Journal of Toxicology

Toxicity of Organometal(loids)

Guest Editors: Elke Dopp, Shelley Bhattacharya,
Alfred V. Hirner, Michael Ashner, and Tanja Schwerdtle

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Editorial

Toxicity of Organometal(loids)

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Biomethylation of metals and metalloids is a process ubiquitously occurring in the environment (under aerobic and anaerobic conditions), which leads to the formation of chemical species with significantly higher mobility and altered toxicity. The alkylation of inorganic metal(loid)s through transfer, for example, of methyl groups, is a significant factor in the biogeochemical cycling of the metal(loid) elements. Biomethylation has been described in natural systems for arsenic, cadmium, germanium, mercury, sulfur, antimony, selenium, tin, tellurium, and lead as well as for bismuth, gold, chromium, palladium, platinum, and thallium under laboratory conditions.

In this special issue biomethylation products of arsenic, bismuth, mercury, lead, and tin are of special interest. Of all metal(loid) species in environmental systems, the element arsenic received the greatest attention worldwide. In this issue, recent research on influences of arsenic methylation on toxicity of arsenic species (M. Hall and M. Gamble), modes of action of arsenic metabolites in human cells (Bartel et al.), and the toxicity of volatile arsenic species compared to volatile species of bismuth, mercury, and tin (E. Dopp et al.) will be presented.

Anthropogenic water pollution by butyltin biocides is a well-documented and a severe environmental problem. Its distribution and accumulation in aquatic organisms and also within the food chain leads to biological effects in different organisms. The immunotoxic effects in mammalian cells is highlighted in this special issue by H. Krug.

Beside carcinogenic and immunotoxic effects, organometal(loid)s can exert neurotoxicity. The best known neurotoxic metal(loid) is methylmercury (MeHg). MeHg affects both, the developing and the mature central nervous systems. Several epidemics resulting from the consumption of food contaminated by MeHg have shown the disastrous effects on living organisms. Mechanisms associated with MeHg exposure and neurotoxic effects are described by P. Kaur et al. in this issue.

It has to be considered that humans not only are exposed to metal(loid) compounds from the environment via inhalation and ingestion, but may also be able to generate these species by endogenous enzymes or/and biomethylation in the colon. Methanoarchaea have an outstanding capability to methylate numerous metal(loid)s therefore producing toxic and highly mobile derivatives which might influence human health. Interesting studies in this field were carried out by the group of R. Hensel and new results are presented in this issue by B. Bialek et al. and B. Huber et al.

Metal(loid)-induced health effects, including carcinogenesis and neurodegeneration, have been reported in numerous publications. However, organisms and cells have developed protective mechanisms to deal with metal(loid) exposure. An overview about mechanisms involved in cellular detoxification of different metals is given in the review of E. Martinez-Finley and M. Aschner. Protein binding of metal(loid)s is also a possibility to detoxify serveral species, for example, lead. This process is highlighted in the review of H. Gonick.

Altogether, this special issue addresses contemporary concentrations of organometal(loids) increasing in dangerous proportions in our environment. Unknowingly, the human population is exposed to such insults, which on the long run may be a point of no return. It is true that there are mechanisms of detoxification which allow the biological systems to survive healthily. In spite of such innate

mechanisms of combating stress, there is an urgent global need to realize the portent of environmental disaster staring at our face. Sooner we heed to the indications spelt out in this special issue better it is for the future of mankind.

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Review Article

Nutritional Manipulation of One-Carbon Metabolism: Effects on Arsenic Methylation and Toxicity

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Exposure to arsenic (As) through drinking water is a substantial problem worldwide. The methylation of As, a reactive metalloid, generates monomethyl- (MMA) and dimethyl-arsenical (DMA) species. The biochemical pathway that catalyzes these reactions, one-carbon metabolism, is regulated by folate and other micronutrients. Arsenic methylation exerts a critical influence on both its urinary elimination and chemical reactivity. Mice having the As methyltransferase null genotype show reduced urinary As excretion, increased As retention, and severe systemic toxicity. The most toxic As metabolite *in vitro* is MMA^{III}, an intermediate in the generation of DMA^{V}, a much less toxic metabolite. These findings have raised the question of whether As methylation is a detoxification or bioactivation pathway. Results of population-based studies suggest that complete methylation of inorganic As to DMA is associated with reduced risk for As-induced health outcomes, and that nutrients involved in one-carbon metabolism, such as folate, can facilitate As methylation and elimination.

1. Introduction

Arsenic (As) is a naturally occurring element commonly present in environmental sources such as air, water, and soil [1]. Through processes that are incompletely understood, As in soil can be mobilized leading to enrichment of As in groundwater. While drinking water is the most common source of exposure, other sources include As from mining and smelting, wood preservatives, pesticides, and foods irrigated and/or prepared with As-contaminated water. Current estimates suggest that roughly 140 million people in Bangladesh, India, Vietnam, Nepal, and Cambodia are drinking water with As concentrations up to 100 times the World Health Organization (WHO) and USA Environmental Protection Agency (EPA) guideline of 10 μg/L [2, 3]. Chile, Mexico, China, and Taiwan also have As in groundwater that is used for drinking. In comparison to the situation in South and East Asia, the magnitude of the problem in the USA is rela- tively small. Nevertheless, the US Geological Survey estimates that 42 million Americans obtain their drinking water from household wells, and roughly 15% of these wells exceed the WHO guideline, indicating that a large number of USA residents are exposed to excess As from household wells [4]. In addition, not all municipalities are yet in compliance with the EPA requirements, with up to 8% of all public water supplies exceeding 10 μg As/L.

Individuals chronically exposed to As are at increased risk for various cancers, including cancers of the skin (Bowen's disease, basal cell carcinomas, and squamous cell carcinomas) [5], lung, bladder, and liver [6]. Chronic As exposure is a major risk factor for ischemic heart disease [7] and Blackfoot Disease, the latter a form of severe peripheral vascular disease associated with systemic atherosclerosis, dry gangrene, and spontaneous amputations of affected extremities [8].

Arsenic is metabolized via methylation and understanding of the importance of As methylation has advanced substantially in the past decade. This paper provides an overview

of what is now known about As methylation, including the details of the methylation pathway and the influence of methylation on As elimination, toxicity, and risk for As-induced health outcomes. We will also discuss the accumulating evidence suggesting that nutritional manipulation of one-carbon metabolism can improve As methylation capacity.

1.1. As Methylation. The predominant forms of As in drinking water are arsenate (As^V) and arsenite (As^{III}). Most species methylate these inorganic As (InAs) species to varying degrees in a process commonly thought to involve alternate reduction and oxidative methylation reactions in a model originally proposed by Challenger roughly 70 years ago [9, 10] (Figure 1). In this model, $InAs^{III}$ serves as a substrate for As methyltransferase (AS3MT), an enzyme identified by Thomas's group in 2002 [11, 12]. AS3MT catalyzes the oxidative methylation of $InAs^{III}$ to methylarsonic acid (MMA^V) using S-adenosylmethionine (SAM) as a cosubstrate. MMA^V is reduced to MMA^{III} in a reaction that can be catalyzed by AS3MT using reducing equivalents provided by thioredoxin [13]. Although earlier reports identified MMA^V reductase, a member of the glutathione-S-transferase superfamily (GSTΩ) [14, 15] as being capable of catalyzing this reduction, studies employing GSTΩ knockout mice indicate this function is not unique to GSTΩ (Chowdhury UK 2006). The methylation of MMA^{III} by AS3MT yields dimethylarsinic acid (DMA^V), which is considerably less toxic than pentavalent or trivalent InAs or MMA. While other methyltransferase enzymes have been identified which are capable of catalyzing these methylation reactions [16, 17], *AS3MT* catalyzes the formation of MMA^V and DMA^V with a kM in the nM range [12]. Although methylation of As is generally considered to be primarily hepatic, AS3MT mRNA has also been detected in rat kidney, adrenal gland, lung, urinary bladder, heart, and brain [12].

There are controversies related to As metabolism and toxicity that warrant discussion. For example, DMA^V can be reduced, and some investigators have reported that DMA^{III} may represent a significant proportion of total urinary As [18–20]; however the potential for artifact is high for several reasons [21]. First, DMA^{III} in urine is highly labile; Gong et al. have demonstrated that it is completely oxidized to DMA^V within 24 hours when stored at $-20^\circ C$ [22]. Second, the method employed in these studies [18, 19] to synthesize the purported chromatographic standard for DMA^{III}, that is, treatment of DMA^V with metabisulfite and thiosulfate, in fact does not generate DMA^{III}, but rather thio-DMA^V [23]. Third, while Valenzuela et al. used appropriate DMA^{III} standards [20], the hydride analyte ultimately detected by their assay could be generated from either DMA^{III} or thio-DMA [21]. This thioarsenical species has recently been found to be present as a relatively minor (roughly 5% of total uri- nary As) arsenical species in urine of Bangladeshi women [21]. For these reasons, it is likely that the chromatographic peak assigned as DMA^{III} in population studies was actually thio-DMA^V. While the toxicity of thio-DMA^V has not been well characterized, Francesconi's group has shown that thio-DMA^V reduces HepG2 cell viability to a greater extent than DMA^V at concentrations ranging from 0.1 to 1 mM [21]. A lesser issue relates to trimethylarsine oxide which can be formed under some assay conditions, but its production is strongly inhibited by the presence of GSH [11], and measurable amounts are generally not thought to be produced by humans *in vivo*.

A second model of As methylation proposed by Hayakawa et al. in 2005 suggests that trivalent arsenical-thiol complexes are the obligate substrates for AS3MT which catalyzes nonoxidative methylation reactions [24]. Work by Naranmandura and colleagues suggests a reductive methylation brought about by the transfer of an unshared electron pair from As to a methylation and concurrent reduction of As by conjugated GSH [25]. Future studies characterizing the structure and function of the AS3MT enzyme will be required to fully characterize the details of this metabolic process.

1.2. The Role of Methylation in As Elimination. Recent work by Thomas's group using AS3MT knockout mice has confirmed the crucial role of AS3MT in the methylation and excretion of As. After a single oral dose of 0.5 mg of arsenic as arsenate per kg body weight, AS3MT knockout mice showed a reduced proportion of methylated arsenicals in both liver and urine at 2 and 24 hours compared to wild-type mice [26]. AS3MT knockout mice also retained a higher percentage of the initial body burden of As in the liver, kidneys, urinary bladder, lungs, heart, and carcass at 24 hours than did wild-type mice. In a subsequent study in which mice received 10 daily oral doses of 0.5 mg of arsenic as arsenate per kilogram body weight, the AS3MT knockout mice showed reduced whole body clearance of As 24 hours after the final dose compared to wild-type mice (40% versus 90% clearance) [27]. During the clearance phase, DMA accounted for 6.9% of urinary As at 240 hours after the first dose in the knockout mice as compared to 84% in the wild-type mice. The AS3MT knockout mice also showed higher fractions of the body burden of As in skin, liver, and urinary bladder as compared to wild type. Although the AS3MT gene resulted in a substantially reduced capacity for As methylation, methylated arsenicals were still observed in urine and tissues of AS3MT knockout mice. In fact, the AS3MT knockout mice had a higher proportion of MMA in both urine and liver than did wild type [26]. In a study which underscores the critical role of AS3MT in As excretion, knockout mice exposed to 100 or 150 ppm arsenite via diet showed severe, and often lethal, systemic toxicity after only one week of exposure [28]. Given that MMA^{III} is the most toxic As metabolite, an interesting question that arises from this work is whether the toxicity observed in the knockout mice is at least in part due to the increased proportion of MMA. The work of Thomas and colleagues also strongly suggests that there are alternate pathways for methylation of InAs to MMA. Indeed, this group recently reported the in-vitro conversion of arsenate to oxy- and thioarsenicals, including MMA^V, by anaerobic microbiota of mouse cecum [29]. There may also be other enzymes capable of catalyzing these reactions.

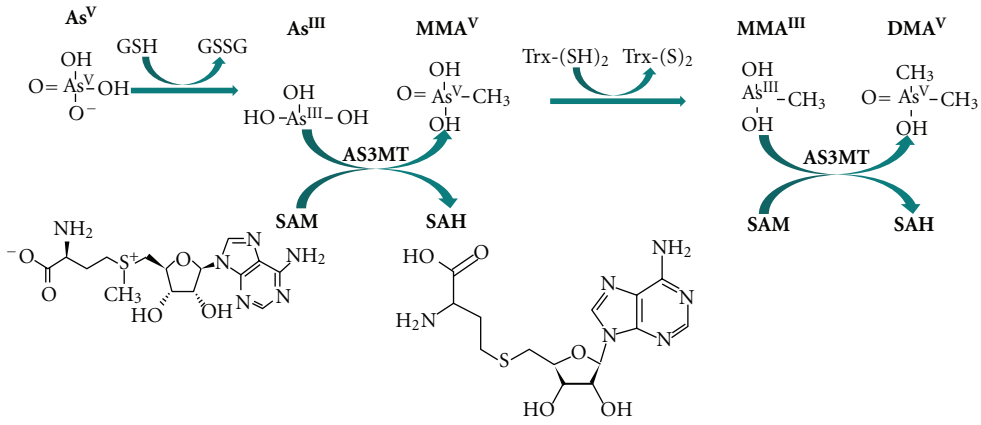

Figure 1: Arsenic metabolism. Arsenic in tubewells in Bangladesh is predominantly arsenite. Arsenic (+3 oxidation state) methyltransferase (AS3MT) catalyzes the oxidative methylation of arsenite using s-adenosylmethionine (SAM) as the methyl donor, forming methylarsonic acid (MMA^V), and s-adenosylhomocysteine (SAH). MMA^V is reduced to methylarsonous acid (MMA^{III}) before a subsequent oxidative methylation step yielding dimethylarsinic acid (DMA^V) and SAH.

