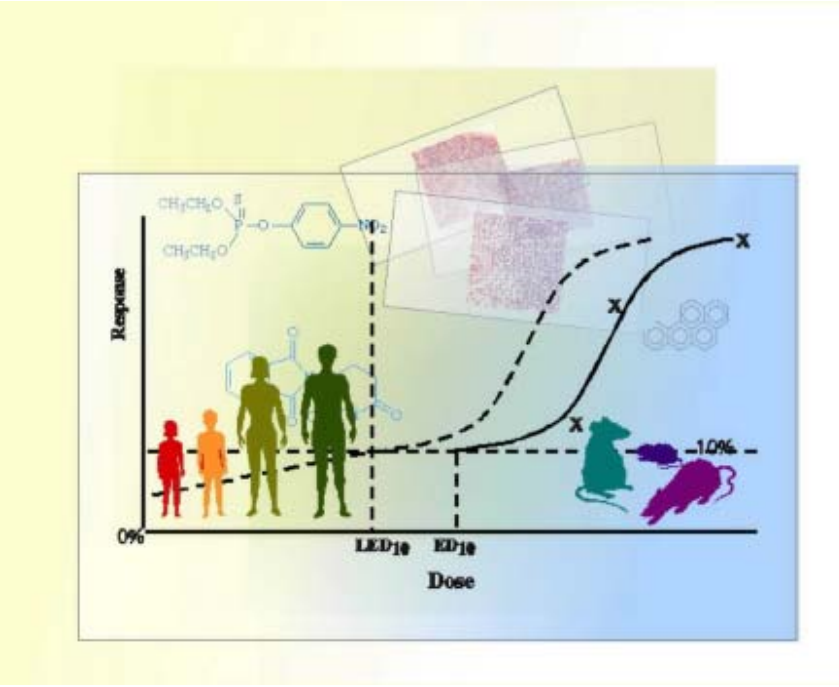
Science Issue Paper: Mode of Carcinogenic Action for Cacodylic Acid (Dimethylarsinic Acid, DMA^V) and Recommendations for Dose Response Extrapolation

July 26, 2005



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PREFACE

By August 2006, under the Food Quality Protection Act of 1996, EPA's Office of Pesticide Programs must review the safety of all existing pesticide tolerances (the legal limit set on the maximum amount of pesticides that may remain in or on foods). As part of this tolerance reassessment process, the risk assessment on cacodylic acid is being updated. For ease of discussion, cacodylic acid will be referred to as DMA^V (dimethylarsinic acid). Cacodylic acid and its sodium salt (sodium cacodylate) are organic arsenical nonselective contact herbicides which defoliate or dessicate a wide variety of plant species. Cacodylic acid and its sodium salt are used in combination, primarily as cotton defoliants, but also for weed control around non-bearing citrus, lawn renovation and edging, and weed control around buildings, sidewalks and driveways, and along utility lines. The only agricultural uses of cacodylic acid are on cotton and non-bearing citrus (i.e. applied when the citrus trees are not bearing their fruit). There are residential exposures to homeowners and children (e.g. toddlers) from the use of cacodylic acid on ornamentals, during lawn renovation and weed control, and around buildings and walkways. Much of this document will focus on and highlight issues related to the cancer hazard assessment of DMA^V. EPA will also be developing a risk assessment for exposures to monomethylarsinic acid (MMA^V). It is important to note that following pesticide applications of MMA^V to citrus and/or cotton plants, residues measured in the fruit and plants are predominately DMA. Thus, whether DMA^V or MMA^V are used as herbicides, the general population is principally exposed to DMA^V in foods.

As part of the reassessment of cacodylic acid, new studies on the metabolism and the animal cancer mode of action were evaluated. These studies are the focus of this special issue paper. A complete health risk assessment developed by OPP includes: hazard identification, dose response assessment, exposure assessment and risk characterization. The current paper focuses only on the carcinogenic mode of action in animals and whether the rat tumor data should be used to estimate human potential risk and if so how does the mode of action understanding informs the dose response extrapolation for cancer risk assessment. A complete hazard characterization including non-cancer endpoints unrelated to the cancer process, exposure assessment, and risk characterization are not included here. Thus, this paper does not represent a complete assessment for evaluating the potential health impacts (for both noncancer and cancer endpoints). These remaining components will be included in EPA's health risk assessment of cacodylic acid expected to be available to the public in late 2005.

The Office of Pesticide Programs (OPP) has provided its analysis and prespective on cacodylic acid's carcinogenic mode of action (Section 3), its relevance to humans (Section 4) and dose response extrapolation approaches for estimating carcinogenic risk (Sections 5 and 6). The SAB should also refer to additional discussion on the mode of carcinogenic action for DMA^V in Appendix E. It should be noted that the parts of Appendix E that refer to the OPP document on cacodyic acid were based on an earlier draft of the OPP paper. Given the issues raised regarding the metabolism and mode of carcinogenic action for arsenicals, OPP is at a point in its assessment of cacodylic acid where external peer review by the Science Advisory Board (SAB) would

facilitate further development and refinement of OPP's health assessment document. The aim of the SAB review is to obtain advice and comment on whether the conclusions drawn in OPP's analysis of metabolism and cancer mode of action are consistent with the current science. This external scientific peer review is viewed as a significant and critical step as OPP proceeds to develop a sound and scientifically credible health risk assessment on cacodylic acid. OPP intends to use the SAB's comments, as well as public comments that are received to further refine this preliminary cancer hazard and dose response analysis.

TABLE OF CONTENTS

List of Figures & Tables

1. Background

When establishing national standards for clean air and water, regulating pesticide products by approval of registration, or setting clean-up standards for hazardous site remediation, EPA must quantify the amount of potential human risk that may be associated with exposure to environmental contaminants. In assessing cancer risk, this necessitates dose-response assessment (i.e., how the frequency of adverse effects changes with decreasing dose), which usually involves extrapolations from high to low doses and from a nonhuman species to human beings. EPA has historically used a low dose linear default approach in estimating the cancer risk associated with environmental exposures, a practice often the subject of debate.

Given advances in understanding carcinogenesis, however, the new EPA 2005 cancer guidelines adopt a view that default options should not be used as the starting point from which departures may be justified by new scientific information, instead "these cancer guidelines view a critical analysis of all of the available information that is relevant to assessing the carcinogenic risk as the starting point from which a default option may be invoked if needed to address uncertainty or the absence of critical information." Moreover, "the use of mode of action in the assessment of potential carcinogens is a main focus of EPA's new cancer guidelines".

The issues of mode of carcinogenic action and low dose-response extrapolation for arsenicals have long been critical to the EPA in its risk assessment practices. With respect to cacodylic acid or dimethylarsinic acid (DMA^V), a great deal of new research has become available on metabolism/pharmacokinetics and carcinogenic mode of action. This new research has raised several important issues. First, information on metabolism/pharmacokinetics has indicated that there are important differences in the efficiency of methylation and cellular uptake depending on whether one is directly exposed to inorganic arsenic (iAs) or DMA^V. This raises the issue to what extent the human cancer epidemiology of iAs might be used for estimating the cancer risk associated with direct exposure to DMA^V, for which there are rat tumor data but an absence of human epidemiology. The metabolism/pharmacokinetic data have also indicated that there are significant differences between rats and humans for exposures to iAs or DMA^V. Thus, if rat tumor data are used for estimating the cancer risk associated with direct exposure to DMA^V, such differences need to be addressed in the risk assessment. The interpretation of the available experimental data for DMAV has raised issues in terms of mode of action. One such important issue is whether there is sufficient information to establish a mode of action, and how does this understanding inform the dose response extrapolation.

To address these issues for DMA^V, the intent of this paper is to provide an evaluation of metabolism/pharmacokinetics and mode of action pertinent to addressing the issues involving the selection of the model used for the cancer dose-response extrapolation. Consistent with the new EPA guidelines, the mode of action information on DMA^V has been evaluated in the context of EPA's "mode of action framework" (see Chapter 2.4 of EPA's 2005 cancer guidelines). The current analysis does not represent a complete cancer risk assessment, which is organized in four areas: hazard identification, dose-response assessment, exposure assessment, and risk

characterization. Nonetheless, it is the mode of action understanding for DMA^V carcinogenesis that is pivotal to the final risk assessment/characterization because that understanding will help inform the hazard characterization of potential human cancer risk, aid in the interpretion human relevancy of the laboratory rodent data, and guide the most appropriate low dose extrapolation in estimating potential cancer risk.

With respect to dose response extrapolation, the new EPA cancer guidelines indicate a preference for the use of biologically-based dose response (BBDR) models. Quite extensive data are required to establish model parameters for such models, however. When data are insufficient to support BBDR model development, the dose response model and/or dose response shape should not be *a priori* defined. Because it was realized that such rich data bases for BBDR model development would not be typically available for most chemicals, the new guidelines direct Agency risk assessors to conduct a two step approach to dose response extrapolation. Such an approach would include as much biological understanding of key events in a mode of action as possible in order to guide more reasonable extrapolations to the lower exposures generally received by humans. The first step in the process involves developing dose-response curves based on tumor incidence or, if available, on key events over the empirical range of observation to establish a point of departure (usually a benchmark dose) that indicates the starting point for the second step of the process—the extrapolation range. Several default options are provided in the guidelines for extrapolation to lower doses--linear, nonlinear, or both. The nonlinear default extrapolation approach is used when there is a sufficient mode of action understanding to support a presumption of nonlinearity and when there is a general lack of support for low dose linearity. A nonlinear approach can be used to develop a reference dose (i.e., a point of departure divided by uncertainty factors) or a margin of exposure. The EPA cancer guidelnes state that "Where alternative approaches have significant biological support, and no scientific consensus favors a single approach, an assessment may present results using alternative approaches (See Chapter 3 of the EPA cancer guidelines for further guidance). Lastly and most importantly, in Chapter 5 of the new guidelines, it is pointed out that "While it is an appropriate aim to assure protection of health and the environment in the face of scientific uncertainty, common sense, reasonable applications of assumptions and policy, and transparency are essential to avoid unrealistically high estimates."

As stated earlier, the purpose of this document is to discuss several key scientific areas that help describe the carcinogenicity of DMA^V:

- ❑ Dataset to quantify the potential human cancer risk from oral exposures to DMA^V (Section 2);
- ❑ Animal mode of carcinogenic action (MOA) for DMA^V including an analysis of the key events for tumor formation in rats (Section 3);
- ❑ Human relevancy of key events in the proposed animal mode of carcinogenic action (Section 4);

- ❑ Dose-response extrapolation approaches that are scientifically supported by available mode of action data (Section 5).

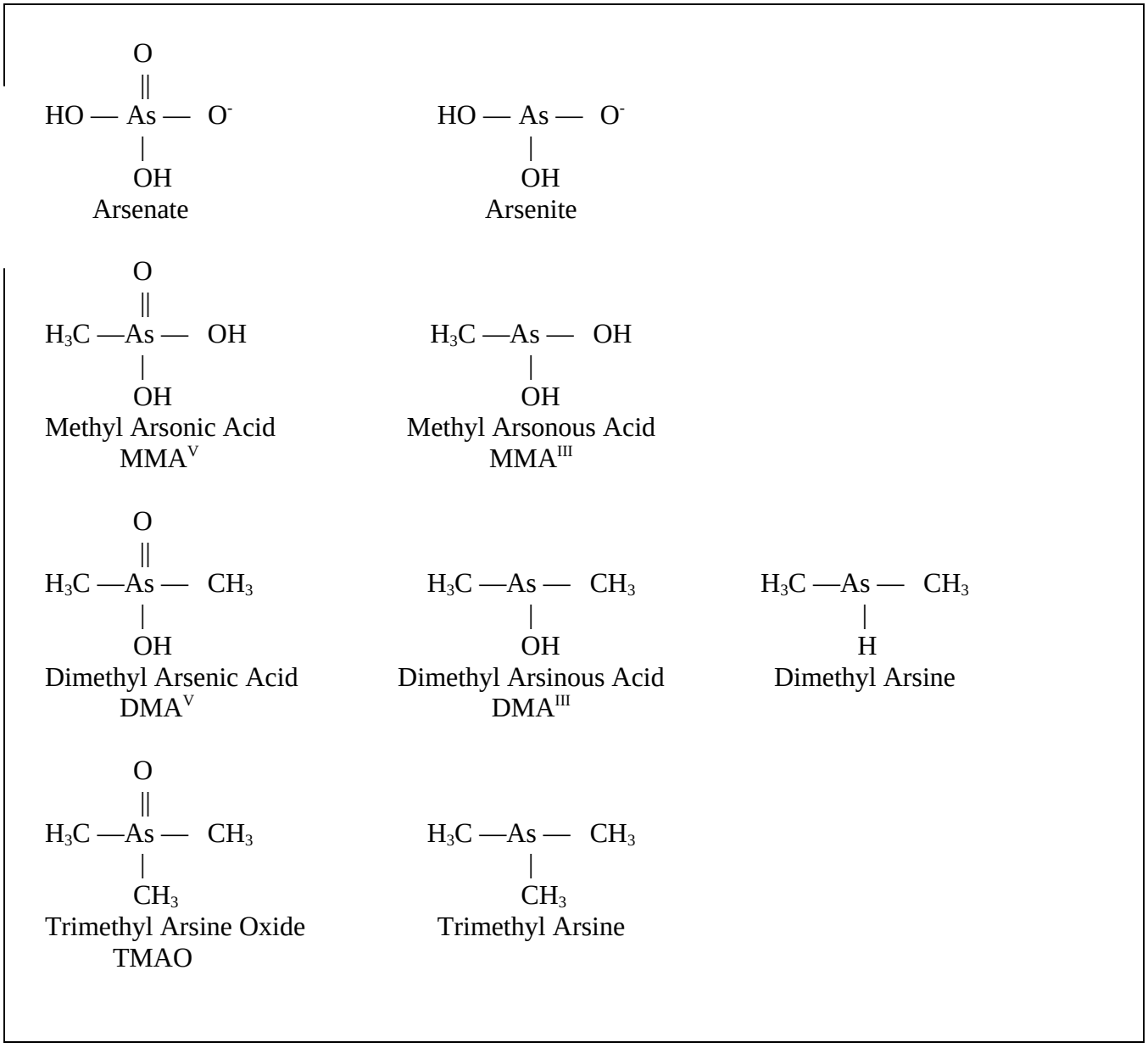
A summary discussion of cancer dose response extrapolation options for estimating the cancer risk associated with direct exposure to DMA^V are presented in Section 6. The scientific strengths of each approach and the limitations of data for each option are also presented for consideration by the SAB.

2. Data for Evaluating Potential Cancer Risk to DMA^V

2.A. Introduction

iAs undergoes a series of methylation and reduction steps in humans and other mammals resulting in the generation of various organic arsenical metabolites (Figures 2.1 and 2.2). Specifically, following exposure to iAs, humans may produce MMA^V, MMA^{III}, DMA^V, DMA^{III} and TMAO. MMA^V and DMA^V are chemicals which are also used as herbicides to treat lawns, citrus or cotton plants. DMA^V can also be found in some foods such as fish (McKiernan *et al.,* 1999; Fricke *et al.*, 2004; Vela *et al.*, 2004). Therefore, exposure to MMA^V and DMA^V can be from exogenous sources or from intracellular metabolism following environmental exposure to iAs. One critical issue for the cancer hazard assessment of DMA^V is the consideration of the dataset for quantifying potential cancer risk. Cancer data are available for iAs (human epidemiological) and for DMA^V (rodent tumors). Because DMA^V is a urinary metabolite produced in humans exposed to iAs, it has been suggested that the epidemiological data from drinking water exposure to iAs be used to quantify the potential cancer risk associated with direct oral exposure to DMA^V. The available studies pertaining to the carcinogenicity, metabolism, and toxicokinetic properties of iAs, MMA^V, and DMA^V have been evaluated and summarized in this Section to determine which dataset provides the most appropriate data for quantifying potential cancer risk associated with direct exposure to DMA^V. The *in vivo* and *in vitro* studies which describe the methylation and/or reduction steps are summarized below in Section 2.C. These studies show that the degree of methylation and reduction varies significantly based on exogenous exposure to different arsenic-containing compounds. As described in Section 2.D, each of the metabolites generated during the methylation/reduction steps express their own toxicity profiles and potency. An important aspect of determining the suitable data to use in the estimation of cancer risk associated with direct exposure to DMA^V is the impact of the mixture of these metabolites on health outcome. Because of the predominately one directional nature of the metabolic pathway in laboratory rodents and humans, only the downstream metabolites would be present from any point where one arsenical compound enters the pathway. Therefore, this would suggest that following ingestion of iAs, the mixture of metabolites is more complex compared to those following ingestion of DMA^V.

Figure 2.1: Structure of selected arsenic containing compounds



2.B. Available Cancer Studies in Humans and Animals

The following text provides an overview of the available studies in humans and animals which characterize the cancer potential for arsenical compounds.

2.B.1. Inorganic Arsenic

Long term oral exposure to iAs has been reported to be associated with several cancers (*e.g.,* skin, lungs, bladder). iAs cancer in the human population may be influenced by a variety of factors including diet (which may influence the extent of methylation of iAs) and by intra-individual and inter-individual variability in arsenic methylation.

The 1999 report by the National Research Council (NRC) of the National Academy of Sciences (NAS) suggests that the bladder and lung cancer human mortality data, particularly from the southwestern Taiwanese studies (Chen *et al.*,1985, 1988, 1992; Wu *et al.*, 1989) provide the best dose-response data for evaluating the long-term effects of oral exposure to iAs. Issues regarding the revised calculations for the iAs slope factor are described in EPA's draft toxicological review for iAs (which has been provided to the SAB).

Historically, standard chronic bioassays with exposure to iAs in rodents have been negative for increased tumor formation (NRC, 2001). However, it has been suggested that iAs has not been studied adequately in the standard rodent cancer bioassay. Huff *et al.(*2000) points out "as we have stated previously (Huff *et al.,*1998a,b), arsenic trioxide and other inorganic (and until now organic) arsenicals have in reality never been tested adequately for carcinogenesis, and never by the inhalation route." There are recent studies at relatively high experimental doses, in transgenic animals, and/or following transplacental exposures which have demonstrated cancer potential in rodents following iAs exposure (Simeonova *et al.*, 2000 & 2001; Santra *et al.*, 2000; Waalkes *et al*.,2000 & 2004). These studies provide qualitative evidence of the cancer potential of iAs.

2.B.2. Monomethylarsonic Acid (MMA^V)

There are no epidemiological studies following chronic exposure to MMA^V. Chronic bioassay studies in rats and mice submitted to EPA for pesticide registration indicate that the large intestine is the primary site of toxicity (Crown *et al.*, 1990; Gur *et al.*, 1991). These studies did not show an increased tumor incidence at any tissue site in either species. Mice were treated at 0, 10, 50, 200 or 400 ppm (approximately 2, 10, 40, 90 mg/kg bw/day). There was no treatment related mortality in mice. Rats were treated with 0, 50, 400 or 1300 ppm (approximately 0, 3, 30, 95 mg/kg bw/day). In the rat study, the highest dose was reduced to 1000

ppm (approximately 73 mg/kg bw/day) at week 53 and further reduced to 800 ppm (approximately 54 mg/kg bw/day) at week 60 due to high mortality, particularly in males. The results of these studies have also been summarized by Arnold *et al.,* (2003).

In a recent study, Shen *et al.,* (2003) exposed male rats to MMA^V at 0, 50, or 200 ppm in drinking water for 104 weeks. Although incidence of GST-P positive foci in the liver and urinary bladder hyperplasia were observed in MMA^V treated animals, there was no increase incidence in tumors at any tissue site.

2.B.3. Dimethylarsonic Acid (DMA^V)

Similar to MMA^V, there are no epidemiological studies for chronic exposure to DMA^V. As described in Section 3, there are, however, a variety of rodent cancer toxicity studies which show the potential for DMA^V to cause cancer in rodents. These studies involve diet or drinking water administration and include standard rodent bioassays, initiation and promotion assays, and transgenic animals. A more detailed description of these studies including the tumor incidence tables and the cancer mode of action analysis for DMA^V is provided in Section 3 of this document. DMA^V causes bladder tumors in the standard rat bioassays performed by Gur *et al*., (1989a) and Wei *et al.* (1999).

2.B.4. Trimethanearsinic oxide (TMAO)

Shen *et al.* (2003) exposed male Fischer 344 rats to 0, 50, and 200 ppm (approximately 0, 2.01, 7.88 mg/kg bw/day) TMAO in drinking water for up to 104 weeks. Incidences of hepatocellular adenomas were 14.3, 23.8 and 35.6% in the 0, 50, and 200 ppm groups, respectively. No other tissue sites exhibited an increased incidence in tumors. There are no epidemiological studies following chronic exposure to TMAO. A mouse bioassay for TMAO is also not available.

In summary, direct oral exposure to iAs is reported to be associated with several cancers in humans. Also differences in tumor profiles are found in rodent studies among several arsenical species (MMA^V, DMA^V, TMAO). These differences suggest that the carcinogenesis associated with iAs is likely to be much more complex compared to direct exposure to any one of its metabolite.

2.C. Toxicokinetics and Metabolism

Toxicokinetic factors (*i.e.,* absorption, distribution, metabolism, and excretion) play critical roles in the evaluation of quantitative dose-response relationships since these factors influence the amount of chemical at the site of action, along with the time course for exposure at that site. The following text summarizes the available studies in humans and animals which characterize the metabolism following administration of iAs, MMA^V or DMA^V. Although species

differences exist, these *in vivo* studies indicate that the methylation/reduction of iAs to MMA^V or DMA^V is highly efficient, particularly in rodents and humans. However, when MMA^V or DMA^V are administered as parent compound, further methylation of MMA^V or DMA^V is less efficient. The results of the *in vivo* studies are further supported by the *in vitro* evidence. Specifically, the *in vitro* evidence indicates that the cellular absorption of MMA^V or DMA^V is less than that of iAs^V, MMA^{III}, or DMA^{III}.

2.C.1. Metabolism Scheme

As originally envisioned by Challenger (1945), the biomethylation of arsenic involves alternating steps in which trivalent arsenic is oxidatively methylated and then reduced from pentavalency to trivalency (Figure 2.2). In mammals, the methylation and reduction reactions are enzymatically catalyzed. Some research suggests that distinct methyltransferases and reductases catalyze each step in the pathway that leads from inorganic arsenic to methylated arsenicals (Zakharyan, *et al.*, 1999, 2001; Zakharyan and Aposhian, 1999). Other investigators have found orthologous genes encoding arsenic (+3 oxidation state) methyltransferase (AS3MT) in the genomes of rat, mouse, and human. AS3MT catalyzes all steps in the pathway from arsenite to mono, di, and tri-methylated products (Thomas *et al.*, 2004; Lin *et al.*, 2002; Waters *et al.*, 2004).

Less is known about the capacity for the reduction of pentavalent methylated arsenicals into trivalent methylated arsenicals. In assays containing AS3MT, the low rates of conversion of substrates containing pentavalent arsenic into the expected methylated products suggest that unknown factors limit the capacity of the enzyme to reduce the substrate. Similarly, in cultured cells, pentavalent arsenicals are converted to methylated products at much slower rates than are trivalent arsenicals. The low rate of conversion could reflect inefficient reduction of the substrate before oxidative methylation or could reflect the relatively slow uptake of pentavalent arsenicals into cells.

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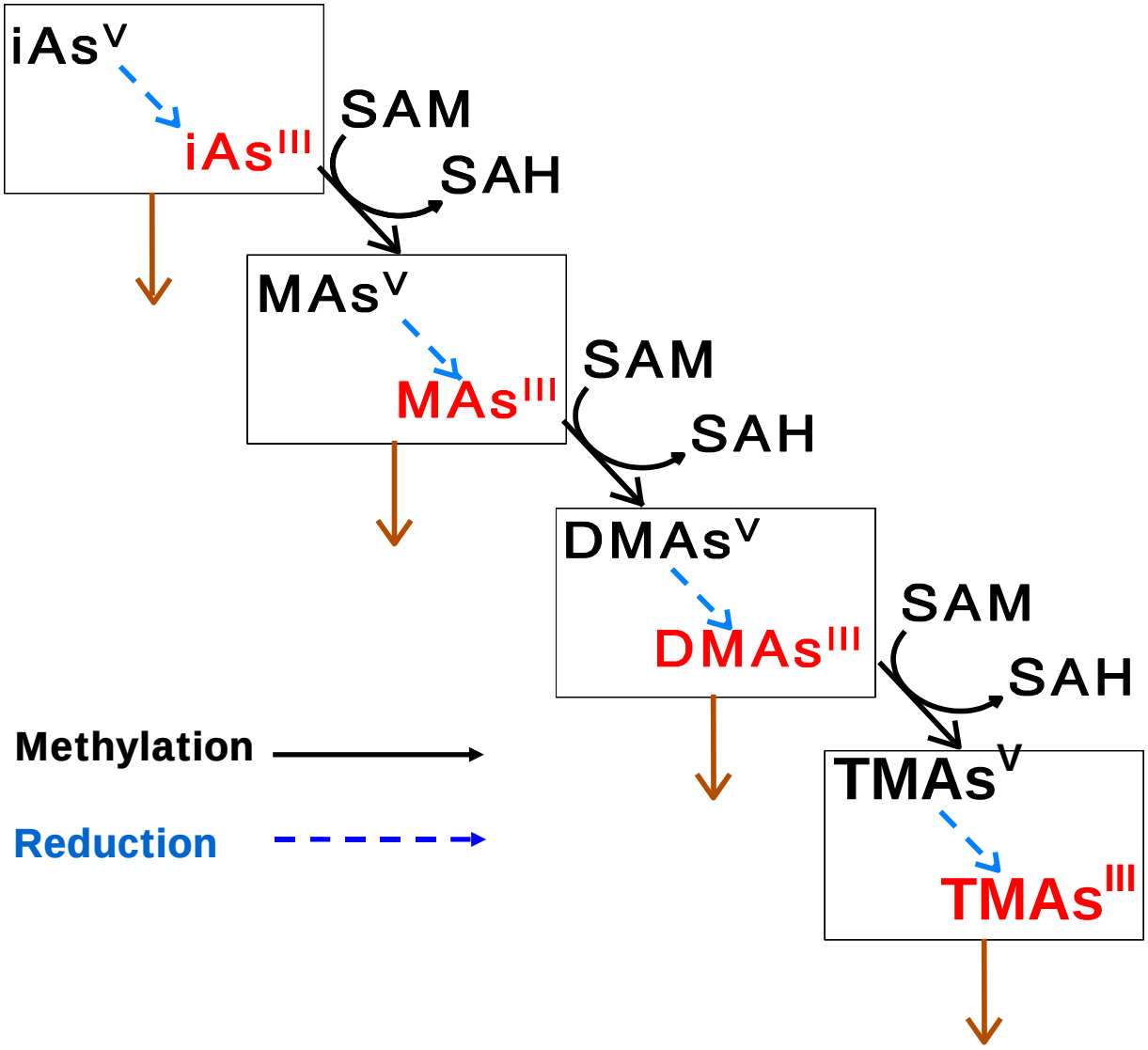
Figure 2.2: Challenger's Scheme for the Methylation of As



2.C.2. *In vivo* Metabolism Studies

Humans and many other mammals efficiently methylate iAs to mono-, di- and trimethylarsenicals. The degree of methylation varies by species (Vahter, 1994; Vahter and Marafante, 1983; Vahter *et al.*, 1995; Healy *et al.*, 1997; Aposhian, 1997; Csanaky and Gregus, 2002). Some species such as the guinea pig, marmoset monkey and chimpanzee do not appear to methylate iAs (Healy *et al.*, 1999, Vahter 1999). When humans are exposed to iAs, urinary concentrations of methylated metabolites vary among populations (Vahter and Concha, 2001; Loffredo *et al.*, 2003; Hsueh *et al.,* 2003). However, when exposed iAs, particularly in drinking water, human urine typically contains 10-20% iAs, 10-20% MMA, and 60-80% DMA (NRC, 2001). Humans tend to excrete higher amounts of MMA^V (10-20%) than do mice (<1%) after exposure to iAs. Rats and hamsters tend to excrete more TMAO than other species following exposure to DMA. Recent studies have detected the trivalent species of MMA and DMA in human urine following exposure to drinking water contaminated with iAs (Aposhian, 2000a&b; Del Razo *et al.*, 2001; Le *et al.*, 2000a&b; Mandal *et al.*, 2001). Following direct exposure to MMA^V and DMA^V, these compounds are methylated to a lesser degree by laboratory animals and humans compared to that of iAs^V. The following describes the *in vivo* metabolism of key arsenical compounds.

There are two studies which measured urinary metabolites in human subjects following a single oral exposure to MMA^V or DMA^V (Buchet *et al.*, 1981; Marafante *et al.*, 1987). In the Buchet *et al.* (1981) study, male subjects working in the laboratory volunteered to participate in the study. Three subjects were administered iAs^{III}; four subjects were administered MMA^V or DMA^V. Each subject received 500 µg arsenic (approximately 0.007 mg/kg arsenic) as iAs^{III}, MMA^V, or DMA^V. Based on total As in the urine, MMA^V and DMA^V were cleared more quickly and retained to a lesser extent than iAs. At 24 hours approximately 72% and 57% of ingested MMA^V and DMA^V, respectively, were excreted compared to only 24% of ingested iAs^{III}. At 4 days post-exposure, approximately 78% and 75% of ingested MMA^V and DMA^V, respectively, were excreted compared to only 49% of ingested iAs^{III}. In the treatment groups exposed to MMA^V and DMA^V, the parent compound was mostly excreted unchanged (87% of total arsenic as MMA in the MMA^V group; >99% of total arsenic as DMA in the DMA^V group). This is in contrast to ingested iAs^{III}, where 75% of the excreted arsenic (approximately 50% of the administered dose 4 days post-exposure) was in methylated forms, the major portion as DMA. The trivalent methylated species and TMAO were not analyzed for in this study. However, the presence of approximately 12% arsenic as DMA^V following treatment with MMA^V infers metabolism to MMA^{III}. Marafante *et al.* (1987) administered DMA^V as 0.1 mg As/kg (approximately 49 mg arsenic) orally to one human male subject. The subject excreted about 4% of the ingested dose as TMAO and the remainder as DMA.

Johnson and Farmer (1991) exposed three volunteers to a single exposure of seafood containing organo-As (arsenobetaine and arsenocholine are typically the most prevalent forms of arsenic in seafood) and two volunteers to a single exposure of iAs. Following the single exposure to seafood containing organo-As, at 6 hours 25% of the dose had been excreted with 50% excreted at 20 hours. Excretion of arsenic was slower in the volunteers administered iAs; at 6 hours only 5.25% of the dose was excreted and 54-70 hours were required to reach 50% excretion. Although Johnson and Farmer (1991) did not evaluate direct exposure to MMA^V or DMA^V, this study comparing the excretion profiles of organo-As in seafood compared to iAs provides additional evidence that the toxicokinetics of organic arsenical compounds differ from iAs.

As part of a larger effort to develop PBPK models for iAs, MMA, and DMA, scientists at EPA's National Health and Environmental Effects Laboratory (NHEERL) have conducted a variety of distribution and kinetic studies in mice. Hughes *et al.* (2003) exposed mice to 0.5 mg/kg iAs^V orally for 1 or 9 days. After a single exposure and after 9 days of exposure to arsenate, iAs, MMA and DMA were detected throughout the animals. MMA was detected in all tissues collected except the bladder. Bladder and lung had the highest percentage of DMA and this increased with duration of exposure. iAs and MMA were found in the lung tissue, although, at lower concentrations than DMA. iAs was also found in the bladder, although at lower concentrations than DMA. In mice exposed to iAs, DMA was the major urinary metabolite in the mice (about 9-10 µg As/day) with smaller amounts of MMA (< 0.5 µg As/day).

Hughes *et al*. (2000 & 2005) and Hughes and Kenyon (1998) have examined the disposition of arsenic after administration of MMA^{III}, MMA^V, and/or DMA^V. Hughes *et al.* (2005) exposed mice to a single dose of MMA^V via gavage at 0.4 or 40 mg As/kg or to 0.4 mg As/kg of MMA^{III}. The oral bioavailability of the MMA^V low dose group was greater than the MMA^V high dose group. Although there were some statistically significant dose-dependent differences in the tissue concentration of MMA^V-derived radioactivity at several time points, most were less than 2-fold. In addition, clearance of MMA^V-derived radioactivity from blood was the same for both doses. By 8 hr post-exposure, 80% of the administered dose (both 0.4 and 40 mg As/kg) of MMA^V had been eliminated. For the 0.4 mg As/kg MMA^V dose, the urinary arsenic consisted of 98% MMA^V and 2% MMA^{III}. For the 40 mg As/kg MMA^V dose, the urinary arsenic consisted of 90% MMA^V, 1% MMA^{III}, 6% DMA^V, 1% DMA^{III} and 2% TMAO. In comparison, more than 90% of the urine in mice treated with MMA^{III} was methylated to DMA^V and DMA^{III}. Similarly, in tissue, < 25% of the tissue As in MMA^V treated mice was DMA compared to 75% or more tissue arsenic as DMA for MMA^{III} treated mice.

Hughes *et al.* (2000) exposed mice to DMA^V by intravenous (iv) injection. DMA^V-derived radioactivity was rapidly distributed in the mice. Radioactivity was measured in blood, liver, kidney and lung as early as 5 minutes after injection followed by rapid decline in the tissues measured. DMA^V was excreted unchanged; demethylation to iAs or MMA or methylation to TMAO was analyzed for but was not observed in this study. Rapid tissue decline is consistent with the results of Hughes and Kenyon (1998) which describe a biphasic elimination of arsenic following i.v. administration to mice with MMA^V or DMA^V. For both compounds, the alpha (distribution) phase was less than 3 hr and the beta (elimination) phase was less than 15 hr. At 2 hours, 50% of the MMA^V and DMA^V doses were excreted in the urine without further methylation. At 24 hours post-dosing, approximately 80% of administered MMA^V and 78% of administered DMA^V were excreted in the urine without further methylation. Seventy-three to 78% of the MMA^V dose was excreted as parent compound with only 2-8% excreted as DMA. Administered dose of MMA^V or DMA^V did not affect the urinary elimination of arsenic. Demethylation was not observed in mice treated with DMA^V. Retention of DMA^V- or MMA^V-derived radioactivity was low; less than 2% of the administered dose of either compound remained in the tissues or carcass 24 hr post-dosing. Suzuzki *et al*. (2004) also observed similar results after i.v. administration of DMA^V and MMA^V to rats. Within 12 hr (last time point), 63-70% of the administered dose was eliminated in urine primarily intact. Less than 0.5% of the dose of DMA^V and MMA^V was eliminated in bile 3 hr post-administration. Cui *et al*. (2004) also observed a minimal biliary elimination of i.v. administered DMA^V and MMA^V in rats. These arsenicals were primarily eliminated in urine. A very low portion of the urinary excreted dose of MMA^V was eliminated as DMA^V.

Stevens *et al*. (1977) examined the disposition of ^{14}C- and ^{74}As-DMA^V in rats after oral, iv, and intratracheal administration. Twenty-four hour post-administration, less than 0.1% of the dose of ^{14}C- DMA^V was recovered as $^{14}CO_2$ and the disposition of ^{14}C- and ^{74}As-DMA^V was very similar, suggesting that demethylation of DMA^V to iAs does not occur in rats. Levels of radiolabeled DMA^V decreased rapidly in all tissues examined, except blood. The major site of body burden of DMA^V was the red blood cells.

Vahter and Marafante (1983) also showed that arsenic is cleared more rapidly when animals are administered DMA^V compared to iAs^V or iAs^{III}. Following injection with DMA^V, the total arsenic was almost completely eliminated by mice and rabbits at 24 hours. In mice, 72 hours were required to almost completely eliminate arsenic following injection with iAs^{III} or iAs^V. In the same study, rabbits injected with iAs^V or iAs^{III} had eliminated only 53-66% of the administered arsenic at 72 hours.

Following oral exposure to DMA^V, laboratory animals excrete TMAO in urine in varying amounts. (Yamauchi and Yamamura, 1984;

Yoshida *et al*.,1997; Yoshida *et al*., 1998; Marafante *et al*., 1987; Lu *et al.*, 2003 ; Salim *et al*., 2003). Yamauchi and Yamamura (1984) administered DMA^V (27 mg As/kg) orally to hamsters and analyzed excreta and tissues for arsenic. The major portion of the administered dose was excreted within 24 hr post-exposure, 45% in urine and 35% in feces. The urinary arsenicals included about 70% DMA, 30% TMAO and <1% iAs and MMA. Trimethylated products were not detected in feces, although about 1% of the fecal arsenic products were in the form of iAs and MMA. Peak tissue DMA concentration was at 6 hr post-exposure. The lung had the highest tissue concentration of DMA (6.8 ug As/g) at this time. Also detected were iAs (1.39 ug As/g) MMA (0.01 ug As/g), and TMAO (3.7 ug/As/g). However, arsenic was rapidly cleared from all of the tissues of the hamster after oral administration of DMA^V.

Marafante *et al*. (1987) administered DMA^V to mice and hamsters. Mice and hamsters were administered 40 mg As/kg orally. Mice and hamsters excreted about 80-85% of the dose as unmetabolized DMA and about 3.5% and 6.4% of the dose as TMAO, respectively. The authors report 13% and 15% identified as a 'DMA-complex" for mice and hamsters, respectively. Yamauchi *et al.* (1988) exposed hamsters to MMA at 5, 50, or 250 mg/kg via gavage. At 24 hours following oral exposure, MMA was excreted in the urine or feces primarily unchanged with a small amount as DMA (0.4 -8% of the administered dose) and about 2% as TMAO only at the 5 mg/kg dose. The MMA in feces was most likely unabsorbed dose, as only <1% of an i.p. dose of MMA^V was excreted in feces. Demethylation of DMA was not observed in hamsters.

Yoshida *et al*.(1997) exposed male rats to a single dose of 50 mg/kg DMA^V orally and by i.p. injection and collected urine for up to 48 hours after administration. In the first 4 hours after administration, unchanged DMA was primarily detected. However, with longer duration post-exposure the relative amount of TMAO in the urine increased with time up to 48 hours. TMAO ultimately accounted for about 30% of the total arsenic excreted.

Yoshida *et al.* (1998) exposed rats to 10 mg/L arsenic as iAs^{III}; 100 mg/L arsenic as MMA^V or 100 mg/L arsenic as DMA^V in drinking water for 1 week or 7 months. Rats efficiently methylated iAs^{III}; with the primary urinary excretion metabolites as DMA (62-85%) and TMAO (6.4-10.8%). Rats exposed to MMA^V excreted 50-65% of total arsenic as unchanged MMA^V and 19-27% as DMA^V and only 4-7% as TMAO. Rats exposed to DMA^V excreted 45-61% of total arsenic as unchanged DMA^V and 24-40% as TMAO with little to no demethylation to MMA.

In the urine of rats fed 100 ppm DMA^V, Lu *et al*. (2003) reported 73µM TMAO compared to 66µM DMA^V. Similarly, Arnold *et al.* (2004) showed a dose-dependant increase in TMAO and DMA^{III} in the urine of rats fed 2-100 ppm DMA^V. In mice following 78 week exposure to DMA^V

in drinking water (0, 50, 200 ppm), 12-17% of the excreted arsenic was in the form of TMAO (Salim *et al*., 2003).

Additional arsenic-containing metabolites, that have not been completely identified, have been observed following direct exposure to MMA, DMA, or TMAO in rats and mice (Yoshida *et al*, 1997; Yoshida *et al*, 1998, Yoshida *et al*., 2001; Salim *et al.*, 2003). These metabolites have been shown to make up about 3-10% of the total arsenic in urine and were initially identified as M-1, M-2 and M-3 (Yoshida *et al*., 1998; Yoshida *et al*., 2001; Yoshida *et al*., 2003). M-2 and M-3 are found in a greater proportion in the feces of rats than urine following a 20-week exposure to DMA^V (100 ppm) (Yoshida et al., 2001). The urinary excretion of M-1 and M-2 is less following ip rather than after oral administration of DMA^V (Yoshida *et al.,* 2001). These results suggested that intestinal bacteria contribute to the formation of these metabolites (Kuroda *et al*., 2004; Yoshida *et al*., 2001; 2003). *Escherichia coli* strain A3-6, isolated from the ceca of DMA^V administered rats, converted DMA^V to M-2 in the presence of cysteine. M-2 was further metabolized to M-3. TMAO was metabolized to M-1 by the bacteria (Yoshida *et al*., 2003). M-2 was found to contain sulfur and appears to be a dimethylarsenic sulfur compound (Kuroda *et al*., 2001; Yoshida *et al*., 2003). M-2 was more cytotoxic to V79 cells than DMA^V, M-1, and M-3 (Yoshida *et al*., 2003; Kuroda *et al*., 2004). M-2 was reported to cause cytogenetic effects in V79 cells, inducing c-mitosis, tetraploids, sister chromatid exchange and chromosomal aberrations (Kuroda *et al*., 2004). Dimethylarsenic sulfur compounds have also been detected in rat liver supernantants following iv administration of DMA^V or a trivalent DMA-cysteine complex (Suzuzki *et al*., 2004b). The metabolites were detected at 10 min, but not 12 hr following administration of compound, indicating they are either rapidly metabolized or redistributed from the liver. Although the role of M-1, M-2, M-3 in the carcinogenic process for DMA is unclear, Kuroda *et al*. (2004) speculate that the known levels of M-2 in urine of rats administered DMA at levels that produce bladder tumors, are high enough to induce cytotoxic and cytogenetic effects in the bladder.

As some microorganisms can demethylate (Sanders, 1979; Hanaoka, 1994; Quinn and McMullan, 1995), it has been postulated that microorganisms in the gut may also be involved in the demethylation of MMA and DMA. For example, Cullen *et al*. (1989) analyzed the cecal contents of mice parenterally administered [^{74}As]-MMA^{III} and detected an unreported level of [^{74}As]-arsenate. Similar results were reported after the cecal contents isolated from untreated mice were incubated with [^{74}As]-MMA^{III}. Stevens *et al*. (1977) reported that <0.1% of a dose of [^{14}C]-DMA^V administered by oral, intratracheal or iv routes was metabolized to $^{14}CO_2$ by 24 hr. Yamauchi and Yamamura (1984) reported low levels of inorganic arsenic in excreta (<1% total excreted) and tissues of hamsters administered a single oral dose of DMA (50 mg/kg). Yoshida *et al.* (1997) exposed rats to 50 mg/kg DMA orally or by ip administration. iAs^{III}

accounted for < 0.1% of the total urinary arsenic over 48 hours. Yoshida *et al.* (1998) showed that after 1 week of oral exposure to 100 mg/L As/L as MMA, 1.5% and <0.1% of the total arsenic was excreted in urine as iAs^{V} and iAs^{III}, respectively. At 7 months of exposure, the iAs^{V} and iAs^{III} were both <0.1%. In the same study, Yoshida *et al.* (1998) exposed rats to 100 mg/L As/L as DMA for 1 week and 7 months. Measurements of iAs^{V} and iAs^{III} in urine were 0.1% or less of the total arsenic excreted at each time point. In a study designed to further evaluate the unidentified metabolites M-1, M-2, M-3, the same laboratory exposed rats to a single dose of 50 mg/kg of DMA^{V} orally (Yoshida *et al.*, 2001). Arsenate and arsenite in urine and feces were below the limit of detection. The authors attributed a small amount of MMA detected to arsenic in the animal feed Other laboratories have not observed demethylation of administered DMA to MMA or inorganic arsenic (Vahter *et al*., 1984; Marafante *et al*., 1987; Hughes and Kenyon, 1998; Hughes *et al*., 2000). Overall, the very low levels of iAs that have been detected in tissues and excreta of rodents administered methylated arsenicals supports the hypothesis that the metabolism of arsenic in mammals is predominately one of methylation/reduction and not demethylation.

After exposure to arsenic compounds, rats accumulate and retain DMA in the RBCs due to binding to rat hemoglobin (Stevens *et al*., 1977; Lerman and Clarkson, 1983; Vahter *et al.*, 1984; Aposhian, 1997; Shiobara *et al.*, 2001; Lu *et al.*, 2004). This binding leads to longer retention time of DMA by rats compared to other species. Lu *et al.* (2004) have recently shown that 15-30 fold more trivalent arsenic species (iAs^{III}, MMA^{III}, and DMA^{III}) were bound to Hb of rat RBC compared to the Hb of human RBC. Rat Hb exhibited more binding affinity as exhibited by the estimated binding constants (*nK*). Specifically, the binding constants were estimated to be 4.69×10^{4} and 2.22×10^{5} for rat Hb compared to 5.00×10^{3} and 1.36×10^{4} for human Hb for MMA^{III} and DMA^{III}, respectively.

Vahter *et al.* (1984) showed that in rats following a single oral dose of 0.4 mg arsenic/kg as DMA, at 20 days post-dose, approximately 50% of the dose remained in the rat. In the same study, after a single oral exposure of DMA (0.4 mg arsenic/kg) to mice, more than 85% was eliminated within the first 24 hours and >99% excreted by 5 days. Similar to the rapid elimination by mice and, in contrast to the lengthy retention by rats, Buchet *et al.* (1981) showed that in human subjects at 4 days after oral exposure to DMA^{V}, that approximately 75% of an oral dose was excreted in the urine.

