Assessment of the Effects of Organic vs. Inorganic Arsenic and Mercury in *Caenorhabditis elegans*

Jessica Camacho, Piper Reid Hunt
FDA Center for Food Safety and Applied Nutrition, Office of Applied Research and Safety Assessment, Laurel, MD

OARSA
Discovering the Future
To Safeguard the Present

Abstract

The FDA Predictive Toxicology Roadmap supports the integration of leading-edge biological models and scientific methods into toxicity testing strategies. The development and qualification of these emerging predictive toxicology tools is a priority for FDA's Center for Food Safety and Applied Nutrition. FDA regulated products from seafood and baby food to skin care and makeup have been found to contain various organic and inorganic forms of mercury and arsenic. The relative toxicities among different forms and metabolites of these toxic elements are not well understood. Many pathways involved in organismal and neuronal development, as well as toxic mode of action, are conserved from worms to humans, indicating that testing in an invertebrate oral toxicity model such as *Caenorhabditis elegans* can provide useful information towards filling data gaps. The effects of inorganic mercury chloride ($HgCl_2$) and sodium (meta)arsenite ($NaAsO_2$) alongside organic methylmercury chloride (meHgCl) and dimethylarsinic acid (DMA) were assessed for *C. elegans* development, stage specific population activity, and adult biomarkers of oxidative stress response (OxStrR). For developmental milestone acquisition in *C. elegans*, meHgCl was 2 to 4-fold more toxic than $HgCl_2$, but DMA was 20-fold less toxic than $NaAsO_2$. In mammals, the nervous system is one of the primary targets for both arsenic and mercury toxicity, and altered motility is a common measure of neurotoxicity. Equitoxic concentrations that induced developmental timing delays of ~15% in *C. elegans* were also associated with significant reductions in spontaneous motor activity with exposure to organic forms of arsenic and mercury but not for inorganic forms, possibly indicating different modes of toxic action. As in mammals, $NaAsO_2$ was a very strong inducer of OxStrR in *C. elegans*, however it was found that 20-fold concentrations of DMA were required to elicit similar levels of response. These findings for mercury and $NaAsO_2$ correlate with findings in mammals, while the DMA data indicates that this organic metabolite may not belong in the same high toxicity category as $NaAsO_2$. This work contributes to the understanding of the accuracy and fit-for-use categories for *C. elegans* toxicity screening and its usefulness to prioritize compounds of concern for further testing.

Introduction

The FDA's Predictive Toxicology Roadmap supports the development and assessment of emerging methods and new technologies to support safety assessments for regulatory purposes. *Caenorhabditis elegans* is a small, non-pathogenic nematode that can be maintained at low cost and handled using standard *in vitro* equipment and techniques, and its 3-day development from egg to egg-laying adult allows for rapid toxicity testing. *C. elegans* assays provide data from a whole animal with intact and metabolically active digestive, reproductive, sensory, and neuromuscular systems. There is significant conservation between *C. elegans* and humans for pathways involved in organismal development and neurotransmission. Conserved alimentary features such as acidic and non-acidic portions of the digestive tract, digestive enzymes, and brush border function make *C. elegans* a potential model for predictive oral toxicity testing. A single *C. elegans* technician can assess a dozen compounds or concentrations in a week for endpoints such as viability, developmental timing, motor activity, or pathway of toxicity specific transgene expression. While this type of medium-throughput *C. elegans* screening cannot replace a descriptive toxicology study in lab mammals, it is very rapid and inexpensive by comparison. Several studies have demonstrated that toxicity ranking screens in *C. elegans* can predict LD50 ranking in rat, indicating that *C. elegans* has the potential to provide a bridge between *in vitro* assays and mammalian *in vivo* oral toxicity testing.