The relative toxicities of the different As species are related to their chemical reactivity, but also to their physiologic half lives. Human retention studies employing single oral doses of ^{74}As as arsenic acid or $AsO(OH)_3$ to human volunteers indicate that arsenicals are eliminated with a three-component exponential decay pattern: 65.9% with a half life of 2 days, 30.4% with a half life of 9.5 days, and 3.7% with a half life of 38 days [30]. However, when considering populations that have been exposed to InAs for many years, these half lives derived from single-dose experiments must be viewed with caution since steady-state tissue concentrations were not achieved, and a "deep compartment" with a much longer half life could have been missed. Indeed, in mice, some InAs deposits in bone [31], suggesting that a longer terminal half life is likely. Similar patterns of elimination are observed in rabbits and hamsters. The initial half lives of MMA and DMA in hamsters are very short (7.4 and 5.6 h, respectively) [32], indicating the importance of As methylation for the facilitation of As elimination. The specific half lives of MMA and DMA in humans have not been determined, and their half lives under steady-state conditions may differ from those calculated using tracer kinetics with single oral doses. Somewhat remarkably, our understanding of the renal mechanisms of As excretion is limited to early studies relating transport of arsenate ($InAs^V$) to phosphate in dogs [33–35]; little is known about renal excretion and/or potential reuptake of different As metabolites.

1.3. Mechanisms of Action of As. InAs is a highly reactive metalloid. As^{III} toxicity is largely attributable to its ability to react with critical sulfhydryl groups of many enzymes. The complex of As with a given protein bestows selectivity to the biological effects of As [36] and As metabolites differ in their protein binding capacity: $InAs^{III}$ has three coordination sites, MMA^{III} has two, and DMA^{III} has only one [37]. A stable structure only forms when As complexes with two sulfhydryl groups in a single protein. For this reason, the stability and specificity of binding between DMA and monothiols are less than that formed between $InAs^{III}$ or MMA^{III} and dithiols [36]. Toxicities of As^V are related to its striking chemical resemblance to phosphate. For example, As^V can serve as a substrate for enzymes that normally utilize phosphate, potentially resulting in disruption of normal biochemical processes [38].

Although As is an established human carcinogen, the carcinogenic mechanism of As is likely to be through a novel process, as As is a poor mutagen in *in vitro* studies. As a notable exception, Waalkes group has determined that *in utero* As exposure during critical periods of development results in various tumors in the adult offspring, indicating that in these circumstances As can act as a complete carcinogen. The carcinogenic mechanism of action of As is not known, but may involve oxidative stress and clastogenicity [39, 40]. Arsenic is considered to be a member of a class of carcinogens known as gene inducers, or indirect carcinogens, due, in part, to its proposed influence on DNA methylation [41]. Recent evidence also indicates that As exposure may be associated with alterations in histone modifications [42–58] and that *in utero* exposure is associated with alterations in stem cell response to carcinogen exposure during adulthood [59].

1.4. Arsenic Methylation as a Detoxification Pathway. Despite decades of research implicating As methylation as a detoxification pathway, the influence of As methylation on As toxicity has been under intense investigation in recent years. Landmark work by Styblo et al. [60] and Petrick et al. [61, 62] in 2000 found MMA^{III} to be the most toxic metabolite both *in vitro* and *in vivo*. Subsequent toxicological studies have confirmed that MMA^{III} and DMA^{III} are at least as cytotoxic [63] and genotoxic [64–68] as $InAs^{III}$. In contrast, data suggesting that DMA^V is a bladder carcinogen in rats [69] has been discounted in terms of human relevance due to the extraordinarily high doses employed [70]. Also, animal models suffer limitations in that there are profound species differences in As metabolism, and animals are less prone to

develop cancer in response to As exposure than humans. Based largely on cell culture studies, the relative toxicities are thought to be $MMA^{III} > DMA^{III} > InAs^{III} > InAs^{V} > MMA^{V} > DMA^{V}$, with the qualification that DMA^{III} is highly unstable and is not likely present in significant quantities *in vivo* [21].

The relatively high toxicity of MMA^{III} leads to a critical question as to whether the overall methylation process is one of detoxification or of bioactivation since this metabolite is thought to be a requisite intermediate in the generation of DMA^{V}. Under conditions of chronic low-dose InAs exposure, the extent to which MMA^{III} arising from endogenous biosynthesis exists as a free moiety within the intracellular milieu as opposed to that which is bound to GSH, AS3MT, or other cellular proteins is an open question and would likely influence its toxicity. Indirect evidence of a beneficial effect of methylation comes from studies indicating a protective role for folate and/or SAM or of enhanced As toxicity under conditions of folate deficiency. For example, McDorman et al. demonstrated that dietary folate deficiency enhances the induction of As-induced micronuclei by 1.3- to 4.5-fold as compared to folate sufficient mice at As doses of 2.4 to 10 mg/kg via oral gavage [71]. Likewise, Ramirez et al. [72] induced micronuclei formation in human lymphocytes with 10 μM sodium arsenite and found that micronuclei formation was attenuated by the addition of 17 nM SAM. Folate deficiency has also been reported to enhance the effects of As on gene expression in a study employing skin biopsies from K6/ODC mice, one of the few rodent models sensitive to As-induced tumorigenesis [73]. While suggestive, these studies lack direct measures of As metabolites, and alternative explanations for the observations cannot be ruled out.

In human populations, case-control studies indicate that individuals with relatively higher proportions of $MMA^{(III+V)}$ and lower proportions of DMA in urine are at increased risk for As-related health outcomes, including skin lesions, skin, lung, and bladder cancers, peripheral vascular disease, and atherosclerosis [5, 74–85]. Note that technology does not yet allow for reliable speciation of MMA^{III} versus MMA^{V} as MMA^{III} is very readily oxidized to MMA^{V} during sample collection, storage, and processing. Some of the above studies were limited in that the number of cases was relatively small (i.e., 26 to 76 cases) [75, 76, 78, 79] and while the odds ratios were not inclusive of one, the 95% confidence intervals tended to be relatively wide (e.g., 1.7–34 for skin cancers) [75]. Two larger studies include one case-control study of urothelial carcinoma ($N = 177$ cases versus 488 controls) in Taiwan and another study of skin lesions ($N = 594$ cases versus 1,041 controls) in Bangladesh. The Taiwan study found that total urinary As, %InAs and %MMA all exhibited significant dose-dependent increased risk for urothelial carcinoma, whereas %DMA was associated with decreased risk [77]. The Bangladesh study found that %MMA in urine was positively associated with risk for skin lesions in a dose-dependent manner, while %DMA was inversely associated with risk [74]. Thus, the weight of the human evidence favors the consensus that incomplete methylation of As to DMA confers increased susceptibility to multiple adverse health outcomes. However, these studies all suffer the common limitation that As metabolites were assessed after disease onset, bringing into question the issue of temporality. In sum, although there appears to be an emerging consensus among epidemiologists that complete methylation of As to DMA is beneficial, the lack of large scale population-based studies analyzing prediagnostic biological samples renders this consensus somewhat tenuous.

2. Nutritional Influences on Methylation Reactions

Methylation of As and numerous other substrates occurs via one-carbon metabolism, a biochemical pathway important in the biosynthesis of purines and thymidylate and the remethylation of homocysteine (Hcys) to methionine (Figure 2). Methionine is activated to SAM, which serves as a methyl donor for a variety of methylation reactions, including the methylation of As. Transmethylation reactions generate s-adenosylhomocysteine (SAH), which can be converted to Hcys. SAH is a strong inhibitor of most methyltransferase enzymes, including AS3MT. One-carbon metabolism is dependent upon folate, vitamin B12, and vitamin B6 for the recruitment and transfer of methyl groups. Other nutrients, including betaine, choline, riboflavin, and serine also contribute to the availability of methyl groups ultimately used in SAM biosynthesis.

2.1. Experimental Evidence of Nutritional Influences on As Methylation and Toxicity. Early studies observed effects of methyl donor deficiency on As excretion and provide experimental evidence that the well-characterized nutritional regulation of one-carbon metabolism can influence As methylation and toxicity. In 1987, Vahter and Marafante reported that methyl donor deficiency in rabbits induced by either choline-, methionine- or protein-deficient diets significantly decreased urinary excretion of As, mainly due to lower DMA excretion. These diets also gave rise to increased retention of As in tissues (e.g., lung) [86], suggesting longer half lives and greater chemical reactivity of the InAs species. Similarly, Tice et al. reported that methyl donor deficiency induced by a choline-deficient diet decreased total urinary As excretion in mice by 28% as compared to mice on a choline-sufficient diet, predominantly due to reduced urinary DMA [87]. This was also accompanied by a shift in target organ As-induced DNA damage from liver and bladder (sites of As methylation and urinary As elimination, respectively) to skin [87], a target tissue in which As has a high affinity for the sulfhydryl groups of keratin.

An elegant series of studies by Finnell's group on As-induced NTDs employed mice heterozygous or nullizygous for folate binding proteins including Folbp-1, -2, and reduced folate carrier (RFC) [88–91]. Each of these binding proteins functions in cellular uptake of folate from the circulation (Folbp1 and 2) and/or enterocytes (RFC). While mice nullizygous for Folbp1 and RFC die *in utero*, the heterozygotes and Folbp2−/− develop normally and Folbp1 embryos can be rescued with folinic acid. The study

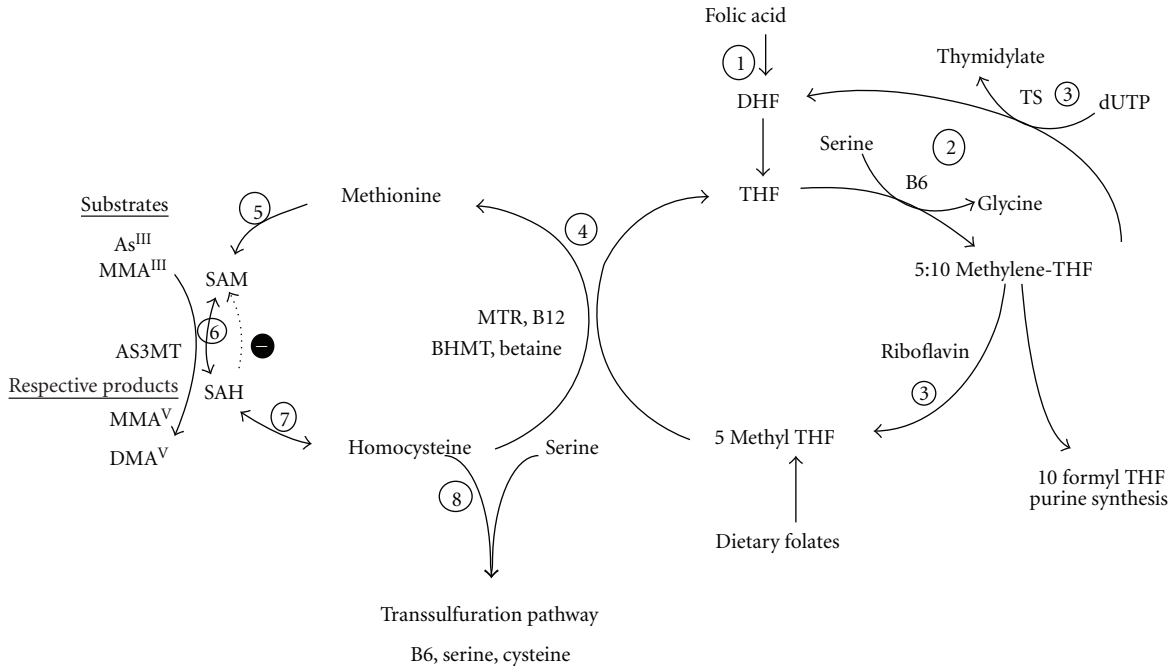

Figure 2: One-carbon metabolism. (1) Folic acid, arising from fortified foods or nutritional supplements, is reduced to dihydrofolate (DHF) and tetrahydrofolate (THF) by dihydrofolate reductase. (2) Serine hydroxymethyl-transferase transfers 1-carbon units from serine to THF, with PLP as a coenzyme, forming 5,10-methylene-THF and glycine. (3) 5,10-methyl THF reductase can reduce 5,10-methylene-THF to 5-methyl-THF. 5,10-methylene-THF can also generate DHF during the synthesis of thymidylate. After absorption from the GI tract, dietary folates can also enter the one-carbon metabolic pathway as 5 methyl THF. (4) In a reaction catalyzed by methionine synthetase and utilizing vitamin B12 as a cofactor, the methyl group of 5-methyl-THF is transferred to homocysteine (Hcys), generating methionine and THF. Alternatively, betaine can donate a methyl group for the remethylation of homocysteine to methionine in a reaction catalyzed by betaine homocysteine methyltransferase (BHMT). (5) Methionine adenosyltransferase activates methionine to form S-adenosylmethionine (SAM). (6) SAM is a methyl donor for a variety of acceptors, including guanidinoacetate (GAA—precursor to creatine), DNA, and As, in reactions that involve a number of methyltransferases. (7) The byproduct of these methylation reactions, s-adenosylhomocysteine (SAH), is hydrolyzed to generate Hcys. (8) Hcys is either used to regenerate methionine or is directed to the transsulfuration pathway.

protocols employed i.p. injections of sodium arsenate (30–40 mg/kg) on gestational days 7.5 and 8.5, that is, a critical period for neural tube closure. These studies demonstrated that for all genotypes studied (including wildtype), dietary folate deficiency caused a reduction in total urinary As excretion, primarily due to a reduction in DMA excretion. Furthermore, folate-binding protein 2−/− mice were more susceptible to As-induced NTDs, a phenotype that was further exacerbated by a folate-deficient diet [90].