There is a recent study that suggests the degree of accumulation of DMAs by the RBCs *in vivo* may vary based on which arsenical compound is administered (Suzuki *et al*., 2003 & 2004a) such that distribution to the RBCs following iAs exposure may be greater than distribution to the RBCs following MMA^{V} or DMA^{V} exposure. Suzuki *et al*. (2003) have shown that iAs^{III} is metabolized within hours to DMA in rats and that DMA is

subsequently redistributed to the RBCs. Suzuki *et al*. (2004a) has recently evaluated the accumulation of DMA by rat RBC exposed directly to MMA^V or DMA^V via iv injection. In this study, following injection of MMA^V or DMA^V, the amount of arsenic which redistributed in the RBCs by 12 hours after injection was lower than the total arsenic distributed in tissues at 10 min. Comparing the results of the Suzuki *et al.* studies (2003 & 2004a), the authors suggest that "DMA^V and MMA^V are redistributed less effectively in RBCs [compared to iAs^{III}] and more efficiently excreted into urine than in the form of arsenite (p. 342)."

As described in Section 5 and Appendix C of this document, work at EPA is on-going to develop a physiologically-based pharmacokinetic (PBPK) model to estimate internal exposure of DMA following oral exposure to mice, rats, and humans. This model accounts for species specific differences in metabolism and potential RBC retention.

In summary, the available *in vivo* metabolism studies indicate that:

- ❑ When exposed directly to iAs, arsenic is efficiently absorbed and methylated in human and laboratory animal tissues.

- ❑ Ingested MMA^V and DMA^V are eliminated more rapidly compared to ingested iAs.

- ❑ Studies in laboratory animals indicate little or no demethylation of either MMA^V or DMA^V (*i.e.,* production of inorganic arsenic from methylated arsenicals is minimal to nonexistent).

Figures 2.3a, 2.3b, and 2.3c summarize the results of *in vivo* metabolism studies described above.

Figure 2.3a provides a visual representation of some of the key differences between humans and rats regarding exogenous exposure to iAs. iAs is efficiently methylated to MMA^V and/or DMA^V.

Figure 2.3a: General metabolic profile following direct exposure to iAs.

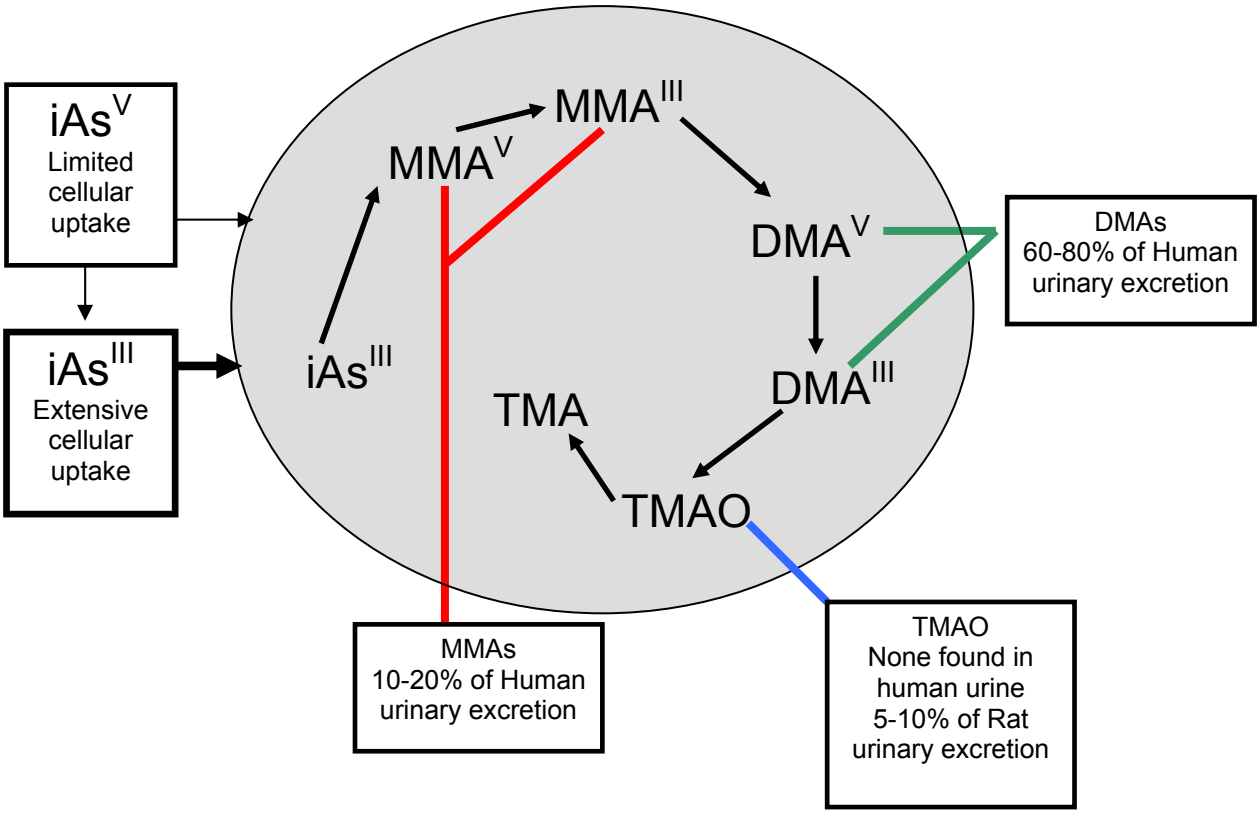


Figure 2.3b provides a visual representation of some key differences between humans and rats regarding exogenous exposure to MMA^V. Following direct exposure to MMA^V, humans and rats excrete MMA^V predominately unchanged. Only a portion of MMA^V is methylated to DMA^V in humans and rats. Excretion of MMA^V occurs rapidly compared to excretion of arsenic following exposure to iAs.

Figure 2.3b: General metabolic profile following direct exposure to MMA^V

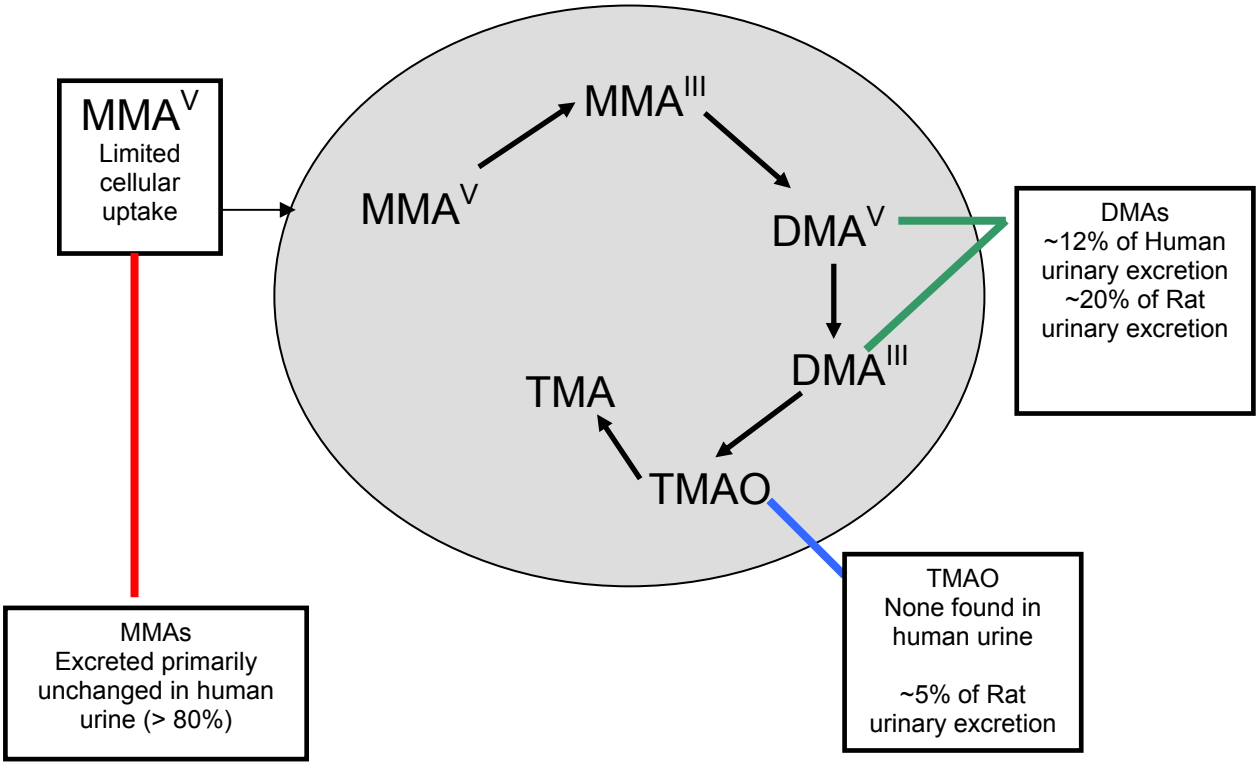
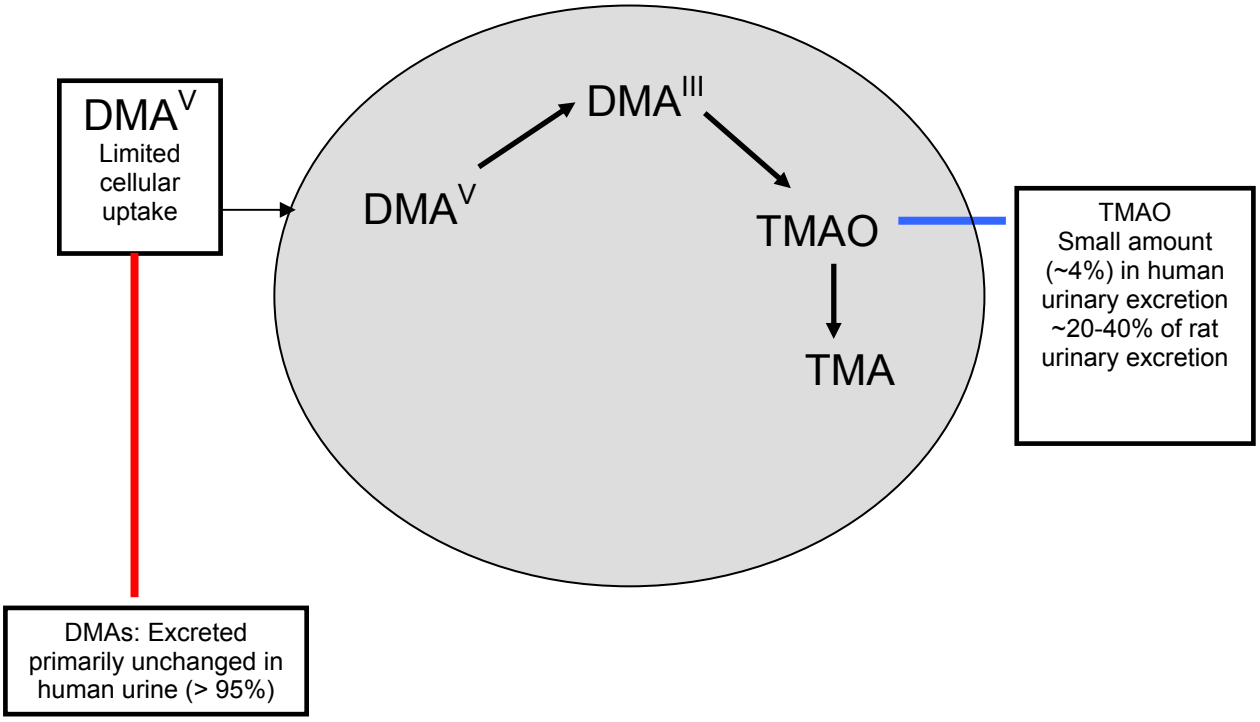


Figure 2.3c provides a visual representation of some key differences between humans and rats regarding exogenous exposure to DMA^V. Following direct exposure to DMA^V, humans excrete DMA^V predominately unchanged. Rats tend to excrete more TMAO than other species. Excretion of DMA^V is more rapid than excretion of arsenic following exposure to iAs or MMA^V.

Figure 2.3c: General metabolic profile following direct exposure to DMA^V



2.C.3. *In vitro* Studies

There is evidence from *in vitro* studies which indicates that iAs is more readily taken up by cells compared to the cellular uptake of MMA^V and DMA^V. Because methylation occurs intracellularly (Lerman *et al.*, 1983; Styblo *et al.*, 1999; Drobna *et al*., 2004), these studies provide characterization of the biological processes leading to the differences observed in the *in vivo* metabolism studies. Furthermore, the differential cellular uptake of iAs, MMA^V, or DMA^V also provides characterization of the potential intracellular exposure contributing to the differential toxicological profiles of the arsenicals.

Delnomdedieu *et al.* (1995) showed that cellular uptake into rabbit erythrocytes at 24 hours of incubation followed the pattern: iAs^{III} > iAs^V > MMA^V > DMA^V. In a recent study using rat heart microvessel endothelial cells, Hirano *et al.* (2004) showed that uptake of MMA^{III} >> iAs^{III} > iAs^V > MMA^V > DMA^V> TMAO. In this study, based on the percent of cellular As/total, uptake of DMA^V into rat cardiac endothelial cells was 2-fold less than MMA^V and 3.5-fold less than As^V (Hirano *et al.*, 2004). Hirano *et al*., (2004) also suggests that the greater cardiac toxicity associated with MMA^{III} (Table 2.1) may be due to the more rapid accumulation by the cells.

Delnomdedieu *et al.* (1995) and Hirano *et al*. (2004) both showed that uptake of MMA^V is greater than uptake of DMA^V. However, Sakurai *et al.* (1998) showed that following incubation at 1mM, uptake of MMA^V in peritoneal macrophages from CDF1 mice was five fold less than DMA^V or TMAO. Sakurai *et al.* (1998) did not include iAs in their studies.

Shiobara *et al.* (2001) incubated DMA^{III} and DMA^V with rat, hamster, mouse, and human red blood cells (RBC) to compare uptake rates among the species and between the forms of DMA. DMA^{III} uptake into human, rat, and mouse erythrocytes was more efficient than DMA^V with the rat > hamster > human. The authors indicate that "DMA^V was practically not or taken up slowly by RBCs of all species." Similarly, using Chinese hamster ovary (CHO) cells, Dopp *et al*. (2004) showed that only 0.03% MMA^V and DMA^V were taken up by cells compared to 2% for MMA^{III}, iAs^{III}, and, iAs^V, and 10% uptake for DMA^{III}. Patterson *et al.* (2003) also showed that uptake of MMA^V and DMA^V by cultured human keratinocytes was very slow compared to that of iAs^{III} and iAs^V. Vahter and Marafante (1983) showed that based on per cent of arsenic bound *in vitro* to plasma and tissues (liver, lung, kidney) of mice and rabbits that substantially more As^{III} and As^V were bound *in vitro* compared to DMA^V.

Drobna *et al.* (2004) showed that the cellular uptake of iAs^{III} MMA^{III}, and DMA^{III} in UROtsa and UROtsa/F35 cells was biphasic with rapid uptake in the first 15 minutes of incubation. In the same study, cytoxicity was considered. In both cell lines the cytoxic potency was MMA^{III}> DMA^{III} >iAs^{III}.

Regarding *in vitro* metabolism, Patterson *et al.* (2003) showed that human keratinocytes reduced As^V but did not reduce MMA^V and DMA^V. Similarly, Styblo *et al*. (1995) have reported that MMA^{III} is readily methylated but MMA^V is not. Consistent with these findings are those of Zakharayan *et al.* (1999) who showed that MMA^{III} is the preferred substrate for MMA-methyltransferase.

In each of the studies described above, cellular uptake of iAs^{III} and/or iAs^V was shown to be greater than cellular uptake for MMA^V and DMA^V. *In vitro* metabolism studies also suggest that reduction of iAs^V occurs at a greater rate *in vitro* than reduction of DMA^V, and that MMA^{III} is readily methylated but MMA^V is not. The *in vivo* metabolism studies described above indicate that methylation is more efficient in humans, mice, hamsters, and rats following direct exposure to iAs compared to methylation rates following direct exposure to MMA^V and DMA^V. The *in vitro* studies suggest that, at least in part, the differences in *in vivo* methylation may be related to the degree to which arsenical compounds are taken up into the cell as well as reduced.

2.D. Toxicodynamic Considerations

2.D.1. *In vivo* Toxicities Associated With Arsenical Compounds

The previous sections (2.B and 2.C) provide the scientific evidence which indicates that the toxicokinetics of ingested iAs are different than the toxicokinetics of MMA^V and DMA^V. Detailed discussion of all the possible toxicities caused by these arsenical compounds are beyond the scope of this document; however, the toxic effects observed in toxicity studies suggest that these chemicals cause different toxic responses and exhibit different dose-response characteristics (Figure 2.4).

In acute toxicity studies in rodents, MMA^V and DMA^V are less toxic compared to iAs. In mice DMA^V is approximately 10-fold less acutely toxic compared to iAs. (Kaise *et al.*, 1985, 1989). Oral LD_{50}s reported in rats range from 821 - 1935 mg/kg and from 644 - 1315 mg/kg for MMA^V and DMA^V, respectively (Gaines & Linder, 1986; Beavers *et al*., 1991; Sabol 1984). The acute oral toxicity of iAs in humans shows lethal effects in the range of 22-121 mg/kg, which is consistent with results of animal studies showing lethality of iAs in the range of 15-175 mg/kg.

Long-term toxicology studies in rodents with iAs^V, MMA^V, and DMA^V do not yield similar effects *in vivo*. Long-term studies with iAs^V suggest that humans develop various adverse health effects including diabetes mellitus, cardiovascular disease, renal disease, vascular skin lesions and cancer, and lung, liver and bladder cancer. Long-term studies with MMA^V suggest that the large intestine is the target organ with no neoplastic lesions observed at any site. DMA^V causes bladder tumors in

rats after feeding or drinking water exposures. Exposure of rats to TMAO in the drinking water resulted in an increase in liver tumors.

In a recent study Tg.AC mice (v-Ha-ras transgenic) were exposed to iAs^{III}, MMA^{V}, or DMA^{V} in drinking water for 17 weeks. After 4 weeks of arsenic treatment, the mice were also treated dermally with TPA (12-O-tetradecanoylphorbol-13-acetate). cDNA microarray and reverse transcriptase-polymerase chain reaction analysis showed altered gene expression from exposure to each of the arsenicals. However, exposure to MMA^{V} and DMA^{V} induced a different pattern of gene expression compared to exposure to iAs^{III} (Xie *et al.*, 2004). Using microarray techniques, Sen *et al.* (2005) have recently shown that qualitatively the genes up-regulated in human urinary bladder epithelial (UROtsa) are similar to those up-regulated in rat urinary bladder epithelial cells (MYP3) exposed to DMA^{V} *in vitro*. The rat cell line, however, was quantitatively more sensitive compared to the human cell line.

2.D.2. Relative Toxicity of Various Arsenical Compounds *In vitro*

A number of *in vitro* studies have evaluated the relative toxicity of arsenic-containing compounds. The toxicities observed vary over several orders of magnitude. The results provided in Table 2.1 are consistent with those of the cellular uptake studies.

- ❑ iAs^{III} and iAs^{V} are more cytotoxic than MMA^{V} and/or DMA^{V}. iAs^{III} and iAs^{V} are also more readily taken up by the cell compared to MMA^{V} and/or DMA^{V}.

- ❑ MMA^{III} and DMA^{III} are more cytotoxic *in vitro* compared to MMA^{V} and/or DMA^{V}. MMA^{III} and DMA^{III} are more readily taken up by the cell compared to MMA^{V} and/or DMA^{V}.

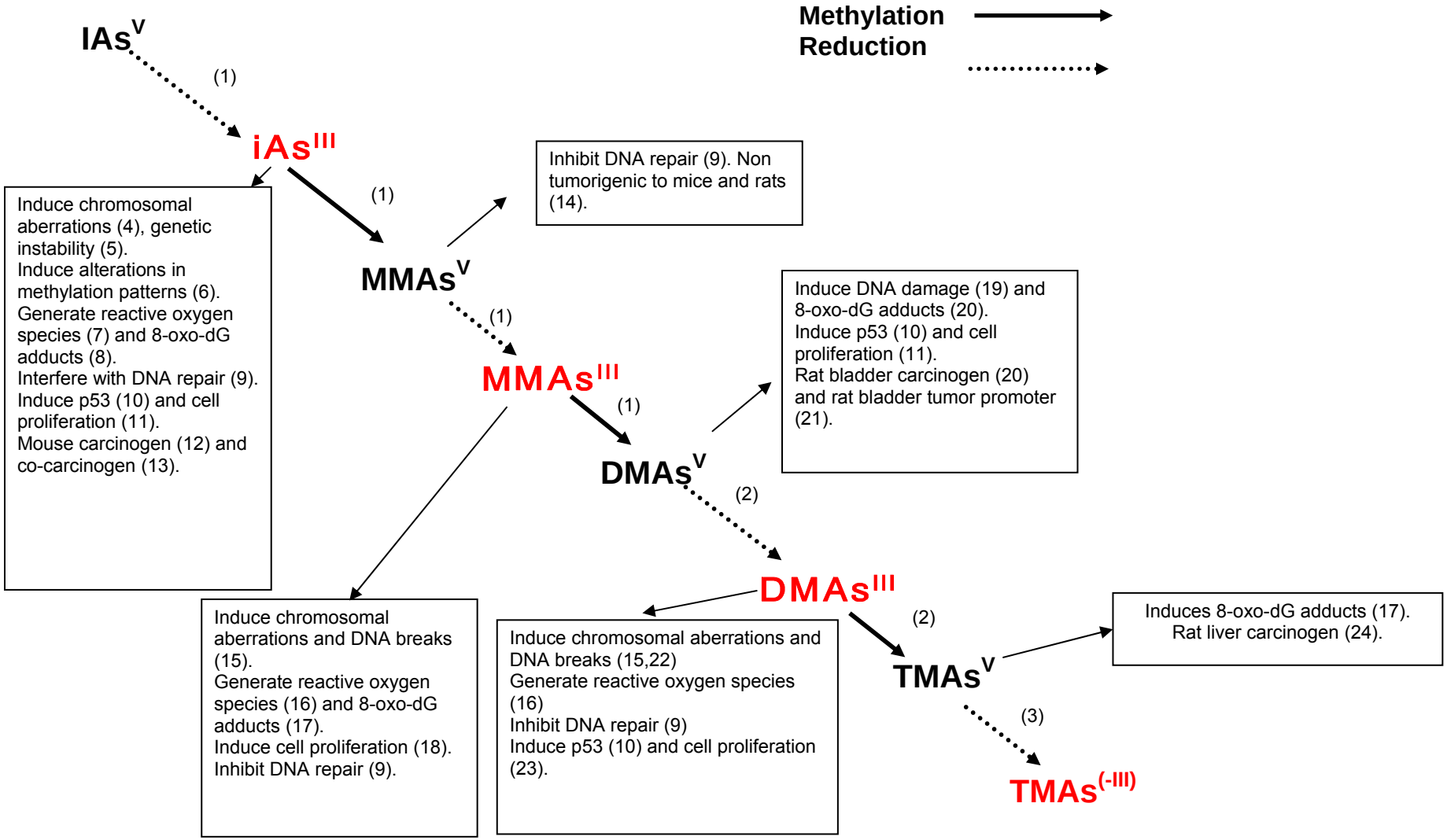
- ❑ MMA^{III} is generally more toxic compared to iAs^{III} and DMA^{III}.

Table 2.1: *In vitro* LC_{50} (mM) values for various arsenical compounds

Reference	Cell line	iAs^{V}	iAs^{III}	MMA^{V}	MMA^{III}	DMA^{V}	DMA^{III}	TMAO
Sakurai *et al.*, 1998	Murine macrophage	0.5	0.005	> 10	NT	5	NT	> 10
Styblo *et al.*, 2000	Primary rat hepatocytes	NR	0.01	NR	0.0028	NR	0.014	NT
	Primary human hepatocytes	NR	>0.02	NR	0.0055	NR	>0.02	NT
	Human epidermal keratinocytes	NR	>0.02	NR	0.0026	NR	0.0085	NT
	Human bronchial epithelial cells	NR	0.003	NR	0.0027	NR	0.0068	NT
	Urotsa cells	NR	0.017	NR	0.0008	NR	0.014	NT
Petrick, *et al.*, 2000	Chang human hepatocytes	1.6	0.068	8.2	0.006	9.1	NT	NT
Cohen *et al.*, 2002a	Rat epithelial bladder (MYP3)	0.0053	0.0004	1.7	0.0008	1.1	0.0005	4.5
	Human epithelial urinary bladder (1T1)	0.031	0.0048	1.7	0.0001	0.5	0.0008	1.7
Hirano *et al.*, 2004	Rat heart microvessel endothelial (RHMVE)	0.22	0.036	36.6	0.0041a	2.54	NTb	>100
Andrewes *et al.*, 2004	Normal human epidermal keratinocytes (NHEKS)	0.1	0.01	>6	0.001	3	0.001	>6

a. MMA^{III} (GS)2
b. NT = Not tested
c. NR = Tested by investigators. Not toxic up to highest concentration tested (20µM).

Figure 2.4: Summary of toxicities observed with arsenical compounds (References provided in Appendix A)



2.D.3. Complicated Mixtures of Metabolites

Figures 2.2 and 2.4 provide the metabolic scheme for arsenicals. The pathway is considered predominately one-directional in most mammals. Environmental exposure to iAs results in an internal mixture of biologically active metabolic products [iAs^{V}, iAs^{III}, MMA^{V}, MMA^{III}, DMA^{V}, DMA^{III}, and TMAO)]. Some of the *in vivo* and *in vitro* biological activities identified for each of these metabolites are noted in Figure 2.4. The results of *in vivo* studies provide different results depending on the administered chemical. *In vitro* studies suggest a variety of biological activities ranging from cytotoxicity to indirect genotoxicity. Hughes *et al.* (2000, 2003, 2005) have shown that the *in vivo* tissue distribution of arsenic varies with time, dose, and which methylated arsenical is administered. Mandel *et al.* (2004) showed that the profile of arsenical compounds varied among different biological samples (hair, nails, plasma, and urine) in 41 people from West Bengal, India exposed to drinking water contaminated with iAs. Thus, qualitatively, the composition of the potential mixture(s) of metabolites in various tissues *in vivo* is currently not known. Similarly, the concentration of these metabolites throughout the body is not known at this time.

Because of the predominately one-directional nature of the pathway in mammals, following direct exposure to DMA^{V} the number of biologically active metabolic products is expected to be smaller compared to direct ingestion of iAs^{V} (Figure 2.3). For example, environmental exposure to iAs^{V} may result in an internal mixture of multiple biologically active metabolic products [iAs^{V}, iAs^{III}, MMA^{V}, MMA^{III}, DMA^{V}, DMA^{III}, and TMAO)]. However, because of the predominately one-directional nature of the methylation/reduction, following ingestion of DMA^{V}, metabolism to DMA^{III} and TMAO can occur (Figure 2.4). Thus, the situation for DMA^{V} is simpler compared to the internal mixture following exposure to iAs.

It has been suggested that intracellularly generated DMA^{V} and/or DMA^{III} may contribute to the carcinogenicity of iAs. However, the ultimate carcinogenic metabolite(s) has not yet been identified for iAs. As shown in Figure 2.4, each of the arsenical metabolites exhibits its own spectrum of toxicities and potencies. The degree to which the toxic metabolites interact at the site of action is unknown. The degree to which this internal mixture of metabolites impacts the final health outcome is not known. Thus, the contribution of DMA^{V} to the carcinogenic potency of iAs in humans can not be quantified reliably. Furthermore, humans tend to excrete more MMA in urine compared to other mammals. It is notable that MMA^{III} is generally more cytotoxic compared to the other metabolites, including DMA^{III} in some *in vitro* systems (Table 2.1).

Chen *et al.* (2003) observed a significant statistical interaction between cumulative arsenic exposure and the ratio of DMA/MMA in urine in relation to risk of bladder cancer in a Taiwanese cohort exposed to iAs

in drinking water, especially when duration of exposure was considered. Specifically, those with bladder cancer tended to exhibit a lower ratio of DMA/MMA compared to those without bladder cancer suggesting that higher levels of MMA urine may be associated with bladder cancer.

2.E. Weight of the evidence and Summary: Data for evaluating potential cancer risk to DMA^V

Exposure to high levels of iAs in the drinking water is associated with increased risk for urinary bladder tumors in humans. Exposure to DMA^V in the diet and drinking water results in urinary bladder tumors in rats. Because DMA^V is a urinary metabolite produced in humans exposed to iAs, it has been suggested that the epidemiological data from drinking water exposure to iAs be used to quantify the potential cancer risk following direct oral exposure to DMA^V. The available dose-response studies, including those that relate to toxicokinetics and toxicodynamics for iAs^V, MMA^V, and DMA^V have been evaluated to determine which dataset should be used for quantifying the potential for human cancer risk from exposure to DMA^V. It was concluded that:

- ***In vivo* metabolism studies in animals and humans indicate important differences in the metabolism of iAs^V, MMA^V, and DMA^V.**

  The general features of the metabolic pathway for iAs are similar in many mammals including rodents and humans. However, there are important differences between the efficiency of the methylation and reduction steps dependent on which chemical is administered orally. When exposed to iAs, urine in humans typically contains 10-20% iAs, 10-20% MMA, and 60-80% DMA. However, when arsenic enters the pathway as MMA^V or DMA^V it is rapidly excreted mainly as the parent organic arsenical without further methylation in the urine of mice and humans. Studies performed in rodents indicate that this pathway is predominately one way in that little or no demethylation of MMA^V and DMA^V occurs so that there would be little to no iAs present when exposed to an organic arsenical.

- ***In vitro* studies provide, in part, a basis for the differences noted in the *in vivo* studies--- poor cellular uptake and limited metabolism of MMA^V and DMA^V.**

  Cellular uptake of iAs^{III} and/or iAs^V has been shown to be greater than cellular uptake for MMA^V and DMA^V. Reduction of iAs^V occurs at a greater rate *in vitro* than reduction of MMA^V and DMA^V. MMA^{III} is methylated at a higher rate compared to MMA^V.

- **Long-term toxicology studies in rodents administered iAs^V, MMA^V, and DMA^V in feed or drinking water do not produce the same adverse effects.**

Long-term studies with iAs suggest that humans develop various adverse health effects including diabetes mellitus, cardiovascular disease, renal disease, vascular skin lesions and cancer, and lung, liver and bladder cancer. Long-term animal studies with MMA^V suggest that the large intestine is the target organ with no neoplastic lesions observed at any site. DMA^V causes bladder tumors in rats after feeding or drinking water exposures. Exposure of rats to TMAO in the drinking water resulted in an increase in liver tumors. Furthermore, preliminary studies using microarray techniques provide support for the results of the long-term studies.

- **Mixture of toxic metabolites possibly generated after exposure to iAs is more complicated than that of DMA^V.**

  The ultimate carcinogenic metabolite(s) is not known for iAs— the parent iAs and each of the toxic metabolite products [iAs^V, iAs^{III}, MMA^V, MMA^{III}, DMA^V, DMA^{III}, and TMAO)] may contribute to the final health outcome. The potential for interaction of these metabolites *in vivo* has not been described and is not known. The internal mixture of metabolites following exposure to DMA^V is simpler, leading to fewer metabolites.

In conclusion, ingested DMA^V (or MMA^V) as the parent compound is not toxicologically equivalent to endogenously generated methylated arsenicals from iAs exposure. Although human data obviate the need for interspecies extrapolation in risk assessment, and thus represent valuable information to dose response assessment, epidemiological data are lacking for DMA^V. Given the kinetic and dynamic differences following exposure to iAs versus DMA^V, rodent (specifically the rat bladder tumors) data specific to direct DMA^V oral exposure is considered to provide a more suitable model for estimating potential cancer risk to humans. The rat can undergo the methylation and reduction steps involved in DMA metabolism like humans. There are, however, quantitative differences between rats and humans. Thus, important quantitative kinetic differences between these two species need to be addressed and characterized in the risk assessment.

3. Mode of Action Analysis for DMA^{V}[1]

As discussed in the previous section, due to the lack of epidemiological data specific to exposure to DMA^{V}, it is proposed that the laboratory rat be used as a model for evaluating the potential human cancer risk associated with exposure to DMA^{V}. When relying on laboratory animal data, two critical assumptions have governed cancer risk assessment for years. In the absence of information to the contrary, it is generally assumed that the experimental data on animal tumors are predictive of human cancer, and that the animal tumor effects found at high experimental doses predict human risk at lower exposures. In the case of DMA^{V}, mode of action data are available to evaluate the human relevance of the animal tumor responses and to guide the most appriopate dose response extrapolation approach for estimating human cancer risk. Thus, the purpose of this section is to present the postulated mode of action (MOA) for DMA^{V} induced carcinogenesis in laboratory animals and the evidence that supports it.

A postulated MOA is a biologically plausible hypothesis for the sequence of events leading to an observed effect (in this case, rat bladder tumors). It identifies "key" cellular and biochemical events—*i.e.,* those that are both measurable (quantifiable) and critical to the observed adverse response. Mode of action contrasts with mechanism of action which generally implies a more detailed description of the molecular and biochemical basis for an effect. The below analysis on DMA^{V} follows a mode of action framework developed by the International Programme on Chemical Safety (IPCS) (Sonich-Mullin *et al*., 2000) and the U.S. EPA (U.S. EPA, 2005), which is used by other regulatory agencies and international organizations (*e.g.,* the World Health Organization, Expert Panel of the Joint Meeting on Pesticide Residues). This MOA framework is based on the Bradford Hill criteria for causality, originally developed for application in epidemiological investigations (Hill, 1965). Both EPA and IPCS have emphasized that this framework "is not a checklist of criteria, but rather presents an analytical approach to considering the weight-of-evidence of an MOA" and whether a precursor event is shown to be causally linked to the tumor response.

This mode of action section will begin with a summary of the available cancer data to inform conclusions about potential human risk associated with exposure to cacodylic acid.

3.A. Summary of Carcinogenic Effects

3.A.1. Epidemiologic Studies

Numerous epidemiological studies show an association between ingestion of iAs and human skin, lung, bladder, kidney, and liver cancers, with lung and bladder cancers being the most common in chronic studies (Yoshida *et al*., 2004). Humans may be exposed directly to the arsenic containing compound DMA^{V}, also known as cacodylic acid, which is used as an herbicide. Six and thirteen poisoning incidents involving cacodylic

[1] It should be noted that this section does not provide an exhaustive review of the literature on arsenicals, but presents the key experimental findings for evaluating the rodent mode of carcinogenic action for DMA^{V} and its applicability to humans.

acid were reported in children under 6 years of age and adults, respectively, by the American Association of Poison Control Centers for the time period 1993-96 (USEPA 2000). Nineteen case reports of accidental worker exposure to cacodylic acid or one of its salts, sodium cacodylate, over a 14-year period (1982-96) have also been recorded as part of the California Pesticide Illness Surveillance Program. Adverse effects were recorded for the skin (contact dermatitis), eyes (swelling), and lungs (USEPA 2000), although doses of exposure are unknown.

Neither epidemiological nor controlled human studies of the adverse effects, including carcinogenicity, from exposure to DMA^V are available on cacodylic acid. As presented below, laboratory animal studies do exist.

3.A.2. Laboratory Animal Cancer Bioassay Studies

Rodent studies have been carried out using oral exposure to DMA^V to determine its carcinogenic potential. These include studies in rats and mice (Gur *et al*., 1989; Wei *et al*., 1999; NCI, 1969; Wanibuchi *et al*., 1996). In the standard rodent bioassay, DMA^V is associated only with bladder tumors in female and male Fischer (F344) rats, but not with neoplastic responses in either sex of B6C3F1 mice or two hybrid strains of mice (Gur *et al*., 1989; Wei *et al*., 1999; NCI, 1969). Although negative findings are found in the standard mouse bioassay, positive findings are reported in genetically engineered mouse strains or strains susceptible for specific tumor types. The rodent cancer data on DMA^V are discussed in more detailed below.

Two key standard bioassay studies demonstrate that DMA^V is a rat bladder carcinogen (Table 2.1.). Gur *et al*. (1989) treated male and female Fischer (F344) rats with 0, 2, 10, 40, or 100 ppm DMA^V in the diet (estimated: males- 0, 0.14, 0.73, 2.8, and 7.3 mg/kg/day; females - 0, 0.16, 0.79, 3.2, and 8.0 mg/kg/day) for 104 weeks (2 years). Only urinary bladder tumors were found to be treatment related. A statistically significant response (papillomas and carcinomas combined = 10/60 or 16.7%) was found in female rats that received 8 mg/kg bw per day. A slight non-statistical increase in carcinomas was observed at 7.3 mg/kg bw per day in male rats (2/59 or 3.4%). In female rats, the first carcinoma was found at week 87 at a dose of 8 mg/kg bw per day DMA^V. Administration of DMA^V in the feed of male and female B6C3F1 mice at doses of 0, 8, 40, 200 and 500 ppm (0, 2, 10, 50 and 126 mg/kg bw per day for males and 0, 3, 13, 62, and 151 mg/kg bw per day for females) for two years had no carcinogenic effects at any site (Gur *et al*., 1989). Additionally, NCI (1969) evaluated DMA^V in a bioassay study in mice. No increased incidences of any tumor type were found when two hybrid strains of mice (derived from C57 X C3H/Anf or AKR) were treated with 121 ppm (46.4 mg/kg bw per day) via gavage on postnatal day 7 until

weaning, and thereafter exposed via the feed for a total treatment period of 18 months.

Wei *et al*. (1999) treated only male F344 rats with DMA^V in the drinking water for 2-years at doses of 0, 12.5, 50, and 200 ppm (estimated: 0, 0.59, 2.7, and 10.7 mg/kg/day) and demonstrated a dose-dependent increase in urinary bladder tumors (papillomas and carcinomas) at 50 ppm (2.7 mg/kg bw per day) and 200 ppm (10.7 mg/kg bw per day) (Table 2.1.). No bladder tumors were observed at 12.5 ppm (0.59 mg/kg bw per day). The first bladder tumor was reported at week 97. Although there was an increase incidence in bladder tumors in this study at 2.7 mg/kg bw per day, there was no increase in tumor incidence when DMA^V was administered in the feed to both sexes of F344 rats at approximately the same daily dose (*i.e.,* 40 ppm or 3.2 mg/kg bw per day). It should be noted, however, that there was a high incidence of hyperplasia in the bladder at 3.2 mg/kg bw per day in the feeding study by Gur *et al*. (1989a—see Appendix B Table B.3.). It is uncertain whether the results of these two bioassays reflect a route of administration difference or simply inter-laboratory variation, substrain differences, or other confounding factors.

Table 3.1: Key Standard Rodent Carcinogenicity Studies with DMA^V: Incidence of bladder tumors in F344 rats

n& ation		Tumor incidence			
	Dose (ppm) **mg/kg/day***	0	2 **0.16**	10 **0.79**	
	Females				
	papillomas	0/59	0/59	0/57	0
	carcinomas	0/59	0/59	0/57	0
	Males				
	papillomas	0/60	0/59	1/59	1
	carcinomas	0/60	1/59	0/59	0
	Dose (ppm) **mg/kg/day**	0		12.5 **0.59**	
ater	Males**				
	papillomas	0/28		0/33	2
	carcinomas	0/28		0/33	0

*estimated for females
**Females were not evaluated.

Wanibuchi *et al*. (1996) treated male F344 rats with 100 ppm (8.68 mg/kg bw per day) DMA^V in the drinking water for 32 weeks. No bladder tumors were found after 32 weeks of exposure.

Although DMA^V showed no carcinogenic effects in the standard mouse bioassay (Gur *et al*., 1989b; NCI, 1969), it did produce positive

findings when evaluated in genetically engineered or tumor susceptible mice. Such strains can be useful for hazard identification and may provide insight into the chemical and gene interactions involved in carcinogenesis. It would be inappropriate, however, to use these strains for cancer risk extrapolation purposes because they are engineered to be highly susceptible to carcinogens. The special studies discussed below support the standard rat chronic bioassay data showing that DMA^V (and sequelae metabolites) is a carcinogen and can function as a tumor promoter.

Chen *et al*. (2000) used a genetically modified mouse skin cancer susceptible model (K6/ODC) and found a low but significant skin tumor response when DMA^V was given via drinking water (10 and 100 ppm) for 5 months. Salim *et al*. (2003) used another approach that involves a model in which the p53 tumor suppressor gene is deleted (knocked-out). This mouse strain is highly sensitive to carcinogens and carries a high background of lymphomas. These authors evaluated the carcinogenicity of DMA^V via drinking water at 50 and 200 ppm in an 18 month study using heterozygous p53 (+/-) knockout mice (estimated to be 3.3 and 12 mg/kg bw per day) and wild-type (estimated to be 2.8 and 10 mg/kg bw per day) C57BL/6J mice. Tumors appeared earlier in the treated p53 (+/-) knockout versus wild-type mice. Thus, the ability of DMA^V to reduce tumor latency for the most common tumor type found with this knockout mouse, malignant lymphoma, is consistent with its ability to act as a tumor promoter. Molecular analysis using PCR-SSCP techniques revealed no p53 mutations in lymphomas from either p53(+/-) knockout or wild-type mice, thereby confirming the genotoxicity studies on the lack of gene mutation induction by DMA^V and sequelae metabolites (discussed later in Section 3.B.6.1.).

DMA^V has also been evaluated in the A/J strain of mouse which carries a lung cancer susceptibility gene(s) (currently unknown) that imparts increased susceptibility to treatment with chemical carcinogens. Hayashi *et al*., (1998) reported that DMA^V caused lung tumors in A/J mice. The only statistically significant endpoint reported in this study was in the number of tumors per mouse at the highest dose (400 µg/ml in drinking water). Dose-related trends in the number of tumor-bearing mice, total number of tumors, and size of tumor were not statistically significant. The authors of the study concluded that their results were not definitive, and that "[F]urther studies using a larger number of animals, including other strains of mice and other species, are required to conclusively demonstrate the carcinogenic potential of DMA."

In conclusion, the rat bladder tumor response is the best model to assess the potential human cancer risk associated with exposure to DMA^V (Gur *et al*., 1989a; Wei *et al*., 1999). The mode of action analysis on the rat bladder tumor response follows.

3.B. Summary Description of Postulated Mode of Carcinogenic Action in Rats

A mode of action analysis begins with a summary description of the hypothesized mode(s) of action and an identification of key events (necessary elements or steps in the mode of action). In evaluating the mode of action for DMA^{V}, the multistep process of carcinogenesis is considered, which involves both mutation and increased cell proliferation.

The events along the path from a normal cell to a metastatic tumor are: a cellular event that results in the production of DNA damage (this can be direct interaction with DNA or indirect effects on DNA); production of genetic alterations (either gene mutations, structural chromosomal mutations, or numerical chromosomal changes); persistent cell proliferation; production of additional genetic alterations (as part of the multistage nature of carcinogenisis). As presented in this section, $DMA^{V/III}$ can potentially produce all of these events. The proposed mode of action is as follows:

- Reductive metabolism of DMA^{V} to DMA^{III} is necessary.

- DMA^{III} causes urothelial cytotoxicity. Regenerative cell proliferation then ensues in order to replace dead urothelial cells. [The amount of cell killing is a function of the severity of the cytotoxicity which is related to the amount of DMA^{III} present. The amount of DMA^{III} is dependent on the conversion of DMA^{V} to DMA^{III}.]

- The oxidative metabolism of DMA^{III} to DMA^{V} leads to production of reactive oxygen species, which in turn may lead to DNA damage.

- Stable chromosomal mutations must be formed for the oxidative DNA damage to be relevant to the carcinogenic process (*i.e.,* clonally expanded). The formation of chromosomal mutations requires DNA replication because chromosomal alterations are produced by errors of replication on a damaged DNA template. Thus, the frequency of chromosomal mutations will be a function of the regenerative proliferative response. With continuous exposure, persistent regenerative proliferation (a result of urothelial cell killing) leads to the production of additional mutations, including those necessary for multistep carcinogenesis.

Urothelial cell killing, regenerative cell proliferation and the production of stable genetic errors all must occur to result in bladder tumors.

The above hypothesized mode of action (MOA) is followed by an evaluation of the measurable key events in the target tissue. A determination of whether the key events are causal is supported by significant biological and/or statistical dose response and temporal associations with the tumor response. The below sections will present the experimental cellular and laboratory animal data evaluated to determine whether there are sufficient data to establish that the

observed rat bladder tumors occur via a cell killing/regenerative proliferation/genetic error process.

In this mode of action analysis, the evidence for cytotoxicity and regenerative proliferation will be presented first where there are quantifiable data available in the target tissue--the rat bladder-- for evaluating these events and for analyzing the dose response and temporal concordance with the tumor response. The data on genotoxicity will follow. Because there is a lack of *in vivo* data in the target tissue and a lack of *in vitro* data in a relevant cell population for genotoxicity, and a lack of clear dose response data for oxidative damage in the target tissue, inferences will need to be made from other assay systems for the involvement of a genotoxic component in the mode of action.

3.B.1. Identification of Key Events

A key event may be defined as an empirically observable, precursor step that is a necessary element of the mode of action, or is a marker for such an element. Based on the postulated mode of action presented above, there are several key events (metabolism, genetic errors via oxidative damage, cytotoxicity, cell proliferation) that are critical to the induction of bladder tumors following ingestion of DMA^V.

The most extensive dose response and temporal data directly related to relevant urinary concentrations of DMA^{III}, urothelial cytotoxicity and cell proliferation, are mostly derived from dietary feeding studies in rats from the laboratory of Dr. Samuel Cohen (University of Nebraska Medical Center). This laboratory primarily used female rats because this was the more sensitive sex in the cancer bioassay feeding study on DMA^V by Gur *et al*. (1989a). Mode of action data are also available from rat drinking water studies from Japanese laboratories, which provide data in support of the tumor promoting properties of DMA, as well as, its ability to result in oxidative damage and regenerative hyperplasia.