Arsenic and mercury are known to be harmful to babies and children, yet differences in the toxicities of their organic versus inorganic forms are not well documented or understood. The bioavailability and effects of these toxic elements varies based on chemical form to the extent that safety and mode of action assessments for one form do not apply to other forms. As a result, the Center for Food Safety and Applied Nutrition has issued two guidance documents that apply specifically to inorganic arsenic (iAs) in foods. Organic arsenic (oAs) in the form of dimethylarsenic acid (DMA) was found in the pups of rodent dams fed iAs, indicating that this metabolized form of arsenic can cross the placenta. While mammalian toxicity data for oAs species is limited, iAs is generally considered more toxic than oAs. However, a few *in vitro* studies have found that some forms of oAs were more toxic than iAs to human cell cultures, raising concerns about the developmental effects of DMA. We assessed the effects of organic DMA and methymercury chloride (meHgCl) along with inorganic sodium (meta)arsenite ($NaAsO_2$) and mercury chloride ($HgCl_2$) on *C. elegans* development, stage specific population activity, and adult transgene expression for markers of oxidative stress response (OxStrR) and unfolded protein response specific to the proteosome and endoplasmic reticulum (UPR_{PS} and UPR_{ER}).

Results and Discussion

Developmental Toxicity and Stage-Specific Activity Changes

C. elegans development goes through 4 distinct larval stages (L1 – L4) prior to adulthood, with a period of lethargus (inactivity during cuticle molting) between each stage. The worm Development and Activity Test (wDAT), which uses infrared beams to assess population activity levels, can track these developmental milestones. A wDAT right shift in peak timing indicates developmental delay, and a change in stage-specific peak height indicates hyper- or hypo-activity (Fig. 1A). Approximately 5% delay in developmental timing was observed at 10µg/mL (76µM) sodium arsenite ($NaAsO_2$) or 200µg/mL (1.4mM) dimethylarsinic acid (DMA) and ~13% delay at 20µg/mL (154µM) $NaAsO_2$ or 400µg/mL (2.8mM) DMA (Fig. 1B), indicating that about 20 times more organic DMA is required to achieve the same level of developmental delay as with inorganic NaAsO2. DMA concentrations that were associated with > 5% delay in developmental timing also induced significant hypoactivity (Fig. 1D). A delay in developmental timing of approximately 5% was observed at 0.5µg/mL (2µM) methylmercury chloride (meHgCl) or 2.0µg/mL (7.5µM) mercury chloride ($HgCl_2$), and ~15% delay at 2µg/mL (8µM) meHgCl or 4µg/mL (15µM) $HgCl_2$ (Fig. 1C), indicating that for developmental delay, organic meHgCl is 2 to 4 times more toxic than inorganic HgCl2. Stage specific activity levels decreased significantly in a dose response manner with meHgCl but not with HgCl2 (Fig. 1E), indicating additional toxicity beyond developmental delay for the organic forms of both arsenic and mercury.

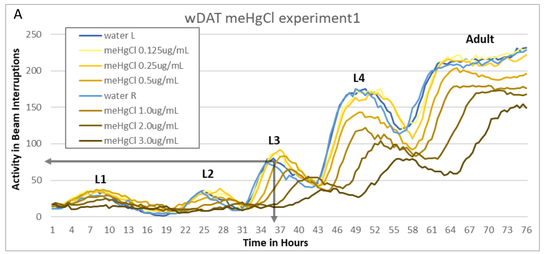

Figure 1. Developmental Toxicity of Inorganic vs. Organic Mercury and Arsenic
A. Mean infrared beam interruption values per well (y-axis) from a single experiment over three replicate wells per condition are graphed over half hour time increments (x-axis) with two separate sets of water controls (L and R) to measure experimental variability. Gray arrows indicate peak timing (delay) and peak height (activity). B-E. Data from four independent experiments, each with 0 L-R indicating the difference between separate but simultaneous control sets of wells. B&C. time to reach the 3rd larval stage (L3) relative to control. D&E. L3 activity peak level relative to control. Not assessed (n/a). Error bars: Standard Deviation (SD). T-test p-values *<0.05, ** <0.005.