While these studies provide strong experimental evidence that nutritional manipulation of one-carbon metabolism influences As methylation, excretion, and toxicity, the As doses were high, and the dietary deficiencies were severe. Moreover, there are marked species variations in the efficiency of As methylation [92].

2.2. Nutritional Influences on As Methylation in Humans. As noted, rodent models suffer two limitations: there are profound species differences in As metabolism, both mice and rats are extremely efficient in methylation of As, and animals are less prone to develop cancer in response to As exposure than humans. Furthermore, it is difficult to mimic chronic low-dose population exposure levels using rodent models. The earliest human data implicating nutritional influences on methylation and toxicity of As came in the form of isolated case reports. For example, there is an interesting case study of a girl with MTHFR deficiency who developed severe clinical signs and symptoms of As poisoning upon exposure to an As-containing pesticide, whereas no other exposed family members developed symptoms [93]. In 2002, in a study of 11 families in Chile, Smith's group reported intra-family associations in As methylation. The father/mother correlation for In As/(MMA+DMA) was low ($r = 0.18$). However, adjustment for plasma folate or homocysteine substantially increased the correlations ($r = 0.33$ and 0.55, resp.) [94]. Although the authors did not conclude that there was a significant effect of nutritional factors on As methylation, the data were highly suggestive.

In 2005, this same group assessed dietary intake of 30 micronutrients by dietary questionnaire in a sample of 87 subjects from two As-exposed regions in the western USA. They found that subjects in the lower quartile for dietary protein, iron, zinc and niacin had higher %MMA and lower %DMA than subjects in the higher quartile [95]. No associations were found for dietary folate, but the study was

conducted several years after mandatory folic acid fortification of the USA food supply and therefore all of the study subjects were essentially folate supplemented.

More recently, Vahter's group analyzed plasma concentrations of folate, cobalamin, zinc, and ferritin in a cross-sectional study of 442 pregnant women from Matlab, Bangladesh. In their analyses, they first stratified by As exposure (tertiles of urinary As) and then compared %InAs, %MMA, and %DMA across tertiles of plasma micronutrient concentrations. In a multivariate adjusted model, only %InAs was found to be lower with increasing plasma folate concentrations and only among the highest As exposure subgroup [96]. In a subsequent study in a subset of 324 women from the same parent study who had urine samples available at gestational weeks 8, 14, and 30, Vahter et al. examined changes in As methylation during the course of pregnancy and whether any observed changes were associated with nutritional status. Gestational week was inversely associated with the percentage of urinary InAs and positively associated with the percentage of urinary MMA and DMA ($P < 0.001$ for all). There were no observed associations between plasma folate or vitamin B12 and the change in urinary %InAs, %MMA, and %DMA over the course of pregnancy [97]. Although the authors concluded from these studies that nutritional status had little influence on As methylation, this is not surprising given that plasma folate concentrations change dramatically over the course of pregnancy, introducing noise to the variable. Also, all of these women had been given prenatal folic acid (400 μg) starting at week 14 in addition to other vitamin and/or mineral supplements. In addition, it is possible that other nutrients which were not examined in this study, such as choline and betaine, play a more substantial role in As methylation during pregnancy; choline biosynthesis is significantly upregulated by estrogen, concentrations of which rise dramatically during pregnancy [98].

Our group has conducted a series of studies in Bangladesh on nutritional influences on As metabolism and toxicity. We first evaluated the underlying prevalence of folate and B12-deficiency and hyperhomocysteinemia (HHcys) in a random sample of 1,650 Bangladeshi adults. This survey revealed that the study population has an extremely high prevalence of HHcys, particularly among males: 63% of males and 26% of females were found to have hyperhomocysteinemia (using NHANES cutoffs of $\geq$11.4 and 10.4 μmol/L, for males and females, respectively) [99]. The data are consistent with a 2000 report in Lancet. In that study of healthy males, plasma total homocysteine (tHcys) concentrations were higher in Indian Asian men residing in the UK than their white European counterparts [100]. Our survey also revealed modest but statistically significant negative correlations between water As and plasma folate concentrations ($r = -0.13$, $P > 0.0001$), suggesting that As may in some way negatively impact folate nutritional status.

We subsequently selected a subset of 300 participants from the survey for measurement of urinary As metabolites for a cross-sectional study on the associations between folate, tHcys, and As methylation [101]. This subset was selected to be representative of the study population for total urinary As after excluding those identified as being cobalamin deficient. The results of these analyses revealed moderate but significant positive correlations between plasma folate and the relative proportion of DMA (%DMA) in urine and negative correlations between folate and both InAs and MMA in urine (Spearman Correlations -0.12, -0.12, and 0.14, for %InAs, %MMA and %DMA, resp.; $P < 0.05$ for all). Concentrations of tHcys were positively correlated with %MMA ($r = 0.21$, $P < 0.001$) and negatively correlated with %DMA ($r = -0.14$, $P < 0.001$).

In this same study, we made the serendipitous observation that urinary creatinine is negatively correlated with %InAs and positively correlated with %DMA in urine ($r = -0.32$ and 0.30, resp., $P > 0.0001$); the correlations remain equally robust with and without control for covariates including body weight, age, and water or urine As concentrations. We have confirmed this observation in several subsequent studies in Bangladesh [53, 102–104] and in an unpublished analysis of data from adults in Mexico provided from Drs. Uttam Chowdhury and H. Vasken Aposhian. Smith's group has subsequently reported similar findings in West Bengal [105]. The underlying mechanism for this observation is not readily apparent, but we note the substantial role of creatine biosynthesis on consumption of SAM-derived methyl groups [106], a role that is downregulated with increased dietary creatine intake.

In 2006, we reported the initial findings from our randomized, controlled trial of folic acid supplementation [103]. For this trial, 200 participants were randomly selected from the 550 participants who fell into the lowest tertile for plasma folate in the survey of 1,650. Participants were excluded if they were cobalamin deficient, pregnant, or taking vitamin supplements. Participants were randomly assigned to receive folic acid (400 μg/day, that is, the USA RDA) or placebo for 12 weeks. Urinary As metabolites were measured at enrollment, after one week and after 12 weeks. Folic acid supplementation resulted in an increase in the proportion of total urinary As excreted as DMA (72% before and 79% after) that was significantly ($P < 0.0001$) greater than that in the placebo group, as was the reduction in %MMA (13% before and 10% after, $P < 0.0001$) and %InAs (15% before and 11% after, $P < 0.001$). Significant treatment group differences were also observed for %MMA and %DMA even after just one week of the intervention.

Based on our understanding that As methylation facilitates urinary As elimination, and our observation that folic acid supplementation increased As methylation, we hypothesized that increased As methylation with folic acid supplementation would lower blood As concentrations. Methodologic advances in our Trace Metals Core Laboratory permitted us to test this hypothesis using blood samples from our folic acid trial by measuring total As and As metabolites in blood, where concentrations are an order of magnitude lower (range: 3–29 μg/L) than those in urine (8–780 μg/L). We measured As metabolites in blood for 130 participants, that is, those participants from our previous trial who had detectable levels of all As metabolites. Results revealed that folic acid supplementation resulted in a decline in total blood As of 13.6 $\pm$ 2.9% as compared to 2.5 $\pm$ 3.2% for the placebo

group ($P = 0.01$) [107]. The decline in blood As was largely due to the decline in MMA in blood. Whereas total blood As (i.e., InAs+MMA+DMA) declined, on average, by 1.7 μg/L, 1.1 μg/L of this was MMA; MMA declined by 22% from baseline.

2.3. Nutritional Impact on Risk for As-Induced Health Outcomes. In 2004, Smith's group conducted a dietary recall study in West Bengal, India, of 192 skin lesion cases and 192 age- and sex-matched controls. The results of this study indicated that participants falling into the lowest quintile for animal protein, calcium, fiber, and folate were at increased risk for As-induced skin lesions [108]. In 2006, this group reported results of a study of plasma concentrations of a series of 17 metabolites (including a series of micronutrients and cholesterol, glucose, glutathione, homocysteine, and transthyretin) and risk for As-induced skin lesions; plasma analyses were done on a subset of 180 of the original 192 cases. No statistically significant odds ratios were observed for any of the parameters studied, including folate and homocysteine. However, approximately half of the samples were stored at 4°C overnight and not aliquoted and frozen until the day after collection [109]. Since folate is highly unstable and would likely be degraded under these conditions, and homocysteine continues to be released into plasma by red blood cells after sample collection, the plasma concentrations for these (and other) metabolites cannot be considered to be accurate due to the sample handling procedures. Additional limitations include lack of statistical control for differences in As exposure which differed by case-control status and lack of As metabolite data.

One of the strongest studies to date on the impact of nutritional status on risk for As-induced health outcomes is the recent aforementioned case-control study of 177 urothelial carcinoma cases and 488 controls in a population in Taiwan exposed to low concentrations of As in drinking water. This study found that higher %DMA in urine and higher plasma folate concentrations were associated with decreased risk. In a multivariate-adjusted model, the odds ratios (95% CI) for increasing quartiles of plasma folate concentrations were 1.0 (referent), 0.33 (0.20–0.54), 0.22 (0.13–0.38), and 0.09 (0.04–0.19), $P_{\text{trend}} < 0.0001$. Furthermore, a significant interaction was observed between urinary As profiles and plasma folate in affecting urothelial carcinoma risk [77].

We have conducted a nested-case control study of 274 skin-lesion cases individually matched to controls for gender and age (within 5 years) and frequency matched for water As (within 100 μg/L). The results of this study indicate that folate deficiency and HHcys are both associated with increased risk for skin lesions [52], as is genomic hypomethylation of leukocyte DNA and low urinary creatinine [Odds ratios (95% confidence interval) were 1.8 (1.1–2.9) for plasma folate <9 nmol/L, 1.7 (1.1–2.6) for HHcys, 1.8 (1.2–2.8) for DNA methylation < median, and 0.7 (0.5–0.8) for a fold increase in urinary creatinine]. Clearly, clarification of the mechanisms underlying urinary As and creatinine interactions warrants further study.

3. Conclusions

The known health effects of long-term exposure to As include an array of health outcomes and increased risk for mortality. In countries with widespread contamination of drinking water, such as Bangladesh, the full impact of this exposure may not be apparent for years to come. Although there has been considerable debate as to whether As methylation is a bioactivation or detoxification pathway, the collective body of evidence from both laboratory and epidemiologic research has clarified several points. First, As methylation to DMA is primarily catalyzed by AS3MT and plays a key role in modulating As excretion and toxicity. Second, a lower capacity to methylate As to DMA, as evidenced by higher proportions of InAs and MMA in urine and blood, is associated with increased risks of several As-associated diseases. Lastly, the methylation and elimination of As are influenced by nutrients involved in one-carbon metabolism, especially folate. Further research is needed to determine whether other nutrients similarly influence As methylation and at what levels of intake the greatest benefit can be obtained. While clearly As-mitigation is of primary importance, nutritional manipulation of one-carbon metabolism may provide an additional approach for lessening the burden of disease resulting from long-term As exposure.

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Research Article

Caspase-10 Is the Key Initiator Caspase Involved in Tributyltin-Mediated Apoptosis in Human Immune Cells

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Tributyltin (TBT) is one of the most toxic compounds produced by man and distributed in the environment. A multitude of toxic activities have been described, for example, immunotoxic, neurotoxic, and endocrine disruptive effects. Moreover, it has been shown for many cell types that they undergo apoptosis after treatment with TBT and the cell death of immune cells could be the molecular background of its immunotoxic effect. As low as 200 nM up to 1 μM of TBT induces all signs of apoptosis in Jurkat T cells within 1 to 24 hrs of treatment. When compared to Fas-ligand control stimulation, the same sequence of events occurs: membrane blebbing, phosphatidylserine externalisation, the activation of the "death-inducing signalling complex," and the following sequence of cleavage processes. In genetically modified caspase-8-deficient Jurkat cells, the apoptotic effects are only slightly reduced, whereas, in FADD-negative Jurkat cells, the TBT effect is significantly diminished. We could show that caspase-10 is recruited by the TRAIL-R2 receptor and apoptosis is totally prevented when caspase-10 is specifically inhibited in all three cell lines.

