenic. In a second mouse model, short-term treatment with inorganic arsenic resulted in urinary bladder urothelial hyperplasia. *In vitro*, inorganic arsenic is highly cytotoxic. The cytotoxicity of methylated trivalent arsenic compounds formed during the metabolism of inorganic arsenic may provide some explanation for the *in vitro* cytotoxicity but since no relationship between methylation

capacity and inorganic arsenic-induced cytotoxicity has been shown, it is not likely that the production of methylated trivalent arsenicals is the only explanation for inorganic arsenic-induced toxicity *in vitro*. Currently most investigators have concluded that inorganic arsenic is a so-called promoter or co-promoter, not a direct genotoxin.

#### **b) Inorganic arsenic low-dose extrapolation**

For inorganic arsenicals, it has been suggested, including by the National Research Council Report in 1999 and again in 2001, that the low-dose extrapolation should be based on a linear process. We believe that the general consensus that inorganic arsenic is not directly genotoxic and the *in vitro* data showing that inorganic arsenic is highly cytotoxic counters such an approach and indicate that the dose relationship for inorganic arsenic in the drinking water related to human cancer risk is not linear.

Some of the evidence that was used by the NRC to conclude that the dose-response relationship for inorganic arsenic was linear was based on epidemiologic findings regarding bladder cancer in various populations, including populations at risk in Taiwan, Chile, and Argentina. The Taiwanese data has been utilized as a basis for setting a water standard for the United States. However, the data from the Taiwanese population shows a risk only at high doses, whereas the lower exposure levels cannot be adequately evaluated.

The major thrust indicating a linear relationship was the report by Moore *et al.* (*Cancer Epidemiol. Biomarkers Prev.*, 6:31-36, 1997) examining the Chilean population suggesting that there was a linear relationship in the formation of micronuclei in urothelial cells in the urine following exposure to inorganic arsenic. However, there are several methodologic difficulties with this paper, and subsequent epidemiologic findings by some of the investigators from this group have not been able to substantiate this original observation.

To begin with, in the experiment involving the Chilean population, the population was divided into quintiles, based on their exposure to inorganic arsenic levels in the drinking water. Unfortunately, the quintiles were clearly not of equal size with respect to the range of arsenic exposure. Also, they were not able to adequately control for cigarette smoking as a confounding factor. Based on their results, they indicated that there was an increase in micronuclei per thousand urothelial cells in the urine increasing in what they conclude is a linear fashion for the four lowest quintiles. However, the highest quintile actually had micronuclei at the same level as lowest quintile. The three middle quintiles had quite similar levels. They tried to explain away the lack of effect at the highest quintile as being related to cytotoxicity, but this is unlikely given the presence of nuclei and urothelial cells in the urine. If there was cytotoxicity, it certainly was not enough to kill all of the cells, and it would not explain the precipitous drop in the number of micronuclei present.

Other work by this group in other populations might provide some of the explanation for this. They were able to show that decreasing arsenic exposure over a period of time led to a decrease in the number of micronuclei in urothelial cells in the urine (Moore *et al.*, *Cancer Epidemiol. Biomarkers*, 6:1051-1056, 1997). However, the reduction occurred to a statistically significant level only for individuals who were cigarette smokers. The decline for non-cigarette smokers was relatively small. This suggests that arsenic may be interacting in some way with agents generated by cigarette smoke, and yet, arsenic-induced bladder cancer appears to occur in non-smokers as well, so this interaction may not be critical. In addition, complicating the interpretation of micronuclei in the urine of cigarette smokers is the fact that cigarette smokers

generally have a more rapidly proliferating, hyperplastic urothelium than non-smokers. This will greatly influence the number of cells that occur in the urine, and potentially the types of cells that are generated, superficial cells versus intermediate cells. The propensity for micronucleus formation in these different cell types is likely to be different, although we are not aware of any systematic examination of this possibility.