The reductive metabolism of DMA^V to DMA^{III} is an obligatory step in cacodylic acid's mode of action. It is this trivalent methylated arsenical (DMA^{III}) that has been shown to be highly toxic to cells from the urinary tract in several *in vitro* assays (Table 2.1) and in *in vivo* studies (discussed later). Cohen *et al*. (2002a) treated F344 female rats with 100 ppm DMA^V fed in the diet for 2 weeks. The authors measured both DMA^V and TMAO (trimethylarsine oxide) in the 24-hour urines of these animals. No DMA^{III} was detected in the urines. Because the urines were analyzed 4 weeks after collection, during this time the highly reactive compound was likely re-oxidized to DMA^V. In a second experiment, the authors treated female F344 rats with 100 ppm DMA^V and then analyzed freshly voided urine (rather than urine collected over a 24-hour period) that was also frozen 2 hours following collection. As shown in Table 3.2., the urine of rats treated with 100 ppm DMA^V for 1, 71, or 175 days had DMA^{III} micromolar concentrations in excess of the *in vitro* LC_{50} (0.5 uM) for rat urinary MYP3

cells (from Table 2.1). In contrast, TMAO, also present in rat urine at micromolar concentrations, was cytotoxic *in vitro* but only at millimolar concentrations. Thus, the rat urinary metabolite data coupled with the *in vitro* rat bladder cell LC_{50} data would suggest that DMA^{III} is the more significant urinary metabolite leading to urothelial cytotoxicity. In a more recent experiment, Arnold *et al*. (2004) confirmed not only the presence of DMA^{III} in the urine following ingestion of DMA^{V} in the diet, but demonstrated a dose-dependent increase in urinary DMA^{III} following dietary exposure to DMA^{V} for both 2 and 3 weeks (Table 3.2). *In vivo* urinary concentrations of DMA^{III} approached the LC_{50} for rat urinary MYP3 cells after 2 or 3 weeks of treatment of 40 ppm DMA^{V} and exceeded the LC_{50} at 100 ppm at both weeks.

Table 3.2: Identification of DMA^{III} in urine of rats exposed to DMA^{V}

Concentration DMA^{III} in fresh voided urine (µM) collected from female F344 rats fed DMA^{V} in the diet for various durations					
DMA^{V} ppm (mg/kg bw/day)	**Day 1**	**2 Weeks**	**3 Weeks**	**10 Weeks (Day 71)**	**25 Weeks (Day 175)**
	Cohen *et al*., 2002a N = 6-9	**Arnold *et al*., 2004 N = 7**	**Arnold *et al*., 2004 N = 7**	**Cohen *et al*., 2002a N = 6-9**	**Cohen *et al*., 2002a N = 6-9**
0	<0.026 (LLD)	<0.013 (LLD)	<0.013 (LLD)	<0.026 (LLD)	<0.026 (LLD)
2 (**0.2)***	---	0.02 ± 0.00	0.03 ± 0.07	---	---
10 **(1)**	---	0.05 ± 0.07	0.12 ± 0.05	---	---
40 (**4**)	---	0.25 ± 0.19	0.28 ± 0.24	---	---
100 **(9.4)**	1.38 ± 1.20[a]	0.92 ± 0.40[a]	0.55 ± 0.40[a]	5.05 ± 3.26[a]	0.80 ± 0.85[a]

Uncertainty expressed as ± S.D. of the mean
LLD=lower limit of detection
[a]*P*<0.05 when compared to respective controls
*mg/kg bw/day dose were estimated by averaging across time points and across studies, because rats eat more during their growth phase, the dose per kg will be higher at earlier timepoints than at later times.

The second key event in the postulated mode of action of DMA^{V} is urotheolial cytotoxicity. Both *in vitro* and *in vivo* assays of urinary tract cells inform the role of urothelial cell killing in the postulated mode of action.

As discussed earlier, Cohen *et al*. (2002a) treated rat MYP3 and human1T1 urinary bladder epithelial cell lines with identical concentrations of the tri- and pentavalent species of inorganic, mono-, di-, and trimethylated arsenic for 1 week. DMA^{III} was the most potent arsenical tested, causing death of 50% of the cells tested at concentrations (LC_{50}) of 0.5 uM and 0.8 uM in rat and human cell lines, respectively. Importantly, DMA^{III} was approximately several orders of magnitude more toxic than DMA^{V} in both rat (2200-fold) and human (625-fold) urothelial cell lines.

Cohen's laboratory also evaluated urothelial cytotoxicity in DMA^{V} treated rats using scanning electron micrographs (SEM). Because the

necrosis caused by DMA^V involved the superficial cell layer of the bladder, cytotoxicity was best observed by SEM. Urothelial cytotoxicity, as detected by SEM, was observed as early as 6 hours following exposure to DMA^V administered in the feed at 100 ppm (approximately 9.4 mg/kg bw/day) to female F344 rats (Cohen *et al*., 2001). The authors noted focal cellular necrosis after 24 hours to 3 days and widespread necrosis after 7 days of treatment at 100 ppm DMA^V. Figure 3.1. is a SEM showing normal rat bladder epithelium with large, flat, polygonal cells. Figure 3.2. is an SEM of rat bladder epithelium just 6 hours after dietary intake of 100 ppm DMA^V. Numerous cellular lesions are evident across the luminal surface of the urothelium, including pitting of individual cell membranes and the beginning of the separation of individual cells.

Figure 3.1: SEM of normal (class 1) rat bladder epithelium (570X original magnification)

Reprinted with permission of the authors (Cohen *et al*., 2001).

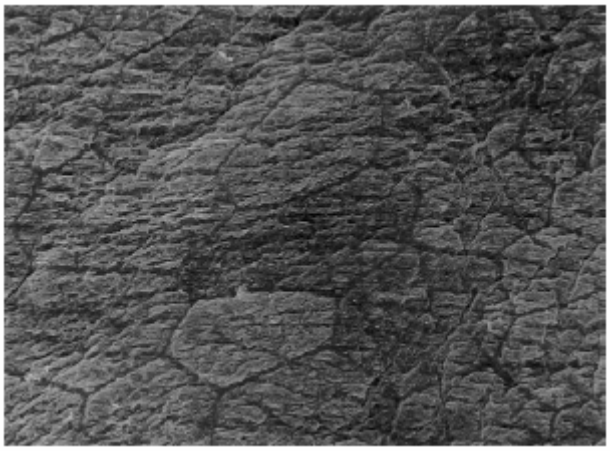


Figure 3.2: SEM of damaged/dying (class 3) rat bladder epithelium (1620X original magnification). Arrowheads: breakdown of intercellular junctions. Arrows: pitting of cells

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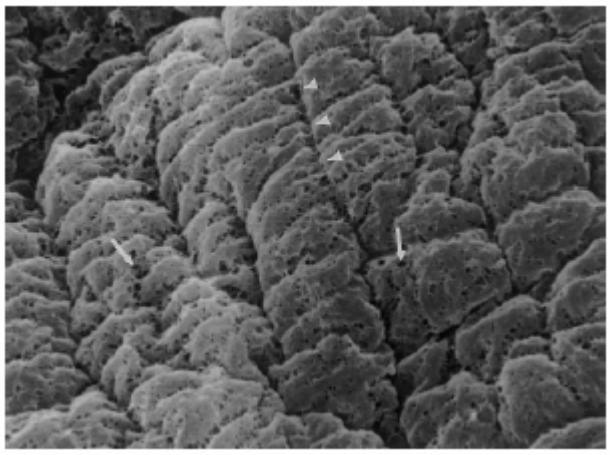


While bladder toxicity can be measured as early as 6 hours following dietary (feeding) administration of 100 ppm (approximately 9.4 mg/kg bw/day) DMA^V (Cohen *et al*., 2001), cytotoxicity to the urothelium is sustained well beyond 6 hours (Table 3.3). As shown in Table 3.3, the severity of urothelial toxicity is dependent on the dose and duration DMA^V treatment. The cytotoxicity found after 3 weeks of treatment (Arnold *et al*., 2004) was less than that after 10 weeks of treatment (Arnold *et al*., 1999) across dose groups. A complete summary of SEM data can be found in the Appendix B.1.

Table 3.3: Urothelial cytotoxicity in female rat bladder following dietary administration of DMA^V

DMA^V ppm (mg/kg bw/day)	Scanning Electron Microscopy (SEM) classification				
	1	2	3	4	
Cohen *et al*., 2001					
0 24 Hours	5	2	0	0	
100 (9.4) 24 Hours	0	3	2	2	
100 (9.4) 6 Hours	0	1	6	0	
Arnold *et al*., 2004 (Week 3)					
0	6	1	0	0	
2 (0.2)	4	3	0	0	
10 (1)	0	5	2	0	
40 (4)	0	0	7	0	
100 (9.4)	0	0	7	0	
Arnold *et al*., 1999 (Week 10)					
0	5	5	0	0	
2 (0.2)	0	4	5	1	
10 (1)	0	2	5	3	
40 (4)	0	5	3	2	
100 (9.4)	0	0	0	4	

[a]*P*<0.05 when compared to respective controls
mg/kg bw/day doses are estimated from an average across time points and across studies.
SEM classification key for bladder toxicity used in Cohen *et al*. (2001; 2002a):
1 = flat, polygonal superficial urothelial cells
2 = occasional small foci of urothelial necrosis
3 = numerous small foci of superficial urothelial necrosis
4 = extensive superficial urothelial necrosis, especially in the dome of bladder
5 = necrosis and piling up of rounded urothelial cells
Normal bladders are usually Class 1 or 2, but occasionally Class 3.

As discussed later, there is evidence that DMA^V can result in oxidative stress via formation of cellular reactive oxygen species (ROS). ROS are highly reactive and can potentially damage nucleic acids, lipids, and proteins. Thus, studies were pursued to determine whether antioxidants would be protective of $DMA^{V/III}$ induced urothelial

toxicity/regenerative proliferation. The effects of five antioxidants on the *in vitro* cytotoxicity of DMA^{V} or DMA^{III} in MYP3 cells was evaluated (Wei *et al*., 2005). Melatonin, Tiron and Troloxis had no effect on the *in vitro* cytotoxicity of either DMA^{III} or DMA^{V}. N-acetylcysteine (NAC) inhibited the cytoxicity of DMA^{III} and DMA^{V}, and vitamin C inhibited the *in vitro* cytotoxicity caused by DMA^{III}. Given these *in vitro* findings, NAC and vitamin C were further studied in a 10 week bioassay using female F344 rats (Wei *et al*., 2005). Although melatonin had no effect protecting against the cytotoxicity of DMA^{V} and DMA^{III} in MYP3 cells, it was included in the *in vivo* study to verify the *in vitro* results. The sodium salt of vitamin C (10,000 ppm), but not melatonin nor NCA, partially inhibited the proliferative effect of DMA^{V} in the bladder epithelium of female F344 rats when a 10 week dose of 100 ppm DMA^{V} was used. Although very few of the antioxidants evaluated by Cohen *et al*., were protective against the cytotoxicity/regenerative proliferation of $DMA^{V/III}$ *in vivo* and *in vitro*, these results, nonetheless, suggest that oxidative stress is at least in part involved in DMA^{V} induced rat bladder toxicity-regenerative proliferation. It is also possible that other pathways can contribute to cytotoxicity (*e.g.,* interaction with cellular proteins via reaction with sulfhydryl groups, and/or depletion of cellular glutathione).

The third key event in the postulated mode of action which follows urothelial toxicity is regenerative proliferation. The studies in female rats via feeding (Cohen *et al*., 2001; Cohen *et al*., 2002a; Arnold *et al*., 1999) provide the most useful and consistent information on the evaluation of cell proliferation because data are available across more doses and times. Arnold *et al*. (1999) administered 0, 2, 10, 40, or 100 ppm (estimated at 0, 0.2, 1, 4, and 9.4 mg/kg bw/day) DMA^{V} in the diet for 10 weeks to female F344 rats and found a clear dose response for measurements of cell proliferation based on incorporation of bromodeoxyuridine (BrdU) DNA labeling. As shown in Table 3.4, the lowest dose showing a statistically significant increase in regenerative cell proliferation was found to be 40 ppm (4 mg/kg bw/day) DMA^{V} in the feed of female rats after 10 weeks of exposure. There appears to be a good dose concordance between increases in the BrdU labeling index for females administered 2, 10 or 40 ppm DMA^{V} for 10 weeks corresponding with the increases in the severity (grade >3) of cytotoxicity observed at 10 and 40 ppm at 3-weeks or at 2, 10, or 40 ppm at 10-weeks (Table 3.3), indicating that increases in the severity of cytotoxicity is followed by increases in regenerative proliferation.

Table 3.4: Dose response of compensatory regeneration in rat bladder at 10 weeks following ingestion (feeding) of DMA^V

Cell proliferation response (BrdU labeling indices) Week 10 (females)		
Ppm	**Diet mg/kg/d**	**Arnold *et al*, 1999 Feeding**
0	0	(0.22 ± 0.14; N = 8)
2	0.2	1X (0.20 ± 0.09; N = 9)
10	1	1.5X (0.33 ± 0.25; N = 10)
40	4	4.3X (0.95 ± 0.42 [a] N = 8)
100	9.4	4.2X (0.93 ± 0.29 [a] N = 7)

Uncertainty expressed as ± S.D. of the mean at week 1
[a] $P<0.05$ when compared to respective controls
mg/kg bw/day estimated based on average across time points and studies

As shown in Table 3.5, regenerative proliferation is found following 100 ppm DMA^V in the diet of female rats for 1 week but not at the earlier time points evaluated. Also, at 100 ppm DMA^V in the diet (feeding) of female rats, cell proliferation begins to increase from day 3 to week 2, and thereafter is persistent (Cohen *et al*., 2001, 2002a; Arnold *et al*., 1999). A complete summary of the BrdU labeling index data can be found in Appendix B Table B.2.

Table 3.5: Time Course for compensatory regeneration in female rats following ingestion (feeding) of 100 ppm (9.4 mg/kg bw/day) DMA^V

Cell Proliferation Response (BrDU labeling Indices)											
	Hour 6	**Hour 24**	**Day 3**	**Week 1**	**Week 2**	**Week 2**	**Week 2**	**Week 10**	**Week 10**	**Week 20**	**Week 26***
	Cohen *et al*., 2001	**Cohen *et al*., 2001**	**Cohen *et al*., 2001**	**Cohen *et al*., 2001**	**Cohen *et al*., 2001**	**Cohen *et al*., 2001**	**Cohen *et al*., 2002a**	**Arnold *et al*., 1999**	**Cohen *et al*., 2001**	**Arnold *et al*., 1999**	**Cohen *et al*., 2002a**
0 ppm	---	0.42 ± 0.05	0.23 ± 0.04	0.44 ± 0.09	0.22 ± 0.03	0.19 ± 0.04	0.16 ± 0.02	0.22 ± 0.05	0.18 ± 0.03	0.25 ± 0.03	0.13 ± 0.02
100ppm	0.22 ± 0.04 (1X)	0.24 ± 0.04 (1X)	0.33 ± 0.11 (1.4X)	0.96 ± 0.14[a] (2.2X)	1.36 ± 0.13[a] (6.2X)	0.94 ± 0.20[a] (4.9X)	0.63 ± 0.10[a] (3.9X)	0.93 ± 0.11[a] (4.2X)	0.61 ± 0.10[a] (3.4X)	0.97 ± 0.11[a] (3.9X)	0.21 ± 0.03[a] (1.6X)

Uncertainty expressed as ± S.E. of the mean in all studies
[a] $P<0.05$ when compared to respective controls
[c]All results in female rats
*At 200 ppm DMAV in the diet of female rats, cell proliferation is increased 4X over controls (Wei *et al*., 2002).

More limited data are available on cell proliferation following drinking water exposures to DMA^V. Wanibuchi *et al*. (1996) administered 0, 10, 25, or 100 ppm DMA^V in the drinking water for 8 weeks to groups of male F344 rats (controls 10, low dose, 5, or high dose, 5) (see Appendix Table B.2.). All rats treated with 100 ppm DMA died during the first half of the study, thus, there are no data on cell proliferation from that dose group. Also, there is an unexplained lack of a dose-response in the Wanibuchi *et al*. (1996) study. Nevertheless, the Wanibuchi *et al*. study does show that a statistically significant increase in cell proliferation occurred as early as 8 weeks at a dose level of 10 ppm in drinking water. Wei *et al*. (2002) also reported a dose response increase in BrdU labeling indices in F344 male rats treated via drinking water at 50 and 100 ppm for 104 weeks. The lowest dose that bladder tumors were detected was 50 ppm in the chronic drinking water bioassay (Wei *et al*., 1999). Appendix B2 provides a summary of all available BrdU labeling index data for DMA^V treated rats.

Simple hyperplasia is an intermediate step in the pathogenesis toward neoplasia in the proposed mode of action of DMA^V-induced rat bladder tumors. It is also a necessary step in the process of urothelial carcinogenesis in general (Cohen, 2002b). Qualitatively, bladder carcinogenesis in the rat begins with an increase in the number of layers of urothelial cells (simple hyperplasia) and then proceeds through a series of steps that includes the appearance of focal papillary hyperplasia, papillomas (which are benign), noninvasive carcinomas, and invasive neoplasms (Cohen, 1998; 2002b). In this process, simple hyperplasia can be either focal or diffuse; metastasis is rare in the rodent (Cohen, 1998).

Appendix B3 summarizes the incidence of simple hyperplasia following treatment of rats with 0, 2, 10, 40, or 100 ppm DMA^V from various studies (Arnold *et al*., 1999; Cohen *et al*., 2001and 2002a; Gur *et al*, 1989a; Wei *et al*., 2002). By week 8 there is a marked increase of simple hyperplasia after dietary (feeding) treatment with 100 ppm DMA^V (Arnold *et al*. 1999) while up to 2 weeks of exposure to 100 ppm DMA^V (administration in feed or drinking water) did not result in a clear increase in hyperplasia (Cohen *et al*., 2001 and 2002a; Wei *et al*., 2004) and at week 3, there is a slight increase in hyperplasia, 2/7 rats which may be related to treatment (Arnold *et al*., 2004). At week 10 the incidence in females was 40% after 40 ppm DMA^V and 90% after 100 ppm DMA^V in feed (Arnold *et al*., 1999; Cohen *et al*., 2001). At week 104, there is no increase in hyperplasia at doses below 40 ppm in drinking water (Gur *et al*., 1989). These data support the presumption that a critical level of cytotoxicity and regenerative proliferation must be attained and sustained for hyperplasia and neoplasia to ensue.

3.B.2. Dose-Response Concordance of Key Events with Tumor Response

Essential to any mode of action analysis is to establish whether the dose-response relationship for the key events in the postulated mode of action parallels that of the tumor response (but not necessarily identical). Key events must be present at similar or lower doses than those needed to induce tumors. Table 3.6 is a summary of the presence or absence of each key event at each dose tested in the DMA^V rat feeding studies described above. Doses are arranged in order of increasing magnitude as one moves down column 1 in Table 3.6. Key events are arranged in increasing temporal sequence as the reader moves from the left side to the right of Table 3.6.

It can be reasonably concluded, therefore, that there is concordance between each dose of DMA^V tested and all key events (responses) measured. Table 3.6 illustrates the dose response concordance in that no key event occurs at a dose that is lower than that at which its antecedent key event occurs. For example, urothelial cytotoxicity (Table 3.3) was measured at every dose tested at week 10. However, urothelial regenerative proliferation, a key event occurring after sufficient cytotoxicity, did not occur after 2 ppm DMA^V. Stated another way, urothelial regenerative proliferation did not occur at any dose lower than those doses at which urothelial cytotoxicity, its antecedent event, occurred. Similarly, hyperplasia of the urothelium occurred at doses not lower than those at which regenerative proliferation was observed. The fact that 100 ppm caused the greatest amount of metabolism of DMA^V to DMA^{III} and cytotoxicity and hyperplasia, taken together with the fact that tumors were formed only after 100 ppm in the feeding chronic bioassay study, shows strong dose-response concordance between DMA^{III}

formation, cytotoxicity, hyperplasia, and tumor formation. As stated earlier there must be a sufficient level of cytotoxicity and proliferation, attained and sustained, to lead to hyperplasia and tumors. It appears that 40 ppm of DMA^V is needed to produce a critical level of cytotoxicity for regenerative cell proliferation to ensue. However, one can not exclude a marginal effect at 10 ppm based both on the Arnold *et al*. (1999) feeding study and the Wanibuchi *et al*. (1996) drinking water study.

Table 3.6: Summary of Key Precursor Events and Urinary Bladder Tumor Formation in Female F344 Rats Administered DMA^V in the Feed

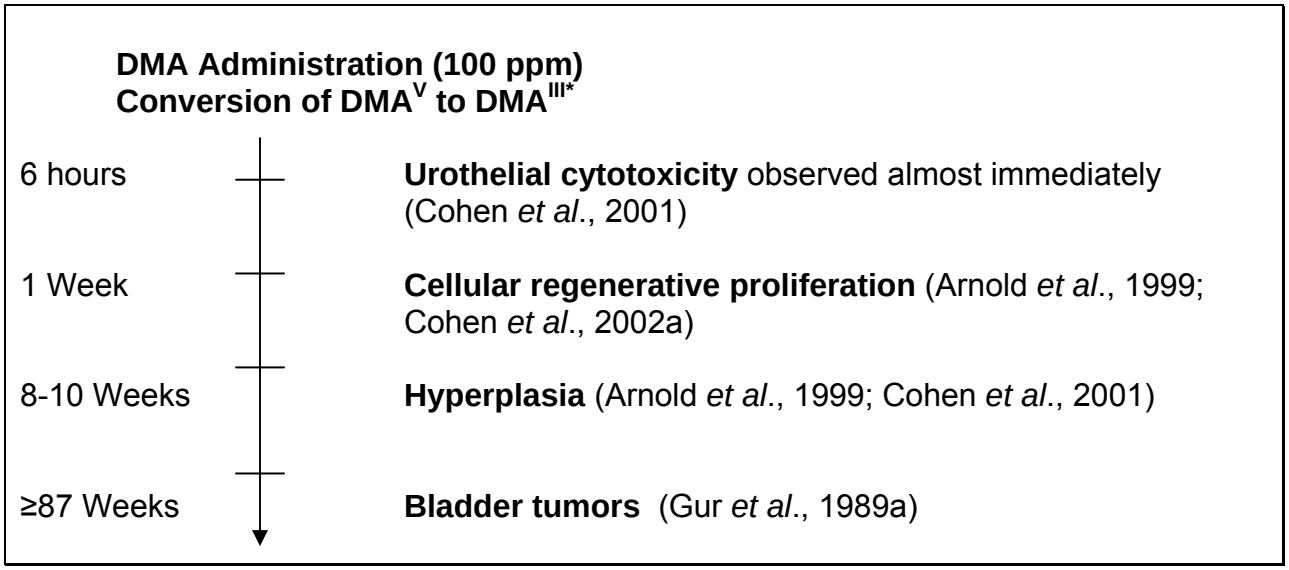
Dose ppm (mg/kg bw/day)	Metabolism of DMA^V to DMA^{III} *	Urothelial toxicity**	Regenerative proliferation response***	Urothelial hyperplasia****	Transitional cell carcinoma
2 (0.2)	**+** (week 3 0.03 ± 0.07uM)	**+** (week 10 6/10, grade 3 or 4)	**-**	**-**	**-**
10 (1)	**+** (week 3 0.12 ± 0.05uM)	**+** (week 3 2/7, grade 3) (week 10 8/10, grade 3 or 4)	**+/-** (week 10 nonstatistical 1.5-fold increase)	**-**	**-**
40 (4)	**+** (week 3 0.28 ± 0.24uM)	**+** (week 3 7/7, grade 3) (week 10 5/10, grade 3 or 4)	**+** (week 10 4.3-fold increase)	**+** (week 10 4/10)	**-**
100 (9.4)	**+** (week 3 0.55 ± 0.4 uM)	**+** (6 hrs 6/7, grade 3) (24 hrs 4/7, grade 3 or 4) (week 2 6/10, grade 5) (week 10 10/10, grade 4 or 5)	**+** (week 1 2.2-fold increase) (week 2 3.9 fold) (week 10 4.2-fold increase)	**+** (week 2 1/10) (week 8 7/10) (week 10 9/10)	**+** (Gur *et al*., 1989a; serial sacrifices not performed but carcinoma first observed at week 87)

* concentration of DMA^{III} in fresh voided urine collected from female rats fed DMA^V in the diet (Arnold *et al*., 2004; Cohen *et al*., 2002a)
**incidence of urothelial toxicity (number of animals affected over total number of animals examined) and SEM classification (Arnold *et al*., 1999; Cohen *et al*., 2001; Cohen *et al*., 2002a)
***BrdU labeling index, fold increase compared to control value (Arnold *et al*., 1999; Cohen *et al*., 2001)
****Simple hyperplasia, number of animals affected over total number of animals examined (Arnold *et al*., 1999; Cohen *et al*., 2002a)
+ present
- absent
mg/kg bw per day estimated by averaging across studies and time points.

2.B.3. Temporal Association

If an event(s) is an essential element of tumorigenesis, it must precede tumor appearance. The studies described in the preceding sections demonstrate a well-defined sequence for the key events in DMA^{V}-induced urinary bladder tumor formation. All key events precede tumor formation, and all key events occur in an ordered sequence relative to one another. This sequence is presented in Figure 3.3. The formation of DMA^{III} was not measured in rat urine collected before 24 hours. However, there was a significant (53-fold) increase (1.38 uM vs. <0.026 µM) at 24 hours following dietary ingestion of 100 ppm DMA^{V} (Table 3.2.). Urothelial cytotoxicity occurs as early as 6 hours after dietary ingestion of 100 ppm DMA^{V} (Table 3.3.) and compensatory proliferation follows cytotoxicity. The earliest time point that proliferation is found is after 1 week of treatment with 100 ppm DMA^{V}, but not before (Table 3.5). Simple hyperplasia occurs at 8 weeks after 100 ppm DMA^{V} administration in the diet, but is not seen before 2 weeks of exposure (Appendix B3). The first first carcinoma was found at week 87 at a dose of 100 ppm DMA^{V} (Gur *et al*., 1989a).

Figure 3.3: Temporal sequence of measurable key events in the target tissue: Postulated mode of action of DMA^{V}-induced urinary bladder tumor formation.



3.B.4. Genotoxicity

As discussed in the EPA 2005 cancer guidelines, there are a number of modes of action that can lead to DNA damage and ultimately may lead to mutation. Genetic alterations can result from direct damage to DNA through a chemical being DNA-reactive or from indirect effects, such as through the production of oxygen radicals that then react with DNA.

Different modes of action and the different types of genetic alterations (either gene mutations or structural or numerical chromosomal changes) may have different influences on the shape of the overall dose response for carcinogenesis.

In case of cacodylic acid's mode of action, the formation of chromosomal mutations is postulated to be a key event. As described below, DNA damage appears to result from an indirect process –*i.e.,* mediated by the production of reactive oxygen species. The weight of evidence indicates that both DMA^{V} and DMA^{III} are not effective gene mutagens, and DMA^{III} appears to primarily result in clastogenicity (chromosomal aberrations). The genotoxicity evidence is discussed below and summarized in Appendix B4.

DMA^{V}

As required for pesticide registrations, a series of guideline studies have been submitted to the Agency on DMA^{V} genotoxicity. These include the Ames assay, mouse lymphoma gene mutation ($L5178Y/TK^{+/-}$) assay, and the mouse micronucleus assay (MRID #41892706, 41892707, and 41892708). DMA^{V} produced negative findings in all of these studies. The conduct of these studies was considered acceptable. The published literature on the genotoxicity of DMA^{V} (summarized in Appendix B4) provides evidence in support of the negative results for the induction of gene mutations by DMA^{V} and DMA^{III}. The published literature, however, indicates that $DMA^{V/III}$ is clastogenic *in vitro*.

DMA^{V} was not mutagenic when evaluated in the Ames assay (Kligerman *et al*., 2003). When DMA^{V} was evaluated in the transgenic "Muta" mouse assay at 10.6 mg/kg bw for 5 days (i.p. injection), only a marginal mutation response (1.3X control) was seen in lung and no increase was seen in liver or bone marrow (Noda *et al*., 2002). In the "Muta" mouse assay, Noda *et al*. (2002) also evaluated micronucleus formation in peripheral blood reticulocytes, and found negative results with DMA^{V}.

In mouse lymphoma cells (L5178Y/TK+/- assay), which can tolerate chromosomal deletions at the TK locus, a low frequency of mutations (generally less than 2-fold) was seen, but at excessive concentrations (5,000-10,000 ug/ml; purity not reported) of DMA^{V} that resulted in approximately 70% cell killing (Moore *et al*., 1997). The majority of mutant colonies were found to be of the "small" type, indicating that they were induced by clastogenic effects (*i.e.,* chromosome breaking). Kligerman *et al*. (2003) reported chromosomal aberrations in human peripheral lymphocytes, but only at a high *in vitro* concentration of DMA^{V} (3,000 and 10,000 uM). Kligerman *et al*. reported cytotoxic effects on cell cycle and mitotic index at these concentrations. Oya-Ohta *et al*. (1996) reported an induction of chromosomal aberrations by high concentrations of DMA^{V}

(>700 uM) in cultured human fibroblasts. The authors did not provide information on cell survival or growth inhibition. DMA^V did not induce chromosomal aberrations in Chinese hamster ovary cells or in mouse L5178Y cells, and only weakly induced sister chromatid exchanges (SCEs) in human peripheral lymphocytes (Kligerman *et al*., 2003; Moore *et al*., 1997). Dopp *et al*., (2004) reported negative results for SCEs in Chinese hamster ovary cells for DMA^V when tested at 10 mM. DMA^V shows little or no ability to damage DNA when assessed *in vitro* using the DNA nicking assay with phage θX174 or by using a human peripheral lymphocyte *in vitro* single cell gel electrophoresis (comet) assay (Mass *et al*., 2001). There is a report of aneuploidy induction in the bone marrow of mice but only at a high i.p. dose (300 mg/kg bw) of DMA^V (Kashiwada *et al*., 1998). The induction of aneuploidy may indicate interference with spindle function (*i.e.,* a nonmutagenic mode of action).

In conclusion, the weight of evidence for DMA^V indicates that it does not lead to gene mutations and does not appear to be an effective inducer of chromosomal changes, particularly in light of the concentrations used to induce positive effects *in vitro*. As discussed below, DMA^{III} does appear to be clastogenic**.**

DMA^{III}

When evaluating the genotoxicity of DMA^{III}, it is important to establish whether or not positive findings are occurring at cytotoxic concentrations given that DMA^{III} is highly cytotoxic *in vitro* at micromolar concentrations.

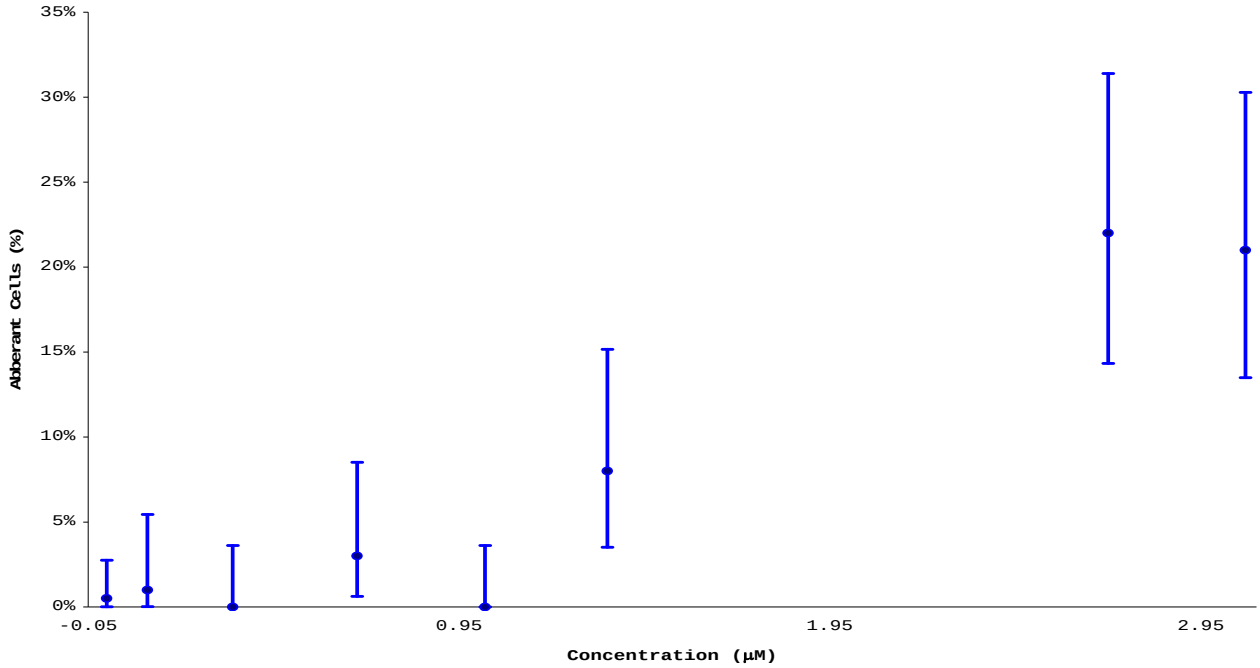
Kligerman *et al*., (2003) evaluated the genotoxicity of DMA^{III} in a series of studies. Their findings indicated that DMA^{III} does not appear to act as a direct DNA reactive gene mutagen. It is negative in bacterial gene mutation tests (*i.e.,* Ames assay and prophage induction assay in E. *coli*). DMA^{III} did elevate the mutation frequency (~2-3 fold) in the mouse lymphoma gene assay (L5178YTK-/+) at 1.29 uM (43% survival). The mutations were predominantly of the “small” colony type, indicating clastogenic effects. This observation is consistent with the chromosomal aberration findings discussed below.

There are a few studies in the literature reporting that DMA^{III} produces clastogenic effects *in vitro* (Kligerman *et al*., 2003; Ochi *et al*., 2003; Dopp *et al*., 2004) Most of these studies provide evaluations of cytotoxicity and found that chromosomal aberrations occurred in the presence of pronounced cytotoxicity (near the LC_{50}). Dopp *et al*., (2004) reported micronuclei formation at 1 -5 uM of DMA^{III} and the induction of chromosomal aberrations at 50 and 100 uM in Chinese hamster ovary cells. DMA^{III} only weakly induced the formation of sister chromatid exchanges at 50 uM. Dopp *et al*. evaluated cytotoxicity using the trypan blue staining and found that the LC_{50} was approximately 1 uM and

complete cell death was found at 100 uM. Ochi *et al*., (2003) found a concentration and time dependent induction of structural and numerical changes of chromosomal aberrations in cultured Chinese hamster V79 cells by DMA^{III} (source was dimethylarsine iodide). When incubated for 24 h, DMA^{III} caused a statistically significant increase in chromomsomal aberrations at 0.375 uM but not at 0.1 or 0.25 uM. When V79 cells were incubated with DMA^{III} at concentrations more than 0.25 M for 24 h, both losses and gains of chromosomes (aneuploidy) were observed in a concentration-dependent manner. The LC_{50} in this lung fibroblast cell line was about 1 uM after 24 hours of incubation.

Kligerman *et al*. (2003) found that DMA^{III} produced a dose-related increase in chromosomal aberrations *in vitro* using mitogen-stimulated human peripheral lymphocytes (in a single replicate study). Kligerman *et al*. also found that DMA^{III} was only a weak inducer of sister chromatid exchanges. The limited ability of DMA^{III} to induce SCEs coupled with its clastogenicity (predominantly chromatid breaks) and cytotoxicity are features of a genotoxin whose mode of action is likely to be via the production of reactive oxygen species (Speit *et al*., 1999; Povcirk, 1996). The concentration- response for induced chromosomal aberrations reported by by Kligerman *et al*., (2003), showed no significant effects at the low end of the dose response curve (0.1 to 1 uM) followed by a very sharp increase in aberrations beginning at 1.35 uM (Figure 3.4.). At 5.4 uM in this study, the cells were reported as “not scorable”. At the lowest dose producing a significant increase in chromosomal aberrations (1.35 uM), there is evidence of cell cycle delay. Also, at the 3.07 uM concentration of DMA^{III}, the mitotic index data are indicative of mitotic arrest which may be due to interference with spindle function.

Figure 3.4: Induction of chromosomal aberrations by DMA^{III} in human peripheral lymphocytes in vitro (data extracted from Kligerman *et al*.,2003).*



*The binomial distribution was used to calculate the 95% confidence intervals (CIs) for percent aberrant cells, where the count of aberrant cells represent the number of "successes" and the total cell count represents the number of "trials." The total cell count for each group was 100, except for the control group where the total count was 200. For proportions, CIs based on the binomial distribution are commonly referred to as "exact" CIs. The CIs for percent aberrant cells (which can be expressed as a proportion) represent "exact" CIs rather than approximate CIs calculated using the normal distribution. The normal approximations were not used since some lower CIs would be negative.

It should be noted that the *in vitro* chromosomal aberration data on DMA^{III} are generated from cell lines that do not represent the relevant target cell population, epithelial cells. These cytogenetic data can be used to state that DMA^{III} can potentially induce chromosomal alterations under specific cellular conditions – this is hazard identification, but not for purposes of quantitative cancer assessment Furthermore, the shape of the dose-response for these *in vitro* chromosomal alterations are irrelevant for considerations of tumor dose-response curve shape, because extensive proliferation is a feature of these assays, irrespective of the concentration of DMA^{III}.

DNA strand breaks are necessary precursor events for the formation of chromosomal aberrations. Thus, the positive findings of DNA strand breakage by DMA^{III} in various *in vitro* assays (summarized in Appendix B4.) are considered supportive of the positive findings of chromosomal aberrations *in vitro*. Flippova and Duerksen-Huges (2003) provides indirect support for the potential DNA damaging effects of DMA^{III} *in vitro* as assessed by the induction of p53 in U2OS cells at concentrations of 20 and 40 uM (human osteosarcoma cells) since the

p53 pathway senses and responds to DNA damage. There are published studies that assessed the induction of DNA strand breaks *in vivo* but mostly at high treatment doses of DMA^V (see Appendix B4). While the studies on DNA strand breakage do not inform the dose-response for tumor induction, they do indicate a potential for DMA^{III} to damage DNA. As discuss below, the DNA strand breakage found following DMA^{III} treatment may be due to the generation of reactive oxygen species.

There is a good deal of evidence that several trivalent inorganic and organic forms of arsenic can produce reactive oxygen species (ROS) and DNA damage when they interact with molecular oxygen and become oxidized to their pentavalent forms. These studies have been carried out with isolated DNA, in cell culture, and *in vivo,* the majority of studies being with arsenite (Huang *et al.*, 2004; Shi *et al.*, 2004). There are several important studies with DMA^{III} that link DNA damage with ROS formation, mostly *in vitro*. In two independent studies using isolated plasmid (BR322) (Ahmad *et al.*, 2002) or phage (ΦX174) DNA (Mass *et al.*, 2001), DMA^{III} was shown to damage DNA at 1 mM and 150 µM concentrations, respectively. Further studies using ΦX174 DNA revealed that the DNA damage was reduced or eliminated using ROS inhibitors, implying an ROS mechanism (Nesnow *et al*., 2002). Moreover, the presence of ROS formed in the DNA damage assays was confirmed as one form of ROS was trapped and was identified by Electron Spin Resonance (ESR) spectrometry as the highly reactive hydroxyl radical (Nesnow *et al.*, 2002). Based on other studies of DNA damage or pyrimidine damage with isolated nucleic acids, three additional reactive forms of dimethylarsenic have been proposed, dimethylarsenic peroxy radical, dimethylarsenic radical, and dimethylarsenic peroxide (Yamanaka *et al.*, 2004). While many of the isolated DNA studies used high µM to mM concentrations *in vitro,* as discussed earlier, DMA^{III} was found to induce chromosomal aberrations in isolated human peripheral lymphocytes at 1.35 µM (Kligerman *et al.*, 2003) and DNA damage (Comet assay) at 10 µM; (Mass *et al.*, 2001). In a related study, the toxicity of DMA^{III} in rat bladder MYP3 urothelial cells was partially inhibited by the ROS scavengers, N-acetylcysteine and vitamin C (Cohen *et al*., 2004). *In vivo*, sodium ascorbate (10,000 ppm) was also found to partially inhibit the bladder epithelial proliferative effects of DMA^V (100 ppm in the diet) when both were administered to rats for 10 weeks presumably through the pathway: $DMA^V \rightarrow DMA^{III} \rightarrow$ ROS (Wei *et al.*, 2005). 8-Oxo-dG is a potential marker of ROS. DMA^V administration to rats (200 ppm in the drinking water for 2 weeks) induced an elevation in the levels of 8-oxo-dG (presumably through the pathway: $DMA^V \rightarrow DMA^{III} \rightarrow$ ROS) in the urinary bladder (Wei *et al*., 2002). It should be noted however, there are uncertainties inherent in the 8-OH-dG adduct results due to methological issues.[2] Nonetheless, the weight of evidence support the notion that

[2] It should be noted that M. Wei has not been able to reproduce the 8-OHdG adduct result in the laboratory of Dr. S. Cohen (Cohen, personal communication). Dr. Cohen's laboratory is in the process of analyzing the urothelium chemically as the immunohistochemical analysis is open to artifactual variations. Dr. Douglas Wolf of EPA's National

DMA^{III} can interact with molecular oxygen to form ROS. DMA^{III} can produce DNA strand breaks and chromosome aberrations via the generation of ROS as a consequence of oxidation of DMA^{III} to DMA^{V}. Oxidative damage to DNA would be an indirect mechanism of genotoxicity.

In conclusion, $DMA^{V/III}$ does not appear to be effective at producing gene mutations based on *in vitro* and *in vivo* genotoxicity studies. When tested *in vitro*, DMA^{III} does appear to induce chromosomal aberrations. The ability of DMA^{III} to induce chromosome aberrations has only been evaluated in actively proliferating systems that used nonrelevant cells (the tumor cells are epithelial). The chromosome aberrations induced by DMA^{III} *in vitro* are predominantly of the chromatid-type; thus, they are produced during the S- or G_2- phases of the cell cycle. Based on extensive literature, the chromosome aberrations induced by almost all chemicals that result from errors of DNA replication lead to chromatid-type aberrations (Preston, 1999). The very few exceptions are chemicals that can induce chromosome aberrations in the absence of DNA replication, thereby leading to chromosome-type aberrations. These chemicals are ones that can induce DNA double-strand breaks directly, and include bleomycin, neocarzinostatin and cytosine arabinoside. They act similarly to ionizing radiation and are called radiomimetic. The chemicals that can induce oxidative radicals (and NOT double-strand breaks) produce chromatid-type aberrations as a result of replication errors at the site of oxidative DNA damage and/or DNA single-strand breaks.

A role for oxidative DNA damage in the formation of chromosome aberrations induced by DMA^{III} in the presence of DNA replication is suggested by studies of DNA damage using *in vitro* systems and by studies that characterize specific oxidative radicals. Thus, the formation of chromosomal aberrations of the chromatid-type requires some form of DNA damage, proposed to be from oxygen radicals, and DNA replication, that is considerably enhanced by DMA^{V}/DMA^{III} induced cytotoxicity and regenerative cell proliferation. The frequency of induced chromosome aberrations will be related to the levels of DNA damage and the probability of misreplication. The degree of misreplication is, in turn, related to the amount of cytotoxicity and regenerative cell proliferation. Given that cytotoxicity and regenerative cell proliferation induced in rat bladder by DMA^{V} are highly non-linear and that a broad database in the general literature on the dose-response curve shape for the production of oxidative DNA damage also provides evidence for non-linearity, it is concluded that

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Health and Environmental Research Laboratory in collaboration with Dr. Jim Swenberg's laboratory has attempted to use the monoclonal antibody developed by a Japanese group to identify 8-OHdG with no success, although they were able to use other methods to confirm the presence of this adduct in the tissues (McDorman et al., 2005). More recently Dr. Wolf's lab has attempted to achieve this same result using a commercially available kit, the Biotrin OxyDNA Test, but this too has been unsuccessful for cell lines and paraffin embedded tissue treated with bromate or DMA^{V}. Thus, there are not reliable data to confirm the linkage between generation of ROS and 8-OHdG adduct formation in the urothelial cells of rats.

chromosomal aberrations induced by DMA^V in rat bladder would be induced as a non-linear function of dose. This conclusion is supported by the observation that the chromatid-type aberrations induced by DMA^{III} in human lymphocytes (in the presence of cytotoxicity and proliferation) are nonlinear with concentration (see Figure 3.4).

3.B.5. Initiation and Promotion Studies

DMA^{III} has been reported to act as a tumor promoter in several organs, including the bladder in rodents (see Appendix Table B5. for a summary of studies), which is not surprising given the ability of $DMA^{V/III}$ to induce rat bladder tumors and its potential to result in cell killing/regenerative proliferation. These studies were viewed as qualitative information supporting the carcinogenic and tumor promoting ability of $DMA^{V/III}$.

3.B.6. Strength, Consistency, and Specificity of Association of Tumor Response with Key Events

There is evidence that supports the role of DMA^{III} as the significant DMA^V metabolite responsible for the cytotoxicity and regenerative proliferation observed in the rat bladder epithlelium. When 2,3-dimercaptopropane-1-sulfonic acid (5600 ppm DMPS), which inactivates trivalent arsenicals via chelation, was co-administered with 100 ppm DMA^V, urothelial cytotoxicity and cell proliferation was inhibited (Cohen *et al*., 2002).

