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Toxicity of three types of arsenolipids: species-specific effects in Caenorhabditis elegans

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Although fish and seafood are well known for their nutritional benefits, they contain contaminants that might affect human health. Organic lipid-soluble arsenic species, so called arsenolipids, belong to the emerging contaminants in these food items; their toxicity has yet to be systematically studied. Here, we apply the in vivo model Caenorhabditis elegans to assess the effects of two arsenic-containing hydrocarbons (AsHC), a saturated arsenic-containing fatty acid (AsFA), and an arsenic-containing triacylglyceride (AsTAG) in a whole organism. Although all arsenolipids were highly bioavailable in Caenorhabditis elegans, only the AsHCs were substantially metabolized to thioxylated or shortened metabolic products and induced significant toxicity, affecting both survival and development. Furthermore, the AsHCs were several fold more potent as compared to the toxic reference arsenite. This study clearly indicates the need for a full hazard identification of subclasses of arsenolipids to assess whether they poses a risk to human health.

Introduction

Arsenolipids represent a large group of various organic lipophilic arsenic species occurring in marine fish, seafood, algae1–6, and freshwater fish7. Based to their chemical structure, arsenolipids are grouped into several subclasses. Among others, arsenic-containing hydrocarbons (AsHCs), arsenic-containing fatty acids (AsFAs), and arsenic-containing triacylglycerides (AsTAGs)5,8–11 represent three food relevant arsenolipid classes, with algae, fish, and shellfish being the food items contributing the majority of human arsenolipid exposure12.

Whereas the toxicity of the human carcinogen inorganic arsenic (iAs) has been extensively characterized, available studies for arsenolipids are rare. After ingestion of cod liver oil, arsenolipids are metabolized in humans, with dimethylarsinic acid (DMAV) being a major urinary metabolite13. In human liver, bladder and neurons, AsHCs exert toxic effects in a similar concentration range as arsenite (iAsIII), however, their toxic modes of action seem to differ14–19. Moreover, AsHCs are likely to cross physiological barriers among others the intestinal as well as brain barriers15,16,20,21. In contrast, AsFAs are less toxic as compared to iAsIII and AsHCs in human liver cells22 and are unlikely to get transferred as intact AsFAs across the intestinal barrier21. There have been no toxicological studies on AsTAGs.

In this study the in vivo model organism Caenorhabditis elegans (C. elegans) combined with advanced analytical techniques were applied to compare the toxicity, bioavailability and metabolism of two AsHCs, an AsFA and an AsTAG as well as iAsIII (Figure 1) in a whole organism. C. elegans is an

optimal model as it is less complex compared to mammalian systems and it is easy to handle. 60 – 80% of human genes have homologous genes in C. elegans and metal homeostasis and transport is conserved 23. Because of their small size, short life-cycle (about 3 days) and high ability to reproduce, these worms provide a robust multicellular and metabolically active experimental model24,25.

Keywords

Caenorhabditis elegans, arsenolipids, arsenic-containing hydrocarbons, arsenic-containing fatty acids, arsenic-containing triacylglyceride, toxicity, metabolism

Abbreviations

AsFA	arsenic-containing fatty acid
AsHC	arsenic-containing hydrocarbon
AsTAG	arsenic-containing triacylglyceride
DMAV	dimethylarsinic acid
DMAB	dimethylarsenobutanoic acid
DMAPr	dimethylarsenopropanoic acid
iAsIII	arsenite

Highlights

- AsHCs and AsTAG are highly bioavailable to C. elegans
- AsHCs are metabolized by C. elegans
- AsHCs but not AsTAG and AsFA affect survival and development in C. elegans

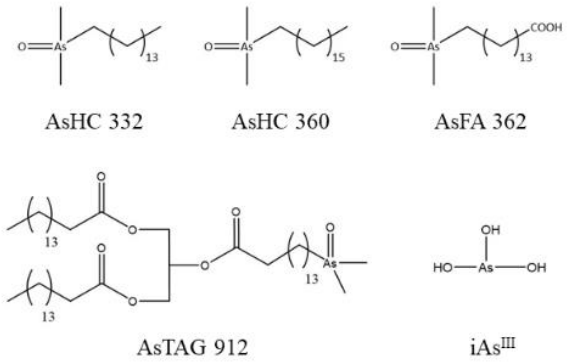

Figure 1: Chemical structure of 1-(dimethylarsinyl)pentadecane (AsHC 332); 1-(dimethylarsinyl)heptadecane (AsHC 360), 15-(dimethylarsinyl)pentadecanoic acid (AsFA 362), 2-((15 (dimethylarsinoyl)-pentadecanoyl)oxy)propane-1,3-diyl dipalmitate (AsTAG 912), and arsenite (iAsIII).