1. Introduction

Tributyltin (TBT) is one of the most toxic compounds still used in antifouling paints for large commercial ships thereby distributed within the aquatic environment. Its distribution and accumulation in aquatic organisms leads to severe effects and has already reduced the number of snail species in the near of sea lanes and harbours [1]. Moreover, the trophic transfer has been demonstrated [2], and the accumulation within the food chain up to the level of marine mammals has reached concentrations that might be biological relevant [3–6]. The most prominent biological effect investigated so far is the so-called imposex within sea snails and dogwhelks [1, 7, 8], and this mechanism is used as biomonitoring tool for organotin compounds [9]. Despite the fact that a lot of studies have been carried out, the underlying molecular mechanism remains unclear [10, 11]. It has been proposed that the inhibition of aromatase activity alters the ratio of the hormones inducing the development of imposex, the imposition of male sex characteristics on female snails [1, 12], but other studies came to other results [11, 13].

As organotin compounds were still used and accumulate in the environment as well as in the food chain, the exposure of mammals and humans increases steadily. Moreover, it has been described earlier that organotin compounds, especially TBT, have a clear immunotoxic effect in mammals [14–16], and this might be due to their exorbitant induction of apoptosis [17, 18]. The effective concentration of TBT to induce apoptosis in the majority of treated cells is around or below 1 μM and has been shown *in vitro* [14, 18–27] as well as *in vivo* [28, 29]. The question whether the disturbance of the intracellular calcium homeostasis is responsible for the onset of apoptosis [22, 27, 30–34] or the direct effect on mitochondrial functions is the first event [18, 20, 24, 32, 33, 35] is under discussion for a long time. Stridh et al. [18, 23] have shown a decade ago that TBT induces apoptosis via the activation of caspases in various human cells, the link for this caspase activation was not yet found. The most obvious players have been discussed to be the increase in calcium concentration or the opening of the permeability pore of the mitochondria. But induction of apoptosis has been demonstrated for very low concentrations of TBT which

do not induce calcium influx [27], and caspases are often inhibited by high calcium concentrations [23]. Some years ago, evidence arose that mitochondria-independent mechanisms contribute to the induction of apoptosis and possibly death receptors or direct caspase activation are involved in the TBT induced effect [36–39].

It is now generally accepted that the programmed cell death can be physiologically induced via death receptors on the surface of the cells, activated by specific ligands that are strictly controlled for instance during development or inflammation [40] leading to the formation of the so-called "death-inducing signalling complex" or DISC [41]. Moreover, it has been shown that at least lymphoid cells can be discriminated into type I and type II cells and only type II cells are strongly dependent on functional mitochondria for their apoptotic machinery [42]. Jurkat T-lymphoblastoid cells are type II cells and present a special tool for the investigation of mitochondrial-dependent cell death characteristics. Additionally, genetic modifications of the DISC within these cells enable a closer look at which point the sequence of events is started after TBT-treatment.

In the present study, the mechanism of TBT-induced apoptosis has been investigated by the use of Jurkat T-cells and two variants, caspase-8 and FADD-deficient Jurkat cells, that provide a direct insight into the death-receptor-coupled mechanisms. The data presented here point to the involvement of initiator caspase activation, especially from caspase-10, and are discussed in terms of the potential immunotoxic role of TBT in exposed mammals.

2. Results

2.1. TBT Induces Apoptosis in Human Jurkat Cells. When human immune cells were treated with TBT, changes of morphological as well as biochemical parameters of apoptosis can be observed. In all experiments done in this study, we used 1 μM TBT, a concentration that induces apoptosis in the majority of the treated cells within 4 hours. After that, time membrane blebbing and the externalisation of phosphatidylserine (PS) occur and chromatin condensation could be observed in Jurkat A3 T-cells (Figures 1(a) and 1(b)). Chromatin condensation was shown by the use of the DNA dye Hoechst 33342, and PS on the outer leaflet of the plasma membrane is detected with Annexin V-FITC by flow cytometry (FACS) and fluorescence microscopy (Figures 1(c)–1(h)). As demonstrated by two typical FACS dot blots, more than 60% of the treated cells undergo apoptosis and exhibit green fluorescence at the plasma membrane without being necrotic as the counter staining with propidium iodide demonstrates clearly. Looking closer to the different proteins that are involved in the apoptotic machinery, the complete sequence of events from initiator caspases down to death substrates is switched on. Focussing at the level of initiator caspases, both, caspase-8 and caspase-10, are cleaved and their active subunits can be detected by western blotting (Figure 1, left). Downstream the initiator caspases, the BID protein is an important linker to the mitochondrial pathway in type II cells and this protein is cleaved after TBT treatment. From the multitude of caspases downstream of the mitochondria we tested for procaspase-9, -7, -6, and -3 and found all these proteases cleaved. As one of the most prominent death substrates poly(ADP-ribose) polymerase (PARP) has also been shown to be cleaved within this series of events (Figure 1, left). Moreover, we tested hepatocytes transfected with a fusion protein of cytochrome c/green fluorescent protein (kindly provided by D. Green, La Jolla Institute for Allergy and Immunology, San Diego, USA) and found the release of cytochrome c after TBT treatment (data not shown).

2.2. Apoptosis Is Diminished in Deficient Cell Lines and Dependent from Caspase Activity. The extrinsic pathway upstream the mitochondria is further characterised by use of two genetically modified Jurkat cell lines where one is caspase-8 deficient and the other is FADD adaptor protein deficient. Furthermore, various caspase inhibitors were used to dissect their roles as possible starting point of the apoptotic sequence of events induced by TBT. Firstly, caspase-8 deficient cells exhibit only a slight reduction of apoptosis in all three cell lines when incubated with 1 or 1.5 μM TBT (Figure 2), whereas Fas-ligand-induced apoptosis was completely abolished (not shown). Without caspase-8, the only measurable protection was found for PS externalisation that is reduced by one third (Figure 3). Secondly, FADD deficiency affords an improved protection against the effects caused by TBT exposure, especially at lower concentrations (Figure 2, 1 μM TBT). This is further corroborated by analysis of PS externalisation and the total caspase activity by a fluorescence assay in living cells. The externalisation of PS was reduced in the same order of magnitude as in the caspase-8-deficient cells (Figure 3), but the CaspaTag assay demonstrates high protease activity in TBT-treated wild-type cells as well as in the caspase-8-deficient variant, whereas in FADD-deficient cells, this activity is obviously reduced (Figure 4).

The importance of caspases for organotin-provoked apoptosis has been investigated by use of several inhibitors. The overall caspase inhibitor zVAD-fmk blocks totally all described effects that normally can be detected after TBT exposure (data not shown). In this study, we used further specific inhibitors of caspases downstream as well as upstream of mitochondria. When caspase-9 and caspase-3 were inhibited as most potent elements of the caspase cascade downstream of the mitochondria, TBT-induced apoptosis is fully prevented in all three cell lines (Figure 2). Preincubation of the Jurkat cells with zLEHD-fmk (caspase-9 inhibitor) and zDEVD-fmk (caspase-3 inhibitor) rescues all viable functions. Nevertheless, a closer look at the western blots revealed often a slight reduction of those elements that were cleaved upstream of the mitochondria, especially BID, even though at slightly higher concentrations of TBT (Figure 2, 1.5 μM). While caspase-8 is activated in fact after TBT treatment of wild-type Jurkat cells, caspase-8 deficient cells undergo apoptosis to a comparable extent. This result suggests that caspase-8 cannot play a substantial role within this concert of effects after TBT treatment. Therefore, we looked closer for caspase-10, the second initiator caspase at the receptor level.

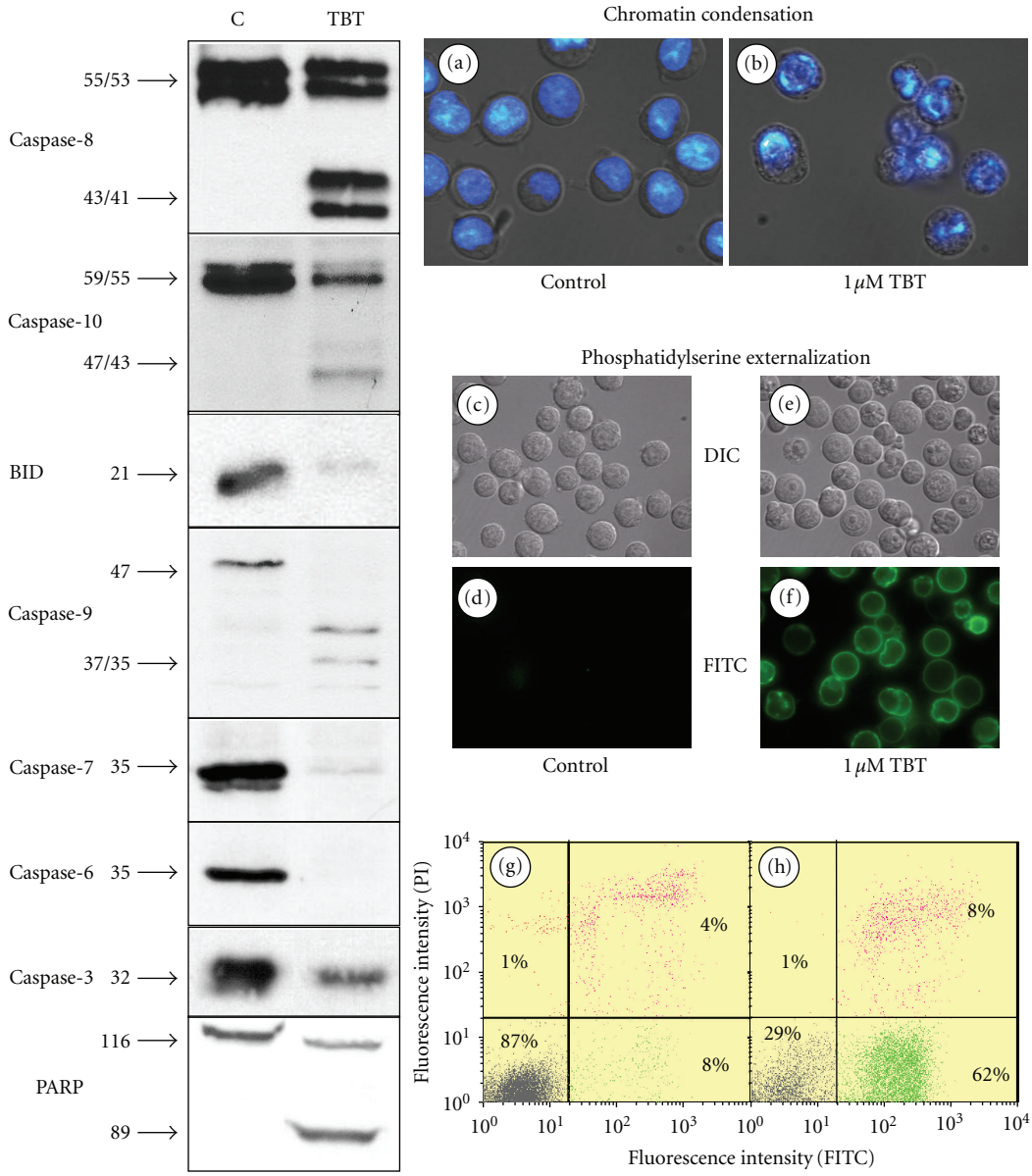

Figure 1: Induction of apoptosis in Jurkat T cells by TBT. Jurkat cells were treated with 1 μM TBT or ethanol (control) for 4 h before samples were analysed. Nuclei of vehicle-treated control cells (a) and after TBT treatment (b) were stained with Hoechst 33342 and analysed with a fluorescence microscope. Both pictures show an overlay of differential interference contrast light microscopy pictures with the fluorescence pictures. From the same experiment, samples were stained with annexin V-FITC for phosphatidylserine externalisation (c–h). Control cells were shown with DIC contrast (c), and no green fluorescence could be detected at 525 ± 12.5 nm (d). TBT-treated cells exhibit ruffled membranes and granular cytoplasm (e) and strong PS-labelling at the plasma membrane (f). The annexin-positive cells were further quantified by flow cytometry. The quadrant analysis of double labelled cells is shown for control (g) and TBT-treated cells (h). 10 000 cells of each sample were counted, and the percentage of viable cells (lower left quadrant, grey dots), apoptotic cells (lower right, green dots), and necrotic cells (upper two quadrants, red dots) were given in the dot blots. On the left side of the figure, immunoblots for 8 different proteins were shown. Protein names and molecular weights are given aside the blots. Left lane: control sample; right lane: TBT-treated sample.