The dose response and temporal data presented in the previous sections provide support for an association of the proposed key events to tumor formation in the rat urinary bladder. The association is strengthened by a stop/recovery study (Arnold *et al*., 1999) which demonstrated the reversibility of urothelial cytotoxicity, proliferation and hyperplasia. In this study, Arnold *et al*. (1999) administered 100 ppm DMA^V in the diet to 10 female rats for 10 weeks. At the end of this period, these same rats were treated with control diets (no DMA^V) for 10 weeks. After sacrifice, urothelial toxicity, compensatory regeneration, and simple hyperplasia were evaluated and compared to results for female rats treated with either 0 or 100 ppm DMA^V for 20 weeks. The results of this study are shown in Table 3.7. After 10 weeks on a DMA^V-free diet following treatment with 100 ppm DMA^V for 10 weeks, both hyperplasia and compensatory regeneration returned to control levels. Urothelial cytotoxicity, as defined as extensive urothelial necrosis (class 4) or necrosis with piling up of rounded urothelial cells (class 5) was still present after 10-weeks of recovery. The authors proposed that the continued morphologic evidence of cytotoxicity was due in part to the very slow rate of cellular turnover in the rat urothelium (15-29 weeks) (Table 3.6.). In addition, because rats accumulate and retain DMA in the RBCs due to binding to rat hemoglobin, it is possible that there is continued low level of

exposure to DMA released from hemoglobin that would result in some cellular toxicity to be present after stopping treatment for 10 weeks. Most importantly, and critical for this proposed mode of action, is the observation that hyperplasia and increased cell proliferation are reversible. Tumors will not arise except through hyperplasia. If hyperplasia is not present then tumors will not subsequently develop.

Table 3.7: Reversibility of Three Key Precursor Events in F344 Female Rats Administered DMA^{V} in the Diet (Arnold *et al*., 1999).

Dose (ppm)	Urothelial toxicity					Regenerative proliferation response	Urothelial simple hyperplasia
	SEM classification					BrdU labeling index (%)	Incidence
	1	2	3	4	5		
0 (20 weeks)	6	4	0	0	0	0.25 ± 0.03	1/10
100 (20 weeks)	0	0	3	6	1	0.97 ± 0.11[a]	4/10
0 (10 weeks)	5	5	0	0	0	0.22 ± 0.05	1/10
100 (10 weeks) followed by 10 week recovery phase	0	0	6	4	0	0.21 ± 0.04[b]	0/10

[a] $P<0.05$ when compared to respective controls
[b] $P<0.05$ when compared to 100 ppm at 20 weeks

Consistency of the association of the key events and tumor formation refers to the repeatability of the key events in different studies (Sonich-Mullin *et al*., 2001). Studies directed at understanding the mode of action for the carcinogenicity of DMA^{V} come from different laboratories and multiple studies with different investigators that have yielded remarkably consistent results.

3.B.7. Biological Plausibility and Coherence

Cytotoxicity and consequent regeneration is a well-known and well-documented mode of carcinogenic action for a variety of chemicals and for a variety of tissues in laboratory animals (*e.g.,* bladder, kidney, liver, nasal) (USEPA, 2001; IARC, 1999; Bogdanffy, 2002; Cohen 2002b; Meek *et al*., 2003). In general, the theory that sustained cell proliferation to replace cells killed by toxicity, viral or other insult, such as physical abrasion of tissues, can be a significant risk factor for cancer is plausible and generally accepted (Correa, 2004). Further, regenerative proliferation associated with persistent cytotoxicity appears to be a risk factor for bladder carcinogenesis in humans (Cohen 1989; 1998; 2002b).

It is generally accepted that the chemically induced neoplasia is a multistep process involving both cell proliferation and mutation (Klaunig and Kaendulis, 2004; Pitot *et al*., 2000). Although there are insufficient data to establish the induction of chromosomal mutations (presumably through the pathway: $DMA^{V} \rightarrow DMA^{III} \rightarrow$ ROS) as a "causal" event in DMA's carcinogenesis, oxidative stress to DNA as a key event in the mode of action $DMA^{V/III}$ is "plausible". DNA strand breaks are induced via the generation of ROS as a consequence of oxidation of DMA^{III} to DMA^{V}. The central biological event in DMA's mode of action, however, is the stimulation of cell proliferation, which is influenced by cytotoxicity, as this determines the probability of converting DNA lesions to "stable" chromosomal mutations. Also, mutations may arise as a result of the regenerative proliferation caused by the urothelial cytotoxicity resulting in the greater likelihood of pre-existing or spontaneous errors being perpetuated that would otherwise be repaired (Trosko and Upham, 2005). Both pathways (spontaneous DNA damage versus newly induced damage) resulting in mutation and/or clastogenic changes may be occurring. There are no quantifiable data in the rat bladder to distinguish between these two possibilities and current views of cancer processes are supportive of both.

3.B.8. Other Modes of Carcinogenic Action

In exploring alternative modes of action, it is important to consider what is understood about the risk factors involve in bladder carcinogenesis. Bladder carcinogenesis may be influenced by a variety of physical, chemical, and biological factors, and the rodent bladder is

generally susceptible to many of the same carcinogenic influences as the human bladder. Bladder tumors can result from an interplay between mutagenic and nonmutagenic processes (Reznikoff *et al*., 1996; Ramchurren *et al*., 1995). As discussed above, $DMA^{V/III}$ is not a direct acting gene mutagen but appears to produce chromosomal aberrations via an indirect mechanism (*i.e.,* oxidative damage). As discussed below, other potential toxicity pathways leading to bladder carcinogenesis have been considered and ruled out.

The formation of urinary solids (*e.g.,* calculi, precipitates or microcrystals) or changes in urinary physiology may be risk factors for bladder cancer development (Cohen 1998). Formation of urinary solids, or changes in chemistry do not appear to be the basis for the urothelial cytotoxicity found following treatment with DMA^{V}. Arnold *et al*. (1999) dosed female F344 rats with 0, 2, 10, 40, or 100 ppm DMA^{V} in the diet for 10 weeks. A group of female rats was also fed 100 ppm DMA^{V} for 20 weeks. Because male rats appear less sensitive to the cytotoxic effects of DMA^{V}, a group of male rats was only fed DMA^{V} at a high concentration, 100 ppm for 10 weeks. Examination of urinary filtrates or observation by scanning electron microscopy did not detect any evidence for the formation of urinary solids (calculus, precipitates, or microcyrstalluria), although dose- dependent increases in urothelial toxicity and hyperplasia were detected. In the same study, urinary pH, volume, and chemistries were also evaluated (Arnold *et al*., 1999). No consistent changes in urinary pH that could be associated with urothelial toxicity and hyperplasia were seen between DMA^{V} treated groups and controls. The observed dose-related increases in urinary volume were most likely associated with increases in water consumption. Most of the urinary constituents (*e.g.,* chloride, magnesium, sodium, and potassium) examined showed decreased concentrations, which would be expected for diluted urine secondary to increases in urinary volume. Increases in urinary calcium and renal calcification were observed in the females treated with 40 or 100 ppm but not until 10 weeks of treatment (Cohen *et al*., 2001). Because cytotoxic and proliferative changes were already present in the bladder by 2 weeks of DMA^{V} administration, changes in urinary calcium/renal calcification do not appear to be critical for the initial development of proliferative changes in the bladder (Cohen *et al*., 2001).

3.B.9. Uncertainties and Limitations

Some uncertainties remain in the database for DMA^{V} despite the clear association of cytotoxicity and regenerative proliferation and tumor response. In themselves, however, they do not discount the scientific support for postulated mode of action via cytotoxicity/regenerative proliferation/replication genetic error process.

- ❑ The cellular target for cytotoxicity is not understood (*e.g.,* interaction with cellular proteins via reaction with sulfhydryl groups).

This level of understanding, however, is informative for understanding the mechanism of toxicity (*i.e.,* detailed understanding of chemical and molecular steps) but is not necessary for establishing a mode of action.

- Three unknown metabolites have been reported (M-1, M-2, M-3). These metabolites may be cytotoxic and produce chromosome breaks (Yoshida *et al*., 1998; Wei *et al*., 2002; Yoshida *et al*., 2003; Kuroda *et al*., 2004). These metabolites may contribute to the urothelial toxicity and clastogenicity, and they may be species specific. At this time, their role in carcinogenicity in humans is unclear. The overall weight of evidence supports DMA^{III} as the significant metabolite of DMA^{V} that is associated with key events leading to the neoplastic response in DMA^{V} treated rats.

- Any inconsistencies in the data noted for cytotoxicity, proliferation and hyperplasia can be accounted for by variability across studies. Although there is an increase in cell proliferation in the male rat bladder following DMA^{V} treatment (drinking water), there is not a clear dose response in the Wanibuchi *et al*. (1996). BrdU labeling index data from Arnold *et al*. (1999) feeding study shows a clear dose response in female rats for compensatory replication that is associated with urothelial cytotoxicity and bladder tumors with respect to time and to increasing doses.

- The dose-response curves for the generation of ROS, oxidative DNA damage and chromosomal aberrations are not available for the bladder. Thus, the probability of chromosomal mutation induction via free radical formation at doses below those which induce urothelial cell killing and regenerative proliferation cannot be precisely predicted. Nonetheless, the available evidence for $DMA^{V/III}$ induced *in vitro* chromosomal aberrations along with the biological understanding of chromosomal mutation formation, as well as the *in vivo* evidence for the dose response of cytotoxicity/regenerative proliferation in the rat bladder suggests that in the absence of induced cell killing and regenerative proliferation, which are clearly nonlinear (perhaps even threshold) phenomena, the impact of $DMA^{V/III}$ induced chromosomal mutations on the cancer process in the bladder will be negligible.

3.B.10. Mode of Action Conclusions

The overall weight of the evidence provides convincing support for the postulated mode of action for DMA^{V} -induced carcinogenesis in rodents. The mechanism of DNA damage and the formation of chromosomal mutations through the production of ROS is plausible, although there are a lack of specific data in target tissue to establish causality. The formation of structural chromosomal alterations for the

great majority of chemicals requires DNA replication because chromosomal alterations are produced by errors of replication on a damaged DNA template. In the human *in vitro* lymphocyte (as well as the other *in vitro* cell systems) used to demonstrate DMA^{III} induced chromosomal aberrations, cell replication is an essential component of the assay and quite extensive proliferation is already present. The likelihood of inducing chromosome alterations in the bladder in the absence of induced cell proliferation is very low because in the urinary bladder of adult rats and humans, normal cell proliferation levels are very low (*e.g.,* on the order of about 0.1% over a 1 hour pulse of BrdU labeling or 1-2% after 4 days of BrdU in the drinking water). Given the importance of stimulated cell proliferation for increasing the likelihood of chromosomal mutation formation, dose response considerations for modeling the tumor response based on the production of genetic alterations requires the use of cell proliferation data. Such data are available for the rat bladder and could reasonably be extrapolated to the human bladder based on plausibility.

To obtain a tumor via the proliferation/replication genetic error process, induced cell proliferation would need to be persistent. There is convincing experimental evidence to indicate that this is the case for the rat bladder. There is a clear association of DMA^{V} treatment and cell killing/regenerative proliferation and bladder tumors. The amount of proliferation (and hence the frequency of chromosomal alterations) would be a function of the amount of cell killing since the tissue will undergo regenerative proliferation in response to cell killing. As the severity of cytotoxicity increases with increasing levels of DMA^{V} (DMA^{III}), regenerative proliferating is the rate limiting step for tumor formation, even though the product is chromosome mutations. Thus, a tumor dose-response curve would be influenced by the induced cell proliferation curve, even though chromosomal mutations may be an output. DMA^{V}-induced tumors would only be produced at treatment durations and dose levels that result in significant cell killing and regenerative cell proliferation in the urothelium of the bladder. Experimental data are available to support the coincidence of key events at similar concentration levels. The levels of DMA^{III} in the urine of rats treated with 100 ppm DMA^{V} range from 0.5 – 5.0uM. The LC_{50} values for DMA^{III} in rat and human urinary epithelial cells *in vitro* are 0.5-0.8uM. There is a significant increase in chromosome aberrations in human lymphocytes *in vitro* at about 1.35 uM DMA^{III}. At 100 ppm, there is significant cell killing and regenerative proliferation in female rat bladders. It appears that chromosomal mutations, cytotoxicity and cell proliferation can potentially occur concurrently at 100 ppm DMA^{V}, the tumorgenic dose in female rats via the feed.

Persistent regenerative proliferation as a result of persistent cytotoxicity would lead to the production of additional mutations, including those necessary for multistep carcinogenesis for bladder tumors. The approach that is clearly indicated by the available data in support of the

key events for tumor formation following exposure to DMA^V would be a biologically-based dose-response model, along the lines of a 2-stage clonal growth model. However, a reasonable alterative default approach would be a nonlinear risk assessment using a reference dose approach and a point of departure based on the rat bladder cytotoxicity or cell proliferation data. A linear extrapolation from the point of departure would only be justified if the mode of action data support this, and they do not.

In summary, the dose-response relationship for DMA^V tumorigenesis based on mode of action considerations will be nonlinear as it is dependent on genetic, biochemical and histopathological events for which dose-response relationships are nonlinear. There must be a sufficient concentration of DMA^{III} in the bladder to produce cell death and regenerative proliferation. The dose-response assessment would ideally be based on use of DMA^{III} dosimetry at the target tissue because it represents the rate-limiting event of reductive metabolism to DMA^{III} to provide a level of exposure that will be protective against the key event of regenerative proliferation. Therefore, the mode of action analysis shows that sufficient DMA^{III} must be present to result in sufficient urothelial cytotoxicity and cell killing to result in increase cell poliferation and associated chromosomal aberrations. All of these events must occur to result in a neoplastic response. Any one event alone is not sufficient to lead to tumors.

The SAB should refer to additional discussion on the mode of carcinogenic action for DMA^V which is provided in Appendix E. It should be noted that the parts of this appendix that refer to the OPP document on cacodyic acid were based on an earlier draft of this paper.

4. Human Relevance

A mod e of action understanding of carcinogenesis greatly improves the ability to rigorously evaluate key assumptions used in cancer risk assessment when extrapolating the tumor results from experiments in laboratory animals to predict and estimate human cancer risk. Two fundamental default assumptions are: 1) the results in the animal bioassay are relevant to humans (interspecies extrapolation); and 2) the doses used in the animal bioassay are relevant for estimating risk at known or expected human exposure levels (dose extrapolation). The metabolism assessment and the mode of action analysis for DMA^V are presented in Sections 2 and 3, respectively. The data and information discussed in these sections is used below to evaluate the human relevance of the DMA^V induced rat bladder tumors. To address the issue of the human relevance of the mode(s) of action determined in rats, a recent Human Relevance Framework approach developed by an expert working group under the Risk Sciences Institute (RSI) of the International Life Science Institute has been used and presented in this section. This framework is published in a 2003 issue of Critical Reviews in Toxicology (Meek *et al*., 2003)[3]. The 2003 Human Relevance Framework is based on three fundamental questions summarized as follows:

- ❑ Is the weight of evidence sufficient to establish the mode of action (MOA) in animals? (Detailed analysis for DMA^V is presented in Section 3)
- ❑ Are key events in the animal MOA plausible in humans?
- ❑ Taking into account kinetic and dynamic factors, is the animal MOA plausible in humans?
- ❑ Conclusion: Statement of confidence, analysis, and implications.

4.A. Human Relevance of Bladder Cancer Produced in Rodents By Xenobiotics: Generic Considerations

The urinary bladder of laboratory mammals and humans share a common development, histology, function, and physiology (reviewed in ILSI, 1995). Bladder cancer develops via common factors, including physical, biological, and chemical stimuli. Bladder cancers are derived from the transitional cell epithelium or are of squamous cell origin (Silverman *et al*., 1996). Moreover, the stages in the carcinogenic process are similar among species (Oyasu, 1995). A number of chemicals, most of which are DNA reactive, are known to produce bladder cancer in both humans and in animals (Cohen, 2002a). These shared attributes indicate that cancer in laboratory animals are relevant indicators of

[3] The WHO/IPCS has recently initiated a project to provide a global perspective and internationally harmonize a conceptual approach on determining the human relevance of animal tumors (see: http://www.who.int/ipcs/methods/harmonization/areas/cancer_framework/en/index.html).

potential cancer development in humans, recognizing that there may also be differences in sensitivity across species, strains and sexes.

4.B. Human Relevance of Bladder Cancer Produced in Rats by DMA

Given the default presumption that bladder tumors in animals produced by xenobiotics may be relevant to humans, the question is whether, taking into account the qualitative and quantitative aspects of key events leading to bladder cancer produced specifically by DMA in rats, humans may be expected to respond to the carcinogenic effects of DMA like the rat. The analytical approach developed by the Risk Sciences Institute (Meek *et. al*., 2003) to answer this question follows.

1. Are Key Events in the Animal MOA Plausible in Humans (qualitative evaluation)?

There is a pauicity of human information specific to the precursor events of cytotoxicity, cell proliferation and DNA damage following DMA^V exposure. DMA^V and DMA^{III} have been detected in human urine following exposure to iAs in drinking water. Although one human subject that administered DMA^V excreted a small amount of TMAO, which suggests only a small amount of DMA^{III} was produced, this information is too limited to draw definitive conclusions. DMA^{III} has been show to produce cytotoxicity in human bladder cells *in vitro* at an LC_{50} comparable to a rat bladder cell line (Table 2.1). Thus, if adequate exposure is achieved to produce a sufficient amount of DMA^{III}, urothelial toxicity would be expected to ensue, and likewise if sufficient cell killing results then regenerative proliferation is plausible given that this would be an expected target organ response to insult in humans. As discussed earlier, regenerative proliferation associated with persistent cytotoxicity appears to be a risk factor for bladder carcinogenesis in humans (Cohen 1989; 1998a,b; 2002b). In the absence of human specific data on DMA, oxidative stress and DNA damage also is qualitatively plausible in humans. As illustrated in Table 4.1, the key events are qualitatively applicable in humans.

2. Taking into account kinetic and dynamic factors, is the animal MOA for DMA induced bladder tumors in rats plausible in humans (quantitative evaluation)?

DMA^{III} has been detected in human urine following exposure to iAs (Aposhian 2000 a, b; Del Razo *et al*., 2001; Le *et al.*, 2000a, b; Mandal *et al.*, 2001). Very little information is available in humans which to characterize the trivalent species in human urine following direct exposure to DMA^V. Although two studies indicate that DMA^V is rapidly excreted (Buchet *et al*., 1981; Marafante *et al*., 1987), these studies are inadequate to draw definitive conclusions regarding the quantitative conversion of DMA^V to DMA^{III}. Thus, it is unknown whether or not a sufficient amount could be present in human urine following direct exposure to DMA^V in order to produce cytotoxicity, regeneration, hyperplasia, or tumors. Although there may be quantitative species differences, there are no scientific data to suggest that if sufficient DMA^{III} were present in urine, key precursor events and

ultimately tumor formation would not occur (Table 4.2). The major uncertainty in this analysis is whether sufficient DMA^{III} would be produced in humans following DMA^{V} exposure.

As discussed earlier in Sections 2 and 3, following exposure of rats to DMA^{V}, the reduced metabolite, DMA^{III}, is believed to be the principal toxicant. An important factor in the dose-response relationship for the toxic response is the uptake into the cell. As described in detail in Section 2.C.3., several rat and human cell types have shown that DMA^{V} is taken up by cells to a lesser degree compared to iAs^{V}, iAs^{III}, MMA^{III}, and DMA^{III}, and that cellular uptake is correlated with cytotoxicity. Similarly, *in vitro* methylation is less efficient for the pentavalent metabolites. *In vivo* metabolism studies in humans and mice indicate small amounts of further methylation following direct exposure to DMA^{V}.

One of the important differences between humans and rats is the binding by rat RBCs to DMA. Shiobara *et al.* (2001) have shown that DMA^{III} is taken up and retained by RBCs but DMA^{V} was practically not or taken up slowly by RBCs of all species [rat, mouse, human]. These results suggest that DMA^{III}, not DMA^{V}, is bound by rat RBC. This binding to Hb leads to longer retention of DMA in the rat compared to humans or mice. Rats also excrete higher proportions of TMAO. The longer retention of DMA in the rat circulation may result in greater metabolism to TMAO in the rat compared to mouse or human. The combined results of *in vitro* and *in vivo* studies in mice and humans suggest that DMA^{V} may not be taken up readily by mouse or human cells or tissues—and thus exogenous DMA^{V} may not be efficiently reduced to DMA^{III} in humans or mice.

Table 4.1: Comparative Qualitative Analysis of Key Events in Rats and Humans

Key Event	Evidence in Rats	Evidence in Humans
Presence of reactive (cytoxic) metabolite(s) in urine (DMA^{III})	Yes	Plausible - Evidence following DMAV exposure too limited to draw conclusions, but DMA^{III} shown to be present following human exposure to iAs .
Oxidative DNA Damage and chromosomal aberrations	No (in vitro evidence only)	No DMA specific data
Urothelial cytotoxicity	Yes	No human evidence but potential to occur in humans if sufficient DMA^{III} is produced
Urothelial regeneration (hyperplasia)	Yes	No human evidence but potential to occur in humans if sufficient cell killing is produced and sustained
Bladder tumor formation	Yes	No epidemiologic data but plausible

Table 4.2: Comparative Quantitative Analysis of Key Events in Rats and Humans

Key Event	Evidence in Rats	Evidence in Humans
Presence of reactive (cytoxic) metabolite in urine (DMA^{III})	Yes - DMA^{III} detected in urine following 26 weeks treatment with 100 ppm DMA^{V} (0.8 to 5.05 umoles); TMAO in urine 36.6 ugrams/ml following administration of 200 ppm in drinking water to F344 rats	Plausible - Evidence following DMAV exposure too limited to draw conclusions, but DMA^{III} shown to be present following human exposure to iAs .
Oxidative DNA damage and chromosomal aberrations	No evidence in rats, in vitro only	No in vivo DMA specific data, in vitro data only in mitogen stimulated human lyphocytes
Urothelial cytotoxicity	Yes - urothelial toxicity observed *in vivo i*n rats at 2 ppm (0.1 mg/kg/day) but not sufficient to produce urothelial regeneration or succesive key events	Potential to occur in humans; However, unknown whether sufficient DMA^{III} is formed in humans to produce urothelial cytotoxicity
Urothelial regeneration	Yes - observed at 10 ppm DMA^{V} (0.5 mg/kg/day)	Potential to occur in humans; However, unknown whether sufficient DMA^{III} is formed in humans to produce sufficient urothelial cytotoxicity that would lead to regeneration
Hyperplasia	Yes - observed at 40 ppm DMA^{V} (2 mg/kg/day or 0.3 to 2 micromoles DMA^{III} in urine)	Potential to occur in humans but unknown if sufficient DMA^{III} formed
Bladder tumor formation	Yes - observed at 100 ppm DMA^{V} (5 mg/kg/day) or 0.8 to 5.05, micromoles DMA^{III} in urine)	No epidemiologic data

4.C. Relevance of Bladder Cancer to Sensitive Human Subpopulations or Lifestages

Although science policy judgments are applicable in general to the human population, it is important to consider potential effects that may occur during different life stages, particularly the young who may be at special risk. Embryologic development of the urinary tract is uniform across mammals. The ureteric bud, an evagination from the primitive cloaca, gives rise to the ureter, renal pelvis, and collecting ducts. The metaneprhic blastema forms around the ureteric bud to develop into excretory portion of the kidney including the gomerulus and proximal and distal tubules. The urinary bladder and urethra form from a second outpouching of the primitive cloaca, the urogenital sinus. Several additional pieces of information are relevant (ILSI, 1995).

- Urinary system anatomy, including the bladder, is basically established by the time of birth.

- Urinary functions, which influence the physiological environment of the bladder, are present at birth although some mature shortly thereafter (*e.g.,* glomerular filtration rate, concentrating ability, glucose reabsorption, ammonia excretion).

- Urinary function continues throughout life in the absence of disease.

- Bladder cell proliferation in the rodent (and primates) is highest *in utero*, then declines after birth to adult levels by 3-4 weeks of age (Cohen *et al*., 1988; Cohen S.M., 1998; Jost, 1989; Kunz *et al*., 1979, 1987; Schreiber *et al*., 1969).

- Bladder cancer is essentially a disease of advanced age (mostly after 65 yr) and is very rare among children (Serrano-Durba *et al*., 1999; Yusim *et al*., 1996).

The urinary bladder and urinary tract are anatomically complete and functionally competent throughout life, which suggests that qualitatively, there are no age dependent differences in susceptibility to chemically-induced bladder cancer among humans. Furthermore, there is no indication that children are at any increased sensitivity, as bladder cancer is very uncommon at early ages in humans and given the late age of onset of bladder cancer, there is no evidence that, in general, there is a shortened latency for tumor development after childhood exposure (ILSI, 1995).

5. Dose-response assessment

5.A. Introduction

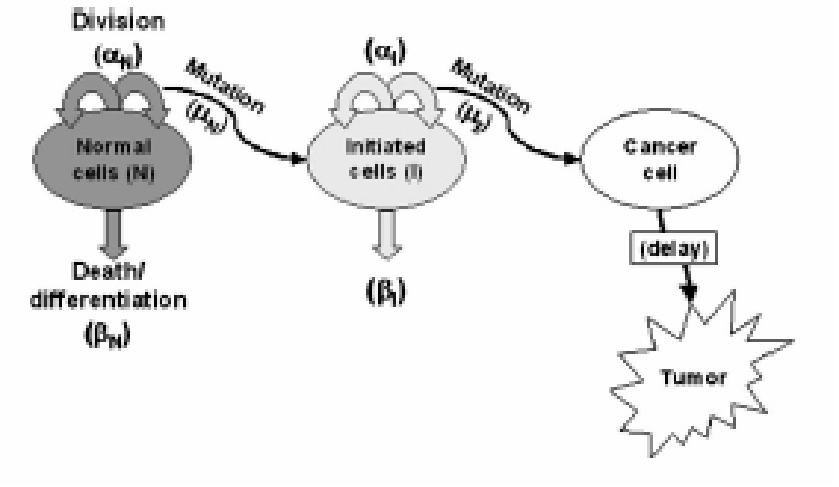
EPA's cancer guidelines (2005) indicate that a critical analysis of all the available data provides the starting point for dose-response assessment, and that default procedures for linear extrapolation are invoked only in the event of absence of critical information or significant uncertainty. Dose-response models are generally classified as either *low-dose linear* or as *nonlinear*. A low-dose linear model shows some response at all doses greater than zero and for a range of doses bounded at the low end by zero dose the response is defined by a straight line with a positive slope. A nonlinear model may show no response over a range of low doses including zero (*i.e.,* have a dose threshold) or show some response at all doses above zero. The cancer guidelines further state that the linear approach is used when: (1) there is an absence of sufficient information on modes of action or (2) the mode of action information indicates that the dose-response curve at low dose is expected to be linear. It should be noted that because it is experimentally difficult to distinguish modes of actions with true "thresholds" from others with a nonlinear dose-response relationship, the nonlinear procedure is considered a practical approach to use without the necessity of distinguishing sources of nonlinearity. It is the practice at EPA to speak of nonlinear dose response relationships rather than thresholds unless there is sufficient evidence defining a true threshold.

This analysis includes a description of biologically based and pharmacokineticly based modeling in addition to standard procedures for establishing points of departure (POD) such as the use of benchmark dose techniques.

5.B. Biologically-Based Dose Response Modeling

Biologically based dose-response (BBDR) and toxicodynamic modeling are the preferred approaches to dose-response assessment since these models can account for the biological processes and key events that lead to the development of a cancer. An example is the two-stage clonal expansion model (Figure 5.1) developed by Moolgavkar and Knudson (1981) and Chen and Farland (1991) which describes cancer as a succession of genetic changes and altered growth behaviors that lead to progressive conversion of normal cells into cancer cells. The two-stage clonal expansion model has been applied to the BBDR model developed for formaldehyde (Conolly *et al.*, 2003; 2004). While the clonal growth model may not be an accurate representation of the actual cellular mechanisms of carcinogenesis, it provides insight into the relative roles of key events in tumor development.

Figure 5.1: Diagram of the two-stage clonal growth model (reproduced from Conolly *et al.*, 2003, 2004).



Toxicodynamic modeling can be used when there are sufficient data to ascertain the mode of action and when there is sufficient information to quantitatively support model parameters that represent rates and other quantities associated with the key precursor events of the mode of action. In the case of DMA^V, the mode of action in rats has been sufficiently elucidated. The dose-response data collected at various durations of exposure needed to evaluate the relationship between cytotoxicity, proliferation, and tumor response are available in the rat. As described in Section 3, although the formation of chromosomal mutations would be expected to be enhanced following cytotoxicity and regenerative proliferation, the contribution of chromosomal aberrations to the tumor response in the normal dividing bladder cell population cannot be unequivocally ruled out. Conceptually, a BBDR model for DMA^V could provide a quantitative tool for evaluating the relative contribution of chromosomal aberrations to the bladder tumor response at low doses (*i.e.,* noncytotoxic). However, sufficient data do not exist at this time to estimate the parameters needed to develop such a model. Specifically, the available chromosomal aberration data were developed in mitogen-stimulated human peripheral lymphocytes. These data are not appropriate for dose-response assessment and for model parameterization given this is a nonrelevant cell line for the epithelial bladder tumor response and there is already stimulated proliferation. Dose response data for chromosomal aberrations in bladder epithelial cells (for example as measured by fluorscent *in situ* hybridization or by micronucleus assay of chromosome alterations in interphase bladder epithelial cells) and a reliable assessment of the dose-response for oxidative DNA damage in the bladder epithelial cells would describe the genotoxicity component of the dose response.

5.C. Physiologically-Based Pharmacokinetic Modeling

As toxicokinetic models generally describe the dynamic relationship between exposure and measures of internal dose over time,

toxicokinetic/pharmacokinetic modeling is the preferred approach for estimating internal dose metrics. A physiologically-based pharmacokinetic (PBPK) model is a mathematical description of the disposition of a chemical and its toxicologically important metabolites in humans or animals. PBPK models typically consist of multiple organs or tissue groups (compartments) linked by blood flow that incorporate actual physiological values for organ volumes and blood flow. These models also incorporate chemical-specific parameters such as measures of tissue solubility, protein binding, and metabolism (Vmax, Km). Use of a PBPK model allows one to define the relationship between external exposure and an internal measure of biologically effective dose in experimental animals or humans.

PBPK models have been published for iAs metabolism and disposition in hamsters, rabbits, mice and humans (Yu, 1999a & b, Mann *et al*., 1996a & b, Gentry *et al*., 2004). The common feature of these models is that they each consist of four separate sub-models describing the kinetics of iAs^{V}, iAs^{III}, MMA^{V} and DMA^{V}. The models differ in terms of chemical-specific parameters, tissue groups included, and assumptions concerning transport into tissues. None of these models are specific to the rat or include urinary bladder, the tumor site for DMA^{V}, as a tissue site.

EPA is undertaking a large effort to develop PBPK models for iAs, MMA, and DMA. The preliminary model for DMA^{V} is provided Appendix C. However, at this time, the development of this model is in its early stages and is not yet sufficiently robust for regulatory application. The PBPK model for DMA^{V} exposure was developed using mouse data and subsequently scaled and parameterized to predict urinary excretion of DMA and metabolism to TMAO in rats and humans. The purpose of this model is to evaluate interspecies differences in various internal dose metrics following oral exposure to DMA^{V} at both low exposure levels and exposure levels used in rodent bioassays at which effects were observed. The PBPK model provides a reasonable quantitative description of key interspecies PK differences, specifically differences in TMAO production and sequestration in rat red blood cells. However, there are some uncertainties associated with deficiencies in the data to support pf PBPK modeling, particularly regarding the conversion rate of DMA^{V} to DMA^{III} in humans.

5.D. Empirical Modeling

5.D.1. Introduction

As mentioned above, a biologically-based model is the preferred approach to estimate potential human cancer risk. When a robust model is not available, simpler approaches may be used. The cancer guidelines (2005) provide a two step approach to dose response assessment to lower exposures anticipated to occur in humans. This approach applies to both linear and non-linear extrapolation. First, an assessment of observed data to derive a point of departure (POD) is performed. A POD marks the

beginning of extrapolation to lower doses and is an estimated dose near the lower end of the observed range, without significant extrapolation to lower doses. The POD is typically expressed as the lower 95% confidence limit. The second step involves extrapolation to lower exposures as appropriate. The linear default approach is a straight-line extrapolation from the POD to the origin (*i.e.,* zero incremental dose, zero incremental response) to give a probability of extra risk. The slope of the line expresses extra risk per dose unit, where risk is the product of the slope and anticipated or measured human exposure. A nonlinear approach can be used to develop a reference dose, a reference concentration, or for determining margin(s) of exposure. As discussed in Section 3, in the case of DMA^V, the mode of action data indicate that the overall dose-response curve for tumors would be highly nonlinear. The tumor response in the rat bladder is closely correlated over the exposure range used with the urothelial cytotoxicity and regenerative proliferation, and for tumors to develop all three events--cytotoxicity, regenerative proliferation and genetic errors--need to occur.

PODs can be estimated using empirical modeling to derive benchmark dose (BMDs) estimates and lower 95% confidence limits (BMDLs). A model can be fitted to data on the tumor incidence or key precursor events. Such models can provide insight into quantitative relationships between tumors and precursor events. The current analysis provides a benchmark analysis for the key events of cytotoxicity, regenerative proliferation measured by BrdU labeling index, and hyperplasia, as well as for the tumor incidence data.

5.D.2. Benchmark dose analysis—methods and data used

A benchmark dose analysis using EPA's Benchmark Dose Software (BMDS) (http://cfpub.epa.gov/ncea/) was performed using following datasets:

- Cytotoxicity of the urothelial (SEM, severity scoring) after dietary exposure to female rats in the feed for 3 and 10 weeks as reported in Arnold *et al*. (2004, 1999);

- Regenerative proliferation (BrdU labeling index) after dietary exposure to female rats in the feed for 10 weeks as reported in Anold *et al*. (1999).

  Additional BrdU labeling index data after drinking water exposure to male rats are available in the studies by Wanibuchi *et al*. (1996) and Wei *et al*. (2002). In the Wanibuchi *et al*. sudy (males rats exposed to DMA^V for 8 weeks at 10 and 25 ppm), there was a lack of dose response for increased cell proliferation---approximately a 7-fold increase over controls was reported at 10 ppm DMA^V but at the higher dose of 25 ppm, the cell proliferation index is less

(approximately 2-fold over control). It is unclear why the BrdU labeling index went down at 25 ppm compared to 10 ppm in the drinking water, other than there can be marked variations in the labeling index, as indicated by the large standard errors reported at 10 ppm in the Wanibuchi *et al*. study. The Wanibuchi *et al*. study is not considered appropriate for modeling given the lack of dose response. Also, insufficient data are provided in the Wanibuchi *et al*. study to reliably convert the 10 ppm dose to mg/kg bw/day to compare with results of Arnold *et al*. (1999) feeding study which found after 10 weeks of exposure to DMA^{V} at 10 ppm (calculated as 0.65 mg/kg bw/day) only a marginal response but not statistically significant in cell proliferation (1.5–fold over control).

The results of the Wei *et al*. (2002) drinking study for cell proliferation, which are described in Section 3 and provided in Appendix B, are also not suitable for POD determination because the data were evaluated only after 104 weeks of exposure. At 104 weeks, there may be age related changes and cell proliferation would be confounded by preneoplastic (hyperplasia) and neoplastic lesions.

- Hyperplasia at 10 and/or 104 weeks as reported in Arnold *et al.* (1999) and Gur *et al.* (1989a) following exposure to female rats in the feed, and Wei *et al.* (2002) following exposure to male rats in the drinking water;

- Total papillomas and carcinomas of the urinary bladder as reported in the dietary studies of Gur *et al.* (1989a) and Wei *et al.* (1999).

Details of the BMD analysis are provided in Appendix D. The EPA's 2005 cancer guidelines suggest that a range of PODs should be estimated ranging from 1% to 10% extra risk. Thus, BMDs and BMDLs for 1% and 10% extra risk are provided below for each endpoint. For the cytotoxicity, hyperplasia, and tumor data, the dichotomous models provided in BMDS were attempted (*e.g.,* gamma, logistic, multistage, quantal linear, quantal quadratic, and Weibulll). For the BrdU labeling index data, each of the continuous models provided in BMDS were attempted (*e.g.,* linear, polynomial, power, Hill). Model suitability was evaluated based on AIC criteria, p-value, and visual fit.

5.D.3. Benchmark dose analysis—results

5.D.3.a. Cytotoxicity

When fitting the SEM-cytoxicity data (Appendix B, Table B1), incidence with scores of 3, 4, and 5 were summed for each

treatment group. (Scores of Class 1 and 2 are observed in normal untreated bladders; Scores of Class 3-5 are considered evidence of treatment related cytotoxicity). For cytotoxicity observed at 3 weeks of DMA^V exposure in feed, the gamma, multistage, quantal linear and Weibull models provided very similar, quality fits and consistent BMD estimates (Table 5.1; Figure 5.2). The multistage model (3^{rd} degree polynomial) provided the best fit.

After 10 weeks of exposure in the feed, cytotoxicity of scores 3-5 was observed in each treatment group including 6/10 animals at the lowest dose. Due to this, each of the BMD models did not perform well using all dose groups or with one dose group dropped. Although it's preferred to use all the available data, as shown in Figure 5.3a-c, removing the two highest doses from the analysis provides a better fit at the low dose levels. As indicated in the cancer guidelines (2005), "when a model's fit is poor, the highest dose is often omitted in cases where it is judged that the highest dose reflects competing toxicity that is more relevant at high doses than at lower doses."

Figure 5.2: Plot of cytotoxicity data from 3 weeks of exposure to DMA^V. (Doses in mg/kg/day)

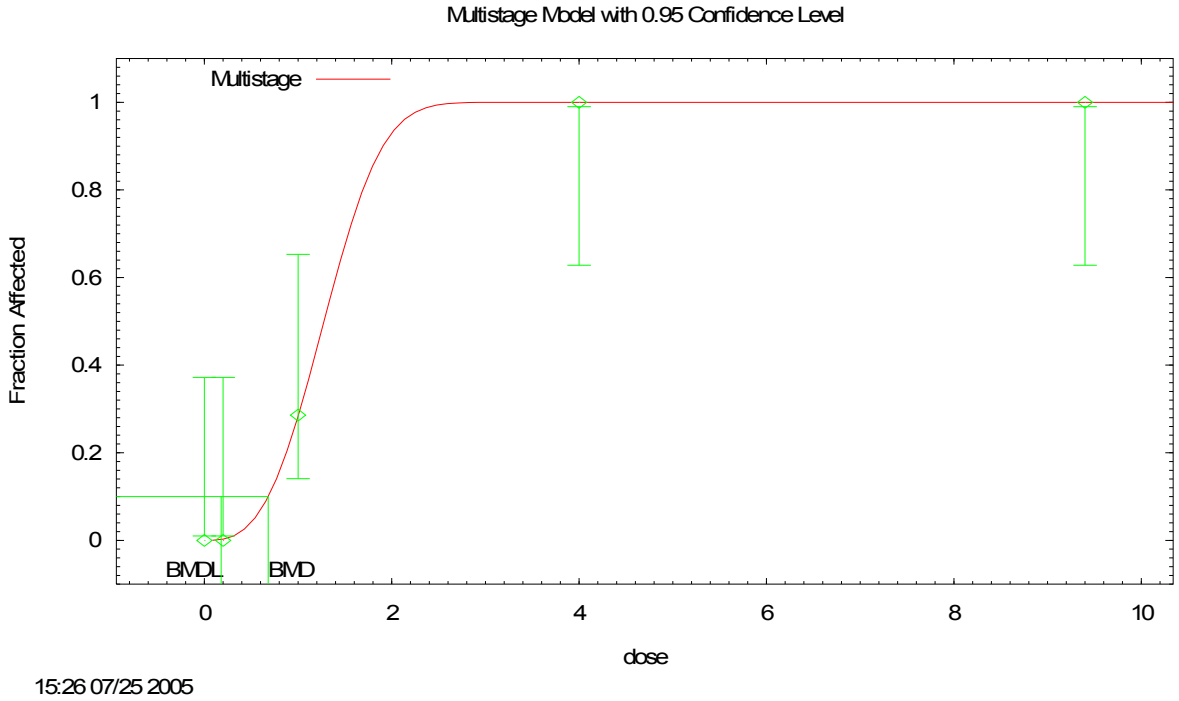
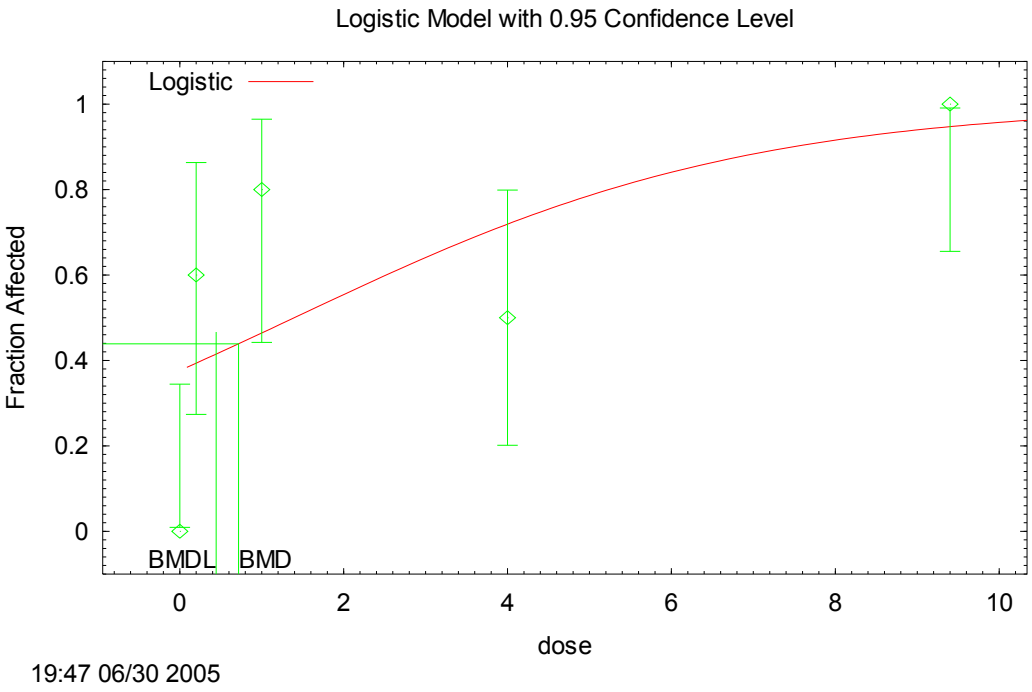


Figure 5.3: Plot of cytotoxicity data from 10 weeks of exposure to DMA^V. (Doses in mg/kg/day)

a. All dose groups included



b. High dose removed

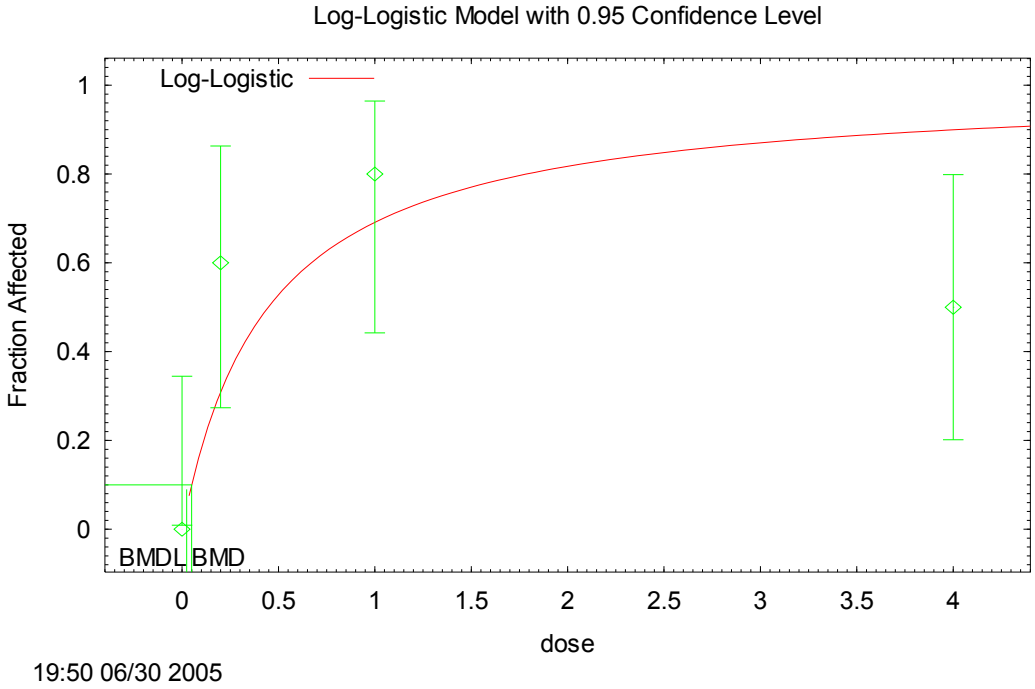
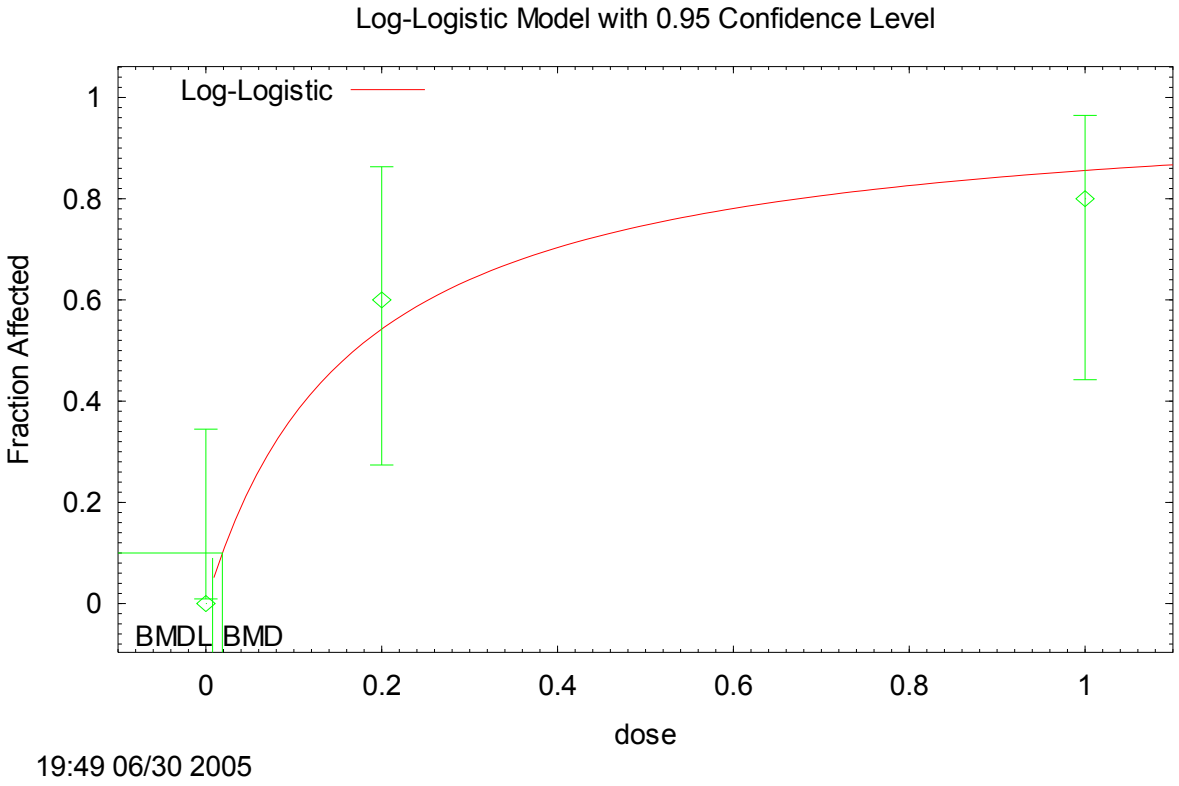


Figure 5.3: Plot of cytotoxicity data from 10 weeks of exposure to DMA^V, cont'd. (Doses in mg/kg/day)

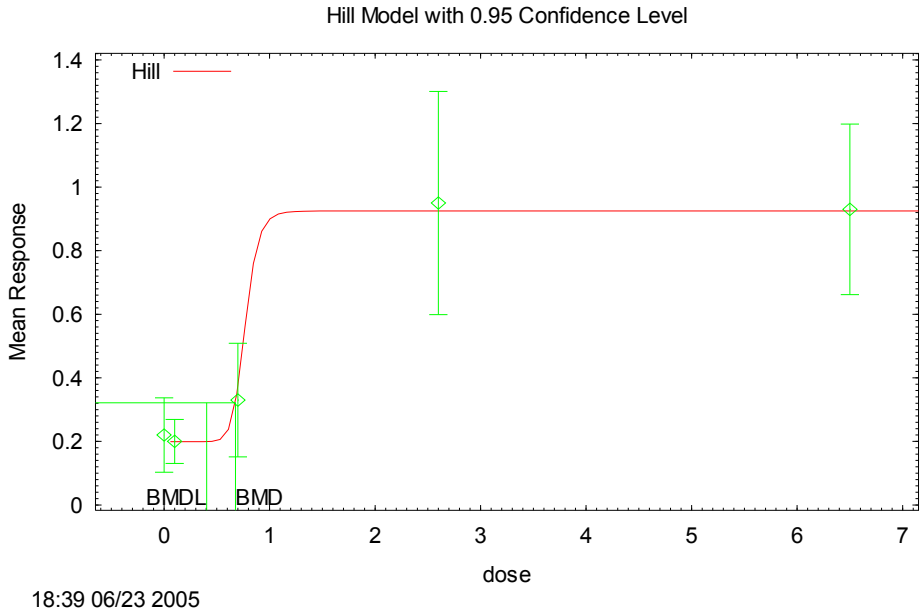
c. Two high doses removed



5.D.3.b. Regenerative proliferation

As shown in Figure 5.4, the Hill model provided the best fit for the BrdU labeling index from Arnold *et al.* (1999).