Further complicating the interpretation of micronuclei in urothelial cells in the urine is the fact that urine is a hostile environment, frequently with osmolalities significantly above or below isotonic levels, greatly affecting the cell membranes and cell integrity. This has been noted by Dr. A. Smith and colleagues in standardizing the collection procedures for urinary cells. Concluding that finding micronuclei in the urine is proof of genotoxicity following arsenic exposure needs to be made with considerable caution. This is particularly true given the observation that there is an increase in micronuclei in urothelial cells in the urine of rats exposed to high concentrations of sodium chloride in the diet. We do not believe anyone would propose that sodium chloride is a genotoxic agent. However, high concentrations of sodium chloride in rats are well known to lead to alterations in urinary composition that prove to be cytotoxic to the urothelium. This is a high dose, rat-specific phenomenon only.

The most informative finding that suggests that the dose response for bladder cancer in humans exposed to arsenic in the drinking water is non-linear comes from the examination of a population in the Western United States by some of the same investigators involved with the Chilean population (Steinmou *et al.*, *Amer. J. Epidemiol.*, 158:1193-1201, 2003). They found no increased bladder cancer risk with exposures up to 200 ppb in the drinking water, although there was a suggestion of a slight increase at the highest concentrations in cigarette smokers assuming a lag time of 40 years. Overall, however, there was no evidence of increased risk, and as indicated by the authors themselves, the incidences were significantly lower than would have been predicted by an extrapolation (presumably based on linearity) from the Taiwanese data. We believe that this is the most telling remark regarding use of the high exposure levels in the Taiwanese population to setting a standard for humans in the United States.

There is also suggestive evidence that supports a non-linear relationship based on the incidence of arseniasis in the American population. In the Southwestern United States there has been a suggestion of some cases of arseniasis still occurring (Tollestrup *et al.*, *Environ. Geochem. Health*, 27:47-53, 2005). The exposure levels for these individuals, however, were not evaluated and might have been a consequence of smelter plants present in that region. Assuming a linear extrapolation, skin arseniasis (including alterations in pigmentation, hyperkeratosis, and hyperplasia) should be relatively commonplace in many states of the United States, including Nebraska, where exposure to inorganic arsenic in the drinking water is frequently above 20 ppb and sometimes over 50 ppb. Although only informal, I have surveyed a number of dermatologists in Nebraska, and they do not see cases of arseniasis. Although the incidence should be considerably lower than occurs in Taiwan or other populations that have high exposure levels, given exposures to 20-50 ppb and a linear dose response, we should be seeing a significant number of cases of arseniasis in the state of Nebraska if the dose response was linear.

In conclusion, the metabolism and kinetics of MMA<sup>V</sup> and DMA<sup>V</sup> produced endogenously during the metabolism of inorganic arsenic differs from the metabolism and kinetics of MMA<sup>V</sup> and DMA<sup>V</sup> from exogenous exposure, thus organic arsenic compounds should be assessed separately from inorganic compounds.

In regard to DMA<sup>V</sup>, based on the well-defined mode of action for the DMA<sup>V</sup>-induced bladder tumors in the rat involving cytotoxicity followed by regenerative cell proliferation, the evidence strongly supports a non-linear dose response relationship for organic arsenic compounds. This information should be incorporated into any risk assessment for the organic arsenicals. Thus, human risk from exposure to organic arsenic compounds is best characterized by a margin of exposure analysis.

In regard to inorganic arsenic, the epidemiological evidence of carcinogenesis in humans exposed to inorganic arsenic also supports a non-linear dose response. This information should be considered in setting safe levels of inorganic arsenic in the drinking water.

We look forward to being able to present before the committee and their deliberations. Thank you for your consideration.

Sincerely yours,



Samuel M. Cohen, M.D., Ph.D.  
Professor and Chair  
Department of Pathology and Microbiology  
Havlik-Wall Professor of Oncology  
University of Nebraska Medical Center



Lora L. Arnold, M.S.  
Department of Pathology and Microbiology  
University of Nebraska Medical Center

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