2.3. Caspase-10 Is Obligatory for TBT-Induced Apoptosis, and Its Inhibition Prevents Apoptosis. When caspase-10 is inhibited by zAEVD-fmk, PS externalisation (Figure 3) and overall caspase activity is drastically reduced in all cell lines investigated in this study (Figure 4). Next, we wanted to know if initiator caspases could be found in an activated DISC and which ones. Immunoprecipitations (IP) with an antibody against the Fas-receptor coprecipitated caspase-8 (data not shown), but this caspase has no substantial relevance for the TBT effect in Jurkat cells as shown above. Therefore, we tested Jurkat cells for other death receptors and found additionally TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, and TNF-R1. As TRAIL-R2 was the dominant form and TRAIL-R3 and TRAIL-R4 are decoy receptors, we

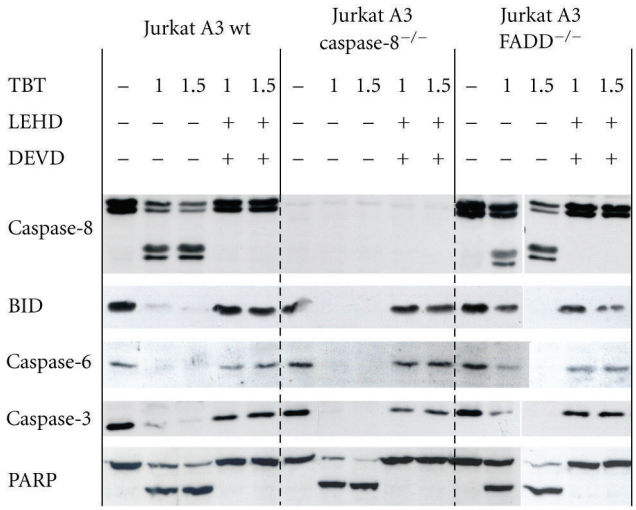

Figure 2: Inhibition of TBT-induced apoptosis by caspase-9 and caspase-3 inhibitors. Western blots of 5 different proteins in Jurkat A3 parental and the two deficient cell lines are shown. Cells were treated with 1 or 1.5 μM TBT for 4 h before proteins were separated on SDS-gels and immunoblotted. Another set of samples was preincubated for 1 h with the inhibitors for caspase-3 (DEVD, 10 μM) and caspase-9 (LEHD, 30 μM) before TBT (1 μM or 1.5 μM) was added. For molecular weights of proteins and cleavage products, compare Figure 1.

used a TRAIL-R2 antibody for our IPs. With this antibody, we could precipitate both initiator caspases after 3 h of treatment with 1 μM TBT (Figure 5(a)). Additionally, we detected the procaspases-8 and -10 in the untreated controls but to a much lesser extent and we never found the activated subunits. Analysing the three different cell lines reveals the fact that caspase-10 could be found in all activated DISC forms even though at different levels (Figure 5(b)).

2.4. Different Roles of Caspase-8 and Caspase-10 in Fas-Ligand and TBT-Induced Apoptosis. A direct comparison of the effects of Fas-ligand and TBT in all three cell lines pretreated with and without the caspase-10 inhibitor zAEVD-fmk provides a detailed insight into the different roles of the two initiator caspases in human Jurkat T cells. Fas-ligand treatment of the two deficient cell lines has no effect at all, and, thus, these data were not included in Figure 6. The Jurkat A3 wild-type cells, however, were driven into apoptosis, and this effect is only to a minor degree diminished by the pretreatment with the caspase-10 inhibitor, and apoptosis still proceeds. TBT treatment, however, has approximately the same effect as Fas-ligand in the absence of zAEVD-fmk, but all consequences of this treatment were prevented in the presence of AEVD. Phosphatidylserine externalisation is reduced to nearly control levels (Figure 3), and activation of caspases is strongly decreased in all three cell lines (Figure 4). In addition, the cleavage of important caspases is prevented (caspase-8 and caspase-3, Figure 6), BID cleavage is drastically diminished, PARP is completely rescued, and DNA fragmentation does not proceed anymore (Figure 6).

3. Discussion

Trialkylated tin compounds, especially TBT, are distributed all over the environment, and were taken up by cells *in vitro* fast and effectively and their toxicity is a function of both concentration and duration of exposure [30]. It has long been discussed that this cytotoxicity of organotin compounds might be the result of a massive alteration of the intracellular calcium concentration $[Ca^{2+}]_i$. Various investigations demonstrated an increase of $[Ca^{2+}]_i$ after exposure to a variety of trialkytins, and this effect should be responsible for their cytotoxicity, immunotoxicity, and neurotoxicity not only in mammalian [14, 17, 30–32] but also in fish cell systems [22]. But more and more evidence has been supplied that alteration of $[Ca^{2+}]_i$ is not the major event in the nonacute cytotoxic scenario [43, 44]. Numerous studies have been carried out during the last two decades indicating the induction of apoptosis in various biological systems without elucidating the starting point of the involved molecular mechanism [14, 17–25, 27, 28, 36]. As early as in 2001, the first publication demonstrated a possible involvement of the death receptors [38], and this was confirmed a few years later [37]. Nevertheless, recently published data connect developmental abnormalities of fish larvae with the induction of apoptosis on the level of caspase 3 [45], and the initiating molecular mechanism by which TBT induces apoptosis is not described. Thus, this study was carried out to enlighten the mechanism in more detail.

There exist two different pathways for apoptosis that can be distinguished from each other, the extrinsic and the intrinsic pathway [46]. The intrinsic pathway is dependent from proapoptotic events on the level of the mitochondria and is mostly affected by environmental chemicals or stress factors. Thus, it seems to be obvious that toxic substances such as TBT exert their effect on mitochondria. A multitude of studies have shown that different parameters of mitochondria were altered after treatment of cells with TBT [18, 32, 37]. Nonetheless, the induction of apoptosis could not be explained sufficiently by all these examinations because mitochondria-independent apoptosis has been described as well [37, 47] and inhibition of the intrinsic pathway by bcl-2 overexpression protects only type II cells but not type I cells from apoptosis although the mitochondrial membrane potential $\Delta\Psi_m$ is still high [48]. Moreover, it has been published earlier that various metal compounds may activate the extrinsic apoptotic pathway [37–39, 49]. A closer look on the formation of the "death-inducing signalling complex" (DISC) reveals its formation within 1 to 3 h after treatment with TBT (Figure 5). Normally, in Jurkat T cells, the DISC consists out of the Fas-receptor molecules to which the adaptor molecules FADD and initiator caspase-8 are bound. But the caspase-8-deficient Jurkat cells showed no or only little reduction in apoptosis after TBT treatment, and solely FADD deficiency decreases substantially the apoptotic cell number although not all. So we looked for other elements as possible constituents of the DISC. As it was published by several groups that not only caspase-8 but also caspase-10 can be recruited to death receptors [50, 51], Apo2L/TRAIL is able to activate both initiator caspases, and caspase-10 is

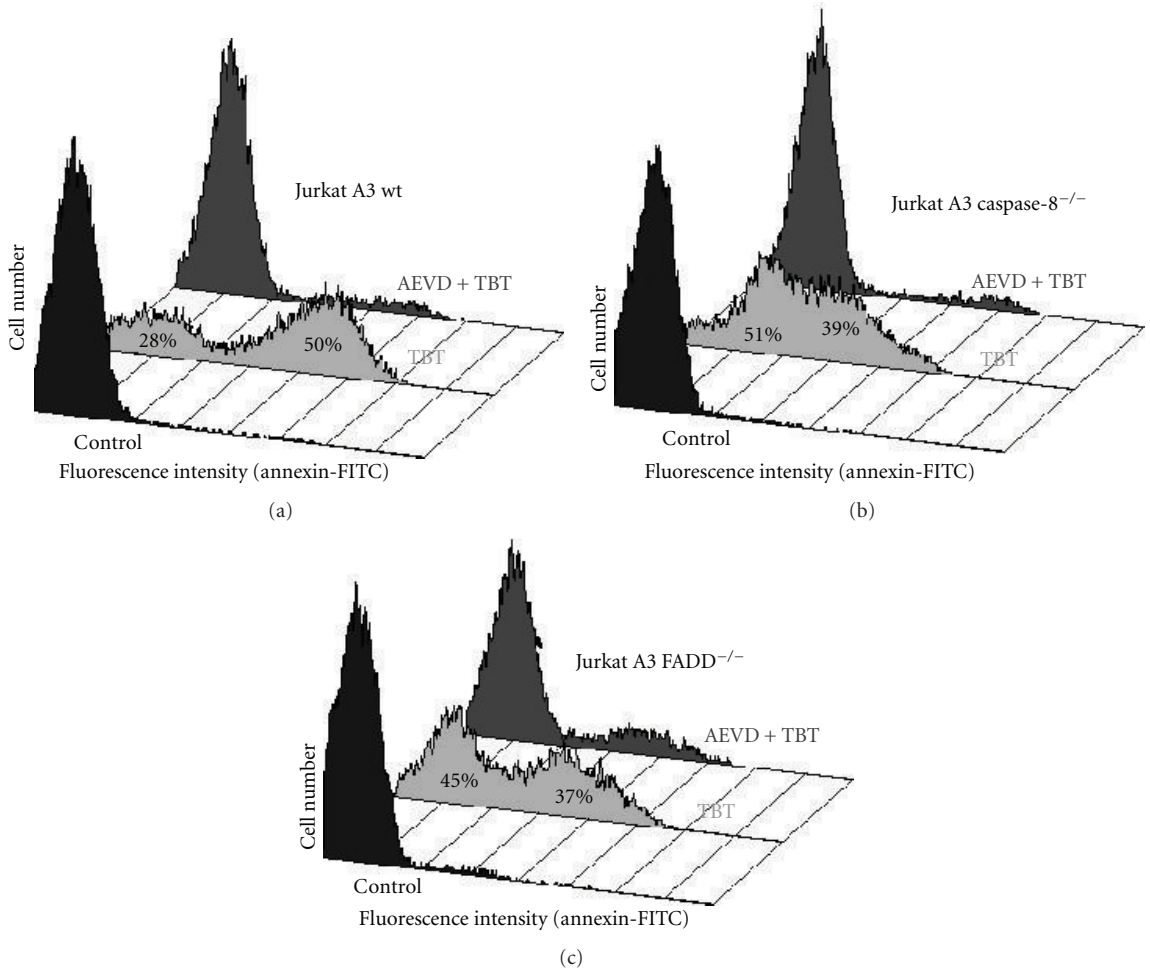

Figure 3: Caspase-10 inhibition prevents TBT-induced apoptosis in all variants of Jurkat A3 cells. Jurkat cells were pretreated with the caspase-10 inhibitor AEVD (8.7 μM, 1 h) before TBT was added (1 μM, 4 h). Then, the cells were stained with annexin V-FITC/PI to separate apoptotic cells from necrotic and viable cells by flow cytometry. The histograms show the fluorescence intensity of PI-negative only (compare lower two quadrants in Figures 1(g) and 1(h)). Numbers given for TBT-treated samples represent the percentage of total cells counted (10 000). Presented are the histograms of all three Jurkat A3 variants.

described as important caspase in HCT 116 colon carcinoma cells [52], we analysed the DISC formation in more detail. As mentioned above, caspase-10 could be found in the DISC and is co-precipitated by anti-TRAIL-R2 antibody. These results were confirmed by measuring various caspase activities in lysates using the substrates IETD-pNA (caspase-8), AEVD-pNA (caspase-10), and DEVD-pNA (caspase-3), respectively. In lysates of TBT-treated cells, all caspases have been found to be active (data not shown). Thus, TBT leads not only to unspecific cleavage of caspases but directly to their activation.

Because caspase-8-deficient Jurkat cells express lower amounts of caspase-10 compared to their parental cell line, these cells might be somewhat less sensitive to TBT as demonstrated here (Figure 3). But determination of all apoptotic markers revealed a nearly unchanged sensitivity to TBT of Jurkat cells lacking caspase-8 when treatment is prolonged to a minimum of 4 h. Another set of experiments focuses on caspases in more detail. Overall inhibition of caspases with zVAD-fmk, an unspecific inhibitor of all cellular caspases, inhibits totally TBT-induced apoptosis in human neutrophils [19] as well as in Jurkat cells (data not shown). A strong evidence for a specific role of the initiator caspase-10 comes from our experiments with its specific inhibitor zAEVD-fmk. While Fas-ligand-induced apoptosis is only slightly prevented after pre-incubation of the cells with AEVD (Figure 6), TBT-treatment has no effect at all, when caspase-10 was inactivated before. Nevertheless, Kischkel and co-workers [50] described FADD as an obligatory adaptor for both initiator caspases to TRAIL receptor; thus, we expected the FADD-deficient cells to be protected against TBT-induced apoptosis. But this is the case only for lower concentrations of TBT up to 1 μM. In this case, the prevention of FADD-deficient cells is apparent (Figure 2), whereas slightly higher concentrations (1.5 μM) overcome this protective effect. This might be due to the fact that these higher concentrations directly affect, on the one hand, the intrinsic machinery of apoptosis or, on the other hand, TBT might be

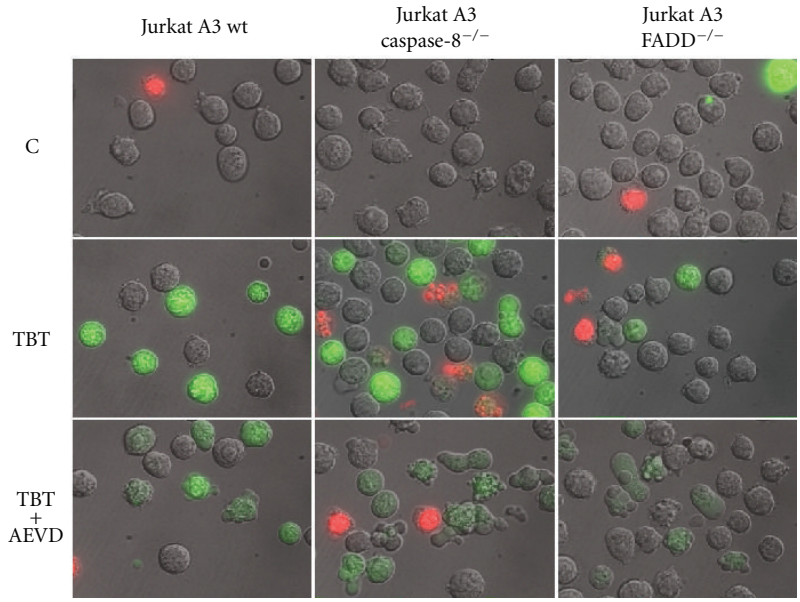

Figure 4: Caspase activity induced by TBT treatment in Jurkat A3 cells. Jurkat cells were pretreated with the caspase-10 inhibitor AEVD (8.7 μM, 1 h) before TBT was added (1 μM, 4 h). At the end of the treatment, FAM-VAD-fmk was added as a caspase substrate that exhibits fluorescence after cleavage. Cells were incubated for further 60 min, counterstained with propidium iodide (red fluorescence), and intracellular fluorescence intensity was analysed by microscopy. C: vehicle-treated control cells; TBT: 1 μM TBT; TBT + AEVD: pretreated with zAEVD-fmk for 1 h and 1 μM TBT for further 4 h.