Figure 5.4: Plot of BrdU data from Arnold *et al.* (1999) (Doses in mg/kg/day)



5.D.3.c. Hyperplasia

Analysis of hyperplasia data are shown graphically below (Figures 5.5-5.7). Quality fits were observed with data from the feeding studies (Arnold *et al.*, 1999; Gur *et al*., 1989a). The quantal quadratic and probit models performed the best for the 10 and 104 week feeding data, respectively. Regarding the hyperplasia from the drinking water study by Wei *et al*. (2002) at 104 weeks, model fit was poor with all doses (all models except logistic had p-values < 0.05). Model fit of the drinking water data at low dose levels based on statistical and visual fit improved by removing the highest dose. BMDs and BMDLs are provided in Table 5.1. After removing the high dose the multistage model (3rd degree polynomial) provided the best fit.

Figure 5.5: Plot of incidence of hyperplasia data from 10 weeks of exposure to DMA^V in the feed (Arnold *et al.*, 1999). (Doses in mg/kg/day)

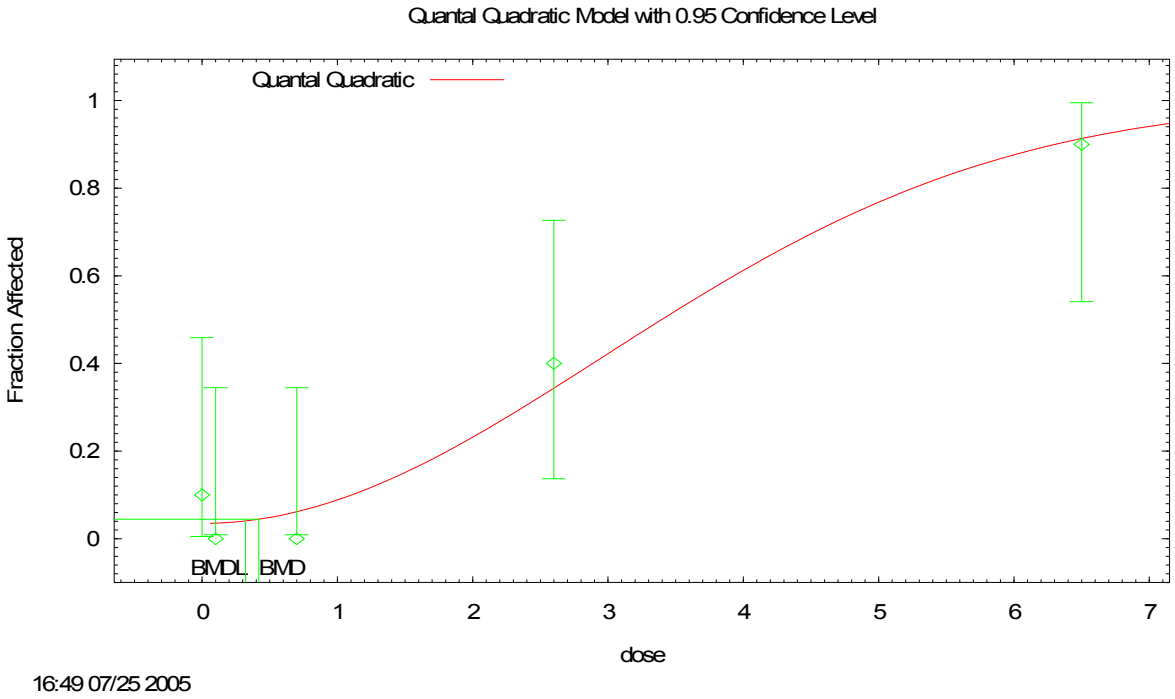


Figure 5.6: Plot of incidence of hyperplasia data from 104 weeks of exposure to DMA^V in the feed (Gur *et al.*, 1989a). (Doses in mg/kg/day)

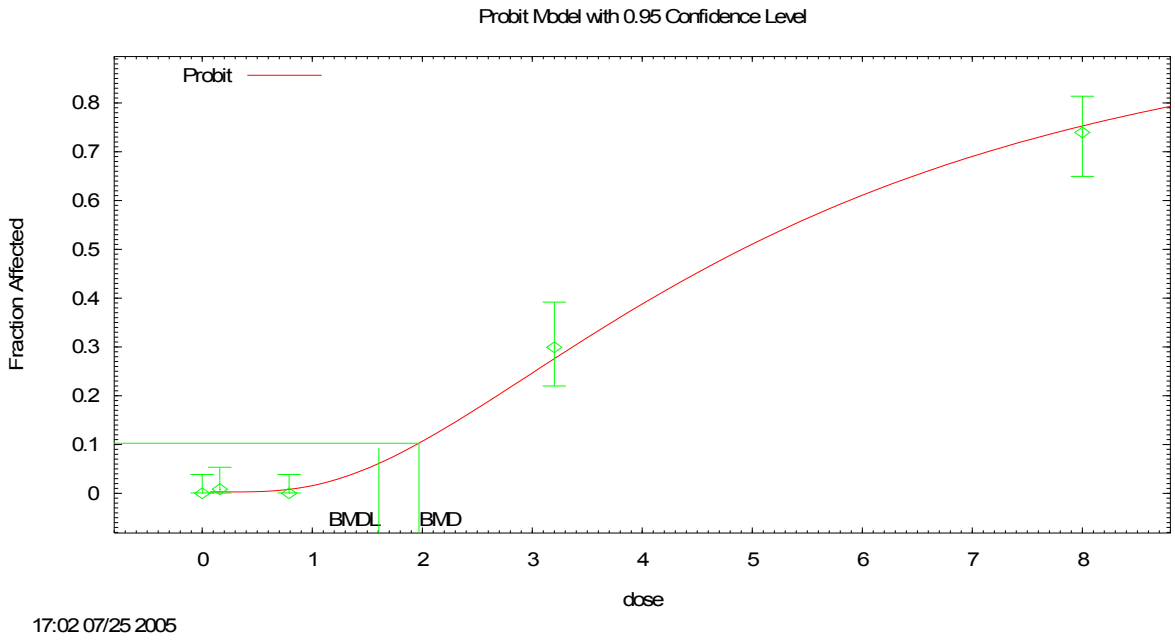
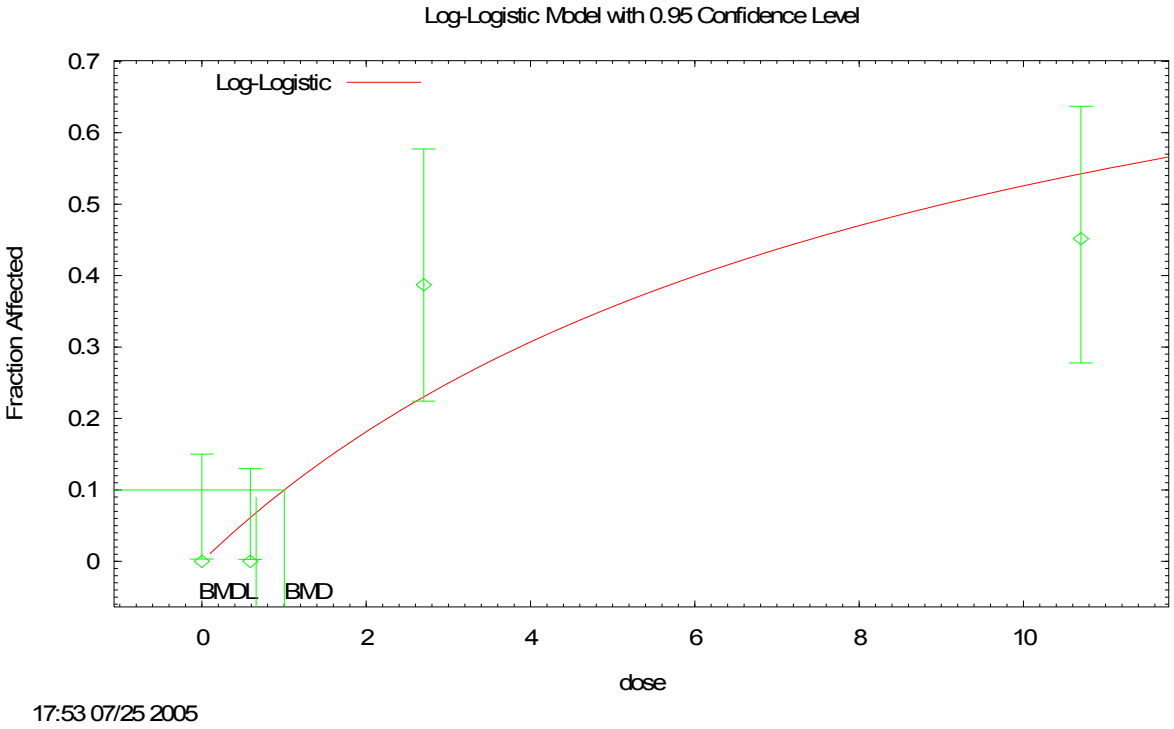
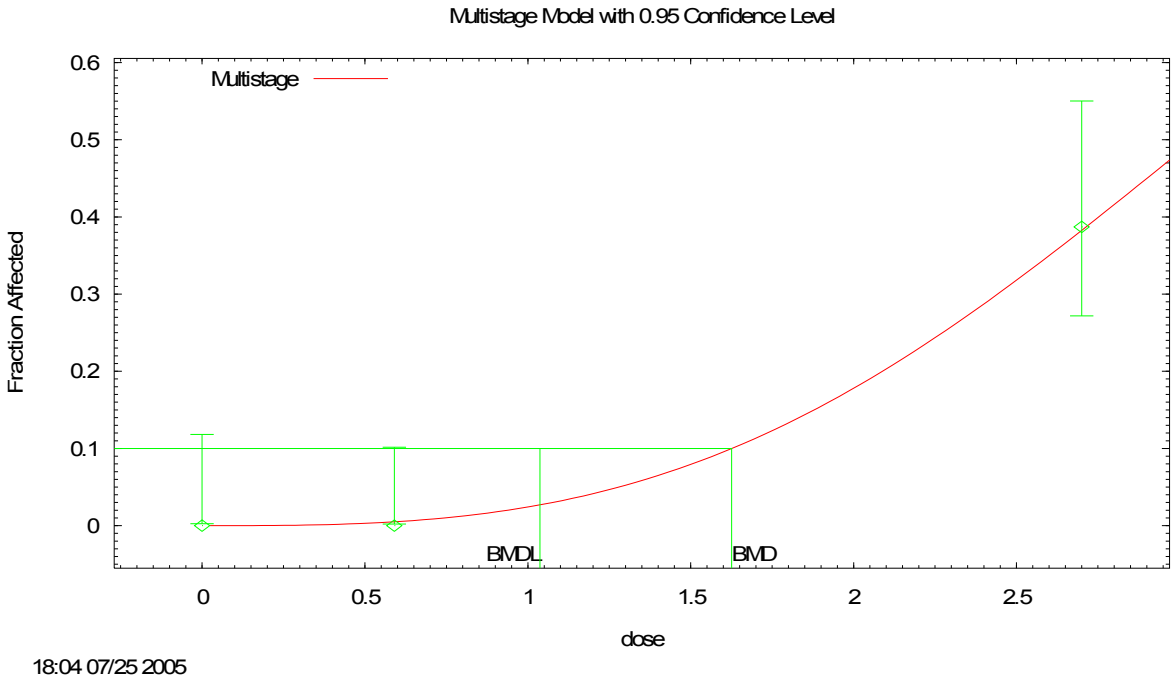


Figure 5.7: Plot of incidence of hyperplasia data from 104 weeks of exposure to DMA^V in the drinking water (Wei *et al.*, 2002). (Doses in mg/kg/day)

a. All dose groups included



b. High dose removed



5.D.3.d. Tumor data

BMD analysis for the tumor data are provided in Figures 5.8 and 5.9 for the feeding and drinking water studies, respectively (Gur *et al*., 1989a; Wei *et al*., 1999).

Tumor data from the feeding study (Gur *et al*, 1989a) were best fit by the Weibull model. Similar to the trend observed in the BMD analysis of the hyperplasia incidence from Wei *et al.* (2002) drinking water study, when all dose groups were included, the visual and statistical fit of the Wei *et al*. (1999) tumor data was moderate to poor for all dichotomous models provided in BMDS. With all the treatment groups included, the statistical fit of the multistage model provided the best fit. Similar to the analysis for the hyperplasia data above from the drinking water study, the highest dose was removed to provide a better fit at lower doses. The multistage model again provided the best visual statistical fit using the three lower dose levels. BMDs and BMDLs are provided in Table 5.1.

Figure 5.8: Plot of rat bladder tumors observed in Gur *et al.* (1989a). (Doses in mg/kg/day)

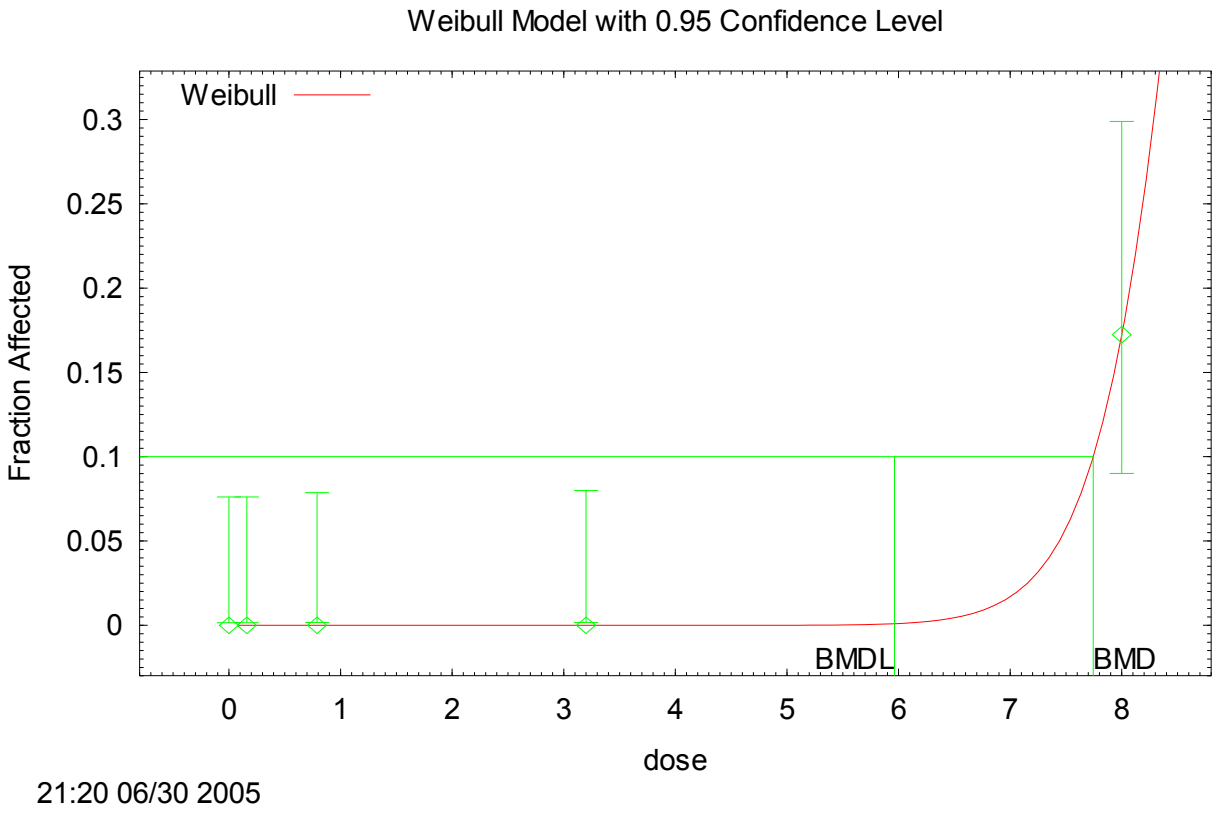
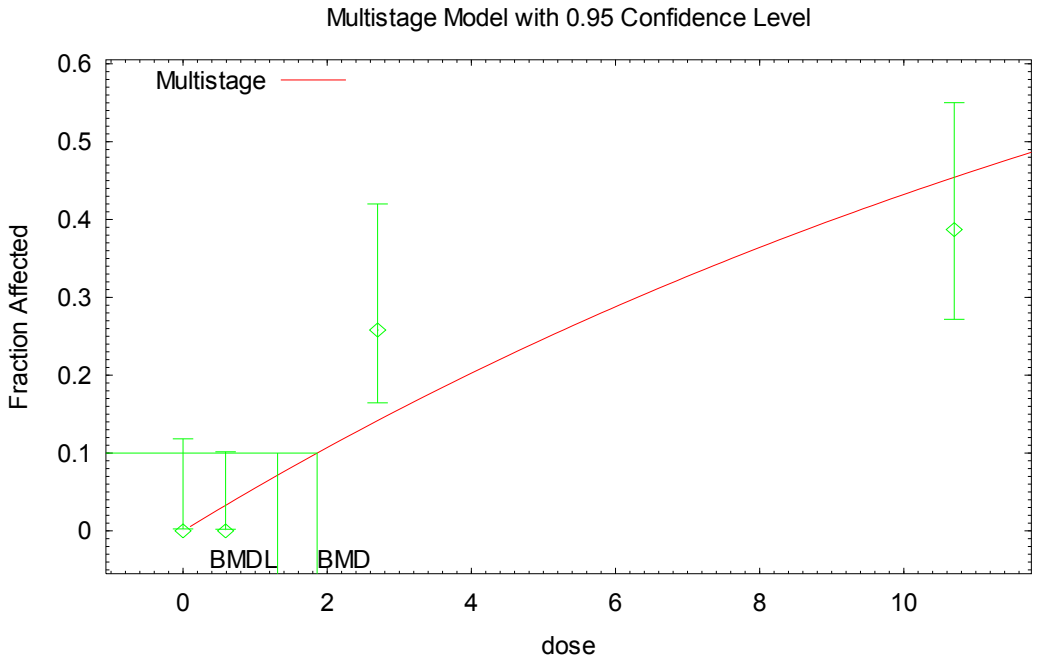


Figure 5.9: Plot of rat bladder tumors observed in Wei *et al.* (1999). (Doses in mg/kg/day)

a. All dose groups included.



b. High dose group removed

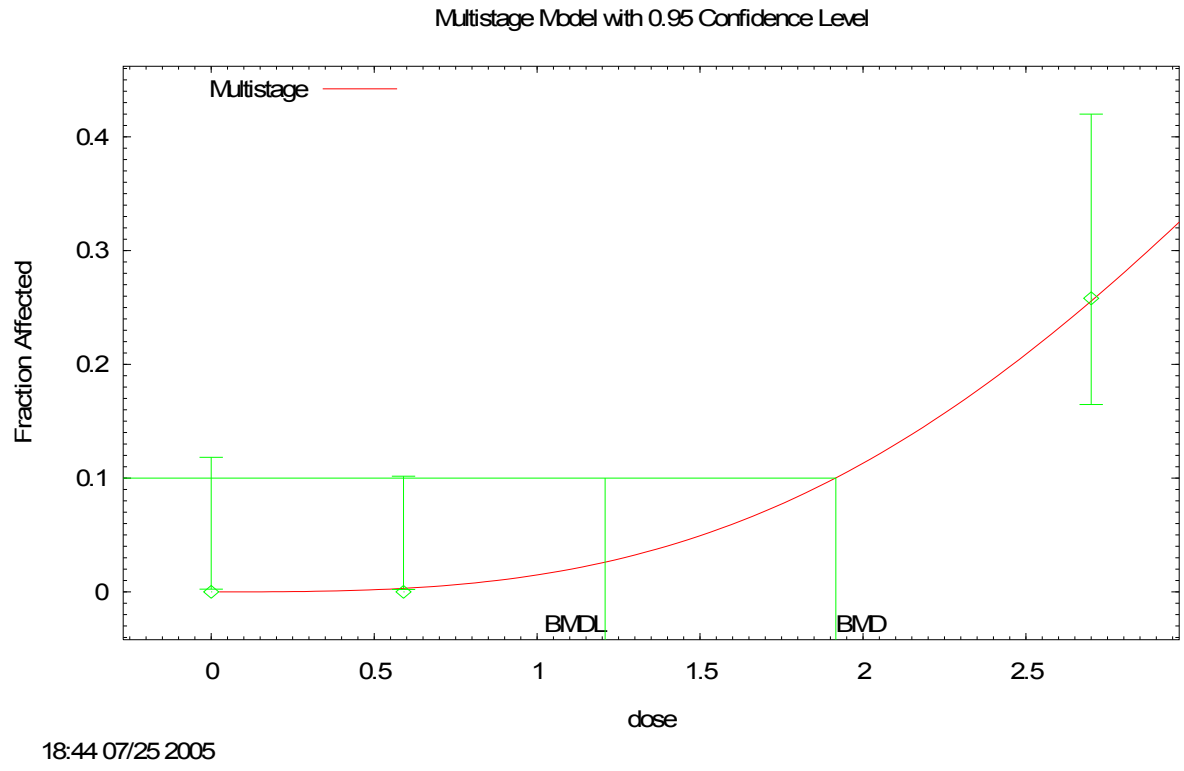


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Table 5.1: Summary of benchmark dose estimates and lower 95% confidence limits for cytotoxicity, BrdU labeling index, hyperplasia and tumor data. (Doses in mg/kg/day)

Biological Event	Feeding					Drinking water				
	Duration	10%		1%		Duration	10%		1%	
		BMD (mg/kg/day)	BMDL (mg/kg/day)	BMD (mg/kg/day)	BMDL (mg/kg/day)		BMD (mg/kg/day)	BMDL (mg/kg/day)	BMD (mg/kg/day)	BMDL (mg/kg/day)
Tumor	104 weeks	7.74	5.96	6.80	2.22	104 weeks	1.92	1.21	0.88	0.14
Hyperplasia	10 weeks	1.36	1.04	0.42	0.32	104 weeks	1.63	1.04	0.74	0.14
	104 weeks	1.97	1.61	0.93	0.66					
BrdU labeling (proliferation)	10 weeks	0.65	0.29	0.54	0.07	Not determined. Available data not suitable for modeling.				
Cytotoxicity	3 weeks	0.68	0.18	0.31	0.02	No reliable dose-response data available				
	10 weeks	0.02	0.008	0.002	0.0007					

5.D.1. Discussion of the Benchmark Dose Modeling

As stated in Section 3, the most robust dose response data directly related to key events (ie, urothelial cytotoxicity and cell) in DMA^V's mode of action are mostly derived from dietary feeding studies in rats (Arnold *et al*., 2004; Cohen *et al*., 2001; 2002). Mode of action information available from rat drinking water studies from Japanese laboratories (Wanibuchi *et al*., 1996; Wei *et al*., 2002) provide supportive information for the tumor promoting properties of DMA, as well as, its ability to result in oxidative damage and regenerative hyperplasia. Although the BrdU labeling index and cytotoxicity data from the drinking water studies are qualitatively consistent with the studies in the feed, the drinking water studies are too limited for reliable benchmark dose modeling.

The benchmark dose analyses of key event and tumor data provided from the DMA^V feeding studies conducted with rats provide support for the results of the mode of action analysis. The BMD/BMDL estimates presented in Table 5.1 are consistent with the dose response concordance of key events in the mode of action and rat bladder tumors as illustrated in Table 3.6. As shown in Table 5.1, there is a correlation between the BMDs/BMDLs for the key events of cytotoxicity, regenerative cell proliferation, hyperplasia, and urothelial tumors (BMDL's 1% at ten weeks: cytoxicity – 0.0007; increased cell proliferation – 0.07; hyperplasia – 0.32; and tumors at 104 weeks – 2.22). Thus, the benchmark dose analyses supports the mode of action conclusions in Section 3.B.10 and the summary of results in Table 3.6. There must be sufficient cytotoxicity in the bladder urothelium to result in an increase in cell proliferation and that there must be a sufficient increase in cell proliferation to produce hyperplasia and bladder tumors. Taken together, the results in Table 3.6 and 5.1 show that there is both a temporal and dose-response concordance for cytotoxicity, regenerative proliferations, hyperplasia and bladder tumor formation.

Although the cytotoxicity and cell proliferation data were not modeled from the drinking water studies (Wei *et al*., 2002; Wanibuchi *et al*., 1996)**,** the hyperplasia and tumor data were modeled. The BMDs/BMDLs at 10% and 1% for hyperplasia in the drinking water study are consistent with the BMDs/BMDLs at 10% and 1% for the dietary study (Table 5.1). Furthermore, visual evaluation of the BrdU and cytotoxicity data suggest that for those key events, administration from feeding and drinking water provide comparable results. The benchmark doses for bladder tumors in the drinking water study are lower than those of the dietary study. The statistical fit of the Wei *et al* (2002) tumor data, however, was moderate to poor for all dichotomous models provided in BMDs, and use of a multistage model provided the best fit only when the top dose was removed. Thus, comparisions of the BMDs from Gur *et al*. (1989a) feeding and the Wei *et al*. (2002) tumor data should be made with caution.

Among the several key events, all of which are necessary for tumor formation, cell proliferation is proposed for deriving a point of departure because it is needed for increasing the likelihood of chromosome mutation formation and for the perpetuation of genetic errors, as well as for hyperplasia. **It is further proposed that a $BMDL_1$ value (0.07 mg/kg/ bw/day) be considered for the point of departure in deriving reference dose or a margin of exposure.** This approach is considered public health protective because a $BMDL_1$ of 0.07 mg/kg bw/day is approximately an order of magnitude lower than the dose (~0.7 mg/kg bw/day or 10 ppm) that resulted in a 1.5-fold nonstatistical increase in cell proliferation after 10 weeks of exposure to DMA^V and about two orders of magnitude lower than the dose (~9.4 mg/kg bw day) resulting in neoplasia in the feeding studies.

6. Summary and Conclusions

Based on consideration of metabolism/pharmacokinetic and mode of action data, a number of dose response extrapolation approaches for DMA have been considered and discussed in this document. These approaches are summarized in Table 6.1.

Given the lack of human data on DMA^V, the use of the cancer slope factor based on the epidemiology of iAs as a surrogate to estimate cancer risk associated with direct exposure to DMA^V was considered but judged to be unsuitable for a number of reasons. First, there are differences in methylation efficiency and cellular uptake between direct exposure to DMA^V versus iAs. Furthermore, metabolism (which is primarily unidirectional) following direct exposure to DMA^V results in fewer arsenical species compared to metabolism following direct exposure to iAs. Thus, exposure to iAs produces a more complex mixture of transformation products, some of which have different biological/toxicity activities and some of which may have similar effects but with different potencies compared to those metabolites resulting from direct exposure to DMA^V. Although DMA^V and DMA^{III} are metabolites of iAs, there is insufficient evidence to establish these as the ultimate carcinogenic species of iAs, if such exists. It is possible that several arsenical species may be involved in various mode of action in different target tissues for inorganic arsenic. Because the ultimate carcinogenic metabolite(s) is not known for iAs and because the mixture of toxic metabolites possibly generated after exposure to iAs is more complicated than that of DMA^V**,** it is concluded human cancer data can not be used to assess the cancer risk associated with direct exposure to DMA^V. Although DMA^{III} is produced by iAs, it is not possible to assign a proportion of the total risk to it because of the potential role of several other metabolites in the total risk for iAs. It is, therefore, proposed that chemical specific data on DMA^V (and its sequelae metabolites) are more appropriate to estimate its cancer risk.

Because human cancer data are not available for DMA^V, the rat tumor data for DMA^V are used to estimate its cancer risk. As discussed in Section 3, a body of data is available on the animal mode of action for DMA^V carcinogenesis. It was concluded, that production of genetic errors, cell wounding/cell death, regenerative proliferation form the key events for tumor induction in the rat bladder and further that these are all plausible in humans (Secton 4). A central biological event in DMA^V's mode of action is stimulated cell proliferation (which in turn can be influenced by DMA^{III}'s cytotoxicity) in cells that would otherwise not divide or divide very slowly. It is this enhanced cell proliferation that determines the probability of converting DNA lesions induced by oxidative radicals into "stable" chromosomal mutations. Under continuous exposure conditions, induced chronic wounding of tissues, the accompanying cell death and persistent regenerative proliferation lead to the production of the additional mutations, necessary for multistep carcinogenesis. Although the animal mode of action for DMA^V is considered qualitatively relevant to humans, there are pharmacokinetic differences between rats and humans that need to be addressed as part of the risk assessment.

The ideal dose response approach to incorporate DMA's mode of action and to address the uncertainty associated with cross species differences in toxicokinetics is by biologically-based (BB) and pharmacokinetic (PK) dose response (DR) modeling. Although sufficient data are available to establish a mode of action for DMA^V, the necessary data for establishing critical parameters (particularly *in vivo* dose response data for oxidative DNA damage and chromosomal aberrations) of a BBDR are not. EPA's Office of Research and Development is currently developing a PK model for arsenicals, but it will not be ready in time for the Office of Pesticide Programs (OPP) to meet its statutory deadline for completing its risk assessment of cacodylic acid. In lieu of these highly sophisticated approaches, OPP has considered the two step process of cancer dose response assessment described in the 2005 EPA's cancer guidelines, in other words modeling in the range of observable data to derive a point of departure and extrapolating to lower doses using a linear, nonlinear or both defaults. It is proposed that the uncertainty associated with differences in pharmacokinetics between rats and humans and with human variability be handled by using standard default uncertainty factors and default allometric scaling.

Based on DMA's mode of action considerations and on the current understanding of the multi step process of carcinogenesis, there is limited scientific support for reliance on a linear default extrapolation. All of the key events in DMA^V's mode of action (cytotoxicity, regenerative proliferation, genetic errors) must occur to result in a neoplastic response in the multi step process of carcinogenesis. Any one event alone is not sufficient to lead to tumors. The likelihood of chromosomal mutations being induced via oxidative DMA damage and thus posing a cancer risk in the absence of cell wounding and regenerative proliferation is very low. Moreover, the assumption of low dose linearity due to chromosome mutations depends on the generation of ROS (via oxidation of DMA^{III} back to DMA^V) which in turn is dependent on sufficient DMA^{III} levels.

Finally, there are defences against the cellular effects of toxicants, including continuous shedding of dead and dying cells and repair of damaged DNA, free radical savangers, etc. A default linear extrapolation from the rat bladder tumor response to zero dose would not take full account of the current biological understanding of DMA's mode of action.

The nonlinear default approach (i.e., derivation of a reference dose or margin of exposure) is regarded as the more appropriate dose response extrapolation approach because DMA^V's mode of action is dependent on genetic, biochemical and histopathological events for which dose-response relationships have been demonstrated to be or are predicted to be nonlinear. Because regenerative proliferation is regarded at the rate limiting key step for tumor formation, it is proposed that a reference dose method be based on a point of departure for cell proliferation ($BMDL_1$ = 0.07 mg/kg bw/day). Because a complete PK modeling approach is not available at this time, default uncertainty factors should be applied to account for intra and inter species differences. Thus, consideration of uncertainty factors should include the 10X for interspecies differences (or ¾ body weight scaling and 3X), 10X for intrahuman variation, and 10X for the FQPA safety factor to protect children. This reference dose approach with its uncertainty factors is regarded as a health protective approach and is used *in lieu* of sophisticated biologically- and pharmacokinetically based modeling at this time.

Table 6.1. DMA^V: Summary of Approaches to Dose Response Extrapolation

Approach	Strength of the Approach	Limitations of the Approach
Biologically-and Pharmacokinetically-Based Models (see Section 5.B and 5.C; Appendix C)	Reduces uncertainty in low dose and cross species extrapolations by accounting for tissue dosimetry, contribution of metabolites, defines relationship between tissue dose and biological response, contribution of key events in the carcinogenic mode of action	Insufficient data to support development of a biologically based model and to support confidence in results. Pharmacokinetic model under development by EPA's Office of Research and Development and currently not fully developed for use by a regulatory program
Two Step Procedure for Default Approaches as Decribed in EPA's 2005 Cancer Guidelines		
Linear cancer slope factor based on epidemiology for iAs (see Section 2.E.)	Human data are used	Chemical specific pharmacokinetic and metabolic characteristics of DMA^V not accounted for (i.e., differences in efficiency of the methylation reaction(s) and cellular uptake between DMA^V and iAs when administered exogenously). Chemical specific pharmacodynamic characteristics of DMA^V not accounted for
Linear Default Extrapolation Based on DMA^V Rodent Data: A line is drawn from a point of departure (based on tumor incidence or precursor response data) that is established from the observable range of data to the origin. See Section 3.B.10.	Accounts for the uncertainty associated with a potential linear component associated with chromosomal damage below noncytotoxic doses	Metabolism resulting from direct exposure to DMA^V is primarily unidirectional Assumes that the carcinogenic effects are directly proportional to dose - that there is a linear relationship between dose and cancer. Does not incorporate all mode of action information--in particular the need for sufficient DMA^{III} to be produced in order to lead to sufficient cell killing which inturn would provoke regenerative proliferation for which the frequency of chromosomal mutation is dependent. Uncertainty remains that the rat may be more sensitive than humans to the carcinogenic effects of DMA^V.
Nonlinear Default Extrapolation Based on DMA^V Rodent Data: Derivation a Reference Dose based on a point of departure for cell proliferation and application of default uncertainty factors to account for uncertainty associated with cross species extrapolation and human variability. See Sections 2.E., 3.B.10 and 5.D.	Incorporates mode of action information and accounts for uncertainty associated with cross species extrapolation and human variability via use of default factors	Uncertainty remains that the rat may be more sensitive than humans to the carcinogenic effects of DMA^V. Does not account for the possibility of a shallow linear slope at doses below those which are cytotoxic
Definition of Dose Response Shape		
Linear Quadratic (see Appendix E)		See Appendix E

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Appendix B Detailed tables for MOA analysis

Table B1. Dose-response relationships across time: Cytotoxicitya

DMA^V (ppm)	Toxicity (SEM)																			
	Hour 6					Hour 24					Day 3					Week 1				
	SEM classification					SEM classification					SEM classification					SEM classification				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
0	---	---	---	---	---	5	2	0	0	0	5	2	0	0	0	2	5	0	0	0
2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
40	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
100	0	1	6	0	0	0	3	2	2	0	0	4	3	0	0	0	7	0	0	0
	Cohen et al. *2001*					et al. *2001*					et al. *2001*					*Cohen* et al. *2001*				

DMA^V (ppm)	Toxicity (SEM)																								
	Week 2					Week 2 *Cohen*					Week 2					Week 2					Week 3				
	SEM classification					SEM classification					SEM classification					SEM classification					SEM classification				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
0	4	3	0	0	0	7	3	0	0	0	6	4	0	0	0	6	4	0	0	0	6	1	0	0	0
2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	4	3	0	0	0
10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0	5	2	0	0
40	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	0	0	7	0	0
100	0	4	0	3	0	0	2	2	0	6^b	0	0	5	3	2^b	0	0	1	9^b	0	0	0	7	0	0
	Cohen et al. *2001*					et al. *2002*					et al. *2001*					*Wei et al. 2004*					*Arnold* et al. *2004*				

DMA^V (ppm)	Toxicity (SEM)																								
	Week 8					Week 10					Week 10					Week 10					Week 20				
	SEM classification					SEM classification					SEM classification					SEM classification (males)					SEM classification				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
0	0	7	3	0	0	5	5	0	0	0	3	7	0	0	0	0	3	7	0	0	6	4	0	0	0
2	---	---	---	---	---	0	4	5	1	0	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
10	---	---	---	---	---	0	2	5	3	0	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
40	---	---	---	---	---	0	5	3	2	0	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
100	0	0	0	2	8^b	0	0	0	4	6^b	0	0	0	1	9^b	0	1	4	5	0	0	0	3	6	1
	Arnold et al. 1999					*Arnold et al. 1999*					*Cohen et al. 2001*					*Arnold et al. 1999*					*Arnold et al. 1999*				

DMA^V (ppm)	Toxicity (SEM)				
	Week 26				
	SEM classification				
	1	2	3	4	5
0	6	4	0	0	0
2	---	---	---	---	---
10	---	---	---	---	---
40	---	---	---	---	---
100	3	6	0	0	0
	Cohen et al. 2002				

[a]All results in female rats, except where noted; [b] $P<0.05$ when compared to respective controls

SEM classification key for bladder toxicity used in Cohen et al. (2001; 2002):

1 = flat, polygonal superficial urothelial cells
2 = occasional small foci of urothelial necrosis
3 = numerous small foci of superficial urothelial necrosis
4 = extensive superficial urothelial necrosis, especially in the dome of bladder
5 = necrosis and piling up of rounded urothelial cells
Normal bladders are usually Class 1 or 2, but occasionally Class 3.

Table B2. Dose-response and temporal relationships: Compensatory Regenerationc

DMA[V] (ppm)	Regenerative response (BrdU labeling index)														
	6 Hour	Hour 24	Day 3	Week 1	Week 2	Week 2	Week 2	Week 2	Week 8 (males)[b]	Week 10	Week 10 (males)	Week 10	Week 20	Week 26	Week 104 (males)
0	---	0.42 ± 0.05	0.23 ± 0.04	0.44 ± 0.09	0.22 ± 0.03	0.19 ± 0.04	0.16 ± 0.02	0.10 ± 0.02	~0.09 ± 0.06	0.22 ± 0.05	0.23 ± 0.03	0.18 ± 0.03	0.25 ± 0.03	0.13 ± 0.02	~0.16 ± 0.1
2	---	---	---	---	---	---	---	---	---	0.20 ± 0.03	---	---	---	---	---
10	---	---	---	---	---	---	---	---	~0.75 ± 0.25[a]	0.33 ± 0.08	---	---	---	---	---
12.5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	~0.22 ± 0.1
25	---	---	---	---	---	---	---	---	~0.21 ± 0.02[a]	---	---	---	---	---	---
40	---	---	---	---	---	---	---	---	---	0.95 ± 0.15[a]	---	---	---	---	---
50	---	---	---	---	---	---	---	---	---	---	---	---	---	---	~0.5 ± 0.25[a]
100	0.22 ± 0.04	0.24 ± 0.04[a]	0.33 ± 0.11	0.96 ± 0.14[a]	1.36 ± 0.13[a]	0.94 ± 0.20[a]	0.63 ± 0.10[a]	1.61 ± 0.22[a]	---	0.93 ± 0.11[a]	0.95 ± 0.05	0.61 ± 0.10[a]	0.97 ± 0.11	0.21 ± 0.03[a]	---
200	---	---	---	---	---	---	---	---	---	---	---	---	---	---	~0.65 ± 0.3a

Cohen et al. 2001; Cohen et al. 2001 ;Cohen et al. 2001; Cohen et al. 2001 ; Cohen et al. 2001; Cohen et al. 2001; Cohen et al. 2002; Wei et al. 2004 ; Wanibuchi et al. 1996 ; Arnold et al. 1999; Arnold et al. 1999; Cohen et al. 2001 ; Arnold et al. 1999; Cohen et al. 2002 ; Wei et al. 2002

Uncertainty expressed as ± S.E. of the mean in all studies, except in Wanibuchi et al. (1996), which used ± 1 S.D. of the mean

[a] $P<0.05$ when compared to respective controls

[b]Results are difficult to interpret, because indices for 0, 10 and 25 ppm are based on 10, 5, and 5 animals, respectively, and all rats treated with 100 ppm died from DMA toxicity after 4 weeks of treatment

[c]All results in female rats, except where noted

Table B3. Dose-response relationships across time: Hyperplasiac

DMA[V] (ppm)	Non-neoplastic changes (Simple hyperplasia)															
Hour	6	Hour 24	Day 3	Week 1	Week 2	Week 2	Week 2	Week 2	Week 3	Week 8	Week 10	Week 10 (males)	Week 10	Week 20	Week 26	Week 104 (males and females)
0	---	2/7	3/7	0/7	1/10	0/7	0/10	0/10	0/7	0/10	1/10	0/10	0/10	1/10	0/10	0/120
2	---	---	---	---	---	---	---	---	0/7	---	0/10	---	---	---	---	1/118
10	---	---	---	---	---	---	---	---	0/7	---	0/10	---	---	---	---	0/120
40	---	---	---	---	---	---	---	---	0/7	---	4/10	---	---	---	---	35/117[b]
100	1/7	0/7	1/7	2/7	0/10	0/7	1/10	0/10	2/7	7/10[a]	9/10[a]	2/10	6/10[a]	4/10[d]	4/9[a]	88/119a,b
	Cohen et al. 2001	*Cohen et al. 2001*	*Cohen et al. 2001*	*Cohen et al. 2001*	*Cohen et al. 2001*	*Cohen et al. 2001*	*Cohen et al. 2002;*	*Wei et al. 2004;*	*Arnold et al. 2004;*	*Arnold et al. 1999*	*Arnold et al. 1999;*	*Arnold et al. 1999; Cohen et al. 2001;*	*Arnold et al. 1999;*	*Cohen et al. 2002*		*Gur et al. 1989a*

[a] *P*<0.05 when compared to respective controls
[b]males: 6/58 and 40/59 at 40 and 100 ppm, resp.; females: 29/59 and 48/60 at 40 and 100 ppm, resp.
[c]All results in female rats, except where noted
dstatistical significance not measured
Dose-response results in bold

Table B4: Summary of DMA^{V} and DMA^{III} Genotoxicity

Endpoint	Study	Result	Reference
DMA^{V} *in vitro* studies			
Mutation	Ames Assay (TA1535,TA1537, TA1538,TA100,TA98)	Negative (up to 10,000 ug/plate) (with/without liver S9 activation)	MRID 41892706
Mutation	Ames Assay (TA104,TA100,TA98)	Negative (up to 15,625 nmole/plate) (with/without liver S9 activation)	Kligerman et al., 2003
Mutation/ Clastogenicity	Mouse Lymphoma Gene Mutation Test (L5178Y)	Negative (1600-6760 ug/ml) (with/without liver S9 activation)	MRID 41892707
Mutation/ Clastogenicity	Mouse Lymphoma Gene Mutation Test (L5178Y)	Weak response but at an excessive concentration of 10,000 ug/ml--predominantly for the induction of small colonies indicative of chromosome breakage)	Moore et al., 1997
Clastogenicity/ SCEs	Human peripheral lymphocytes	SCEs and Chromosomal Aberrations: weakly positive but at high *in vitro* concentrations, 3,000 and 10,000 uM	Kligerman et al., 2003
Clastogenicity	Human fibroblasts	Positive (primarily chromatid breaks) at 700 uM	Oya-Ohta et al., 1996
Clastogenicity/ SCEs	Chinese hamster ovary cells	SCEs and Chromosomal aberrations: Negative up to 10,000 uM for chromosomal aberrations Micronuclei: Negative up to 500 uM	Dopp et al., 2004
Mutation/ Clastogenicity	Mouse Lymphoma (L5178Y)	Negative up to 10,000 ug/ml	Moore et al., 1997
DNA strand breakage	Phage θX174 naked DNA nicking assay	Negative (up to 300 mM)	Mass et al., 2001
DNA strand breakage	Single cell comet assay in human peripheral lymphocytes	Negative (up to 1000 uM)	Mass et al., 2001
DMA^{III} *in vitro* studies			
Mutation	Ames Assay (TA104,TA100,TA98)	Negative (up to 2133.17 nmole/plate) (with/without liver S9 activation)	Kligerman et al., 2003
Mutation	Prophage induction in E.*coli*	Negative (up to 1 uM) (with/without liver S9 activation)	Kligerman et al., 2003

Endpoint	Study	Result	Reference
Clastogenicity	Mouse Lymphoma Gene Mutation Test (L5178Y)	Positive, primarily the induction of small colonies indicative of chromosome breakage (1.3 -2.56 uM)- (without liver S9 activation)	Kligerman et al., 2003
Clastogenicity	Peripheral human lymphocytes	Positive at 1.35 and 3.09 uM	Kligerman et al., 2003
Clastogenicity	Chinese hamster cells	Positive at 1 -5 uM for micronucleus formation Positive for chromosomal aberrations at 50 and 100 uM (mostly chromatid breaks) Approximately 45% cell death at 1 uM; 100% cell death at 100 uM	Dopp et al., 2004
Sister Chromatid Exchange	Peripheral human lymphocytes	Weakly positive at 1.35 uM	Kligerman et al., 2003
Sister Chromatid Exchange	Chinese hamster ovary cells	Weakly positive at 50 uM	Dopp et al., 2004
DNA strand breakage	Phage θX174 naked DNA nicking assay	Positive at 37.5 uM (damage was prevented in the presence of antioxidants and ROS inhibitors)	Nesnow et al., 2002
DNA strand breakage	Phage θX174 naked DNA nicking assay	Positive at 10 uM	Mass et al., 2001
DNA strand breakage	Single cell comet assay in human peripheral lymphocytes	Positive at 23 uM	Mass et al., 2001
DNA strand breakage	Human cell line (HELA S3)	Positive at >10 uM : damage found in the form of formamidopyrimidine-DNA glycosylase sensitive sites which is indicative of oxidative DNA damage by ROS	Schwerdle et al., 2003
DMA^V *in vivo* studies			
Mutation	"Muta" Mouse assay (10.6 mg/kg bw once daily for 5 days ip injection)	Negative	Nado et al., 2002
Clastogenicity/ Aneuploidy	Mouse Micronucleus Assay (gavage)	Negative (up to 586 mg/kg bw)	MRID 41892708
Aneuploidy	CD-1 mice (ip injection)	Positive at 300 mg/kg bw in bone marrow cells	Kashiwada et al 1998

Endpoint	Study	Result	Reference
Micronucleated reticulocytes	Mouse (ICR) (single IP injection) study	DMA (10.6 mg/kg bw) only induced micronucleated reticulocytes after co-administration of glutathione	Kato et al., 2003
DNA strand breakage	Rat gavage study using alkaline elution	Positive at 387 mg/kg bw in lung cells	Kitchin and Ahmad, 2003
DNA strand breakage	Mouse (B6C3F1) gavage study using alkaline elution	Weakly positive in liver but not lung tissue at 720 mg/kg bw. Decreases in GSH content also found.	Ahmad et al., 1999
DNA strand breakage	Mouse gavage study	Positive in lung tissue at 1500 mg/kg bw	Yamanaka and Okada, 1994
Oxidative DNA adducts	Rat (oral-0,.5, 10, and 20 mg/kg)	Positive in kidney at 10 mg/kg bw	Vijayanahavan et al., 2001
Oxidative DNA adducts	Rat drinking water study	8-OHdG adducts found in bladder at 200 ppm (1.21 versus 1.76 /10^5dG) Wei could not replicate this finding in a repeat study in the laboratory of S. Cohen (personal communication, S. Cohen)	Wei et al., 2002
Oxidative DNA adducts	mouse drinking water study	8-OHdG adducts in lung (1.23 versus 1.79 /10^5dG) and liver tissue (1.17 versus 2.22 /10^5dG) at 400 ppm	Yamanaka et al., 2004

ROS = reactive oxygen species; 8-OHdG = 8-hydroxy deoxyguanosine.