Experimental

C. elegans and handling

The N2 Bristol strain (wild type) was obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota). The worms were propagated at 20 °C either on Nematode Growth Medium (NGM) or 8 P plates seeded with Escherichia coli strains OP50 or NA22, as described previously26, by isolating embryos from gravid worms using a bleaching solution (1% NaOCl and 0.25 M NaOH), and allowing these embryos to hatch in M9 buffer for 18 h. Synchronous L1 populations were obtained and used for the experiments.

Preparation of standard solutions

Synthesis and purification of the two arsenic-containing hydrocarbons (AsHC 332, 1-(dimethylarsinyl)pentadecane; AsHC 360, 1-(dimethylarsinyl)heptadecane), the arsenic-containing fatty acid (AsFA 362, 15-(dimethylarsinyl)pentadecanoic acid) and the arsenic-containing triacylglyceride (AsTAG 912, 2-((15-(dimethylarsinoyl)pentadecanoyl)oxy)propane-1,3-diyl dipalmitate) was carried out as described before8,27. The purity of the compounds (>98%) was established by NMR spectroscopy and HPLC/mass spectrometry stock solutions of the respective AsHCs, AsFA, and AsTAG were prepared in 100% EtOH (Carl Roth GmbH + Co KG, Karlsruhe, Germany) with a final concentration of 10 mM and stored at 4 °C28; additional dilutions were prepared shortly before each experiment with EtOH. For all experiments with lipid-soluble arsenicals, the final EtOH concentration was set to a constant level of 1%, which did not induce any toxic effects (data not shown). Stock solution of the water-soluble compound iAsIII (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, 99% purity) was freshly prepared in sterile, deionized water shortly before each experiment.

Treatment and toxicity testing

For treatments, 30,000 L1 stage worms were exposed to the arsenicals for 4 h in 85 mM NaCl. Afterwards, worms were pelletized by centrifugation at 3,400 x g for 2 min and washed two times with 85 mM NaCl. For toxicity testing, the worm pellet was re-suspended in M9 buffer and 20 – 30 worms were transferred to OP50-seeded NGM plates in triplicates. 48 h post-treatment, the total number of surviving worms was manually counted and scored as percentage of the original plated worm count. For developmental toxicity testing, their developmental stage was recorded 48 h after treatment, and worms reaching the L4 stage were counted to calculate the L4 score.

Bioavailability

To evaluate the bioavailability of the arsenicals, an aliquot of the treated worms for toxicity testing was taken and pelletized by centrifugation. For analysis, the worm pellets were subjected to a freeze/thaw cycle three times in liquid nitrogen, re-suspended in deionized water and homogenised by sonication (three times 20 s, 100%, with 20 s on ice between each round). For standardization, the protein content was determined according to Bradford29,30. Afterwards, Rh was added as internal standard and the worm samples were processed by microwave-assisted acid digestion (Mars 6, CEM GmbH, Kamp-Lintfort, Germany). Arsenic content was determined by inductively coupled plasma tandem mass spectrometry (ICP-MS/MS; Agilent 8800 ICP-QQQ, Agilent Technologies Deutschland GmbH, Waldbronn, Germany) as described elsewhere14. To calculate the bioavailability, the arsenic concentration per pellet was normalized to the protein content.

Metabolism studies

For metabolism studies in L1 stage worms, incubation was carried out as described above. Exposed worms were pelletized, frozen in liquid nitrogen and stored at -80 °C until analysis.

For analysis the worm pellets underwent 5 cycles of freezing in liquid nitrogen followed by 10 minutes sonication at room temperature. Then 500 µL pure EtOH were added to the pellets and they were extracted by overhead shaking for 2 hours. After centrifugation at 23,000 x g for 15 min, the supernatant was directly used for HPLC-ICP-MS/MS-ESIMS analysis. Instrument settings and conditions are listed in supplementary Table S1 and Table S2. Extraction efficiency was checked by repeating the extraction with EtOH. Total As was determined after microwave assisted acid digestion in the second extract and the remaining pellet. The concentrations determined in these fractions were less than 1% of total As extracted in the first step.

Statistics

All worm experiments were carried out independently at least three times on different days. Mean and standard deviation (SD) were calculated from raw data and statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Dunnett's test. Statistically significant levels are indicated in the figure legends as *p < 0.05, **p < 0.01, ***p < 0.001.