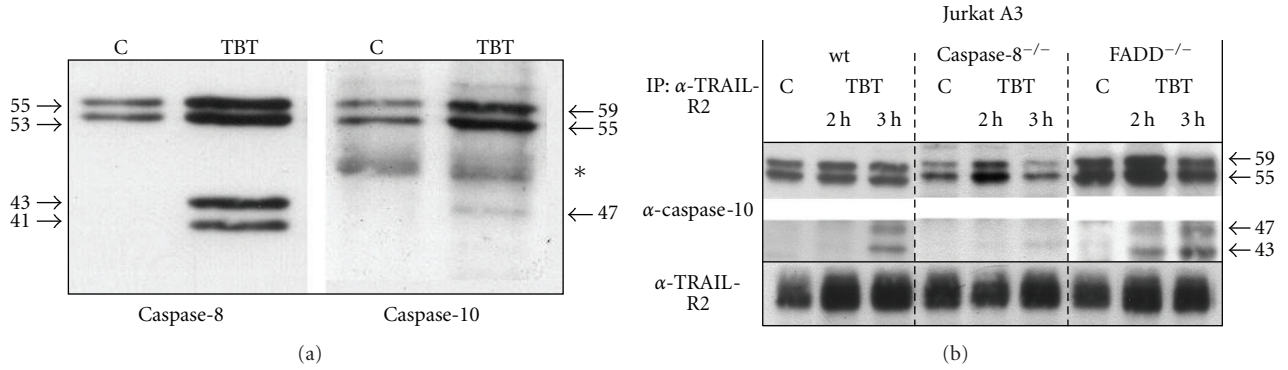

Figure 5: TBT induces recruitment of caspase-8 and caspase-10 by TRAIL-R2. Jurkat A3 wt cells were treated with or without TBT for 3 h before cells were lysed and immunoprecipitation (IP) was carried out with anti-TRAIL-R2 antibody. Initiator caspase-8 and caspase-10 were detected by western blotting (WB) in the precipitates (a). Both antibodies recognise full length procaspases and the processed subunits. Asterisk indicates an unspecific band. Caspase-10 was further investigated in all three cell lines after IP with anti-TRAIL-R2 antibody (b). After 2 or 3 h of incubation with TBT, cells were analysed by IP/WB. In all three cell lines, the amount of procaspase-10 and/or its cleavage products increase over time in the precipitate. Loading control for TRAIL-R2 is shown below. Arrows and numbers give the molecular weight of procaspases and cleaved subunits. Both parts of the western blot (upper part containing the procaspase-10, lower part with the cleavage products) are differentially exposed to visualize the weak bands of the cleavage products.

able to activate directly caspases as has been demonstrated earlier [36, 47]. Another evidence for caspase-10 dependency with no or only less involvement of FADD-adaptor protein has been described recently for another chemical but with the same set of Jurkat cells [53]. This group found the same total inhibition of all effects by the caspase-10 inhibitor zAEVD-fmk and no reduction in caspase-8-deficient cells. Moreover, FADD recruitment was not involved because the FADD-deficient Jurkat cells exhibited DNA fragmentation and other signs of apoptosis; thus, these results are obviously congruent with the data presented here.

Furthermore, it has been published lately that caspase-10 may cleave specific substrates, as the proapoptotic protein BID, without being cleaved before into its active subunits [54]. This may be the reason why type II cells are more sensitive to bcl-2 overexpression than type I cells, as type II cells are dependent on BID cleavage and the activation of the mitochondrial pathway. In our hands, the type I cell line

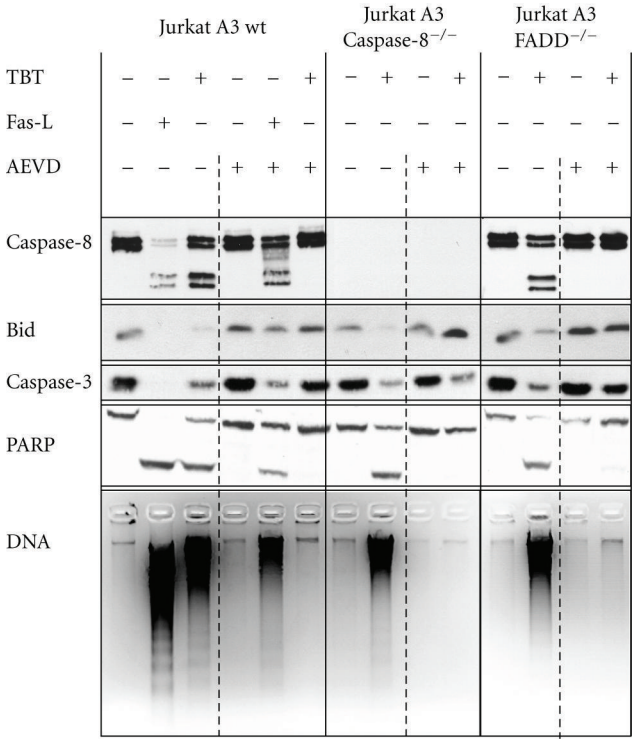

Figure 6: Caspase-10 inhibition prevents all TBT-induced apoptotic effects in Jurkat A3 cells. Jurkat A3 parental and the two deficient cell lines were analysed as shown in Figure 2 except the pretreatment was accomplished with zAEVD-fmk. Jurkat A3 wt cells were treated with 1 μM TBT or Fas-ligand (Fas-L) as positive control for 4 h, and the deficient cell lines were treated with TBT only before proteins were separated on SDS-gels and immunoblotted. A second set of samples was preincubated for 1 h with the caspase-10 inhibitor AEVD (8.7 μM) before TBT or Fas-L were added. Additionally, DNA fragmentation was recorded from the same samples after agarose gel electrophoresis.

SKW has a higher level of caspase-10 expression, and this is cleaved at the DISC in both variants, the wild type as well as in the bcl-2 overexpressing line (data not shown).

The question to what extent the extrinsic or the intrinsic pathway is responsible for the TBT-induced apoptosis in the absence of a functional FADD adaptor protein can be answered by the concentrations of TBT used within the experiments. Lower concentrations not disturbing the lysosomal or mitochondrial systems are more or less totally dependent on the formation of an activated DISC, whereas higher concentrations overcome this mechanism and stress the intracellular machinery via lysosomes or/and mitochondria leading to caspase-independent responses [55–57]. The here described results indicate that TBT in principle activates initiator caspase-10 leading to BID cleavage and activation of the mitochondria inducing the downstream apoptotic machinery (Figure 7).

Besides mammalian system cells from other species were affected by TBT as well. In trout blood cells, 1–5 μM TBT induces apoptosis within 1 h [35], and, in gill tissue of the mussel *Mytilus galloprovincialis* treated with 1 μg/g bw TBT ($\approx$3 μM), apoptosis could be detected after 24 h incubation [28]. The strongly discussed immunosuppressive properties of TBT *in vivo* might be the consequence of specific induction of cell death in immunocompetent cells. Such a killing of lymphocytes by TBT can be observed as a loss in thymus weight or thymus atrophy [29, 58] that debilitates the immune function of animals, making them vulnerable to infectious diseases [59–63].

On the background of these findings, the deadline for banning TBT must be possibly reconsidered as not only sea snails but also open water mammals and humans might be affected and new regulatory strategies have to be discussed independent from market forces [64]. Whereas the International Maritime Organisation (IMO) has banned TBT since 2008, the European Commission has forbidden its use "after 1st of July 2010 in articles where the concentration in the article, or part thereof, is greater than the equivalent of 0.1% by weight of tin," but "articles treated with such biocides may still be imported into the Community" [65].

4. Materials and Methods

4.1. Materials. All cell culture reagents were purchased from Life Technologies (Eggenstein, Germany), petri dishes and multiwell plates were obtained from Nunc (Wiesbaden, Germany). Annexin-FITC is from BD Pharmingen (Heidelberg, Germany), PI and Hoechst 33342 from Sigma (Deisenhofen, Germany), and the inhibitors of caspase-10 (zAEVD-fmk; FMK009), caspase-9 (zLEHD-fmk; FMK008), and caspase-3 (zDEVD-fmk; FMK004) were from R&D Systems (Wiesbaden, Germany).

The primary antibodies anti-caspase-6 (Cat no. 9762), anti-caspase-7 (Cat no. 9492) and anti-caspase-9 (Cat no. 9502) were purchased from Cell Signaling Technology (Frankfurt, Germany), anti-caspase-3 (Cat no. C31720), and anti-caspase-8 (Cat no. 551242 clone 3-1-9) from BD Pharmingen (Heidelberg, Germany), anti-caspase-10/a (Cat no. M059-3) from MoBiTec (MBL) (Göttingen, Germany), anti-TRAIL-R2 (DR5) (Cat no. PC392) from Calbiochem (Darmstadt, Germany), anti-BID (Cat no. AF846) from R&D Systems (Wiesbaden, Germany), and anti-PARP (Cat no. 1835238) from Roche Biochemica (Mannheim, Germany). As secondary reagents, we used: horseradish-peroxidase-(HRP-) conjugated goat anti-mouse IgG1 (Cat no. P 0447) from DakoCytomation (Glostrup, Denmark) and HRP-conjugated donkey anti-rabbit (Cat no. NA934) from Amersham Biosciences (Freiburg, Germany).

4.2. Cell Cultures. The Jurkat cell line A3 as well as the FADD and caspase-8-deficient cell lines were kindly provided by J. Blenis (Harvard Medical School, Boston, USA) and were maintained in RPMI 1640 supplemented with 10% FCS and 1 mM HEPES. The cultures were grown with 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. During experiments, 10 mM glucose were additionally included in the incubation medium.