Table B5: Initiation and Promotion Studies Conducted on DMAV

Study	Doses	Results	Reference
Multiple Organs: 30 week drinking water study of F344/DuCrj male rats pretreated with five mutagens (DMBDD model) followed by DMA^V exposure	Group 1 Pretreated with the DEN, MNU and DMH, BBN, DHPN but not DMA^V Group 2 Pretreated with DEN, MNU, and DMH, BBN, DHPN, and then 50, 100, 200, and 400 ppm DMA^V Group 3 Treated with 100 or 400 ppm DMA^V only	DMA^V acted as a promoter, but not as an initiator of carcinogenesis In the initiated-mutagen pretreated groups, DMA^V enhanced the tumor induction in bladder, kidney, liver and thyroid In animals treated only with DMA^V, no tumors were observed as well as no preneoplastic lesions.	Yamamoto et al., 1995
Bladder: Drinking water study with NCI Black Reiter male rats pretreated with BBN for 4 weeks and then given 100 ppm DMA^V for 32 weeks	Pretreated with BBN followed by 0 or 100 ppm DMA^V	DMA^V acted as a promoter DMA^V enhanced the development of preneoplastic lesions, BrdU labeling index in urinary epithelial cells., and bladder tumor incidence.	Wei et al, 1998
Bladder: Drinking water study with F344 rat treated with BBN for 4 weeks then given DMA^V for 32 weeks	Pretreated with BBN s then given 2, 10, 25, 50, or 100 ppm DMA^V	DMA^V acted as a promoter	Wanibuchi et al., 1996
Skin: Drinking water study with UVB-induced skin tumorigenesis hairless mouse model	UVB treated mice given 0, 400, 1000 ppm DMA^V for 25 weeks	DMA^V acted as a promoter by enhancing the development of skin tumors	Yamanaka et al., 2000
Skin: K6/ODC transgenic mice initiated by DMBA followed by DMA^V treatment	Pretreated DMBA mice followed by topical administration of 3.6 mg of DMA^V	DMA^V acted as a promoter by enhancing the development of skin tumors	Morikawa et al., 2000

Study	Doses	Results	Reference
Lungs: Drinking water study of F344 male rats pretreated with DHPN followed by DMA^V treatment for 30 weeks	pretreated with DHPN for 1 week followed by 0. 100, 200, or 400 ppm DMA^V	DMA^V did not act as a promoter Development of alveolar epithelial hyperplasias, adenomas, and carcinomas in lung not enhanced by DMA^V treatment.	Seike et al., 2002
Lungs: Mice pretreated with 4-nitroquinoline 1-oxide		DMA^V acted as a promoter of lung carcinogenesis	Yamanaka et al., 1996
Liver: F344 male rats pretreated with DEN followed by DMA^V in drinking water for 6 weeks	Rats pretreated with 200 mg/kg bw DEN (i.p.) followed by 100 ppm DMA^V	DMA^V acted as a promoter DMA^V (as well as MMA and TMAO) increased the development of GST-P- positive foci in rat liver at a similar potency	Nishikawa et al., 2002

Appendix C Physiologically-Based Pharmacokinetic (PBPK) Modeling for Dimethylarsinic Acid (DMA^V)

SUMMARY

Given the quantitative importance of DMA^V as a metabolite of arsenate and its activity as a complete bladder carcinogen and multi-organ tumor promotor in rodents, a physiologically based pharmacokinetic (PBPK) model is currently underdevelopment to describe its tissue distribution and metabolism. It is important to note that in this appendix, DMA (as distinct from DMA^V or DMA^{III}) refers to both together, i.e. DMA^V + DMA^{III}. In this Appendix we present a PBPK model for DMA^V exposure that was developed using mouse data and subsequently scaled and parameterized to predict urinary excretion of DMA and metabolism to trimethylarsine oxide (TMAO) in rats and humans. The purpose of this model is to evaluate interspecies differences in various internal dose metrics following oral exposure to DMA^V at both low exposure levels and exposure levels used in rodent bioassays at which effects were observed. The PBPK model provides a reasonable quantitative description of key interspecies PK differences, specifically differences in TMAO production and sequestration in rat red blood cells. Model predictions are consistent with limited data available for model evaluation.

Selection of the most appropriate dose metric is determined by the toxic endpoint(s) of concern. In the case of DMA^V, a major endpoint of concern is bladder tumors that have been observed in lifetime bioassays of rats. In this case, the target tissue is urinary bladder epithelium (Cohen et al., 2001 & 2002, Arnold et al., 1999). Based on the much greater toxicity of DMA^{III} compared to DMA^V for rat and human urinary bladder epithelial cells (Cohen et al., 2002), it seems likely that DMA^{III} is the toxic metabolite in this case. Thus, since the target tissue is bladder surface epithelium (urothelium), the ideal dose metric would be a measure of DMA^{III} delivered to the bladder urothelium both via excretion in urine and via blood to bladder tissue. Given that the paucity of *in vivo* data available do not allow reliable development of a DMA^{III} sub model, a reasonable surrogate dose metric might be amount of DMA metabolized to TMAO since DMA^{III} is an intermediate in this pathway. A key observation is that model predictions are linear across exposure levels for each species. This suggests that pharmacokinetic differences among species can be accounted for by a single factor across species. The greatest impact of interspecies PK differences accounted for in this model, especially sequestration in rat red blood cells, is that the time to steady state differs across species. In a practical sense, this difference would have the greatest impact under exposure scenarios of short duration.

In this Appendix we present a PBPK model for DMA^V exposure that was developed using mouse data and subsequently scaled and parameterized to predict urinary excretion of DMA and metabolism to trimethylarsine oxide (TMAO) in rats and humans. The purpose of this model is to evaluate expected interspecies differences in various internal dose metrics following oral exposure to DMA^V at both low exposure levels and exposure levels used in rodent bioassays at which effects were observed. **[Note: This model is in it's preliminary stages of development and has not been used in the development of proposed PODs or proposed regulatory endpoints.]**

MATERIALS AND METHODS

Model Structure.

The structure of the DMA^V model is shown in Figure App.C-1. The tissue compartments in the model include lung, liver, kidney, bladder, skin and residual body. Arterial and venous blood are separated as explicit compartments. DMA transport into lung, liver and kidney was modeled as membrane-limited (a combination of transport by blood flow and diffusion through a membrane) transport kinetics, whereas all other tissue transport was modeled as blood flow limited. The model structure accommodates both oral and intravenous routes of exposure, and includes urinary excretion of DMA, as well as first order metabolism of DMA to trimethylarsine oxide in the liver (TMAO) and sequestration of DMA in rat red blood cells. We chose not to model biliary excretion at this time since urine is the major route of excretion and studies in mice (Hughes and Kenyon, 1998) and rats (Stevens et al., 1977; Suzuki et al., 2004) suggest that biliary excretion is a minor excretory route. The rationale for the structural and physiological features of the model is provided in Table App.C-1.

Mass balance differential equations for the model are similar to those used in other models (*e.g.,* Dedrick et al., 1973). An explanation of the mass balance equations are included in the Addendum to this Appendix.[4]

Model Parameterization and Calibration.

Parameters for cardiac output, organ volumes and blood flows were obtained from the literature and are presented in Table App.C-2. Chemical-specific parameters are given in Table App.C-3. Initial estimates for partition coefficients were determined using the area method of Gallo et al. (1987) applied to pharmacokinetic data for the 0-45 minute time period following a single bolus i.v. dose of DMA^V (see also Gabrielsson et al., 1984). Subsequently, partition coefficients and diffusion constants for lung, kidney and liver were re-estimated and optimized using tissue concentration-time course data from i.v. kinetic studies in mice for the respective tissues (Hughes et al., 2000). Urinary excretion of DMA-derived radioactivity is dose-independent in mice intravenously administered DMA^V (Hughes and Kenyon, 1998); thus, urinary elimination of DMA was modeled as a first order process and was scaled allometrically across species. The GI absorption coefficient was estimated using blood time-course data from mice administered DMA^V orally at two different dose levels (Hughes et al., 2005) and assumed to be the same across species.

Species-specific data were used to estimate first order metabolic rate constants for metabolism of DMA^V to TMAO in liver. Metabolism was assumed to be first order over the relevant range of exposure concentrations based on rat data. Wei et al. (2002) measured urinary concentrations of arsenic compounds in rats exposed to 0, 12.5, 50 and 200 ppm DMA^V in drinking water at week 100 of a 104 week exposure. The concentration of TMAO in urine increased linearly with dose over the entire dose range. No studies were available in humans or mice in which multiple doses or exposure levels of DMA^V were used and TMAO measured in urine. Urinary excretion was assumed to

[4] The model was initially implemented in ACSL (Simusolv®, Ver 3.0). It is currently being implemented in updated software (MatLab Ver. 7.0.4, Release 14, The Mathworks, Natick, MA; AcslXtreme Ver. 2.0, Aegis Xcellon, Huntsville, AL).

be constant across doses based on i.v. kinetic data in mice (Hughes and Kenyon, 1998). Using the assumption of first order metabolism from DMA to TMAO and dose independence for urinary excretion, species-specific estimates for ktma were derived from urinary excretion data for TMAO in mice and humans (Marafante et al., 1987) and rats (Yoshida et al., 1997; Vahter et al., 1984) following oral administration of DMA^V.

A significant species-specific difference in rats, compared to mice and humans, is that whole body clearance of DMA^V is much slower in the rat (Vahter et al., 1984, Buchet et al., 1981). For an oral dose of ^{74}As-DMA (0.4 mg As/kg), Vahter et al. (1984) reported that in the mice 85% of the dose was cleared with a half-life of 2.5 hrs and about 14% with a half-life of 10 hours, whereas in the rat 45% of the dose was cleared with a half-life of 13 hours, and the remaining 55% with a half-life of ~50 days. Stevens et al. (1977) earlier attributed this difference to the propensity of rat erythrocytes to accumulate DMA. Subsequent mechanistic studies have demonstrated that binding of DMA^{III} to rat hemoglobin (Hb) at reactive cysteine residues in α and β chains of Hb accounts for this species difference (Shiobara et al., 2001; Lu et al., 2004).

Sequestration in red blood cells was incorporated in the PBPK model by inclusion of separate red blood cell and plasma compartments. In essence, transport of DMA is occurring in the plasma and there is a parameter that controls diffusion across the red blood cell membrane (pabc) and then another (prbc) that describes partitioning in the red blood cell compartment. These parameters were estimated for mice using plasma and erythrocyte time course data following intravenous administration of DMA^V at two different dose levels (100-fold difference). These parameters were assumed to be the same in humans as in mice because DMA^V is rapidly cleared in both humans and mice (Buchet et al., 1981; Vahter et al., 1984). For rats, pabc and prbc were estimated using whole body retention data from Vahter et al. (1984). The rationale for using these data is that the major determinant accounting for slow whole body clearance in the rat is retention in red blood cells (Stevens et al., 1977, Vahter et al, 1984).

Model Evaluation

In the context presented here, model evaluation refers to determination of the ability of the model to predict the behavior of experimental data sets that are different from the data used to estimate parameters and develop the model. For the mouse, time course data in whole blood, liver, lung and kidney were available following a single intravenous dose of DMA^V (Vahter et al., 1984). Data suitable for model evaluation are severely limited for the rat and human. However, it is possible to evaluate how consistent the model is in terms of general predictive characteristics for rat and human, e.g. time to steady state in the rat (Vahter et al., 1984) and rapidity of clearance in the human (Buchet et al., 1981).

Model Analysis

The model was used to simulate drinking water exposure scenarios for mice, rats and humans in order to evaluate inter-species differences in internal dose metrics. Exposure levels simulated were in the range of 0.001 to 200 ppm (rats, humans) and 500 ppm DMA^V (mice). This range was selected to span the range from extremely low (100-fold lower than concentration used in bioassays) to the highest exposure level

used in lifetime bioassays conducted in rodents. Although drinking water exposure was simulated, the model is also applicable to oral intake in food; however, the expectation would be that bioavailability from food might be lower compared to drinking water. Dose metrics were calculated at steady state using the same exposure scenario for all species (6 equal drinks per day spaced 4 hours apart) since the goal for this modeling exercise was to evaluate the effect of interspecies differences in pharmacokinetics following exposure to DMA^V.

Dose metrics of interest include daily area under the arterial blood concentration curve for free DMA (μg-h/L), daily area under the bladder tissue concentration curve (μg-h/L) for DMA, and amount of DMA metabolized to TMAO (μg/day). Metabolism to TMAO was selected as a surrogate for DMA^{III} production because DMA^{III} is an intermediary metabolite in the pathway to TMAO and available data suggest that DMA^{III} may be the toxic metabolite (Cohen et al., 2002).

RESULTS

This model was developed mainly using data from pharmacokinetic studies in mice exposed to DMA^V by the intravenous route and, to a lesser extent, the oral route, of exposure. Chemical-specific parameters estimated using these data are shown in Table App. C-3. Time course data for liver, lung, kidney and blood were used to determine that accumulation of DMA in the tissues was membrane-limited. A membrane-limited PBPK model is more complex than a blood flow limited model in that one needs to estimate solubility parameters for each organ (partition coefficients), as well as permeability values (related to diffusion), for each organ. Partition coefficients were initially estimated using the area method of Gallo et al. (1987). The i.v. data generated at U.S. EPA were then used to estimate permeability coefficients and to refine partition coefficient estimates for liver, lung and kidney.

In addition to organ time course data, we also had for the mouse, cumulative urinary excretion time course for two i.v. dose levels. Simulations of these data turned out to be very sensitive to the kidney permeability coefficient, and thus it was used to narrow down this value. Renal excretion was then estimated from the kidney time course data. Parameters were varied until they led to a significant change in the simulated curves. In all cases, final parameter estimates were selected based on their sensitivity, i.e. when their values made a contribution to the shape observed in the tissue concentration time curve for the particular tissue.

Figures App.C-2 and App.C-3 illustrate how specific sets of data were used to estimate selected parameters. For example, time course data for blood following oral administration of DMA^V at two different dose levels were used to estimate the coefficient for oral absorption (Figure App.C-2). Figure App.C-3 illustrates use of whole body retention data in rat to estimate parameters governing uptake and retention in rat erythrocytes. In Figure App.C-4, model predictions are shown for data generated in mice by the i.v. route of exposure (Vahter et al., 1984) that were not used to parameterize the model for purposes of model evaluation. The model gave a good visual fit to whole blood data, but consistently slightly over-predicted kidney concentration. This latter finding may be related to the fact that these data were generated in male NMRI mice, whereas the data used to calibrate the model, were generated in female B6C3F1 mice. Messow et al., (1980) and Hackbarth and

Hackbarth (1981) have reported sex-and strain-dependent differences in kidney morphology and glomerular filtration rate in mice and this may provide a partial explanation of this difference.

Time to steady state is dose-independent (does not differ across dose levels) for mouse, rat and human. This is expected because major governing rates are all modeled as first order, i.e. GI absorption and metabolism to TMAO, and urinary excretion. Figures App.C-5, -6A and –7, illustrate the time to steady state for an exposure level of 1 ppm DMA^V (0.543 μg As/L) in drinking water. This would most accurately be termed pseudo-steady state because plasma concentration oscillates due to the episodic nature of the modeled exposure, i.e. 6 equal exposures per day, four hours apart. In the mouse, pseudo-steady state is achieved within 10 hours (Figure App.C-5). This is consistent with what would be expected on the basis of the whole body clearance half lives calculated by Vahter et al. (1984) in mice, i.e. 85% of the dose was cleared with a half-life of 2.5 hrs and about 14% with a half-life of 10 hours. In the rat, pseudo-steady state is achieved by ~4200 hours or 180 days (Figure App.C-6). This is again consistent with what would be expected on the basis of whole body clearance half lives calculated by Vahter et al. (1984) in rats, i.e. 45% of the dose was cleared with a half-life of 13 hours, and the remaining 55% with a half-life of ~50 days. Predicted time to pseudo-steady state in humans (Figure App.C-7) is ~24 hours and this is consistent with rapid urinary clearance for DMA^V reported by Buchet et al. (1981). Figure App.C-6B illustrates the difference in predicted concentration in rat plasma and RBCs for the same time scale (0-120 hours) used in the mouse and human graphs. Plasma concentration is oscillating because of the episodic nature of the exposure, while RBC concentration is continuing to increase.

With respect to using the model to evaluate interspecies differences for various dose metrics, Figures App.C-8A and App.C-8B are illustrative of general behavior for AUC metrics for various tissues. In Figure App.C-8A, DMA^V exposure in drinking water is plotted against the AUC for plasma DMA concentration (log-log scale). This illustrates that the model is linear across relevant exposure levels, as one would expect, i.e. rates are all modeled as first order. That the lines are essentially co-incident with one another is a function of scaling, i.e. exposure rate and clearance processes (*e.g.,* urinary excretion, metabolism, etc) scale allometrically across animal species. In Figure App.C-8B, the average daily intake dose of DMA^V (μg As/kg/day) is plotted against the same AUC. This figure illustrates that different average daily doses are required to achieve equal AUC across species. This is because the same exposure scenario (1 ppm DMA^V in six equal daily drinking events 4 hours apart) leads to different daily intakes in the rat, mouse and human.

Table App.C-4 compares average daily intakes at specific exposure concentrations of 2, 10 and 50 ppm DMA^V in drinking water with TMAO formation (μg/day/kg of BW). An important factor to realize in this comparison is that the same exposure concentration results in a different intake dose across species due differences in BW and water consumption. For the same exposure level, humans are predicted to form ~4-fold less TMAO compared to rats. This 4-fold difference remains the same across exposure levels within species because the major metabolic processes in the model are first order. Mouse and rat are relatively similar in terms of their formation of TMAO. The best explanation for this is that although the estimated first order metabolic

rate constant for rats is 2.4-fold higher than the mouse, sequestration in rat red blood cells results in less DMA being available in the liver for metabolism to TMAO.

DISCUSSION

A PBPK model has been developed to describe DMA pharmacokinetics in mice, rats and humans. The model structure used here is more complex than that typically used for chemical classes such as volatile organic compounds because there is a mixture of blood-flow limited and membrane-limited transport into tissue compartments. The mouse database developed at U.S. EPA is the most extensive available in the same gender, strain and species of laboratory animal, with time course data for liver, kidney, lung, whole blood, plasma and erythrocytes and urinary elimination of DMA (total DMA = DMA^{III} + DMA^{V}) after intravenous administration at two dose levels that differ by 100-fold. The time course data for each of these organs was used to determine that accumulation of DMA in the tissues was membrane-limited. The i.v. dataset is the most useful time course data because it allows for estimation of partition and permeation coefficients using the simplest PBPK model possible. An oral dataset, for example, adds the complications of multiple processes to consider when interpreting and utilizing the pharmacokinetic data for parameter estimation, e.g. gastrointestinal absorption and potential first pass effect in the liver. Typically, it is simplest to start with i.v. data, if available, to determine partition and permeation parameters for non-volatile compounds, and then continue model development with data from other routes of exposure, such as oral and dermal.

There are several key assumptions in the extrapolation exercise using the PBPK model for DMA^{V}. Partition and permeability coefficients estimated on the basis of mouse data were assumed to be the same across species with the exception of the erythrocyte compartment in rats as noted previously. This assumption is commonly used in PBPK models and has, in general, proven to be adequate unless there are known or expected interspecies differences in intracellular protein binding or protein binding in blood. Estimation of these parameters from *in vivo* data is necessary in this case because, unlike the case with VOCs, there is not a relatively simple method (vial equilibration) to obtain independent estimates of partition coefficients (Krishnan and Andersen, 1994). No validated *in vitro* technique to measure *in vivo* permeability coefficients exists to our knowledge, and permeability coefficients are typically estimated from tissue time course data. Metabolic rate constants for TMAO production are specific for each species rather than being scaled allometrically from one species to another; this is a clear strength of this model in terms of reducing extrapolation uncertainty. Renal excretion rate was estimated on the basis of mouse data and scaled allometrically across species. The oral absorption rate was estimated on the basis of mouse data and assumed to be the same across species.

One key pharmacokinetic difference in DMA^{V} for rats compared to mice and humans is that rat hemoglobin (Hb) very effectively binds DMA, probably as DMA^{III} and that rats appear to metabolize DMA^{V} to trimethylated metabolites more extensively than other species. The effect of DMA binding to rat Hb is to increase amount of dose retained and the retention time in rats relative to other species. For example, 42% vs. 99.7%of a single oral dose of DMA^{V} (0.5 mg As/kg) was eliminated in the urine and feces of rats and mice, respectively, in a 48 hour period. In rats in this same study, 45%

of the dose was eliminated with a half-life of 13 hours and the remaining 55% with a half-life of approximately 50 days; the corresponding figures for mice are 85% of the dose with a half-life of 2.5 hours and 14% with a half-life of 10 hours (Vahter et al., 1984). Recent studies indicate that DMA is bound to rat hemoglobin as DMA^{III} and thus Hb represents a storage depot for the more toxic form of DMA (Shiobara et al., 2004; Lu et al., 2004). Once released, as a result of red blood cell death and Hb recycling (Stevens et al. 1977), DMA^{III} may be available for reaction with target sites or for metabolism to TMAO. Thus for the same intake dose, one would expect the rat bladder epithelium to experience lower concentrations of DMA^{V} and DMA^{III}, but over a longer time period compared to other animal species.

The analysis presented in this Appendix allows for a quantitative comparison of the impact of known interspecies differences in metabolism and disposition on dose metrics of interest. One important observation is that the predictions are linear across exposure levels within each species. This suggests that pharmacokinetic differences among species can be scaled by a single factor across species. The greatest impact of interspecies pharmacokinetic differences accounted for in this model, especially sequestration in rat red blood cells, is that the time to steady state differs across species. In a practical sense, this difference would have the most impact under exposure scenarios of short duration.

Uncertainties and Research Needs

Uncertainties in this analysis come from two principle sources: (1) limitations in the data available to develop and evaluate the PBPK model and (2) uncertainties concerning the most appropriate dose metric to use based on target organ toxicity.

The model presented here does not distinguish between DMA^{V} and DMA^{III}, but rather treats them as a single chemical species. This is the same approach taken in models that have been developed for inorganic arsenic (Mann et al., 1996a & b; Yu, 1999). This approach was dictated by the paucity of data that distinguishes between DMA^{V} and DMA^{III} in tissues and urine. However, this approach may not be optimal given the known differences in cellular uptake and binding between pentavalent and trivalent arsenicals in general and that exist for DMA^{III} and DMA^{V} as well (*e.g.,* Shiobara et al., 2001, Hirano et al., 2004, Lu et al., 2004). In order to develop a reliable expanded model, i.e. two complete sub-models, one for DMA^{V} and one for DMA^{III}, time-course tissue concentration data, ideally at multiple doses, in which both forms were measured, would be needed, as well as corresponding urinary elimination data.

Recent studies indicate that rats may metabolize DMA^{V} more extensively than other rodent species. Marafante et al. (1987) reported that mice and hamsters given a single oral dose of DMA^{V} (73.7 mg /kg) excreted 3.5% and 6.4% of the dose as TMAO in urine within 48 hours. Rats administered a single oral dose oral dose of 50 mg/kg DMA^{V} excreted over 50% of the arsenic in urine in the form of TMAO between 6 and 24 hours after dosing (Yoshida et al., 1997). In another study, rats exposed to DMA^{V} at 100 mg/L (ppm) in drinking water for one week excreted 44.9% of the arsenic in urine in the form of DMA and 40% as TMAO (Yoshida et al., 1998). Following nearly 2 years exposure to DMA^{V} in drinking water at 12.5 to 200 ppm, 30-40% of the arsenic present in urine was in the form of TMAO. Collectively, these data suggest that in rat there is a

higher flux of metabolism through the pathway leading to formation of trimethylated metabolites compared to hamsters or mice.

While these data are highly suggestive, they have clear limitations. One is that the available data in rats is quantified in terms of metabolite concentrations in urine (Yoshida et al, 1997 & 1998, Wei et al., 2002) rather than as percentage of the dose eliminated in urine as particular metabolites, as is the published data for mice and hamsters (Marafante et al., 1987). Given that rats have slower overall clearance compared to mice and hamsters, comparisons in which the data are not expressed on the same basis could be misleading. Such comparisons are most appropriately made on both a percentage of the dose excreted basis and concentration excreted in urine over the same time interval for both species. In such studies it would be important to use analytical methodologies that have been optimized to detect trimethylated arsenicals, as well as DMA^{III}; this has been a limitation in many commonly used analytical methods in the past.

There are only two studies in the literature that specifically examined the disposition of DMA^{V} in humans after exposure to DMA^{V} (Buchet et al., 1981, Marafante et al., 1987). Buchet et al. (1981) evaluated urinary excretion of DMA in 4 human subjects over a period of 4 days and found that 75% of the dose was excreted in urine; metabolism to TMAO and fecal excretion of DMA were not quantified. Marafante et al. (1987) reported urinary excretion of DMA and TMAO in a single human subject administered DMA^{V} orally (0.1 mg As/kg body weight). They reported that ~80% of the dose was excreted in urine over a 3 day period with ~76% as DMA and ~3.5% as TMAO. Data on human metabolism of DMA^{V} are obviously severely limited, although results from these two reports are at least consistent with one another in terms of overall urinary elimination. In light of the limited human data, model estimates for TMAO metabolism must be considered uncertain and this clearly points to the need for additional metabolism studies in humans.

Selection of the most appropriate dose metric is determined by the toxic endpoint(s) of concern. In the case of DMA^{V}, a major endpoint of concern is bladder tumors that have been observed in lifetime bioassays of rats exposed to DMA^{V} in diet or drinking water (Gur et al., 1989; Wei et al., 1999). In this case, the target tissue is urinary bladder epithelium (Cohen et al., 2001 & 2002, Arnold et al., 1999). Based on the much greater toxicity of DMA^{III} compared to DMA^{V} for rat and human urinary bladder epithelial cells (Cohen et al., 2002), it seems likely that DMA^{III} is the toxic metabolite in this case. Thus, since the target tissue is bladder surface epithelium (urothelium), the ideal dose metric would be a measure of DMA^{III} delivered to the bladder urothelium both via excretion in urine and via blood to bladder tissue. Given that the paucity of *in vivo* data available do not facilitate development of a reliable DMA^{III} sub model, a reasonable surrogate dose metric might be amount of DMA metabolized to TMAO since DMA^{III} is an intermediate in this pathway. It should be noted that this metric provides an estimate of the minimum amount of DMA^{III} that would have been produced, but does not account for DMA^{III} that may not have been fully metabolized to TMAO. It is known that this does happen since DMA^{III} has been identified in both rat and human urine (Lu et al., 2003; Cohen et al., 2002; Mandal et al., 2001; Valensuela et al., 2005).

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Figure App.C-1 Schematic Diagram of Pharmacokinetic Model

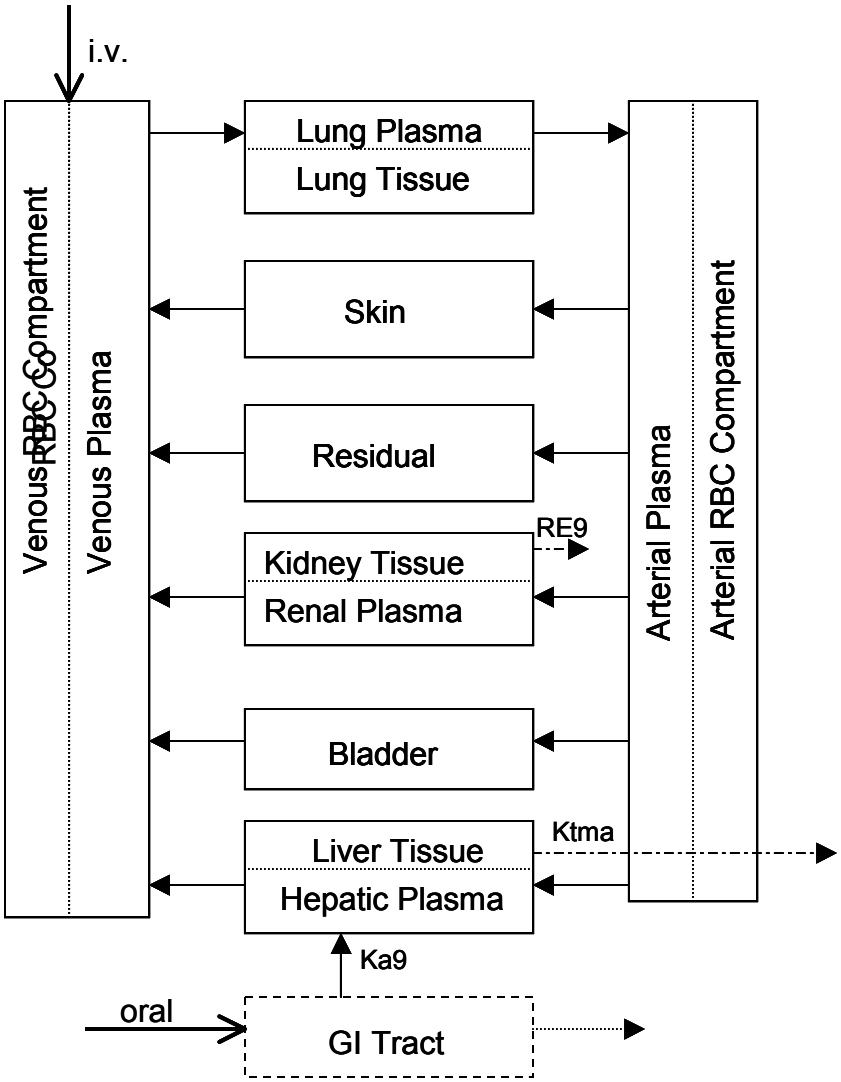


Table App.C-1 DMAV Model Description

Characteristic	Description and Rationale
Tissue Transport	Blood flow limited in organs or tissue groups for which tissue time course data were unavailable. This is a simplifying assumption. Membrane-limited description necessary to fit tissue time course data in mouse liver, kidney and lung.
DMA^V GI absorption	first order used as simplest initial case
DMA^V metabolism	first order due to linear increase in TMAO concentration in urine with dose in lifetime rat bioassay (Wei et al., 2002)
Urinary excretion	first order due to dose independence following i.v. administration over a 100-fold difference in dose range in mice (Hughes and Kenyon, 1998)
Hemoglobin binding/sequestration	Sequestration mechanism for DMA that is unique to rat (Shiobara et al., 2001; Lu et al., 2004) and accounts for long biological half-life in rat compared to mouse (Vahter et al., 1984)

Table App.C-2 Physiological Parameters for DMAV Model

Parameter, units	Symbol	Species			Footnote
		Mouse	Rat	Man	
Body Weight, kg	BW	0.03	0.24	70	default - 1
Cardiac Output, L/hr-kg$^{0.75}$	QCC	16.1	16	12.9	2
Hematocrit	hemcrt	0.415	0.45	0.45	
Flow (fraction QC)					2
Lung	QCC				3
Liver	QLC	0.161	0.183	0.227	
Kidney	QKC	0.091	0.141	0.175	
Bladder	QDC	0.0033	0.0005	0.0005	4
Skin	QIC	0.058	0.058	0.058	
Residual	QRC	1-ΣQi	1-ΣQi	1-ΣQi	
Volumes (fraction BW)					2
Arterial Blood	VAC	0.027	0.022	0.026	5
Venous Blood	VVC	0.051	0.040	0.049	5
Lung	VNC	0.007	0.005	0.008	
Liver	VLC	0.055	0.034	0.026	
Kidney	VKC	0.017	0.007	0.004	
Bladder	VDC	0.0009	0.00035	0.00064	4
Skin	VIC	0.165	0.19	0.037	
Residual	VRC	1-ΣVi	1-ΣVi	1-ΣVi	
Volume fraction of blood					
Lung	VNBC	0.5	0.36	0.2	2
Liver	VLBC	0.31	0.21	0.11	2
Kidney	VKBC	0.24	0.16	0.36	2

[1] These are default body weights, i.e. what is used if actual body weight is not available for experiment or scenario of interest.
[2] Taken from Brown et al., 1997. Note that cardiac output is scaled by $BW^{0.75}$.
[3] Lung receives all cardiac output. See model diagram for reference.
[4] Data for blood flow and volume of the urinary bladder in rats and mice are from Stott et al., 1983. Tissue volume for human urinary bladder is from ICRP, 1975. Data for bladder blood flow in humans was not located in the literature - for now, using rat bladder blood flow.

Table App.C-3 Chemical Specific Parameters DMAV Model

Parameter	Symbol	Units	Mouse	Rat	Human	Comments
liver:plasma PC	p9l	none - ratio	1.0	1.0	1.0	fitted[1]
skin:plasma PC	p9i	none - ratio	0.87	0.87	0.87	area method, initial liver[2]
resid:plasma PC	p9r	none - ratio	0.83	0.83	0.83	area method, initial lung[2]
lung:plasma PC	p9n	none - ratio	5.0	5.0	5.0	fitted[1]
kidney:plasma PC	p9k	none - ratio	10	10	10	fitted[1]
bladder:plasma PC	p9d	none - ratio	0.87	0.87	0.87	area method, initial liver[2]
liver diffusion constant	palc	none - ratio	0.3	0.3	0.3	fitted[1]
kidney diffusion constant	pakc	none -ratio	1.0	1.0	1.0	fitted[1]
lung diffusion constant	pafc	none - ratio	0.107	0.107	0.107	fitted[1]
1st Order GI Absorption Rate	ka9	hr-1	0.5	0.5	0.5	fitted[3]
Renal Excretion Rate for DMA	RE9C	$\mu g/hr/kg^{0.75}$	0.1	0.1	0.1	fitted[4,]
1st Order metabolism to TMAO	ktma	L/hr	0.00073	0.00178	0.00049	calculated, see text
hematocrit (proportion)	hemcrt	none - ratio	0.415	0.45	0.45	literature
RBC diffusion coefficient	pabc	none-ratio	51.05	0.0133	51.05	Fitted[5]
RBC binding/partition coeff	prbc	none-ratio	0.50	11530	0.50	Fitted[5]

[1]Parameters estimated using tissue-time course i.v. data in mice administered DMA^V (Hughes et al., 2000) and assumed to be the same across species. PC is partition coefficient.
[2]Initial estimate for partition coefficient (PC) using area method of Gallo et al. (1987).
[3]Estimated using blood time course data in mice after a single oral dose of DMA^V (Hughes et al, 2005) and assumed same across species.
[4]Estimated using data for time course of DMA excretion in urine after i.v. administration to mice (Hughes and Kenyon, 1998) and assumed same across species.
[5]Estimated using plasma and red blood cell time course data in mice after i.v. administration of DMA^V (Hughes et al, 2000) and assumed to be the same in humans. Rat parameter values were optimized based upon time course whole body retention data of Vahter et al. (1984).

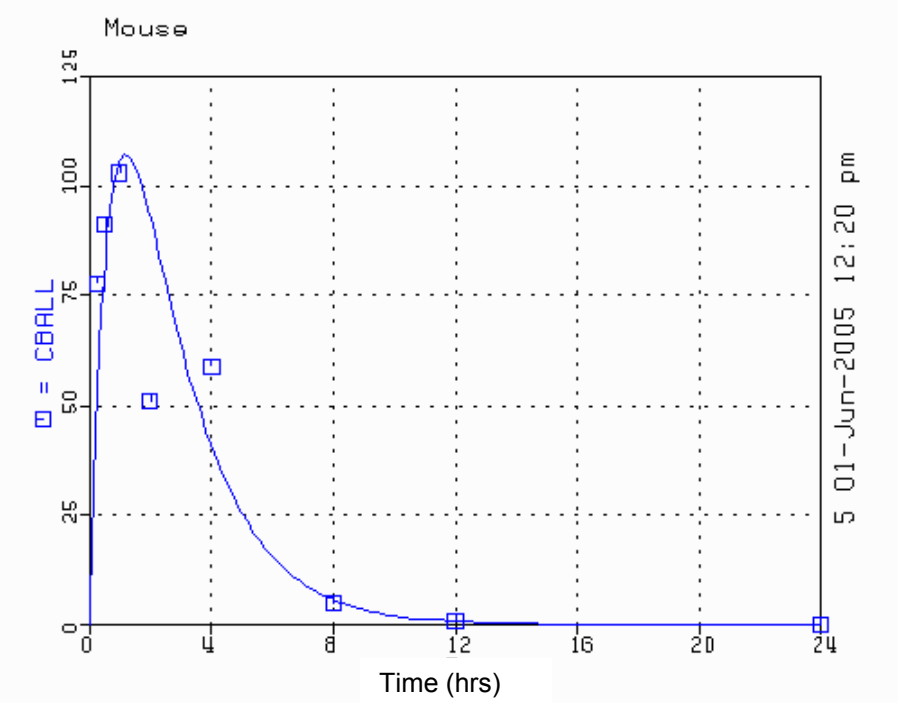
Table App.C-4. Comparison of TMAO formation for mice, rats and humans at different exposure concentrations to DMAV in drinking water.1

Exposure Concentration		Species	Intake Dose	TMAO Formed
DMA^V (ppm)	As (μg/L)		(μg As/kg BW/day)	(μg/kg BW/day)
2	1086	mouse	266	0.011
		rat	158	0.013
		human	38	0.003
10	5430	mouse	1331	0.055
		rat	791	0.065
		human	191	0.014
50	27150	mouse	6654	0.275
		rat	3957	0.325
		human	957	0.068

[1]Daily drinking water consumption is calculated as $0.102BW^{0.75}$. This yields daily drinking water volumes of 7 mL for a 0.030 kg mouse, 35 mL for a 0.240 kg rat and 2.46 L for a 70 kg human.

Figure App.C-2. Data (squares) and model predictions for time (hours) course in whole blood (μg/L) of mice administered a single oral dose of (A) 600 μg As/kg or (B) 60,000 μg As/kg as DMA^V. Data are from Hughes et al. (2005). The optimized value for oral absorption (ka9) was 0.5hr-1.

A.



B.

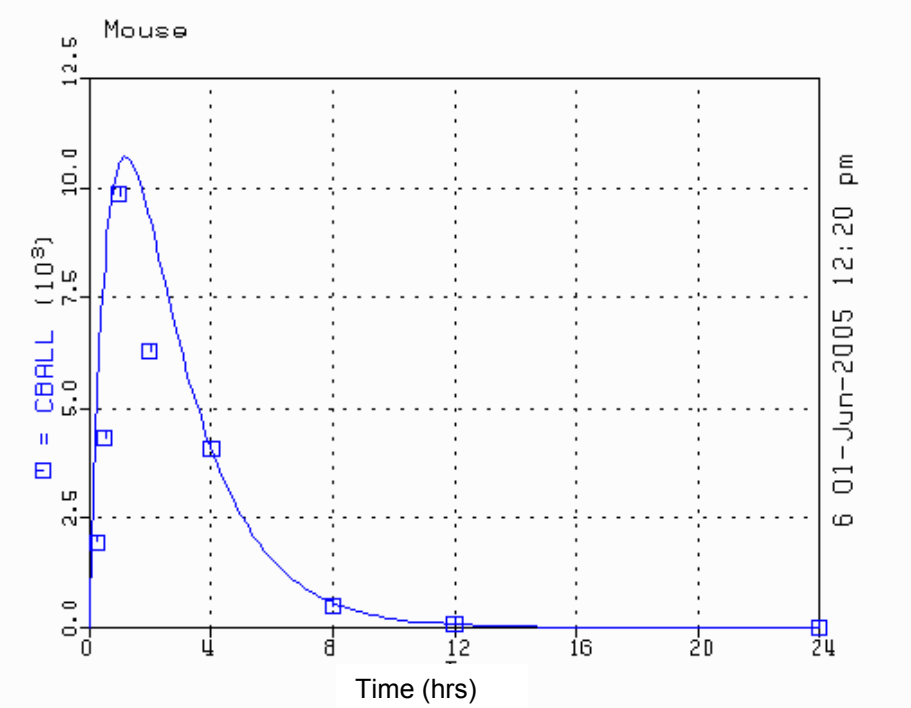
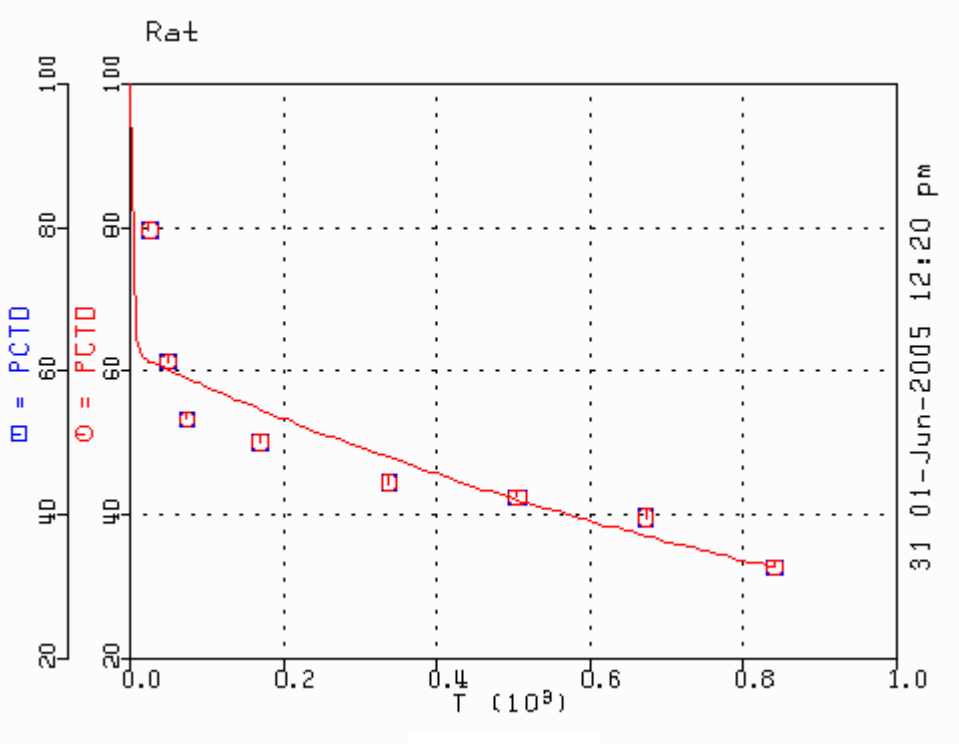


Figure App.C-3. Data (squares) and model predictions for time (hours) course of whole body clearance as percent of the dose retained (pctd) in rats administered a single oral dose of 0.4 mg As/kg body weight as DMA^V. Data are from Vahter et al. (1984). The optimized values for pabc and prbc were 0.0133 and 11530, respectively.