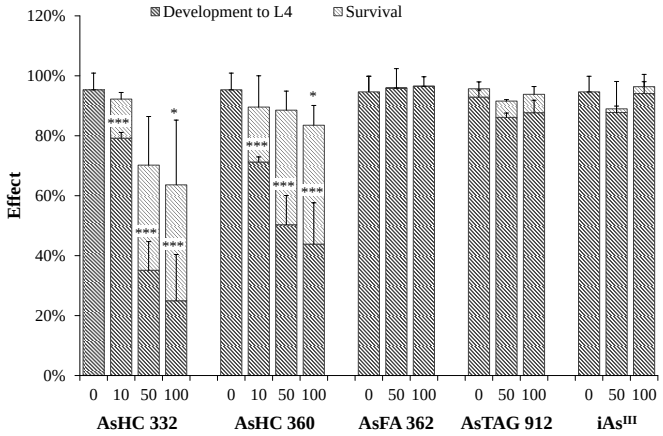

Figure 2: Survival and developmental effects on C. elegans after exposure (in µM) to arsenic-containing hydrocarbons AsHC 332 and AsHC 360, arsenic-containing fatty acid AsFA 362, arsenic-containing triacylglyceride AsTAG 912, and arsenite (iAsIII) for 4 h in the L1 larvae stage. After 48 h, the number of worms alive and their larvae stage were recorded. All values were compared to the number of seeded worms. Shown are mean values of at least three independent determinations +SD. One-way ANOVA followed by Dunnett's test *p < 0.05, ***p < 0.001.

Results and discussion

Toxicity and bioavailability of arsenicals

At the investigated concentration range, up to 100 µM, AsFA 362, AsTAG 912, and iAsIII affected neither survival nor development in C. elegans. In contrast, both AsHCs significantly affected survival and especially development, with the smaller compound (AsHC 332) showing greater potency than the larger one (AsHC 360) (Figure 2).

Upon incubation, uptake of compounds in C. elegans can take place via their mouth, pharynx and consequently the intestine but also via the cuticle31,32. Because of the long hydrocarbon chain of the AsHCs, they might easily interact with lipophilic structures in the nematode and be taken up by crossing the cuticle and the intestine.

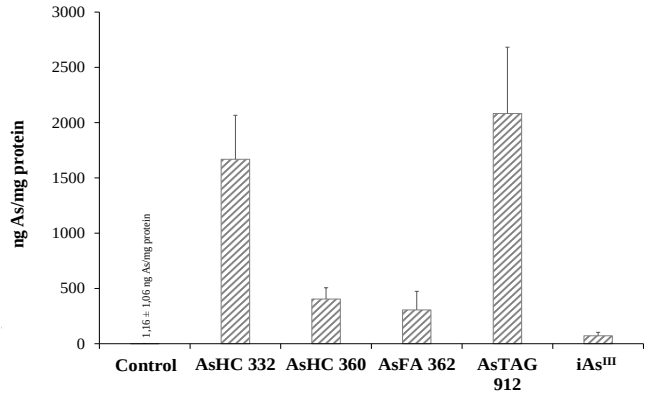

Figure 3: Bioavailability of arsenic-containing hydrocarbons AsHC 332 and AsHC 360, arsenic-containing fatty acid AsFA 362, arsenic-containing triacylglyceride AsTAG 912, and arsenite (iAsIII) in C. elegans after exposure to 100 µM for 4 h in the L1 larvae stage normalized to the respective protein content. Shown are mean values of at least three independent determinations +SD.

Bioavailability studies showed that AsTAG 912 and AsHC 332 increased the arsenic content in worms up to 2082 ± 601 ng As/mg protein and 1668 ± 397 ng As/mg protein, respectively (Figure 3). Treatments with AsHC 360 and AsFA 362 also increased the arsenic content in C. elegans, but not to the same extend. In general, the bioavailability of arsenic was much higher upon uptake of the arsenolipids compared to iAsIII. This was also observed in another in vivo model organism (Drosophila melanogaster). Here, after feeding with AsHCs total As concentrations were much higher as well33.