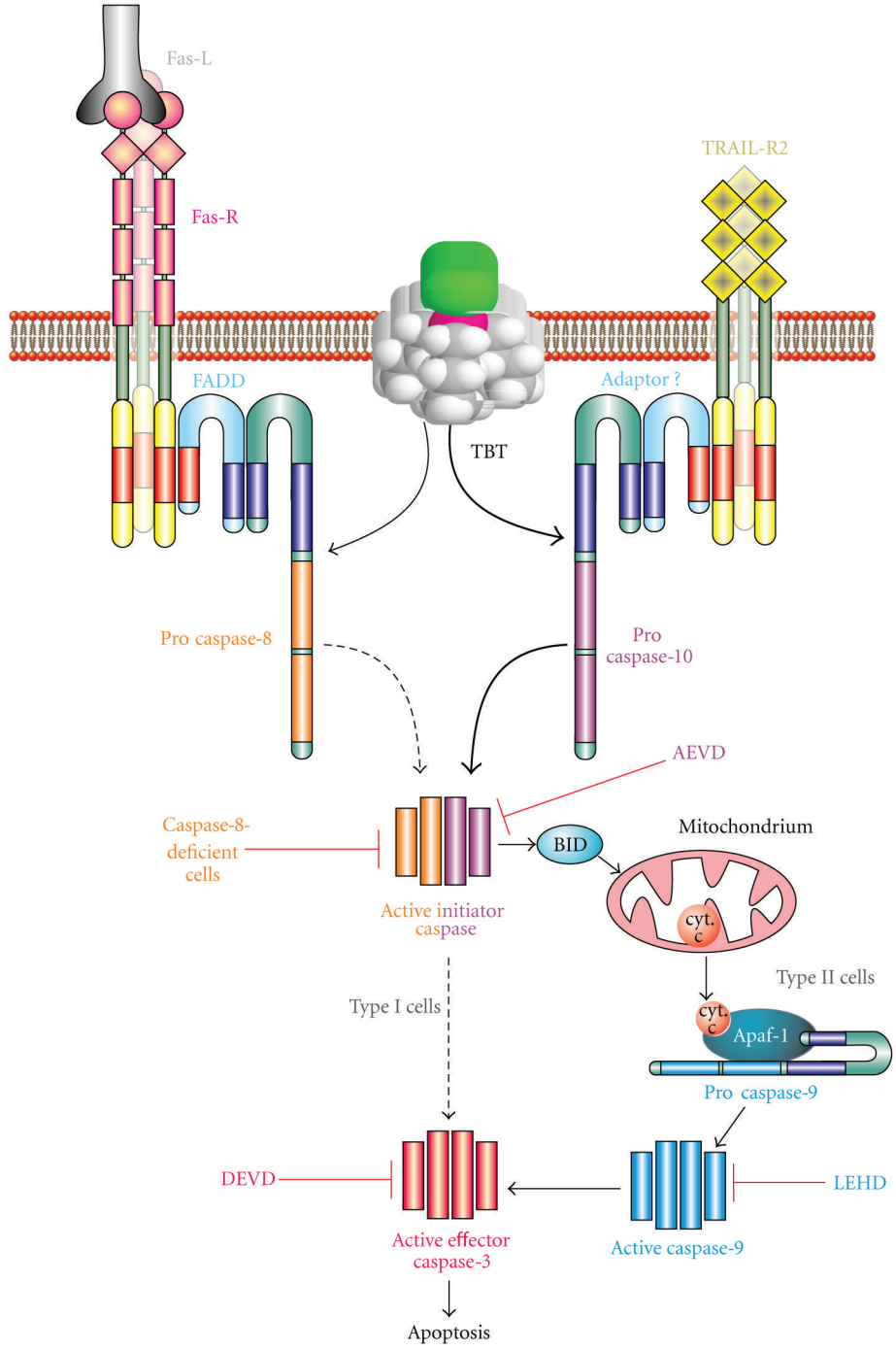

Figure 7: Proposed scheme of TBT-induced apoptosis in Jurkat cells. TBT activates caspase-10 upstream of mitochondria which leads to BID cleavage, and activation of the mitochondria. Downstream caspases were then cleaved and the caspase-cascade is provoked. Caspase-8 deficiency cannot prevent apoptosis, but inhibition of caspase-10 (AEVD) or inhibition of the two important caspases downstream of the mitochondria, caspase-9 and caspase-3 (LEHD and DEVD), totally suppress cell death.

4.3. Methods

4.3.1. Treatment of the Cells. For induction of apoptosis, 2×10^6 cells per mL medium were incubated with 1 μL/mL of a 1 mM stock solution of TBT (Merck) in ethanol to give the final concentration of 1 μM. As a positive control, 100 ng Fas-ligand plus 1 μg enhancer (Alexis, San Diego, USA) per mL incubation medium were used. All controls were incubated

with the same amount of vehicle (1 μL ethanol/mL) to exclude side effects of the solvent.

4.3.2. DNA Fragmentation. Apoptotic DNA fragments were isolated according to the following procedure. 2×10^6 were disrupted in 500 μL lysis buffer (20 mM EDTA, 1% NP 40, 50 mM Tris/HCl, pH 7.5). After centrifugation at 1600 g for 5 min, supernatants containing the apoptotic DNA were transferred into Eppendorf tubes. After addition of 1% SDS, samples were treated for 2 h with RNAse-A (5 μg/μL) at 56°C and subsequently for further 2 h with proteinase K (2.5 μg/μL). The DNA was precipitated by the addition of 50 μL of 10 M ammoniumacetate and 250 μL ice-cold ethanol, stored overnight at −20°C followed by centrifugation. The pellet was resuspended in 25 μL of TE-buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), and the DNA-fragments were analysed by agarose gel electrophoresis (1.8% agarose containing ethidium bromide as DNA stain), and pictures were taken with a MWG Gel documentation system.

4.3.3. Caspa-Tag Assay. The assay was carried out as described by the manufacturer (Intergen, Heidelberg, Germany). From each treated sample, 150 μL containing 3×10^5 cells were washed with fresh medium and 10 μL of 30x solution of the caspase substrate FAM-VAD-fmk were added to give the final concentration of 10 μM. The mixture was further incubated for 1 h in an incubator at 37°C. Then, 1 mL of washing solution was added, the suspension was centrifuged at 400 g for 5 min at room temperature. The resulting cell pellet was washed once with washing buffer and finally resuspended in 400 μL washing buffer including 1 μL PI as counterstain for necrosis. Cells were left for 15 min on ice, and then the microscopic pictures were taken.

4.3.4. Immunoprecipitation. TRAIL-associated caspase-10 and caspase-8 were immunoprecipitated as follows: 10^7 cells (2×10^6/mL) were treated with 1 μM TBT and then lysed in 500 μL cell lysis buffer (30 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin and 10 μg/mL leupeptin, 1% Triton and 10% glycerol). The lysates were centrifuged at 14 000 g for 10 min at 4°C. The supernatants were then incubated for 4 h with protein A-Sepharose (Sigma, Deisenhofen, Germany) and 1.5 μg anti-TRAIL-R2. The beads were centrifuged at 7000 g for 6 min at 4°C, washed once with the same amount of lysis buffer, and centrifuged again. After having removed the supernatants carefully, the pellets were resuspended in 100 μL electrophoresis buffer and 15 μL of each sample were analysed by SDS-gel electrophoresis and western blotting.

4.3.5. Apoptosis Assays. Apoptotic and necrotic cells were determined either microscopically or by flow cytometry using recombinant annexin V conjugated to FITC and propidium iodide. The determination of apoptosis is based on the binding of annexin V-FITC on the phosphatidylserine exposed at the surface of apoptotic cells. Necroses were determined by staining with the membrane impermeable DNA-intercalating dye PI. For the assay, 1×10^6 cells were pelleted at 1500 g and resuspended in 100 μL binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl and 5 mM $CaCl_2$) containing 5 μL annexin V-FITC and 100 ng PI. After 15 min of incubation at 4°C in the dark, cells were diluted in 400 μL binding buffer and immediately analysed by flow cytometry. Fluorescence was analysed at 530 ± 14 nm (FITC) and 610 ± 10 nm (PI) and quantified with the CELLQuest Prosoftware (BD, Heidelberg, Germany).

For microscopic assessment of apoptosis and necrosis, 6×10^4 cells were diluted 1 : 1 with 2x binding buffer, containing 1 μL annexin V-FITC and 3 ng PI. After 15 min of incubation in the dark, cells were analysed with a 63x oil objective and a Zeiss Axiovert S100 microscope (Carl Zeiss GmbH, Jena, Germany), connected to a Hamamatsu CCD Camera (C4880-80). Fluorescence and differential interference contrast pictures were taken using an automation procedure and merged using Openlab software (Improvision, Coventry, UK).

Chromatin condensation was determined after staining of the cells with Hoechst 33342. In short, after treatment, 1×10^6 cells were washed with phosphate-buffered saline (PBS) incubated with a final concentration of 10 μM Hoechst 33342 for 10 min, washed again with PBS to reduce background fluorescence, and finally visualised with the same system described above with excitation at 364 ± 15 nm and emission at 460 ± 10 nm.

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Research Article

Toxicological Characterization of the Inorganic and Organic Arsenic Metabolite Thio-DMA^V in Cultured Human Lung Cells

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We synthesised and toxicologically characterised the arsenic metabolite thiodimethylarsinic acid (thio-DMA^V). Successful synthesis of highly pure thio-DMA^V was confirmed by state-of-the-art analytical techniques including ^{1}H-NMR, HPLC-FTMS, and HPLC-ICPMS. Toxicological characterization was carried out in comparison to arsenite and its well-known trivalent and pentavalent methylated metabolites. It comprised cellular bioavailability as well as different cytotoxicity and genotoxicity end points in cultured human A549 lung cells. Of all arsenicals investigated, thio-DMA^V exerted the strongest cytotoxicity. Moreover, thio-DMA^V did not induce DNA strand breaks and an increased induction of both micronuclei and multinucleated cells occurred only at beginning cytotoxic concentrations, indicating that thio-DMA^V does not act via a genotoxic mode of action. Finally, to assess potential implications of thio-DMA^V for human health, further mechanistic studies are urgently necessary to identify the toxic mode of action of this highly toxic, unusual pentavalent organic arsenical.

1. Introduction

Inorganic arsenic is a well-documented human carcinogen (IARC, Group 1) causing tumors in the lung, skin, and bladder [1, 2]. However, the underlying molecular mechanisms of inorganic arsenic-induced carcinogenicity are still to be elucidated, especially since inorganic arsenic, unlike other classical chemical carcinogens, does neither induce direct DNA damage nor mutagenicity at exposure-relevant concentrations [3]. Besides the contribution of its metabolism, a variety of further potential mechanisms are discussed, including the induction of genetic damage via oxidative mechanisms [4–6], epigenetic dysregulation [7], and interaction with the cellular DNA damage response and DNA repair [8], resulting in comutagenic and cocarcinogenic effects [9].

For the general population, human diet is the primary source of both total arsenic and inorganic arsenic intake. The European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain and the Joint Food and Agriculture Organization/World Health Organisation (FAO/WHO) Expert Committee on Food Additives (JECFA) have concluded in their recent scientific opinions on arsenic that risks to human health related to the presence of inorganic arsenic in food cannot be excluded. In 2010, the JECFA withdrew the previous provisional tolerable weekly intake (PTWI) [9–11]. Furthermore, the EFSA Panel emphasized the necessity for toxicological characterization of seafood and fish-related organic arsenicals, including arsenosugars and arsenolipids, for which to date no toxicological data exist [9]. In contrast to arsenobetaine, which is the main arsenic compound in fish, but is not metabolized by humans, arsenosugars and arsenolipids are extensively biotransformed to a multitude of arsenic metabolites [12, 13]. Some of these compounds are believed to be highly toxic and thus it cannot be excluded that seafood and fish-related organic arsenic species present risks to human health.

Regarding *in vitro* toxicity of the well-known and partly toxicologically characterised human inorganic arsenic metabolites monomethylarsinous (MMA^{III}), dimethylarsinous (DMA^{III}), monomethylarsonic (MMA^V), and dimethy-

larsinic (DMA^V) acid, the trivalent metabolites exert stronger cytotoxicity, as well as direct and indirect genotoxicity as compared to arsenite [14–21] in most cellular and subcellular test systems. Therefore, trivalent methylated arsenicals are generally believed to strongly contribute to inorganic arsenic-induced genotoxicity and, most likely, carcinogenicity.

Thiodimethylarsinic acid (thio-DMA^V, $[(CH_3)_2As(S)OH]$, also named dimethylmonothio-arsinic acid, $DMMTA^V$ or $DMTA^V$) is the pentavalent sulfur analogue of DMA^V and a metabolite of organic as well as inorganic arsenicals. The first identification of thio-DMA^V as a mammalian arsenic metabolite was obtained in urine and wool extract from a sheep naturally consuming large amounts of arsenosugars through seaweed [22]. In this paper, the group of Feldmann also discussed the serious problem that thio-DMA^V may have been misidentified as DMA^{III} in human urine samples before and, therefore, might have escaped detection in many samples so far [22, 23]. Indeed, thio-DMA^V has later been identified in human urine after exposure towards arsenosugars as well as inorganic arsenic-contaminated drinking water [12, 13, 23]. In a recent study investigating the arsenic metabolites in urine samples of 75 inorganic arsenic-exposed women in Bangladesh, thio-DMA^V has been shown to be a common metabolite, being detected in 44% of the samples [23]. Furthermore, thio-DMA^V might also directly occur in food, which has been postulated before for rice [24].

Probably because thio-DMA^V is not commercially available, in the literature no *in vivo* toxicity studies for thio-DMA^V (except for toxicokinetic studies) and only few *in vitro* toxicity studies exist. Nevertheless, these few studies point to a quite strong cellular toxicity of thio-DMA^V in mammalian cells in culture. Thus, in most studies, thio-DMA^V showed much higher cytotoxicity as compared to MMA^V and/or DMA^V [23, 25] and comparable effects to trivalent arsenicals [26, 27]. In some studies, thio-DMA^V even exerted stronger cytotoxicity as compared to arsenite [27–29]. Moreover, Ochi et al. provided evidence for a genotoxic potential of thio-DMA^V in cultured hamster cells [25], whereas no detailed data exist regarding the genotoxicity of thio-DMA^V in human cells.

The aim of the present study was to further investigate the toxicity of thio-DMA^V in cultured human A549 lung cells. Therefore, we synthesised and analytically characterised highly pure dimethylthioarsinic anhydride, which in aqueous solution immediately forms thio-DMA^V. Subsequently, cytotoxicity, cellular uptake, as well as for the first time genotoxicity at the DNA and chromosomal levels were examined in cultured human cells, while comparing effects of thio-DMA^V with effects of arsenite, MMA^{III}, DMA^{III}, MMA^V, and DMA^V.

2. Materials and Methods

2.1. Caution. Inorganic arsenic is classified as a human carcinogen. The following chemicals are hazardous and should be handled with care: sodium arsenite, methyloxoarsine (precursor to MMA^{III}), iododimethylarsine (precursor to DMA^{III}), dimethylthioarsenic anhydride (precursor to thio-DMA^V), MMA^V, and DMA^V.