Time (hrs)

Figure App.C-4. Data (squares) and model predictions for time (hours) course in whole blood (CBALL, μg/L) and kidney (C9K, μg/L) for mice administered 0.4 mg As/kg body weight as DMA^V intravenously (Vahter et al., 1984).

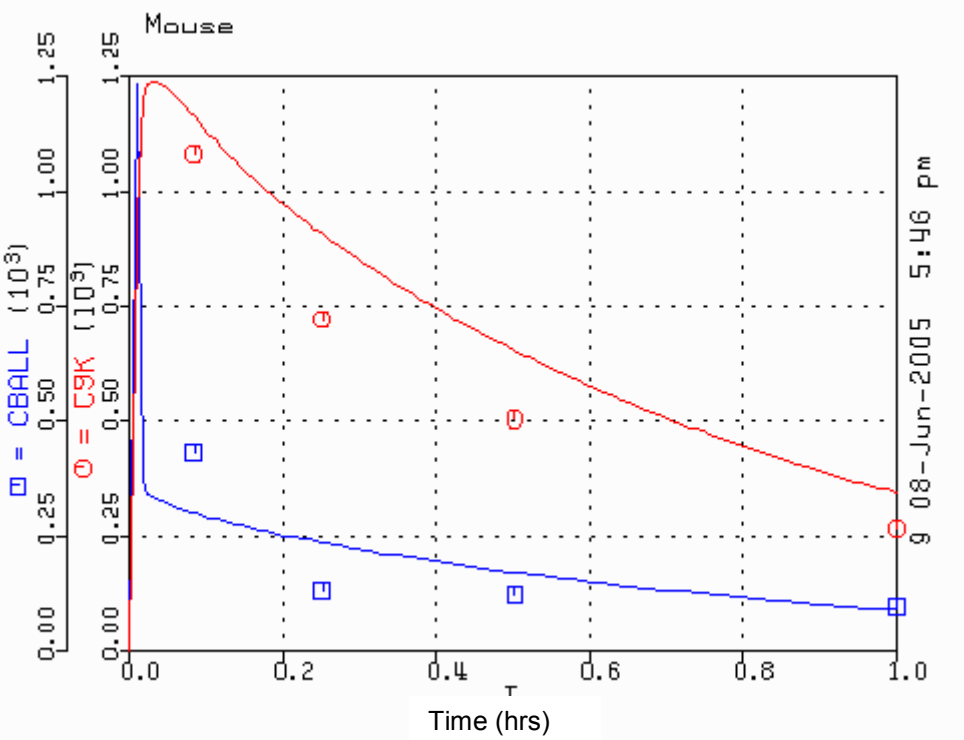


Figure App.C-5. Time course predictions for DMA concentration in venous plasma (CV9, μg As as DMA^V/L) in the mouse using an exposure scenario consisting of six equal exposures occurring every four hours in drinking water at 1 ppm DMA^V (543 μg As as DMA^V/L). This equates to an intake dose of 133 μg As/kg BW/day in the mouse.

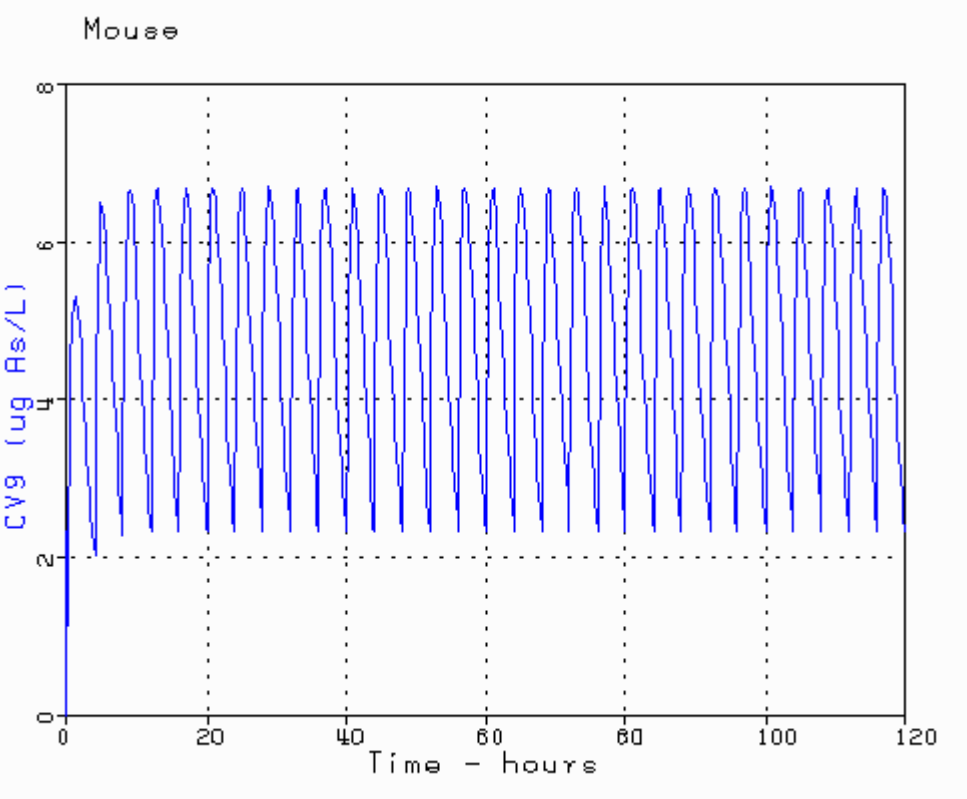


Figure App.C-6A Time course predictions for DMA concentration in venous plasma (CV9, μg As as DMA^V /L) in the rat using an exposure scenario consisting of six equal exposures occurring every four hours in drinking water at 1 ppm DMA^V (543 μg As as DMA^V/L). This equates to an intake dose of 79.1 μg As/kg BW/day in the rat. The time scale in this graph (0 to 6500 hours) illustrates the longer time required to reach steady state blood concentration in the rat.

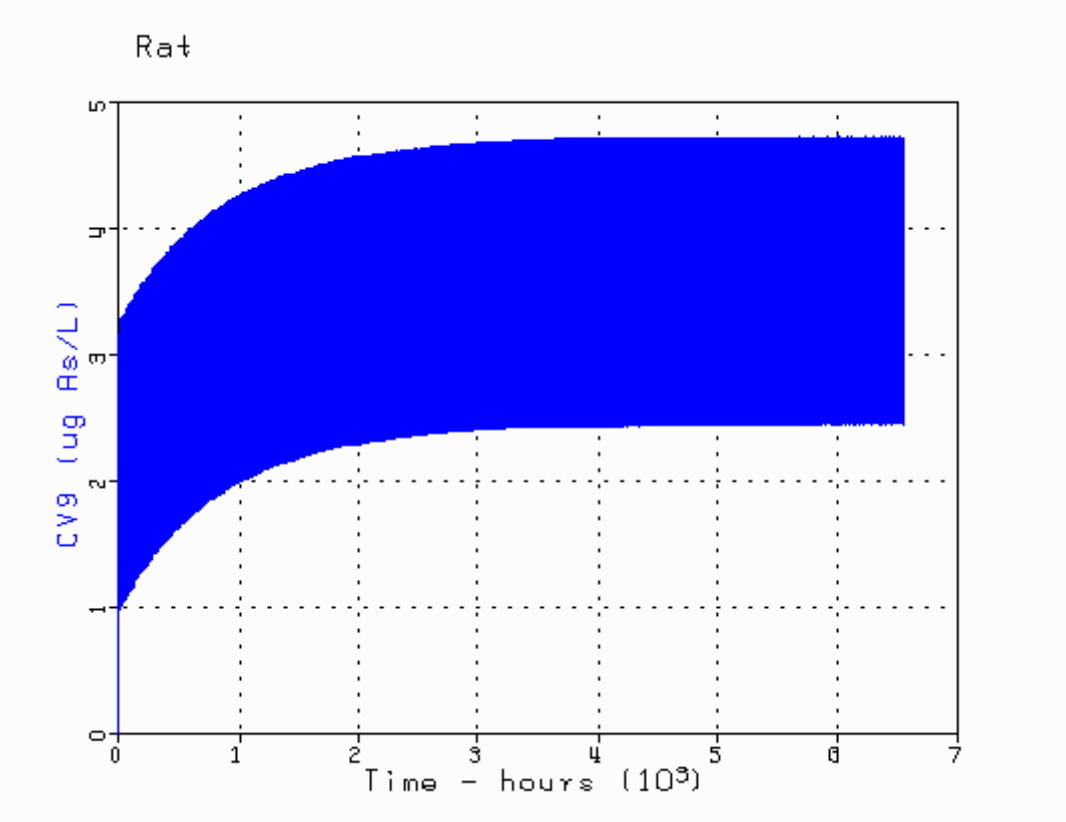


Figure App.C-6B Time course predictions for DMA concentration (μg As as DMA^V /L) in venous plasma (CV9, oscillating line) and arterial red blood cells (C9VBL, straight line) in the rat using an exposure scenario consisting of six equal exposures occurring every four hours in drinking water at 1 ppm DMA^V (543 μg As as DMA^V/L). This figure is shown on the same time scale as figures for rat and human to illustrate predicted accumulation of DMA in rat red blood cells.

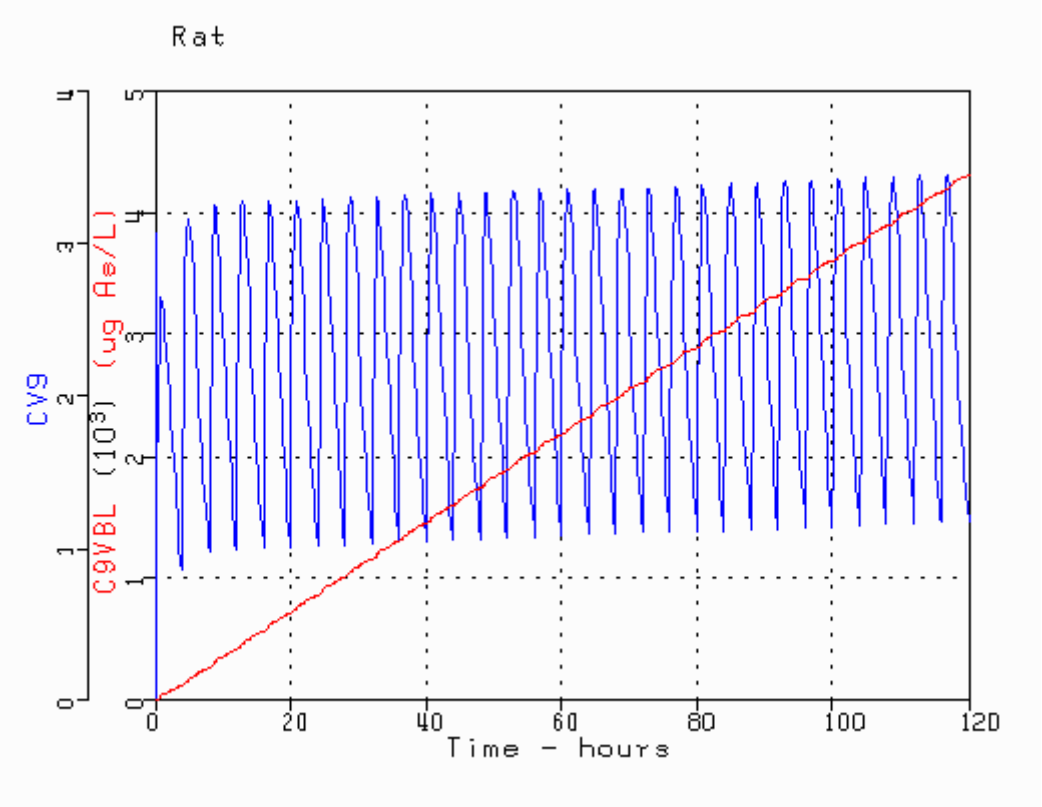


Figure App.C-7. Time course predictions for DMA concentration in venous plasma (CV9, μg As as DMA^V/L) in the human using an exposure scenario consisting of six equal exposures occurring every four hours in drinking water at 1 ppm DMA^V (543 μg As as DMA^V/L). This equates to an intake dose of 19.2 μg As/kg BW/day in the human.

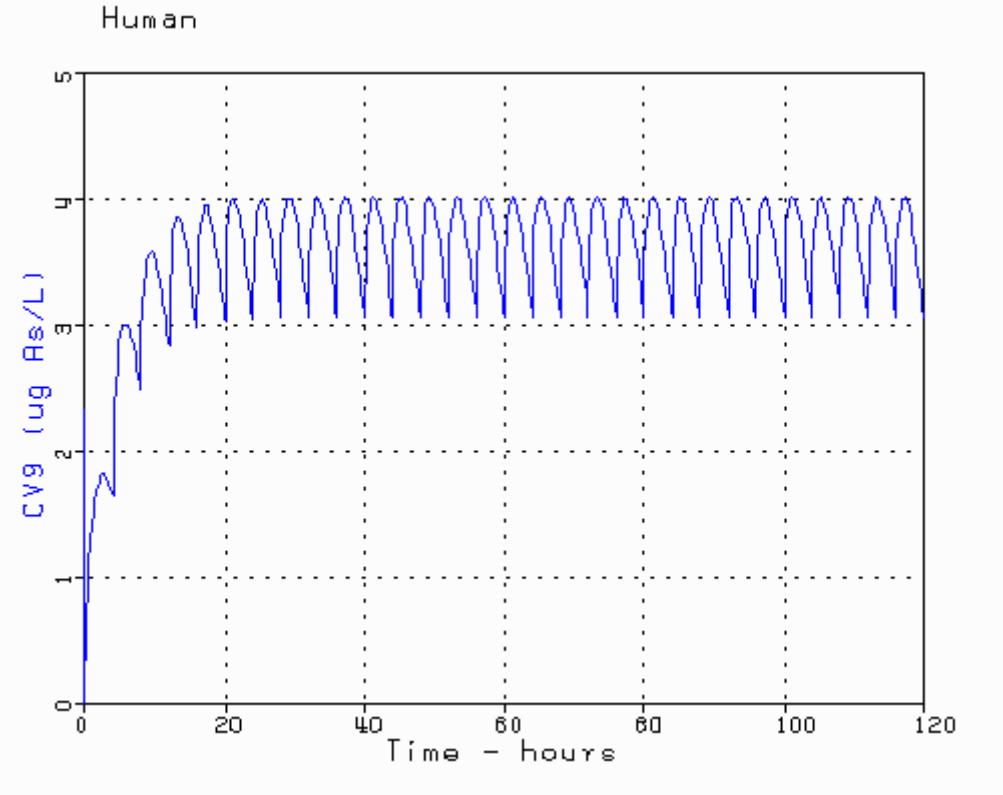


Figure App.C-8A.

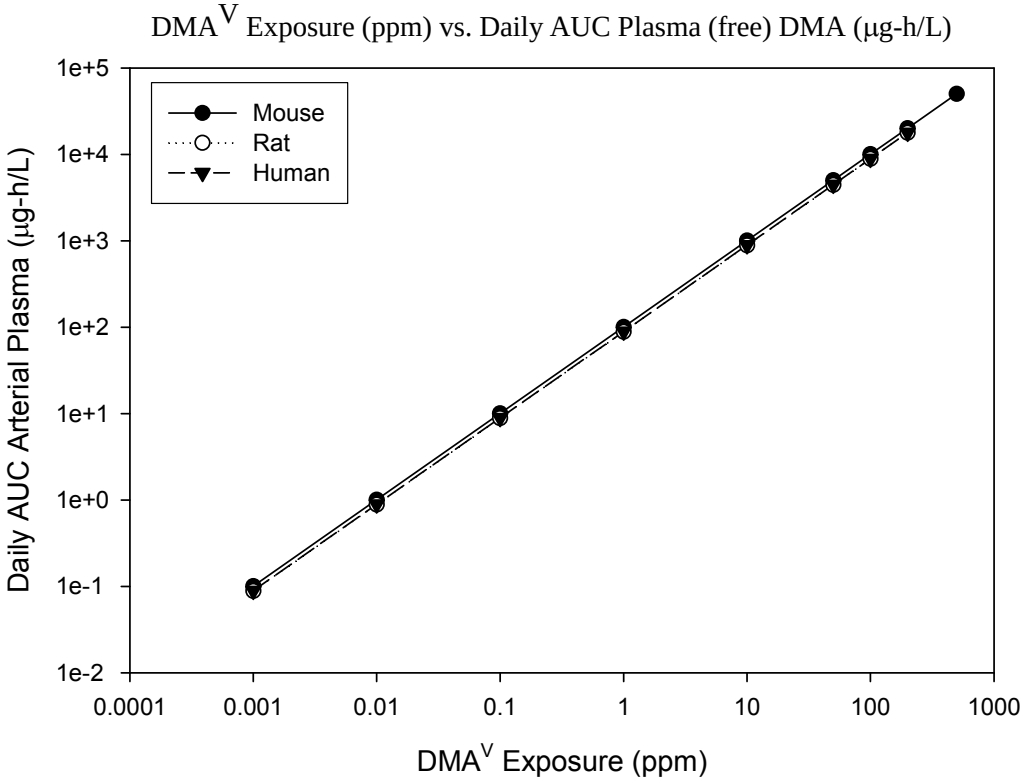
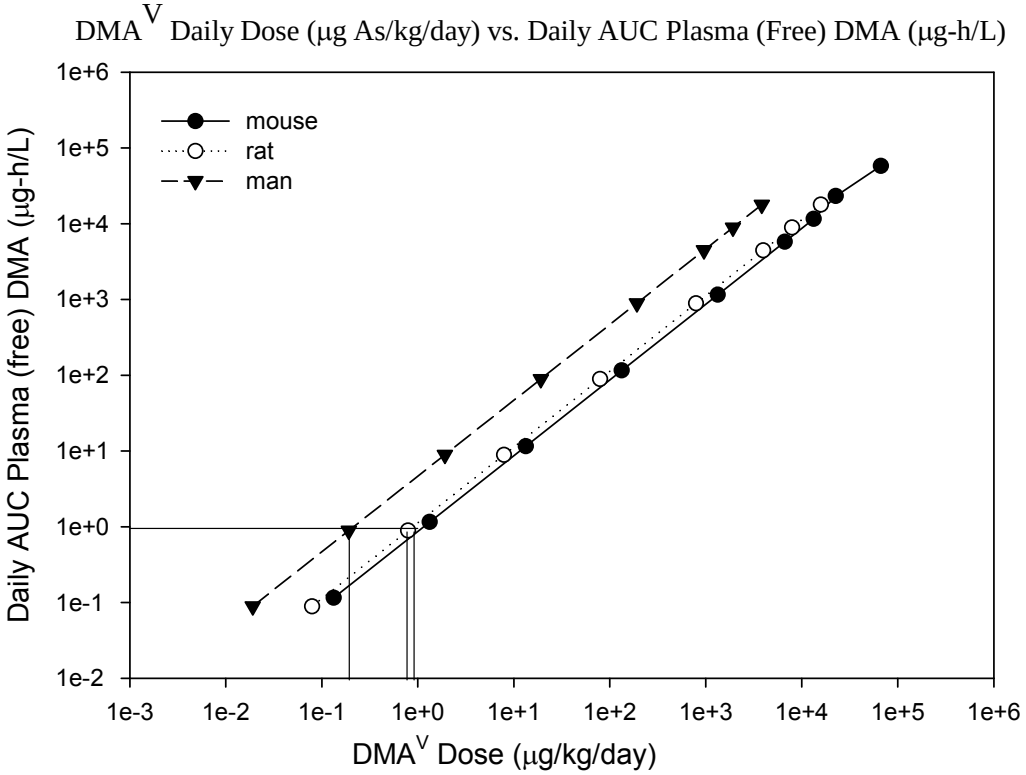


Figure App.C-8B.



ADDENDUM: Differential equations for organs included in the DMA^V PBPK model

The membrane-limited lung is divided into two components, the blood and tissue compartments:
Lung Blood:
dAbl/dt = Qc(CV9-CA9)+Paf(cn9/p9n-Ca9)
Lung Tissue:
dAlung/dt = Paf(Ca9-cn9/p9n)

The membrane-limited liver is also divided into two components, blood and liver tissue compartments:
Liver Blood:
dAbl/dt = Ql(Car – Cv9l)+Pal(C9lt/p9l – Cv9l)
Liver Tissue:
dAliver/dt = Pal(Cv9l – C9lt/p9l) – Cv9l*ktma*Vl

The membrane-limited kidney also has two components, blood and kidney tissue:
Kidney Blood:
dAbl/dt = Qk(Car – Cv9k)+Pak(C9kt/p9k – Cv9k)
Kidney Tissue:
dAkidney/dt = Pak(Cv9k – C9kt/p9k) – re9(C9kt/p9k)

Arterial blood was divided into plasma and RBC compartments:
Arterial Plasma:
dAapl/dt = Qc(Ca9 - Car)+PAB(C9art/prbc - Car)
Arterial RBC:
dAaRBC/dt = PAB(Car – C9art/prbc)

Venous blood was divided into plasma and RBC compartments:
Venous Plasma:
dAvpl/dt = (dmaon)(ivdr)ivdma) – Qc(Cv9) + Qr(Cv9r) + Qi(Cv9i) + Qk(Cv9k) +Qd(Cv9d) +Ql(Cv9l) +PAB(Cv9vbl/prbc – Cv9)
Venous RBC:
dAaRBC/dt = PAB(Cv9 – Cv9vbl/prbc)

Rate in residual compartment is blood flow limited:
dAr/dt = Qr(Car – Cv9r)

Rate in skin is blood flow limited:
dAi/dt = Qi(Car – Cv9i)

Rate in bladder is blood flow limited:
dAd/dt = Qd (Car –Cv9d)

Appendix D Results of Benchmark dose analysis forDMA^V tumor and mode of action data

D.1. Urothelial cytotoxicity

BMR level	10%		AIC
	BMD	BMDL	
3 week SEM (Arnold et al, 2004)			
Gamma	0.83	0.24	12.38
Logistic	0.91	0.35	12.38
Multistage (3^{rd} degree polynomial)	0.68	0.18	10.41
Probit	0.85	0.32	12.38
Quantal linear	0.19	0.10	14.20
Quantal quadratic	0.55	0.35	10.63
Weibull	0.83	0.23	12.38
10 week SEM (Arnold et al, 1999)			
Gamma	0.04	0.03	28.53
Logistic	0.02	0.008	25.83
Multistage (1st degree polynomial)	0.04	0.03	28.53
Probit	0.06	0.04	28.20
Quantal linear	0.04	0.03	28.53
Quantal quadratic	$P < 0.05$		37.32
Weibull	0.04	0.03	28.53

Urolethial Cytotoxicity at 3 Weeks

```
================================================================
==
        Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
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DOCUMENTS\BMDS, HOME\DMA, BMD HOME\CYTOTOX_3WEEK.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\CYTOTOX_3WEEK.plt
                                                Mon Jul 25 15:26:17 2005

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 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
-beta1*dose^1-beta2*dose^2-beta3*dose^3)]

   The parameter betas are restricted to be positive


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

 Total number of observations = 5
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 0
 Degree of polynomial = 3


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008


                Default Initial Parameter Values
```

Background = 1
Beta(1) = 1.20444e+019
Beta(2) = 0
Beta(3) = 0

Asymptotic Correlation Matrix of Parameter Estimates

(*** The model parameter(s) -Background -Beta(1) -Beta(2)
have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	Beta(3)
Beta(3)	1

Parameter Estimates

Variable	Estimate	Std. Err.
Background	0	NA
Beta(1)	0	NA
Beta(2)	0	NA
Beta(3)	0.333304	0.44473

NA - Indicates that this parameter has hit a bound
implied by some inequality constraint and thus
has no standard error.

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-4.18789			
Fitted model	-4.20664	0.0375068	4	0.9998
Reduced model	-24.1314	39.8871	4	<.0001

AIC: 10.4133

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Chi^2 Res.

i: 1
0.0000 0.0000 0.000 0 7 0.000
i: 2
0.2000 0.0027 0.019 0 7 -1.003
i: 3
1.0000 0.2834 1.984 2 7 0.011
i: 4
4.0000 1.0000 7.000 7 7 1.000
i: 5
9.4000 1.0000 7.000 7 7 0.000

Chi-square = 0.02 DF = 4 P-value = 1.0000

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 0.681207

BMDL = 0.179603

Benchmark Dose Computation

Specified effect = 0.01

Risk Type = Extra risk

Confidence level = 0.95

BMD = 0.311253

BMDL = 0.0169775

Urolethial Cytotoxicity at 10 Weeks

```
================================================================
==
        Logistic Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:20 $
        Input Data File: C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\CYTOTOX_10WEEK2HD.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\CYTOTOX_10WEEK2HD.plt
                                                Mon Jul 25 15:54:56 2005

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 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = background+(1-background)/[1+EXP(-intercept-slope*Log(dose))]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Slope parameter is restricted as slope >= 1

   Total number of observations = 3
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



   User has chosen the log transformed model


              Default Initial Parameter Values
                background =           0
                 intercept =     1.65702
                     slope =           1
```

Asymptotic Correlation Matrix of Parameter Estimates

(*** The model parameter(s) -background -slope
have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	intercept
intercept	1

Parameter Estimates

Variable	Estimate	Std. Err.
background	0	NA
intercept	1.78475	0.519069
slope	1	NA

NA - Indicates that this parameter has hit a bound
implied by some inequality constraint and thus
has no standard error.

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-11.7341			
Fitted model	-11.9156	0.362876	2	0.8341
Reduced model	-20.7277	17.9871	2	0.0001242

AIC: 25.8312

Goodness of Fit

Dose	Est._Prob.	Expected	Scaled Observed	Size	Residual
0.0000	0.0000	0.000	0	10	0
0.2000	0.5437	5.437	6	10	0.3573
1.0000	0.8563	8.563	8	10	-0.5074

Chi-square = 0.39 DF = 2 P-value = 0.8249

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 0.0186487

BMDL = 0.00757688

Benchmark Dose Computation

Specified effect = 0.01

Risk Type = Extra risk

Confidence level = 0.95

BMD = 0.00169534

BMDL = 0.000688808

D.2. Regenerative Proliferation

10 week BrdU labeling (Arnold et al,1999)			
BMR level	**10%**		**p-value**
	BMD	**BMDL**	
Hill	0.36	0.080	0.39
Linear	Not estimated (poor fit)		p< 0.05
Polynomial	non-monotonic shape		
Power	Not estimated (poor fit)		p< 0.05

Regenerative Proliferation at 10 Weeks

```
================================================================
==
        Hill Model. $Revision: 2.1 $ $Date: 2000/10/11 21:21:23 $
        Input Data File: C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\BRDU_ARNOLD_10WEEK.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\BRDU_ARNOLD_10WEEK.plt
                                                Mon Jul 25 16:15:43 2005

================================================================
==

 BMDS MODEL RUN
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~~~

   The form of the response function is:

   Y[dose] = intercept + v*dose^n/(k^n + dose^n)


   Dependent variable = MEAN
   Independent variable = COLUMN1
   Power parameter restricted to be greater than 1
   The variance is to be modeled as Var(i) = alpha * mean(i) ^ rho

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                Default Initial Parameter Values
                      alpha =     0.150474
                        rho =      1.43744
                  intercept =         0.22
                          v =         0.73
                          n =      3.71355
                          k =      3.71855
```

Asymptotic Correlation Matrix of Parameter Estimates

	alpha	rho	intercept	v	n	k
alpha	1	0.79	0.074	-0.39	-9.9e-005	-0.00029
rho	0.79	1	0.34	-0.39	-6.2e-005	-0.00049
intercept	0.074	0.34	1	-0.33	4.6e-005	0.00056
v	-0.39	-0.39	-0.33	1	-0.00083	0.0016
n	-9.9e-005	-6.2e-005	4.6e-005	-0.00083	1	-1
k	-0.00029	-0.00049	0.00056	0.0016	-1	1

Parameter Estimates

Variable	Estimate	Std. Err.
alpha	0.140747	0.0618874
rho	1.40814	0.426711
intercept	0.200808	0.0311312
v	0.72786	0.101919
n	13.25	1966.84
k	0.76643	10.3147

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	7	0.22	0.14	0.201	0.121	0.158
0.1	7	0.2	0.09	0.201	0.121	-0.00667
0.7	7	0.33	0.25	0.369	0.186	-0.21
2.6	7	0.95	0.42	0.929	0.356	0.0599
6.5	7	0.93	0.29	0.929	0.356	0.00374

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model A3: Yij = Mu(i) + e(ij)
Var{e(ij)} = alpha*(Mu(i))^rho

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	31.699722	6	-51.399445
A2	40.257731	10	-60.515462
A3	38.643043	7	-63.286087
fitted	38.279552	6	-64.559103
R	12.356825	2	-20.713651

Explanation of Tests

Test 1: Does response and/or variances differ among Dose levels?
(A2 vs. R)
Test 2: Are Variances Homogeneous? (A1 vs A2)
Test 3: Are variances adequately modeled? (A2 vs. A3)
Test 4: Does the Model for the Mean Fit? (A3 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	55.8018	8	<.0001
Test 2	17.116	4	0.001835
Test 3	3.22938	3	0.3576
Test 4	0.726983	1	0.3939

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels
It seems appropriate to model the data

The p-value for Test 2 is less than .05. A non-homogeneous variance model appears to be appropriate

The p-value for Test 3 is greater than .05. The modeled variance appears to be appropriate here

The p-value for Test 4 is greater than .05. The model chosen seems to adequately describe the data

Benchmark Dose Computation
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 0.649313

BMDL = 0.292341

Benchmark Dose Computation
Specified effect = 0.01

Risk Type = Extra risk

Confidence level = 0.95

BMD = 0.541824

BMDL = 0.0677298

D.3. Hyperplasia

BMR level	10%		AIC
	BMD	BMDL	
10 week from feeding study (Arnold et al, 1999)			
Gamma	1.49	0.63	35.09
Logistic	1.57	0.73	34.94
Multistage (2^{nd} degree polynomial)	1.36	0.54	33.46
Probit	1.55	0.75	34.81
Quantal linear	0.48	0.30	37.53
Quantal quadratic	1.36	1.04	33.46
Weibull	1.38	0.57	35.46
104 week from feeding study (Gur et al, 1989a)			
Gamma	1.92	1.49	303.9
Logistic	1.94	1.55	302.9
Multistage (1st degree polynomial)	1.85	1.40	305.0
Probit	1.97	1.61	300.7
Quantal linear	P < 0.05		
Quantal quadratic	P < 0.05		
Weibull	P < 0.05		
104 week from drinking water study (Wei et al, 2002)			
Gamma	2.10	1.02	43.38
Logistic	2.33	1.01	45.38
Multistage (3rd degree polynomial)	1.63	1.04	43.7
Probit	2.05	0.95	45.38
Quantal linear	0.75	0.48	49.56
Quantal quadratic	1.29	1.04	44.88
Weibull	2.37	1.05	45.38

Hyperplasia at 10 Weeks (feed)

```
================================================================
==
        Quantal Quadratic Model $Revision: 2.2 $ $Date: 2000/03/17 22:27:16 $
        Input Data File: C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD
HOME\HYPERPLASIA_ARNOLD_10WEEK.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD
HOME\HYPERPLASIA_ARNOLD_10WEEK.plt
                                                Mon Jul 25 16:41:27 2005

================================================================
==

 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(-slope*dose^2)]


   Dependent variable = COLUMN3
   Independent variable = COLUMN1

   Total number of observations = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



            Default Initial (and Specified) Parameter Values
              Background =     0.136364
                   Slope =    0.0436882
                   Power =            2   Specified


        Asymptotic Correlation Matrix of Parameter Estimates

        ( *** The model parameter(s)  -Power
             have been estimated at a boundary point, or have been specified by the user,
             and do not appear in the correlation matrix )
```

	Background	Slope
Background	1	-0.13
Slope	-0.13	1

Parameter Estimates

Variable	Estimate	Std. Err.
Background	0.0350837	0.0342502
Slope	0.0569959	0.0194463

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-13.2318			
Fitted model	-14.731	2.99842	3	0.3919
Reduced model	-29.6477	32.8318	4	<.0001

AIC: 33.462

Goodness of Fit

Dose	Est._Prob.	Expected	Scaled Observed	Size	Residual
0.0000	0.0351	0.351	1	10	1.116
0.1000	0.0356	0.356	0	10	-0.6079
0.7000	0.0617	0.617	0	10	-0.8106
2.6000	0.3436	3.436	4	10	0.3755
6.5000	0.9132	9.132	9	10	-0.1479

Chi-square = 2.43 DF = 3 P-value = 0.4873

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 1.35962

BMDL = 1.04058

Benchmark Dose Computation

Specified effect = 0.01

Risk Type = Extra risk

Confidence level = 0.95

BMD = 0.419922

BMDL = 0.321386

Hyperplasia at 104 Weeks (feed)

```
================================================================
==
        Probit Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:53 $
        Input Data File: C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\HYPERPLASIA_GUR_104WEEK.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\HYPERPLASIA_GUR_104WEEK.plt
                                                Mon Jul 25 17:02:31 2005

================================================================
==

 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = Background
            + (1-Background) * CumNorm(Intercept+Slope*Log(Dose)),

   where CumNorm(.) is the cumulative normal distribution function


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Slope parameter is restricted as slope >= 1

   Total number of observations = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



   User has chosen the log transformed model


               Default Initial (and Specified) Parameter Values
                 background =           0
                  intercept =     -1.72126
```

slope = 1

Asymptotic Correlation Matrix of Parameter Estimates

	background	intercept	slope
background	1	-0.032	0.016
intercept	-0.032	1	-0.94
slope	0.016	-0.94	1

Parameter Estimates

Variable	Estimate	Std. Err.
background	0.00285466	0.0028502
intercept	-2.23191	0.253509
slope	1.40138	0.153389

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-145.409			
Fitted model	-147.365	3.91132	2	0.1415
Reduced model	-304.307	317.796	4	<.0001

AIC: 300.729

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Scaled Size	Residual
0.0000	0.0029	0.343	0	120	-0.5861
0.1600	0.0029	0.337	1	118	1.144
0.7900	0.0080	0.965	0	120	-0.9862
3.2000	0.2757	32.257	35	117	0.5675
8.0000	0.7531	89.625	88	119	-0.3454

Chi-square = 3.07 DF = 2 P-value = 0.2159

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 1.97024

BMDL = 1.6053

Benchmark Dose Computation

Specified effect = 0.01

Risk Type = Extra risk

Confidence level = 0.95

BMD = 0.93483

BMDL = 0.663668

Hyperplasia at 104 Weeks (drinking water)

```
================================================================
==
        Logistic Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:20 $
        Input Data File: C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\HYPERPLASIA_WEI_104WEEK.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\HYPERPLASIA_WEI_104WEEK.plt
                                                Mon Jul 25 17:53:49 2005

================================================================
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 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = background+(1-background)/[1+EXP(-intercept-slope*Log(dose))]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Slope parameter is restricted as slope >= 1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



   User has chosen the log transformed model


              Default Initial Parameter Values
                background =           0
                 intercept =     -2.9454
                     slope =     1.40268
```

Asymptotic Correlation Matrix of Parameter Estimates

(*** The model parameter(s) -background -slope
have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	intercept
intercept	1

Parameter Estimates

Variable	Estimate	Std. Err.
background	0	NA
intercept	-2.19866	0.257386
slope	1	NA

NA - Indicates that this parameter has hit a bound
implied by some inequality constraint and thus
has no standard error.

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-42.0326			
Fitted model	-46.5393	9.01344	3	0.02911
Reduced model	-63.4412	42.8173	3	<.0001

AIC: 95.0786

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Scaled Size	Residual
0.0000	0.0000	0.000	0	28	0
0.5900	0.0614	2.028	0	33	-1.47
2.7000	0.2305	7.146	12	31	2.07
10.7000	0.5428	16.827	14	31	-1.019

Chi-square = 7.48 DF = 3 P-value = 0.0580

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 1.00144

BMDL = 0.660359

```
================================================================
==
        Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
        Input Data File: C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\HYPERPLASIA_WEI_104WEEK.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\HYPERPLASIA_WEI_104WEEK.plt
                                                Mon Jul 25 17:57:51 2005

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==

 BMDS MODEL RUN
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Observation # < parameter # for Multistage model.
   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
-beta1*dose^1-beta2*dose^2-beta3*dose^3)]

   The parameter betas are restricted to be positive


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

 Total number of observations = 3
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 0
 Degree of polynomial = 3


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008


                Default Initial Parameter Values
                  Background =           0
                    Beta(1) =            0
```

Beta(2) = 0.0686727
Beta(3) = 0

Asymptotic Correlation Matrix of Parameter Estimates

(*** The model parameter(s) -Background -Beta(1) -Beta(2)
have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	Beta(3)
Beta(3)	1

Parameter Estimates

Variable	Estimate	Std. Err.
Background	0	NA
Beta(1)	0	NA
Beta(2)	0	NA
Beta(3)	0.0245206	0.0115514

NA - Indicates that this parameter has hit a bound
implied by some inequality constraint and thus
has no standard error.

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-20.6904			
Fitted model	-20.8578	0.334743	2	0.8459
Reduced model	-35.6235	29.8663	2	<.0001

AIC: 43.7155

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Chi^2 Res.
i: 1					
0.0000	0.0000	0.000	0	28	-1.000

i: 2
0.5900 0.0050 0.166 0 33 -1.005
i: 3
2.7000 0.3828 11.868 12 31 0.018

Chi-square = 0.17 DF = 1 P-value = 0.6810

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 1.62573

BMDL = 1.03709

Benchmark Dose Computation

Specified effect = 0.01

Risk Type = Extra risk

Confidence level = 0.95

BMD = 0.742819

BMDL = 0.13723

D.4. Bladder tumors

BMR level	10%		AIC
	BMD	BMDL	
Feeding study (Gur et al, 1989a)			
Gamma	7.33	5.82	55.33
Logistic	7.73	5.90	55.32
Multistage (1st degree polynomial)	6.73	5.65	56.66
Probit	7.50	5.66	57.32
Quantal linear	6.92	4.29	64.12
Quantal quadratic	6.49	5.10	58.64
Weibull	7.74	5.96	55.32
Drinking water study (Wei et al, 2002)			
Gamma	2.28	1.17	39.40
Logistic	2.45	1.16	39.40
Multistage (3rd degree polynomial)	1.92	1.21	37.61
Probit	2.24	1.14	39.40
Quantal linear	1.21	0.71	41.21
Quantal quadratic	1.65	1.26	38.32
Weibull	2.47	1.19	39.40

Bladder tumors (feed)

```
================================================================
==
        Weibull Model $Revision: 2.2 $ $Date: 2000/03/17 22:27:16 $
        Input Data File: C:\BMDS\DATA\GURTUMOR_TOTAL.(d)
        Gnuplot Plotting File:  C:\BMDS\DATA\GURTUMOR_TOTAL.plt
                                                Tue Jun 21 15:20:17 2005

================================================================
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 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(-slope*dose^power)]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Power parameter is not restricted

   Total number of observations = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



            Default Initial (and Specified) Parameter Values
              Background =   0.00833333
                   Slope =   0.00148258
                   Power =      2.32781


        Asymptotic Correlation Matrix of Parameter Estimates

        ( *** The model parameter(s)  -Background    -Power
             have been estimated at a boundary point, or have been specified by the user,
             and do not appear in the correlation matrix )
```

Slope

Slope 1.$

Parameter Estimates

Variable	Estimate	Std. Err.
Background	0	NA
Slope	1.0505e-017	1.#QNAN
Power	18	NA

NA - Indicates that this parameter has hit a bound implied by some inequality constraint and thus has no standard error.