Metabolism of arsenicals in C. elegans

To further probe the metabolism of arsenolipids, arsenic speciation analysis was performed. The results establish that C. elegans was able to metabolize arsenolipids. In addition to the original incubated compound, several other peaks appeared in the ICP-MS/MS chromatogram (Figure 4). The identification of the main peaks was possible with high resolution molecular mass spectrometry, which provided the exact mass (Figure S1, table S4.). The obtained chromatograms after worm treatments with the two AsHCs showed the same main peak pattern. In addition to the incubated (original) arsenicals, their thioxo-analogues was found, as already described in in vitro studies34. Most of the peaks in the HPLC chromatogram, representing less polar compounds (Fig 4; RTs <1200 s) were identified as AsFAs. The major AsFA product was the fatty acid corresponding to the incubated hydrocarbon in which the end of the hydrocarbon chain is oxidized to a carboxylic acid group. The other identified AsFAs were consistently two carbon units shorter. In the case of AsHC 332 incubation, the products were AsFA 362, followed by AsFA 334, AsFA 306, and so on. This is consistent with transformation by β-oxidation, which is the common fatty acid metabolism pathway. This catabolic process breaks down fatty acids, such as those stored in triglycerides, to generate acetyl-CoA. The citric acid cycle starts with this molecule and it is important in energy production from lipids35.

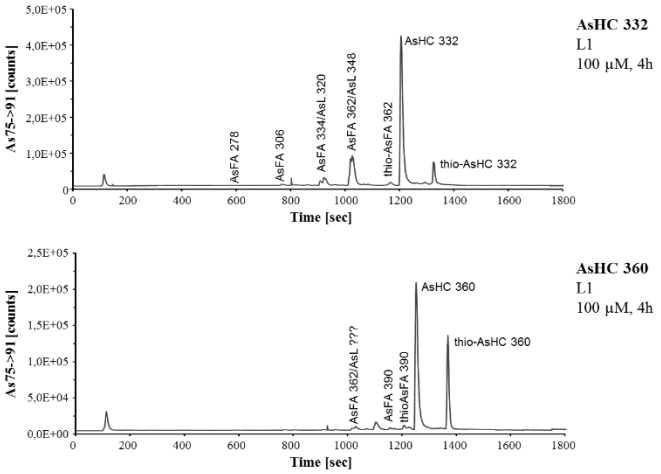

Figure 4: Representative LC-ICP-MS/MS chromatograms of arsenic speciation analysis of L1 stage C. elegans after 4 h exposure to 100 µM AsHC 332 or 100 µM AsHC 360.

After treatment with AsFA 362, also the thiolated species could be detected, but no significant concentrations of shorter As-containing fatty acids were observed. Interestingly, after incubation with AsTAG 912 no significant thioxylated, hydrolyzed or shortened metabolic products could be observed, but only the applied compounds as such (Figure 5). The unknown compound at RT 15.5 minutes accounting for less than 10% of the total As could also be observed in the applied compound. Trace amounts of oxo- and thio-AsFA 362 could be detected in samples incubated with AsTAG 912. Quantitative results and mass balance are shown in table S4.

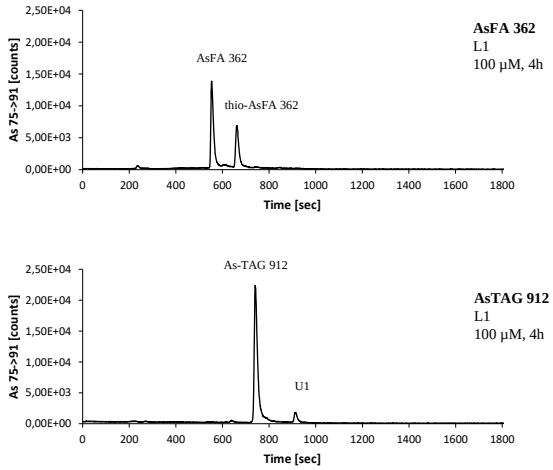

Figure 5: Representative LC- ICP-MS/MS chromatograms of arsenic speciation analysis of L1 stage C. elegans after 4 h exposure to 100 µM AsFA 362 and AsTAG 912.

Conclusions

Our novel study, is the first to provide toxicity data on species-specific effects of arsenolipids in a whole multicellular organism. Our novel findings demonstrate the need for the parallel use of speciation analysis and toxicity testing as to best characterize the toxicity of various arsenolipids. Despite their moderate (AsTAG) or high (AsFA) bioavailability, the former was poorly metabolized and the latter was only partly thiolated. In contrast, AsHCs were extensively metabolized. We conclude that AsTAG and AsFA themselves are non-toxic with respect to survival and development in the worms at the tested concentration range. Whether the substantial toxic effects induced by the AsHCs are caused by the parent compounds or their respective metabolites has yet to be determined. This study points towards a potential risk to humans related to the presence of

arsenolipids in fish and seafood, and highlights the need for further toxicological studies of these compounds.

Conflicts of interest

"There are no conflicts to declare".

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