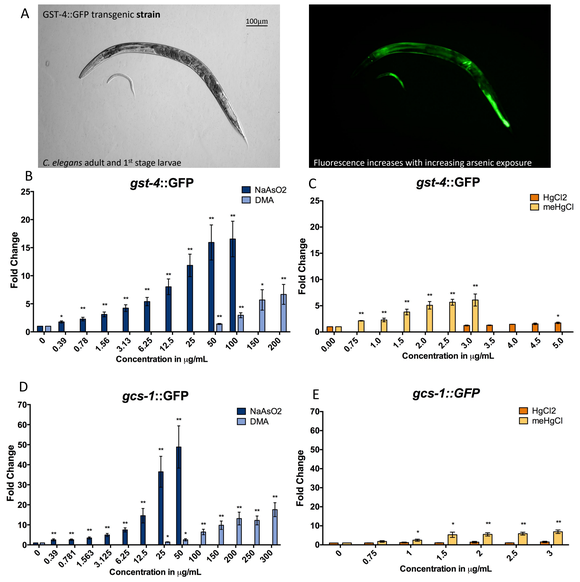

Figure 2. Assessment of oxidative stress response to inorganic and organic Arsenic and Mercury
A. Bright field and fluorescence images of a *C. elegans* strain carrying a promoter for phase II enzyme *GST-4* fused to GFP that can be used to monitor Nrf mediated OxStr. B-E. OxStr response biosensor fold change (detected using a COPAS-flow cytometer) compared to water control, from at least three replicates per exposure group. Top concentrations are maximum concentration not altering adult length and optical density below 80% of control. Error bars: Standard error of the mean (SEM), T-test p-values *<0.05, ** <0.005.

Oxidative Stress and Unfolded Protein Response

C. elegans transgenics can provide *in vivo* bioassays for conserved genes and/or responses to oral exposures, yielding quick assessment of reporters of interest. We utilized adult transgenic *C. elegans* strains to assess expression of biomarkers for oxidative stress (OxStrR) and unfolded protein response (UPR) after exposure to inorganic ($NaAsO_2$, $HgCl_2$) and organic (DMA, meHgCl) forms of arsenic and mercury.

OXIDATIVE STRESS RESPONSE: GST-4 *(*an oxidative stress response gene used to monitor Nrf mediated OxStr (Fig. 2A)), and GCS-1 (the *C. elegans* ortholog of human GCLC that catalyzes the first rate-limiting step of glutathione synthesis) were used to assess oxidative stress response. $NaAsO_2$ strongly induced both transgenes, while 20x more DMA was required to induce similar levels of expression (Fig. 2B&D). $HgCl_2$ only affected expression of the *GST-4* oxidative marker expression at 5µg/ml (18µM), with a 1.7-fold change in expression, while meHgCl induced a 2 to 6-fold increase in expression at 1 to 3µg/mL (4-12µM) (Fig. 2C&E), indicating that methylation reduces oxidative stress for arsenic but increases it for mercury.

UNFOLDED PROTEIN RESPONSE: To assess unfolded protein response, we used *C. elegans* AIP-1 (ortholog to Human AIRAP, proteasome specific unfolded protein response (UPR_{PS})) and HSP-4 (a close homolog of human HSPA5, a regulator of endoplasmic reticulum unfolded protein response (UPR_{ER})). 50 to 250µg/mL (0.4-2mM) of $NaAsO_2$ increased UPR_{PS} expression, while DMA had no effect at any tested concentration (Fig. 3A). $NaAsO_2$ significantly decreased expression of UPR_{ER} marker only at its highest concentration of 150µg/ml (1.15mM), while expression of UPR_{ER} marker was significantly decreased with 25-250µg/ml (180µM-1.8mM) DMA (Fig. 3C). $HgCl_2$ slightly, but significantly, increased UPR_{PS} marker at 3-4µg/ml (11-15µM) while meHgCl increased it at its highest concentration of 2.5µg/ml (10µM) (Fig. 3B). The UPR_{ER} marker induced opposite effects between $HgCl_2$ and meHgCl, with $HgCl_2$ significantly increasing expression at 3-4µg/ml (11-15µM) and meHgCl decreasing expression between 0.5-3µg/ml (2-12µM) (Fig. 3D). These results further highlight the distinct stress responses of different species of the same toxic elements.