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-26.6622			
Fitted model	-26.6622	1.45652e-006	4	1
Reduced model	-43.4634	33.6024	4	<.0001

AIC: 55.3244

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Scaled Size	Residual
0.0000	0.0000	0.000	0	59	0
0.1600	0.0000	0.000	0	59	0
0.7900	0.0000	0.000	0	57	-2.486e-009
3.2000	0.0000	0.000	0	56	-0.0008534
8.0000	0.1724	10.000	10	58	7.609e-007

Chi-square = 0.00 DF = 4 P-value = 1.0000

Benchmark Dose Computation

Specified effect = 0.1
Risk Type = Extra risk
Confidence level = 0.95
BMD = 7.7439
BMDL = 5.96421

Specified effect = 0.01
Risk Type = Extra risk
Confidence level = 0.95
BMD = 6.79619
BMDL = 2.21743

Bladder tumors (drinking water)

```
================================================================
==
        Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
        Input Data File: C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\TUMOR_WEI.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\TUMOR_WEI.plt
                                                Mon Jul 25 18:34:40 2005

================================================================
==

 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
-beta1*dose^1)]

   The parameter betas are restricted to be positive


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 2
 Total number of specified parameters = 0
 Degree of polynomial = 1


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values
Background = 0.0391232
Beta(1) = 0.0449182

Asymptotic Correlation Matrix of Parameter Estimates

(*** The model parameter(s) -Background
have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	Beta(1)
Beta(1)	1

Parameter Estimates

Variable	Estimate	Std. Err.
Background	0	NA
Beta(1)	0.0566331	0.0223482

NA - Indicates that this parameter has hit a bound
implied by some inequality constraint and thus
has no standard error.

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-38.3921			
Fitted model	-41.224	5.66385	3	0.1292
Reduced model	-54.6069	32.4297	3	<.0001

AIC: 84.448

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Chi^2 Res.
i: 1					
0.0000	0.0000	0.000	0	28	0.000
i: 2					

0.5900 0.0329 1.084 0 33 -1.034
i: 3
2.7000 0.1418 4.396 8 31 0.955
i: 4
10.7000 0.4545 14.088 12 31 -0.272

Chi-square = 5.13 DF = 3 P-value = 0.1623

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 1.86041

BMDL = 1.31032

```
================================================================
==
        Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
        Input Data File: C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\TUMOR_WEIHD.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\OWNER\MY
DOCUMENTS\BMDS, HOME\DMA, BMD HOME\TUMOR_WEIHD.plt
                                                Mon Jul 25 18:40:10 2005

================================================================
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 BMDS MODEL RUN
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Observation # < parameter # for Multistage model.
   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
-beta1*dose^1-beta2*dose^2-beta3*dose^3)]

   The parameter betas are restricted to be positive


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

 Total number of observations = 3
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 0
 Degree of polynomial = 3


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008


                Default Initial Parameter Values
                  Background =           0
```

Beta(1) = 0
Beta(2) = 0.0418719
Beta(3) = 0

Asymptotic Correlation Matrix of Parameter Estimates

(*** The model parameter(s) -Background -Beta(1) -Beta(2)
have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	Beta(3)
Beta(3)	1

Parameter Estimates

Variable	Estimate	Std. Err.
Background	0	NA
Beta(1)	0	NA
Beta(2)	0	NA
Beta(3)	0.0149713	0.0105152

NA - Indicates that this parameter has hit a bound
implied by some inequality constraint and thus
has no standard error.

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-17.7017			
Fitted model	-17.8038	0.204243	2	0.9029
Reduced model	-27.1804	18.9574	2	<.0001

AIC: 37.6077

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Chi^2 Res.

i: 1

0.0000	0.0000	0.000	0	28	0.000
i: 2					
0.5900	0.0031	0.101	0	33	-1.003
i: 3					
2.7000	0.2552	7.912	8	31	0.015

Chi-square = 0.10 DF = 2 P-value = 0.9498

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 1.91634

BMDL = 1.20834

Benchmark Dose Computation

Specified effect = 0.01

Risk Type = Extra risk

Confidence level = 0.95

BMD = 0.875603

BMDL = 0.137567

Appendix E Cancer Risk Assessment of Organic Arsenical Herbicides: Comments on Mode of Action, Human Relevance and Implications for Quantitative Dose-response Assessment

I. Introduction

This discussion provides advice from the U.S. Environmental Protection Agency's (EPA) Office of Research and Development (ORD) staff on key issues pertaining to mode of action (MOA) and inference about dose-response for human exposure to organic arsenic compounds, such as may result from herbicide usage. We have considered the data available for the MOA of dimethylarsinic acid (DMA^V) (and DMA^{III}) and believe that a reasonable, hypothesized MOA for DMA-induced bladder tumors in rats involves chromosomal alteration, cytotoxicity, and increased rates of cell division. The EPA Office of Pesticide Programs (OPP) draft Science Issue Paper on DMA, in the latest version available, places a reliance on cytotoxicity and regenerative cell proliferation in the production of rat bladder cancers. We have taken a more inclusive approach and are suggesting that genotoxicity warrants emphasis as a key event. In this discussion we attempt to frame a statement for the hypothesized MOA with some specificity, address its relevance to humans, and examine the implications of the MOA information for risk assessment. We also recognize that this is one of the first chemicals to be evaluated under EPA's final Guidelines for Carcinogen Risk Assessment (Cancer Guidelines) and that many of the issues we have raised are not limited to DMA, and will inform a broader, on-going, dialogue within the Agency.

II. Context for Our Review

In order to maintain the logic behind our approach, we will through necessity repeat quite a bit of information contained in the OPP draft Science Issue Paper. Our approach is to build on specific definitions from the revised Cancer Guidelines and develop a systematic analysis of how available data on the toxicity of DMA^V are consistent with the MOA proposed for rodent bladder carcinogenesis.

Two definitions from EPA's final Cancer Guidelines [1] provide a context for discussion of MOA and dose response:

> The Term "*mode of action*" is defined as a sequence of key events and processes starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation. A "*key event*" is an empirically observable precursor step that is itself a necessary element of the mode of action or is a biologically based marker for such an element. Mode of action is contrasted with "*mechanism of action*", which implies a more detailed understanding and description of events often at the molecular level, than is meant by mode of action.

and

> The term "*nonlinear*" is used here in a narrower sense than its usual meaning in the field of mathematical modeling. In these cancer guidelines, the term "*nonlinear*" refers to threshold models (which show no response over a range of low doses that include zero) and some non-threshold models (e.g., a quadratic model, which shows some response at all doses above zero). In these cancer guidelines, a nonlinear model is one whose slope is zero at (and perhaps above) a dose of zero. A *low-dose-linear* model is one whose

slope is greater than zero at a dose of zero. A low-dose-linear model approximates a straight line only at very low doses; at higher doses near the observed data, a low-dose-linear model can display curvature. The term "*low-dose-linear*" is often abbreviated "linear," although a low-dose-linear model is not linear at all doses. Use of nonlinear approaches does not imply a biological threshold dose below which the response is zero. Estimating thresholds can be problematic; for example, a response that is not statistically significant can be consistent with a small risk that falls below an experiment's power of detection.

The revised Cancer Guidelines also provide a graphic schematic (see Figure 1) to guide consideration of MOA information. The MOA framework requires that one evaluate the available data to determine: (1) MOA in animals, (2) relevance to humans, (3) life-stage implications, and (4) extrapolation approach (dose response). Section III of this paper (Mode of Action Evaluation) focuses on both the biological plausibility of an MOA and its relevance to humans. Sections IV and V (Quantitative Dose-Response; Relationships Between Risk Assessment for Inorganic and Organic Arsenicals) focus on the extrapolation step.

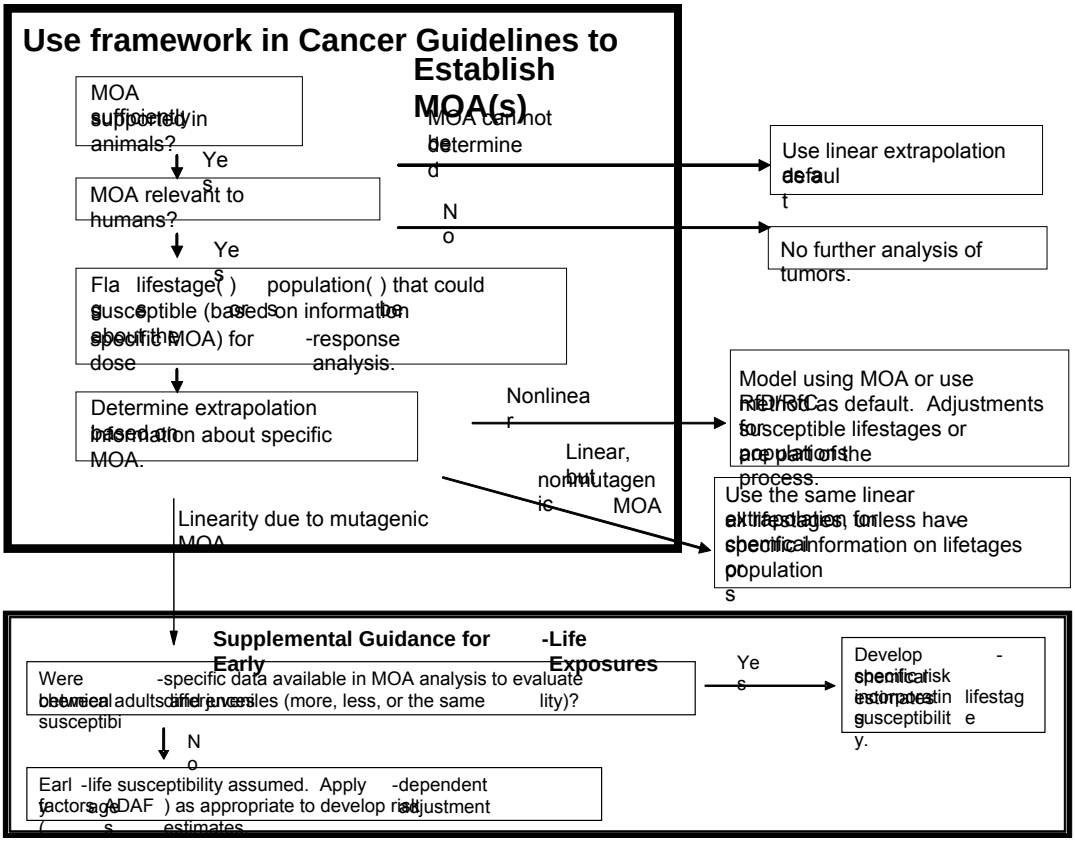

Figure 1. Flow chart for early-life risk assessment using MOA framework

III. Mode of Action Evaluation

a. Biological Plausibility: Proposed MOA for DMA Induction of Rat Bladder Tumors

To describe an MOA for DMA^V (and its metabolite DMA^{III}), we took an approach similar to that presented by Preston and Williams [2] for direct DNA-reactive chemicals, and adapted this to the anticipated production of DNA damage by reactive oxygen species (ROS) for DMA. In this approach, a set of key events is developed for the production of a tumor, and the data available for DMA^V/DMA^{III} cellular effects are matched against this set to determine the plausibility of this MOA for DMA-induced tumors. The postulated key events for tumor development are:

1) Production of reactive oxygen species
2) Reaction with DNA in target cells to produce DNA damage
3) Conversion of DNA damage into chromosomal mutations through process of DNA replication
4) The frequency of conversion of DNA damage to chromosomal mutations likely to be substantially influenced by cell replication rate
5) Cytotoxic and cell proliferation in target tissue increase production of chromosomal damage
6) DNA damage and cell replication are sustained during chronic exposure
7) Subsequent events of mutation induction and clonal expansion lead to tumor formation

It should be noted that in our review of the available data, there is a lack of direct empirical evidence for DMA's MOA in humans and/or in vivo for animals on many aspects of the proposed MOA. As a result, much of our conclusion is based on inference from related data based on principles of extrapolation. We now consider the data that are available to support the key events needed for tumor formation by DMA.

- Based on experimental cellular and laboratory animal data, DMA^{III} is generally regarded to be the most potent of the arsenical species in producing adverse effects (cytotoxicity and genotoxicity). Metabolism of DMA^V to DMA^{III} substantially increases the potency of DMA for both genotoxic and cytotoxic effects [3]. The biologically effective target tissue dose of DMA^{III} will be a balance between the competing processes of metabolism, sequestration, and elimination.
- The interaction with DNA appears to be indirect and could be mediated by the production of ROS. There is experimental evidence in vitro and in vivo to support the production of reactive oxygen species by DMA^V and DMA^{III} [4].
- Elevated levels of the hydroxyl radical (a ROS) has been detected in solutions of DMA^{III} and the hydroxyl radical has been shown to fragment DNA by hydrogen radical abstraction [5]. The mechanism of DNA damage through the production of ROS is plausible, but the nature of the dose response is not established.
- In the urinary bladder of adult rats and humans, normal cell replication rates are relatively low—0.3% over a 24h period after a single pulse dose of BrdU [6,7] or 1–2% after 4 days of BrdU in the drinking water [8]. Treatment of rats with 100 ppm DMA^V resulted in an increased cell proliferation in bladder cells as measured by BrdU incorporation [6,9,10]. The increase in cell replication was of the order of fourfold at 100 ppm, a dose at which rat bladder tumors were observed. While there is a probability that DMA-induced DNA damage could be converted into a genetic alteration from normal cell

replication processes, this probability is expected to be substantially enhanced when the cell proliferation rate is increased.

- Cytotoxicity was observed in rat bladders following exposure to DMA^V at various concentrations [6,9]. An important role for cytotoxicity in the production of cancer is that it can lead to regenerative cell proliferation, as indeed was observed. In addition, cells that contain cytotoxic damage but are not killed can form part of the proliferative cell population. DMA-induced cytotoxicity was observed in rat bladder cells at earlier times than cell proliferation, which is temporally predicted for the order of key events.

- In regard to coincidence of key events, data are available to support their coincidence at similar concentration levels. The levels of DMA^{III} in the urine of rats treated with 100 ppm DMA^V range from 0.5 – 5.0 µM [10]. The LC_{50} values for DMA^{III} in rat and human urinary epithelial cells in vitro are 0.5 – 0.8 µM [10]. There is significant urothelial cell cytotoxicity in female rat bladders at 100 ppm DMA^V. There is a clearly significant increase in chromosome aberrations in human lymphocytes in vitro at about 1 – 3 µM [3]. Thus, it appears that genotoxicity, cytotoxicity, and cell proliferation can occur at a dose of 100 ppm DMA^V.

- Mutagenicity is a key event in the formation of tumors whatever the overall MOA and so there is a necessity to establish how this arises following DMA treatment. The available data support the conclusion that chromosomal mutations are induced by DMA^{III}, but point mutations within single genes are not [11]. Chromosomal alterations have been observed in mitogenically stimulated human lymphocytes treated with DMA^{III} and small colonies (indicative of chromosomal mutations but not point mutations) have been observed in the mouse lymphoma assay [3,11]. Chromosomal alterations are produced by errors of DNA replication on damaged DNA templates, and thus their frequency would be expected to increase with DNA damage and/or increase with cell proliferation. The form of their dose-response curve would be influenced by the dose-response curves for DNA damage and cell proliferation. Together with the fact that the induction of chromosome alterations is generally a "two-hit" process (though both events need not be produced by DMA), the dose-response curve is predicted to be a linear-quadratic type depending upon the extent of background processes of DNA damage and cell proliferation.

- As noted above, the formation of structural chromosomal alterations for the majority of chemicals requires DNA replication – the chromosomal alterations are produced by errors of replication on a damaged DNA template. For the human lymphocyte system used [3], cell replication is an essential component of the assay and quite extensive proliferation is designed into the protocol by the addition of a mitogenic agent. Thus, data from this assay cannot be directly applied to estimate levels of genetic damage that occur in bladder tissue exposed to DMA.

- For DMA, it is suggested that ROS are the proximate cause of DNA damage and that cytoxicity and regenerative proliferation are the cause of enhanced cellular proliferation. Other mechanisms of action may be contributing to enhanced cellular proliferation (e.g., cell signaling pathway disruption), though there are limited supporting data.

b. Relevance of MOA for Humans

The available data in support of an MOA in human bladder cells for DMA treatment is relatively sparse. DMA^{III} is observed in the urine of individuals exposed to inorganic arsenic (iAs) [12], supporting the general view that DMA^{III} is potentially present in the bladder following DMA^{V} treatment. Given the similarity of the physiology and cellular structure of rat and human bladders, it is reasonable to propose that the MOA postulated for the rat (i.e., DNA damage, chromosomal alterations, cytotoxicity, and regenerative cell proliferation) is also plausible in humans. ROS in particular have been shown to play a role in carcinogenesis in humans in other contexts [13,14]. Also, there appear to be no specific factors that would indicate that such an MOA would be ruled out on kinetic or dynamic factors in humans. There are however many uncertainties regarding translating observed rodent and in vitro findings to humans exposed to environmental levels of arsenic. Further discussion of uncertainties in addressing inference for humans follows in the sections addressing dose response, and relationships between the effects of organic and inorganic arsenic. Perspectives on these issues are included as attachments.

As discussed further below, rodents have not been judged to be reliable models for iAs carcinogenesis [15]. The biological reasons for this species difference are not yet resolved. As DMA^{III} may play an important role in iAs carcinogenesis, there are also qualitative and quantitative questions concerning whether or not the DMA-induced carcinogenic response observed in the rat for DMA would accurately represent the human response to this agent.

IV. Quantitative Dose Response

a. Based on Empirical Evidence (Observable Range)

As we noted above, the available body of data present a biologically plausible MOA, which can reasonably be viewed as relevant to humans. As a result, one might suggest that we have a descriptive model that lays out the process by which tumors are formed in both rats and, by extension, potentially humans. However, there are no direct empirical data in humans for the effect of interest (bladder cancer) or to describe the specific MOA. There are also uncertainties or data gaps informing interspecies differences. As a result, formulating and parameterizing a descriptive model based on the rat MOA to allow quantitative dose-response modeling in humans is difficult, and overall we believe the current body of evidence is not sufficiently robust to allow quantitative human dose-response modeling based on MOA data at this time. Additional data to inform the quantitative dose-response assessment could reduce the uncertainties in risk assessment for organic arsenicals, reduce the error bounds on any estimate, and increase the overall confidence in the estimate. Making an inference concerning the dose response for induction of bladder cancer by DMA is complicated by the need to consider the dose response for DNA damage, dose response for effects on cell division rates on normal cells and cells with chromosomal alterations, and the interplay between these factors (and other potential key events in the process). Ideally, these phenomena could be addressed through a biologically based dose-response model, along the lines of a two-stage clonal growth model. We have considered the data set available for MOA of DMA^{V} (and DMA^{III}) in rats and humans and

find that it is not sufficiently robust to allow a quantitative risk assessment to be conducted using a biologically based dose-response approach (see Attachment 1). Clonal growth models can be highly sensitive to assumptions, inputs, and model specification choices.

The above discussion suggests that the relationship between DMA dose and tumor risk is likely to be curvilinear, but that it would be expected to include a low-dose linear component. An empirical model with both linear and higher order dose components (e.g., a linear-quadratic function or a multistage model with a polynomial that has both linear and higher-power terms) provides a reasonable approach for modeling DMA cancer data. In an attempt to explore the relationship of DMA and tumor risk, benchmark dose modeling using a linear-quadratic model was conducted on observed tumor data in the rodent model (see Attachment 2). ORD staff wanted to give further consideration to alternative modeling approaches that make greater use of MOA data in predicting tumor response. As discussed above, further research will be required to support investigation of two-stage clonal growth models that could incorporate both chromosome mutation and cellular proliferation into cancer risk modeling. Further benchmark modeling of available experimental endpoints (e.g., cytotoxicity and cellular replication data) may provide additional useful information for the assessment. However, development of appropriate models involves some relatively complex issues of statistics and interpretation. In the case of the cell replication data, it will be important to interpret models so as not to exclude some degree of cancer risk at baseline cell replication rates. ORD staff are willing to pursue these avenues if this can be accommodated with the Agency's needs for a completed assessment of the organic arsenicals.

b. Extrapolation to Lower Doses / Exposed Human Population – Risk Assessment

As stated above, the dose response for DMA, given the proposed MOA, is likely to be described via a linear-quadratic type of model. However, there are many qualitative and quantitative uncertainties in translating these results to humans in a practical risk assessment, thus multiple approaches to characterizing human risks due to DMA warrant consideration. Several factors limit ability to make more detailed inference about the shape of the expected dose-response curve in humans based on the existing biological data.

- While cytotoxic effects of DMA and observed increases in cell proliferation in rat bladder tissue would thus be predicted to increase production of chromosomal damage, this does not imply that some DMA-induced chromosomal damage and resulting chromosomal mutations would not occur in the absence of induced increases in cell replication.

- In the absence of tissue-relevant dose-response data for DNA damage induced by DMA, it is difficult to predict the extent of the role for normal cell replication as compared with induced cell proliferation in the fixation of DNA damage.

- Some publications predict nonlinear responses for ROS-mediated events [16]. However, a thorough review of relevant data would be needed to support advice on the state of the science. A consideration is whether ROS-mediated DNA damage may be more likely to be nonlinear due to "buffer" capacity and biological protective mechanisms. There is no

convincing experimental evidence either way on this point. Kinetically, at baseline, there is a large flux of ROS formation, a large flux of ROS being deactivated, and a constant background of ROS-induced damage at any given time point. Thus, addressing an additivity hypothesis, the additional ROS from DMA exposure (in rodents or humans) may result in a positive dose-response relationship in spite of a large capacity for deactivation of ROS.

- Justifications for the use of a model that includes a linear term are based on both mechanistic and statistical principles. Mechanistically, a plausible MOA for DMA bladder carcinogenicity observed in the rat includes evidence of genotoxicity (chromosomal aberrations), cytotoxicity, and increased rate of cellular proliferation. Statistically, a model with a linear component is possible, as exposure to DMA may lead to incremental increases in DNA damage and chromosomal aberrations.

- Currently, risk assessment can be performed by using a point of departure based on the rat tumor data. A linear extrapolation to the origin from an appropriately selected point of departure should be presented as an approach consistent with the procedures in the Cancer Guidelines. Given the uncertainties in several key steps leading to tumor formation by exposure to DMA, several dose-response models that permit curvature of the dose-response curve in the observed range may be applicable. EPA's Risk Characterization Policy advises that biologically plausible alternatives be presented in the risk characterization component of the risk assessment to inform readers of the full range of plausible alternatives and to make clear the choices selected for the recommended approach and the impact of these choices on the assessment. Further, the Cancer Guidelines advise that in the absence of sufficient information on the MOA, the agency generally takes default positions that protect the public health regarding the interpretation of toxicological and epidemiological data: animal tumor findings are judged to be relevant to humans, and cancer risks are assumed to conform to low-dose linearity.

V. Relationships Between Risk Assessment for Inorganic and Organic Arsenicals

There are several distinct scientific issues that arise in considering relationships among risk assessment issues for inorganic and organic arsenicals. This section considers different aspects of these relationships.

There is a large body of data that links human exposures to iAs to various tumors in the lung, bladder, and skin, and to other adverse health effects [17,18]. The metabolism of iAs in humans occurs through alternating steps of reduction and methylation including formation of DMA^{V}, DMA^{III}, monomethylarsonic acid (MMA^{V} and MMA^{III}), and trimethanearsinic oxide (TMAO) [19]. Many of the major metabolites MMA, DMA and TMAO have been subjected to a variety of toxicological tests in vivo and in vitro. However, there are no epidemiological data on the chronic human health effects of the specific metabolites.

The trivalent species MMA^{III} and DMA^{III} have recently been identified as the most toxic and genotoxic forms in several assay systems [3]. Therefore, it is reasonable to anticipate that the effects of both organic and inorganic arsenicals are substantially dependent on the production of these metabolites. On the other hand, approaches to determining the relative contributions of

the organic metabolites, together with iAs species themselves, as drivers of iAs toxicity and carcinogenesis are uncertain. While DMA^{III} has been shown to be the most toxic metabolite for some important endpoints, including genotoxicity, each of the arsenical metabolites exhibits its own toxicity, possibly via similar and/or separate MOAs that are responsible for DMA toxicity and tumor formation [20] (see Figure 2). Therefore, exposure to DMA^{V} results in a subset of metabolite exposures, as does exposure to iAs, and thus potentially different biological effects.

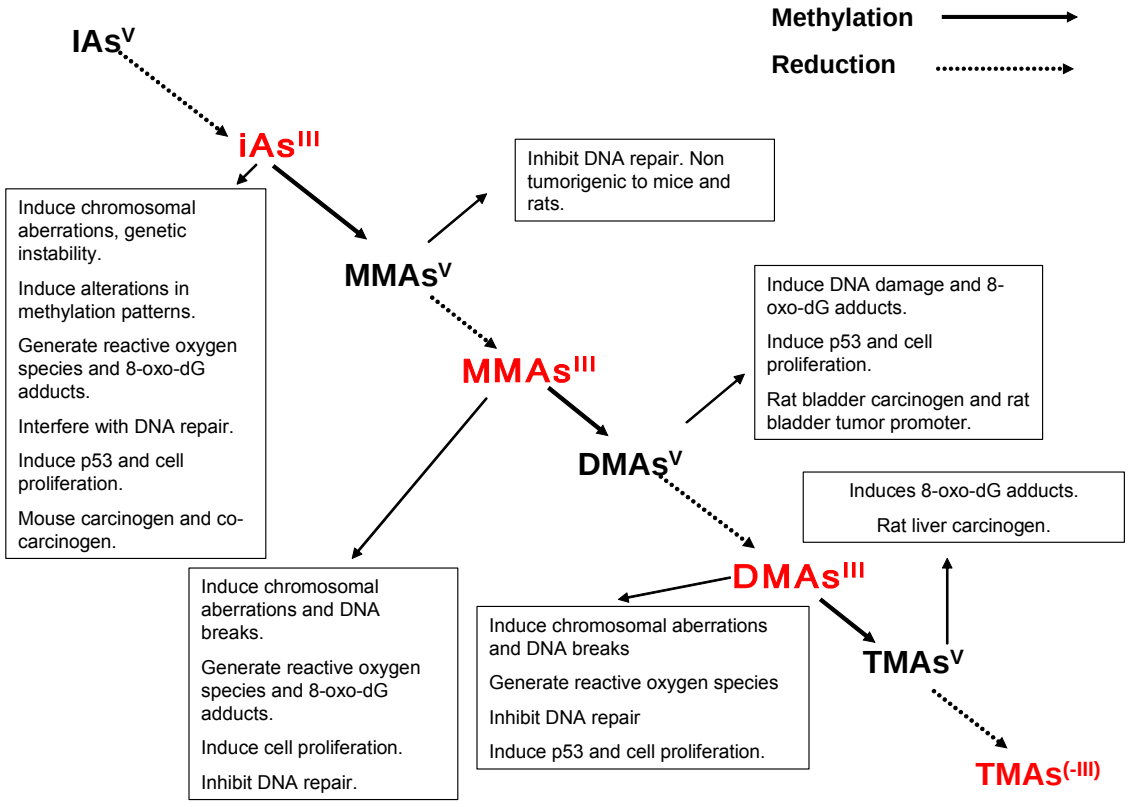

Figure 2 Summary of Arsenical Compounds and Observed Toxicities

a. Addressing Cumulative Exposure

The strong association between iAs exposure and DMA as a metabolite suggests the need to account for the contribution of iAs exposures to the total human tissue exposure to DMA. Thus exposure to organic arsenicals from herbicide use occurs in conjunction with "background" tissue exposures resulting from iAs exposure. A DMA risk assessment should include consideration of this cumulative tissue exposure.

Because substantial evidence suggests that DMA^{III} may be the form of arsenic associated with the greatest relative effect and DMA^{III} is an important urinary metabolite in both humans and rats, a source apportionment based upon DMA^{III} may provide useful information. Basically, this analysis would estimate the relative contribution from ingestion of DMA^{V}, MMA^{V}, and iAs exposures to the level of DMA^{III} produced in humans from a cumulative exposure.

The presence of multiple sources of exposure can affect where exposed individuals would fall on a dose-response curve for health effects. For example, if only one source of exposure existed, individuals so exposed may fall below a health criterion for the agent or may fall on a "shallow" portion of a dose-response curve. When other "background" sources of exposure are included, an individual's exposures may exceed the health criterion or may fall on a steeper range of the dose-response curve.

b. Considering Relevance of DMA Data to iAs Risk Assessment

The DMA data provide evidence or inform the assessment of iAs from the perspective of effects, such as the concordance of observed bladder tumors in those exposed to iAs. However, it would not be appropriate to utilize the existing data on DMA as the basis of a risk assessment for iAs. As stated earlier, iAs exposure in humans results in multiple tumor types while experimental studies in rodents have been negative (with the exception of multiple tumors in transplacentally exposed mice). There is also a large database specific to iAs, in a wide range of test systems, on the biological effects of these substances. Other metabolites "upstream" of DMA exhibit their own toxicity via incompletely established MOAs indicating that DMA data cannot be directly applied to address inorganic risks. Assessment of iAs risks should be grounded in the existing epidemiological database for iAs.

c. Considering Relevance of Data on iAs for Risk Assessment of Organic Arsenicals

ORD staff have discussed at some length the reverse question of how data on the human health effects of iAs can inform the assessment for organic arsenicals. We have considered potential approaches that are alternately more qualitative and more quantitative in nature.

An approach to explore more qualitatively the contribution of DMA^{III} to the production of bladder tumors in humans would be to establish the relationship of bladder tumors in humans exposed to iAs with reported levels of DMA^{III} in human urine and the relationship of bladder tumors in rats with reported levels of DMA^{III} in rat urine. If there is agreement between the dose-response relationship between humans and rats for bladder tumors, this may increase the confidence in the DMA MOA and the ability to predict bladder tumors. If, however, significant differences were to be observed, this might indicate the following possibilities: 1) species differences of the carcinogenicity of DMA^{III}); 2) the contribution of additional metabolites from iAs to the tumor response; or 3) the contribution of other modes of action. Alternative inferences could be derived based upon the comparison of these two relationships. If there are large differences between the relationships, that fact should be informative to the assessment and reviewers.

A more quantitative approach is to treat joint exposure to arsenical metabolites as a "mixtures" assessment and seek to apply measure of relative effect for the different components of the mixture in producing key events. A goal could be to develop a measure of "delivered effective dose" for the joint effect of arsenicals. Currently available measures of relative effect would likely rely on in vitro endpoints with associated uncertainties. Available information on relative "potency" would likely be based upon in vitro studies (addressing endpoints relevant to the MOA such as formation of ROS, genotoxicity, or cytotoxicity), with associated uncertainties

inherent in these data. ORD Staff have not reached a consensus recommending the validity and utility of this type of analysis, but recommend that this question be considered in further review of the assessment. Attachments 3, 4 & 5 to this document discuss perspectives on these issues in greater depth.

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Attachment 1: Inputs and Parameter Considerations: Potential for Two-Stage Model of DMA^V Carcinogenesis

Inputs	Does Parameter Depend on DMA^V?	Can We Estimate Parameters?
n_N (number of normal target cells)	Yes, probably, due to hyperplasia, etc.	Pathology data may support cell counts. It can be estimated over time if α_N, β_N are known or estimated.
α_N, β_N (rates of birth, and differentiation, death for N-cells)	Yes, probably	Yes, can be inferred from labeling data that are used to calculate labeling index (LI). Need original labeling data, not LI here.
α_I, β_I (rate of birth, and differentiation, death for I-cells)	Yes, probably	• Not now. Can be done if cells with chromosome alterations can be marked and studied. This is a research need to develop a lab method for doing this. • One potentially useful approach is to use data on preneoplastic lesions such as hyperplasia, but current data are not sufficient.
μ_N (rate of transition, mutation, from N to I)	Yes, probably	• Might approach estimate using overall rate chromosomal mutation + adjustment for relevant sites. • One possible assumption is $\mu_N = \mu_I$ for background rate, but μ_I may have different dose relationship than μ_N.
μ_I (rate of transition, mutation, from I to M, maligant)	Maybe	

N = normal cells, I = initiated cells.

Remark: Under the framework of the two-stage model, it is important to distinguish between N- and I-cell populations in terms of the birth and death rates. This is because the model is constructed in such a way that risk is much more sensitive to change in the rates for the I-cells than N-cells. In addition to this mathematical relationship, there is also strong biological reason to distinguish between these two cell populations.

Attachment 2: Benchmark Dose Analysis of Rat Bladder Tumors in DMA^V Studies

Dose-response analyses using EPA's Benchmark Dose Software (BMDS) are developed here for two tumor incidence studies cited in the OPP draft Science Issue Paper on DMA, one study conducted by Gur et al. (1989) and another by Wei et al. (1999). The Gur study used both female and male rats while the Wei study used only male rats. The Gur study only showed significant increase of tumor incidences for females at the highest dose of 100 ppm. The Gur study was negative for male rats. The rest of the doses did not produce any papillomas or carcinomas. The Wei study showed incidences of tumors at the 50 and 200 ppm levels for the male rats.

A linear-quadratic analysis using the following formula was used to fit the data from both studies using the BMDS software. The linear quadratic equation used is:

$$P[\text{Response}] = \text{background} + (1 - \text{background})(1 - e^{-\text{beta}_1 \cdot \text{Dose} - \text{beta}_2 \cdot \text{Dose}^2})$$

where beta_1 and beta_2 are the slopes for the linear and quadratic components of the fitted equation, respectively. Using the above formula to fit the tumor incidences for both the Gur and Wei studies, the estimated beta's are as follows

Study	**beta_1(standard error)**	**beta_2 (standard error)**
Gur (1989)	0	9.3×10^{-6} (1.27×10^{-5})
Wei (1999)	0.0027(0.0011)	0

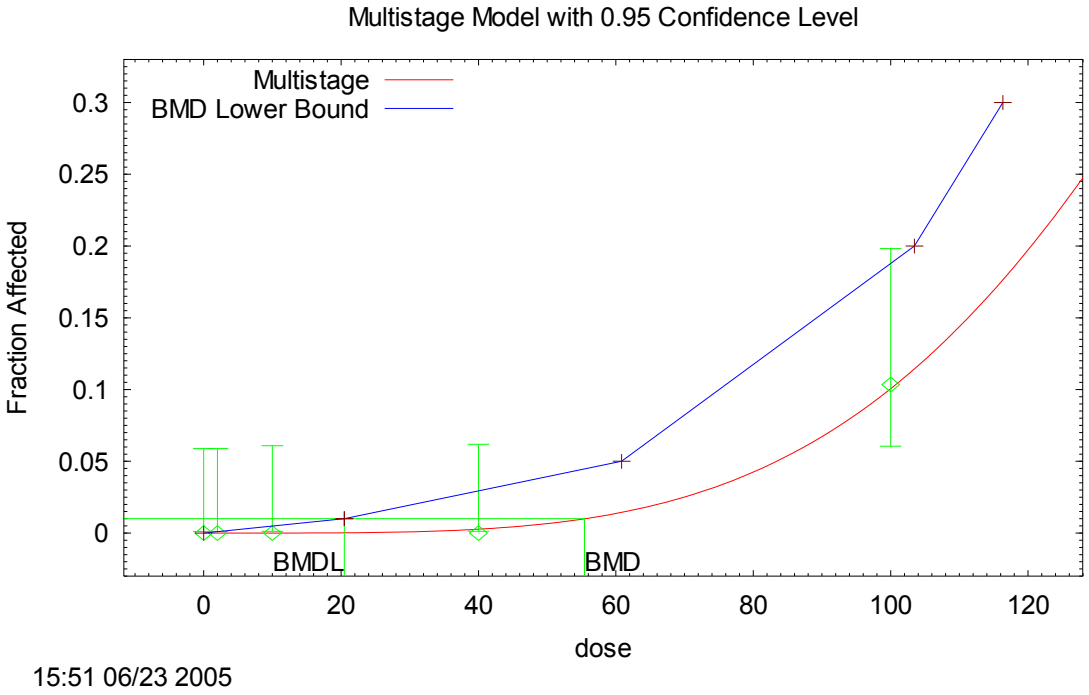
The table below shows the input data and BMD estimates from these analyses, followed by graphs showing the curve fits for the Gur et al. (1989) and Wei et al. (1999) studies.

We note that the apparent dose-response pattern in the two experiments differed (as well as the differing overall response observed for the male rats). Data from the Gur et al. study yielded a best fit with a model having only a dose-squared component. The upper bound on risk from this fit (or equivalents to the lower bounds on dose) allows for a linear component to be present in the response – as indicated by the "BMD Lower-Bound" curve. The Wei et al. data were best fit by a model with only a linear term (no dose-squared term in the maximum likelihood estimate [MLE] fit).

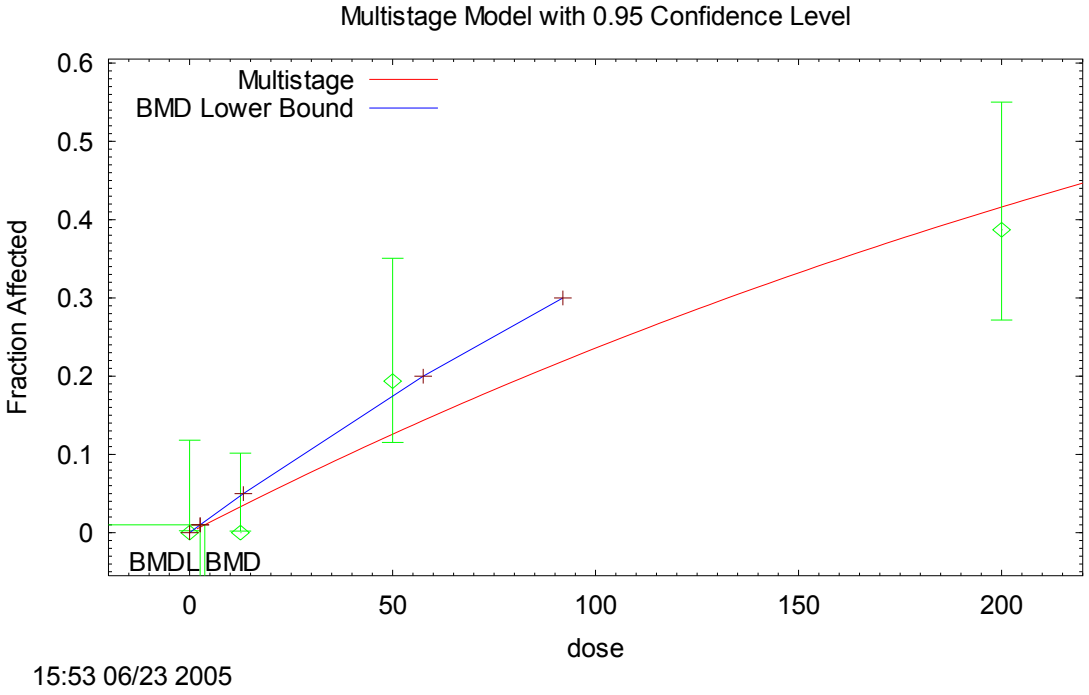
F344 Rat Bladder Tumor Data Sets
Input Data for Benchmark Dose Analyses and resulting BMD Estimates

Study	Duration & Administration	Tumor Incidence						BMD_{10} ($BMDL_{10}$)
Gur et al., 1989	2-year Feeding	Dose (PPM) (mg/kg/day)	0	2 (0.16)	10 (0.79)	40 (3.2)	100 (8)	106 PPM (78 PPM)
		Females						
		Carcinomas	0/59	0/59	0/57	0/56	6/58	
Wei et al., 1999	2-year Drinking water	Dose (PPM) (mg/kg/day)	0		12.5 (0.59)	50 (2.7)	200 (10.7)	39 PPM (27 PPM)
		Males						
		Carcinomas	0/28		0/33	6/31	12/31	

a) Data from Gur et al. (1989) -- Bladder Carcinomas in Female F344 Rats



b) Data from Wei et al. (1999) -- Bladder Carcinomas in Male F344 Rats



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Attachment 3: PBPK Modeling for Arsenicals

Physiologically-Based Pharmacokinetic (PBPK) models are useful to the risk assessment for DMA^V and its comparisons to iAs risks. There are adequate pharmacokinetic information in the literature in addition to data generated by EPA scientists to develop models for DMA^V and iAs. Two published models for iAs for humans are also available in the literature (Mann et al., 1996) (Yu, 1999). Biochemical parameters used in published models can be helpful in developing an updated PBPK model for iAs for humans that considers recent biochemical and exposure data in the literature. For example, this model can be calibrated against available published levels of total arsenic excreted in urine from exposed populations (Valentine et al., 1979). Another useful study can be used to calibrate the model to estimate the levels of DMA^{III} and MMA^{III} for population exposed to iAs (Mandal et al., 2001). Once calibrated against available data, PBPK models can be used to estimate tissue (bladder), or urine, levels of DMA^{III}, and the other metabolites generated from exposures to DMA^V and iAs. Quantitative information on the urinary levels of DMA^{III} under different exposure scenarios (ingested DMA^V or iAs) and for different species can be useful to compare risk values obtained for DMA^V to estimates for exposure to iAs risk in humans.

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Attachment 4: A Perspective That Data on iAs Should Not Be Used to Support the Quantitative Risk Assessment for DMA

Inorganic arsenic is unique as it is one of a small group of demonstrated individual chemicals that is carcinogenic to humans and produces tumors at multiple sites (bladder, lung, skin, liver, and possibly kidney). Rodents are generally nonresponsive to the tumorigenic effects of iAs except for a recent transplacental mouse study where arsenite gave liver, lung, ovarian, and adrenal cortical tumors. After decades of research on arsenic, we have learned a great deal about how arsenic interacts with biological systems and is affected by biological systems, but we still do not know how arsenic induces human cancer. Humans are more responsive to arsenic in terms of breadth of effects than any single rodent species. To date, we do not have enough information to explain these differences.

The biotransformation and pharmacodynamics of iAs are complex in mammalian systems with arsenite being biotransformed through a series of reduction and methylation steps in a cascade to form the final urinary metabolite, trimethylarsine oxide, and possibly its reduced form, trimethylarsine. Arsenical forms of greater instability are produced within each step, and these forms have greater reactivity toward biological and biochemical intermediates, and biological macromolecules. Each intermediate arsenical form has the potential to induce cancer (genotoxicity) or to affect the promotion and progression of cancer such as affecting signal transduction pathways and gene expression. Many of these forms have been detected in the urine of humans exposed to iAs and in rodents exposed to inorganic and organoarsencials. Moreover, the exposure of mammalian cells and organs to mixtures of these intermediates brings to the forefront potential synergistic interactions between these forms that could enhance the tumorigenesis process. To even further complicate these processes, there is a growing body of evidence that implicates arsenic-induced reactive oxygen species (ROS) and the downstream effects of arsenic-induced oxidative damage and oxidative stress in the mechanisms of cellular injury, toxicity, and carcinogenic activity. This implies that some (if not many) of the toxicological effects of arsenic are mediated indirectly through ROS. ROS are known to induce DNA damage, lipid peroxidation, and protein oxidation. ROS themselves are not stable forms. ROS can interconvert between themselves, can react with nitric oxide to become reactive nitrogen species (RNS) which have their own spectra of biological activities, and high-energy ROS can cascade down to lower-energy forms and in that process can radicalize other biological molecules. Moreover, ROS and sequelae radicals are affected by cellular defenses that can ameliorate their activities.

Therefore, the metabolic, pharmacokinetic, pharmacodynamic, and cellular processes that are taking place within these cascades are seemingly complex and it is believed to be difficult, if not impossible, to apportion risk at this time to any one of these arsenical intermediates by any known scientifically defensible method. While there are dose-response data on iAs-induced human tumors, this perspective suggests that these relationships cannot be used in DMA^V risk assessment as a human endpoint for the reasons cited above.

Clearly, iAs represents a mixtures issue, with numerous metabolites, each of which has its own spectrum of toxicity via similar or different modes of action. If one were to use the iAs epidemiological data to support the quantitative risk assessment of DMA, one must account for both the pharmacokinetics and the pharmacodynamics of all metabolites. While DMA has been shown to be most toxic in several in vitro assays, it is believed not currently possible to assign a

proportion of the observed human bladder tumor frequency to DMA^{V} or more particularly to DMA^{III}, especially since metabolites may be acting via not only similar but different modes of action, or to assign relative toxicity or potency to the individual metabolites, or even to know what is the appropriate endpoint for any relative potency estimation. This is not to say that such modeling exercises are not useful from an exploratory hypothesis-generating perspective, only that it is not viewed as scientifically defensible to conduct such quantitative dose-response modeling at this time.

Attachment 5: A Perspective That Human Cancer Findings for iAs Can Inform Quantitative Risk Assessment for Organic Arsenicals

While the database on arsenicals is extremely complicated, there are strong biological reasons to believe that human exposures to organic arsenicals may lead to a related pattern of cancer risks as does human exposure to iAs. Human exposure to iAs, MMA^V, or DMA^V will lead to tissue exposures to DMA^{III} (with iAs and MMA^V also leading to tissue exposure to MMA^{III}). The methylated trivalent species, DMA^{III} and MMA^{III}, have recently been identified as the most toxic and genotoxic forms of arsenic in several assay systems. There is strong reason to anticipate that the human health effects of both inorganic and organic arsenicals are substantially dependent on the production of these metabolites.

The perspective presented here is that analysis is needed to develop further quantitative comparison of potential human risks from inorganic and organic arsenic exposures. An analysis of exposures to different arsenicals as a mixtures problem at the tissue level, together with application of MOA data on the relative biological effects of the different metabolites, can provide a comparison between the human data on iAs and rodent data on MMA^V and DMA^V that is not otherwise attainable at this time.

It is not anticipated that the outcome of these exercises would necessarily be applicable as a basis for quantitative risk assessment of human exposure to organic arsenicals. Rather the goal would be to allow quantitative insight on the similarities and differences in risk assessment approaches now being considered for inorganic versus organic arsenic when evaluated using plausible dosimetric approaches. While a mixtures approach to considering relative effects of different arsenicals involves inherent uncertainties, a risk assessment for organic arsenicals that omits such a comparison has a different, significant set of uncertainties. In particular, as has been stated above, rodent bioassay models for iAs have not been reflective of human risks; thus a question remains whether rodent bioassay data for organic arsenicals can be considered as reliable for assessing human risks to these compounds. In the case of DMA^V, positive bioassay results have been reported for the rat bladder, a site concordant with risks from human exposure to iAs. Relevant uncertainties here include mode of action (i.e., roles of induced genotoxicity and cytotoxicity) and likelihood for quantitative differences. However, the DMA^V bioassays did not produce evidence for multi-site carcinogenesis seen in humans exposed to iAs. A significant question is whether humans exposed to DMA^V would be at risk of cancer at sites beyond the bladder, and in particular for cancer of the lung, which has been a strong finding in humans exposed to iAs. This concern is supported by some animal data in a sensitive mouse strain where DMA^V dosing led to increased multiplicities of lung adenomas. A potentially greater concern regards MMA^V, similarly with iAs; there is an absence of positive bioassay findings. Exposure to MMA^V will result in the formation of metabolites MMA^{III} and DMA^{III}, which, again, can be judged likely to be important contributors to iAs cancer risks.

Human data can also be used to cast light on the reasonableness of risk numbers calculated from animals to humans. If one were to assume that bladder cancer observed in human studies is induced by DMA^{III} only, the human-based potency for DMA^{III}-induced bladder cancer would serve as an upper-bound risk estimate for DMA^{III}, under the assumption that there are no inhibition effects from other metabolites.

Published PBPK models, as well as PBPK models under development in ORD, can be helpful in estimating tissue levels of arsenical species in rodents and humans. There are several models in the literature that can be considered for that purpose. Estimated urinary concentrations of arsenical species may provide the basis for meaningful and feasible dosimeters for the evaluation of bladder tumor risks from exposure to arsenicals.

Trivalent inorganic arsenic (arsenite) itself, while less effective in comparative tests, is also known to lead to the generation of ROS, and to produce chromosome damage and cytotoxicity, and thus may contribute to a mixtures assessment.

A mixtures approach to comparisons of arsenicals considers plausible contributions of each metabolite to risks using relative response from the metabolite in producing key events in the proposed mode of action (DNA damage, chromosomal aberrations, or cytotoxicity). Major differences in the ability of metabolites to produce DNA damage or chromosomal mutagenesis have been demonstrated in the work of Kligerman et al. (2003) and other investigators. The relative biological response for the trivalent methylated arsenicals, MMA^{III} and particularly DMA^{III}, are likely to dominate mixtures comparisons, however trivalent iAs (arsenite) is also active in test systems and may make a quantitative contribution in a mixtures analysis.

For a given a human exposure to arsenic (organic or inorganic), tissue levels of different metabolites would be estimated and then, allowing for differences in relative response amongst the metabolites, a dose-additive formula can be applied to estimate a total Delivered Effective Dose (DED). The DED levels, likely indexed as "DMA^{III} equivalents," could be used to correlate risks from iAs, MMA^{V}, and DMA^{V} exposures. The main assumptions of this analysis would be that the different metabolites of iAs follow a similar MOA for carcinogenesis and that, at least when considered at the relatively low levels relevant to environmental risk assessment, dose-additivity can be assumed. Molecular, chemical, and physical methods were used to show that reactive oxygen species (ROS) are intermediates in the DNA-damaging activities of MMA^{III} and DMA^{III}. The generation of ROS is judged a likely MOA for the chromosomal damage produced by these compounds. Arsenite is also known to lead to the generation of ROS and to produce chromosome damage and cytotoxicity. A reasonable hypothesis may be made that the trivalent arsenicals will follow the same MOA of DMA^{III} since they are capable of generating ROS – which per the above discussion is judged key to the action of that compound. Pentavalent arsenicals, have been shown to have much lower effect in test systems and are not likely to contribute significantly in a DED analysis. The principle of additivity in response is also based on the assumption of low tissue doses (below enzyme saturation capacity), which is also reasonable given that exposures associated in risk estimates are in the low-dose region of the dose-response curve and that tissue levels will always be lower than exposure levels.