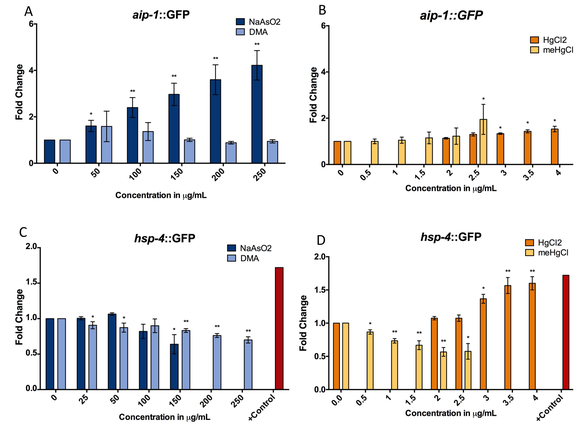

Figure 3. Assessment of Unfolded Protein Response to inorganic and organic Arsenic and Mercury
A-D. UPR response biosensor fold change (detected using a COPAS-flow cytometer) compared to water control, from at least three replicates per exposure group. Top concentrations shown are the maximum concentration not altering adult length and optical density below 80% of control. A&C. UPR_{PS} response. B&D. UPR_{ER} response. Error bars: Standard error of the mean (SEM) T-test p-values *<0.05, ** <0.005.

Conclusions

- In *C. elegans*, developmental toxicity was ranked meHgCl > $HgCl_2$ > $NaAsO_2$ >> DMA.
- iAs is generally considered more toxic than oAs, and we found that relative to NaAsO2, 20-fold higher concentrations of DMA were required in *C. elegans* to induce similar levels of developmental delay, indicating that:
  - *C. elegans* data reflects human epidemiological findings, and
  - limited *in vitro* data for high toxicity with oAs may not be consistent with *in vivo* oral toxicity.
- The primary sequelae of methylmercury exposure in humans is neurotoxicity, for which altered motor activity is an established endpoint. In rodents and *C. elegans*, developmental exposure to meHgCl induced hypoactivity, indicating conservation of toxic effect.
- In developing *C. elegans*, concentrations equitoxic for delay of milestone acquisition timing were associated with significant hypoactivity for organic but not inorganic forms of arsenic and mercury, indicating additional modes of toxic action for the organic forms.
- Relative to NaAsO2, ~20x DMA is needed to induce similar increases in biomarkers of oxidative stress response transgene expression.
- In contrast to arsenic, only the methylated form of mercury induced oxidative stress.
- For both developmental toxicity and oxidative stress response in *C. elegans*, monomethylation increases mercury toxicity while dimethylation decreases arsenic toxicity.
- ER specific UPR transgene expression was reduced for both species of arsenic, while iHg and oHg influenced transgene expression in opposite directions.
- Proteosome specific UPR transgene expression was induced by iAs and iHg, but not by oAs and only slightly for oHg.
- Our findings in *C. elegans* reflect findings in mammals for which there is data, stress the disparity of effects from exposure to different chemical forms of toxic elements, and indicate low oral toxicity for DMA relative to inorganic arsenic in this model organism.

Mission Relevance

As mandated by the 2016 update to the Toxic Substances Control Act, the FDA is actively promoting the development and assessment of alternative, non-vertebrate alternative models for predictive toxicology. New, validated toxicological tools that predict human response at reduced time and expense will allow rapid evaluation of many more compounds and mixtures of concern and thus support safety assessment and regulatory decision making. *C. elegans* is a model organism that shows promise for providing rapid and inexpensive predictive toxicity information. In contrast to the high toxicity of inorganic arsenic and both organic and inorganic forms of mercury, we found that oral exposure to an organic form of arsenic is toxic to developing and adult *C. elegans* only at very high concentrations, which is consistent with epidemiological observations.