2.2. Materials. Dulbecco's modified Eagle medium (DMEM), foetal calf serum (FCS), trypsin, and penicillin-streptomycin solutions were obtained from Sigma-Aldrich (Steinheim, Germany). The culture dishes were supplied by Biochrom (Berlin, Germany). Sodium(meta)arsenite (≥99% purity) and Alcian Blue were purchased from Fluka Biochemika (Buchs, Germany). Methyloxoarsine ($CH_3As^{III}O$, ≥99% purity) and iododimethylarsine [$(CH_3)_2As^{III}I$, ≥99% purity] (storage at −80°C) were kindly provided by Professor Dr. W. Cullen (University of British Columbia, Vancouver, Canada). DMA^V (≥99% purity) and MMA^V (99% purity) were purchased from Sigma-Aldrich (Steinheim, Germany). A549 cells (CCL-185) were obtained from the American Type Culture Collection (Bethesda, MD, USA).

Giemsa dye and acridine orange were bought from Roth (Karlsruhe, Germany). The ICPMS elemental standard (As, 1 mg/L) was purchased from SPETEC (Erding, Germany). Hydrogen peroxide solution (30%, Suprapur) and nitric acid (65%, Suprapur) were products of Merck (Darmstadt, Germany). Triton X-100 was bought from Pierce (Oud-Beijerland, The Netherlands), hydroxyapatite (high resolution) from Calbiochem (Bad Soden, Germany) and Hoechst 33258 from Merck (Darmstadt, Germany). All other proanalysis chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) or Merck (Darmstadt, Germany).

2.3. Synthesis of Dimethylthioarsinic Anhydride. Dimethylthioarsinic anhydride, which dissociates in water to thio-DMA^V, was synthesized according to Fricke et al. [30]. Briefly, DMA^V was dissolved in ethanol (30%) and hydrogen sulfide was bubbled into the solution and stirred over night. After removing the solvent, the residue was extracted with chloroform/water (3 : 1) and the chloroform layer was washed with water to remove the remaining water-soluble arsenic compounds. Finally, the solvent was removed and dimethylthioarsinic anhydride was recrystallized from methanol/hexane.

2.4. Analysis and Purity Control of Dimethylthioarsinic Anhydride. HPLC-FTMS (Thermo Accela, Thermo LTQ Orbitrap XL), HPLC-ICPMS (Shimadzu LC-10, Perkin Elmer ELAN 6000), and electrothermal AAS (Perkin Elmer, AAnalyst 600) were applied for identification and quantification as well as to obtain information about purity of the arsenic species. Thio-DMA^V solutions in water were prepared directly for each experiment; in order to compensate for sensitivity differences, the sample used for HPLC-FTMS (20 mg/L) was diluted by a factor of 400 for HPLC-ICPMS analysis. Briefly, for chromatographic separation, PTFE autosampler vials, a reversed-phase column (Waters Atlantis T3, 2.1 × 150 mm, 5 μm), and the eluent 13.2 mM ammonium acetate in water/10% methanol (pH 4.6) [30] were used. The flow rate was 0.3 mL/min. The m/z range for HPLC-FTMS analysis was set from m/z 80 to 1000. Fragmentation experiments were carried out with collisionally induced dissociation

(CID) using normalized collision energy, and data analysis was performed using Xcalibur software. For quantification of arsenic species by HPLC-ICPMS, chromatographic data were collected by monitoring m/z 75 (As and $^{40}Ar^{35}Cl$) and 77 ($^{40}Ar^{37}Cl$) with 100 ms dwell time. The results of the chromatography were analyzed with the data analysis software from OriginLab. Quantification of total As in thio-DMA^V solutions was carried out by electrothermal AAS, applying an ICPMS elemental standard.

Furthermore, ^{1}H-NMR spectroscopy (Bruker DCX-400, 400 MHz) was used to obtain additional purity information. Arsenic species were dissolved in deuterium oxide (D_2O), and the chemical shift values were observed for structural information. The obtained results were evaluated with the NMR data software MestReNova (Mestrelab Research) and compared with data from the literature.

2.5. Cell Culture and Incubation with the Arsenicals. Since the lung is an important target organ for inorganic arsenic-induced carcinogenicity, human A549 epithelial lung adenocarcinoma cells were used as *in vitro* model system. A549 cells were grown in culture dishes as monolayer in DMEM containing 10% FCS, 100 U penicillin/mL, and 100 μg streptomycin/mL. The cultures were incubated at 37°C with 5% CO_2 in air and 100% humidity.

Arsenical stock solutions were prepared in sterile deionised water. All stock solutions were prepared shortly before each experiment, among others to prevent oxidation of trivalent arsenicals. Logarithmically growing A549 cells were incubated with the arsenicals for 1 h or 24 h as described for the respective experiments.

2.6. Cytotoxicity Testing of Thio-DMA^V. The cytotoxicity of thio-DMA^V was elucidated by quantifying its effect on cell number and colony forming ability. Cell number and colony forming ability testing were exactly performed as described before for inorganic arsenic, MMA^{III}, DMA^{III}, MMA^V, and DMA^V [31]. Briefly, after 24 h of incubation with the respective arsenicals, cells were washed with phosphate buffered saline (PBS) and trypsinized. Subsequently, cell number and cell volume were measured by an automatic cell counter (Casy-1, Roche Innovatis AG, Bielefeld, Germany). These measurements are based on noninvasive (dye-free) electrical current exclusion with signal evaluation via pulse area analysis. To assess the impact of thio-DMA^V on colony forming ability of A549 cells, after cell counting of each sample, 300 cells/dish were seeded. After 7 days of incubation, colonies were fixed with ethanol, stained with Giemsa (25% in ethanol), counted and calculated as percent of control.

2.7. Cellular Bioavailability. To compare cellular bioavailability of thio-DMA^V with cellular bioavailability of inorganic arsenic and its related methylated metabolites, cellular bioavailability studies were carried out by exactly the same protocol as previously reported [31]. Briefly, logarithmically growing cells ($1 * 10^6$) were exposed to thio-DMA^V for 24 h, trypsinized, collected by centrifugation, washed with ice-cold PBS, and cell number as well as cell volume were measured by an automatic cell counter in each sample as described before. After incubation with the ashing mixture (65% HNO_3/30% H_2O_2 (1/1, v/v)) at 95°C for at least 12 h, samples were diluted with bidistilled water, and arsenic was measured by electrothermal atomic absorption spectrometry (AAnalyst 600, Perkin Elmer).

2.8. Determination of DNA Strand Breaks. DNA strand breaks were quantified by alkaline unwinding as described previously [32]. Briefly, $1 * 10^5$ cells were seeded, allowed to attach for 24 h and incubated with thio-DMA^V for 1 or 24 h. Subsequently, the medium was removed, cells were washed with PBS and an alkaline solution containing 0.03 M NaOH, 0.02 M Na_2HPO_4, and 0.9 M NaCl was added. After neutralisation and sonication, separation of single- and double-stranded DNA was performed on 0.5 mL hydroxyapatite columns at 60°C. Single- and double-stranded DNA were eluted with 1.5 mL of 0.15 M and 0.35 M potassium phosphate buffer, respectively. The DNA content of both fractions was determined by adding Hoechst 33258 dye to a final concentration of $7.5 * 10^{-7}$ M to 1 mL of each sample and measuring the fluorescence with a microtiter fluorescence reader (FLUOstar Optima, BMG Labtechnologies, Jena, Germany) at an excitation wavelength of 360 nm and an emission wavelength of 455 nm. DNA strand breaks were quantified by calibration with X-rays as described previously [33].

2.9. Formation of Micronuclei and Multinucleated Cells. By the early 1990s, the micronucleus assay was shown to be suitable to investigate arsenic-induced chromosomal alterations as a biological marker of its genotoxicity [34]. In recent years, the *in vitro* micronucleus assay has become an attractive tool for genotoxicity testing in general [35]. Therefore, this endpoint has been (and is) strongly used to characterize the genotoxic potential of arsenicals in epidemiological studies [36, 37] as well as in cultured mammalian cells (e.g., [38]). To investigate the induction of micronuclei and multinucleated cells, in this study A549 cells were seeded in 6-well plates on Alcian blue coated glass coverslips. After 24 h, cells were incubated with the respective arsenicals for 24 h, fixed with an ice-cold fixation solution (90% methanol/10% PBS, −20°C) for 10 min, dried in the air at room temperature, stained with acridine orange (125 mg/L in PBS) for 10 s, and finally analyzed by fluorescence microscopy. Per coverslip, at least 1000 cells were counted and categorized in mononucleated, binucleated, and multinucleated cells as well as cells with and without micronuclei.

3. Results

3.1. Synthesis, Analysis and Purity Control of Dimethylthioarsinic Anhydride. Colorless, highly pure dimethylthioarsinic anhydride crystals were obtained by the reaction of DMA^V with H_2S in ethanol, followed by extraction with chloroform and recrystallisation from methanol/hexane (Figure 1). After dissolving dimethylthioarsinic anhydride in

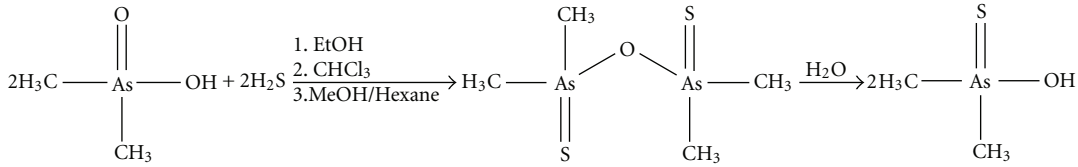


Figure 1: Scheme of the synthesis of dimethylthioarsinic anhydride and its conversion into the acid form thio-DMA^V in water.

water, formation of thio-DMA^V was analysed by means of hyphenated techniques and ^{1}H-NMR.

The mass spectrometric data and the ^{1}H-NMR results confirmed the conversion of the synthesized anhydride in water into the acid form thio-DMA^V. Structural elucidation of thio-DMA^V was performed by HPLC-FTMS analysis and by determination of the exact mass. The calculated mass of the acid form is m/z 154.9512 $[M+H]^+$and the detected mass was m/z 154.9509. Fragmentation experiments with collisionally induced dissociation of the parent ion m/z 154.95 $[C_2H_8OAsS]^+$ gave a fragment ion of m/z 136.9403 $[C_2H_6AsS]^+$ and further fragmentation led to m/z 108.9089 $[H_2AsS]^+$. The corresponding HPLC-FTMS total ion chromatogram (Figure 2(a)) demonstrates thio-DMA^V as a protonated molecular ion with a retention time of 7.3 min, whereas neither starting material nor further reaction products were detected.

HPLC-ICPMS analyses further verified the purity of thio-DMA^V by retention time matching of known arsenic species, including DMA^V as the starting material. Thus, after dissolving thio-DMA^V in water, HPLC-ICPMS chromatograms showed only one compound (Figure 2(b)). Under additional consideration of the quantification of thio-DMA^V by electrothermal AAS, the purity of dimethylthioarsinic anhydride was assessed to be ≥98%.

^{1}H-NMR measurements of thio-DMA^V (Figure 2(c)) in D_2O resulted in a chemical shift value of 2.12 ppm which is similar to the value of 2.11 ppm reported by Fricke et al. [30]. DMA^V showed a chemical shift of 1.98, the range of which is consistent with a pentavalent arsenical. The ^{1}H-NMR data of thio-DMA^V showed no impurities, and the desired compound was obtained in analytically pure form based on ^{1}H-NMR spectroscopy.

3.2. Cytotoxicity of Thio-DMA^V. Cytotoxicity of thio-DMA^V was determined by investigating its effects on cell number and colony forming ability (Figure 3) after 24 h incubation. The cell volume (Figure 4) was determined as well, however, principally to calculate cellular arsenic concentrations later on. Regarding both endpoints, cell number and colony forming ability, thio-DMA^V exerted higher cytotoxicity as compared to arsenite and especially to the pentavalent methylated metabolites MMA^V and DMA^V, whereas effects were about twofold lower as compared to MMA^{III} and DMA^{III} (Table 1). Thio-DMA^V affected colony forming ability stronger as compared to cell number, which is comparable to the trivalent methylated metabolites. In case of arsenite, MMA^V and DMA^V, both cytotoxicity endpoints showed similar sensitivity.

Table 1: Cytotoxic effects of the arsenicals in A549 cells after 24 h incubation. Shown are IC70 values for the endpoints cell number and colony forming ability. In case of arsenite, MMA^{III}, DMA^{III}, MMA^V, and DMA^V IC70 values were generated from the data originally published in Ebert et al. 2011 [31]; IC70 values represent the respective inhibitory concentrations of the compounds that are required for 30% reduction of cell number or colony forming ability *in vitro*.

Arsenic species	IC70 (cell number)	IC70 (colony forming ability)
Arsenite	57.2 μM	58.8 μM
MMA^{III}	5.6 μM	3.8 μM
DMA^{III}	5.1 μM	3.2 μM
Thio-DMA^V	12.1 μM	7.2 μM
MMA^V	>500 μM	>500 μM
DMA^V	>500 μM	>500 μM

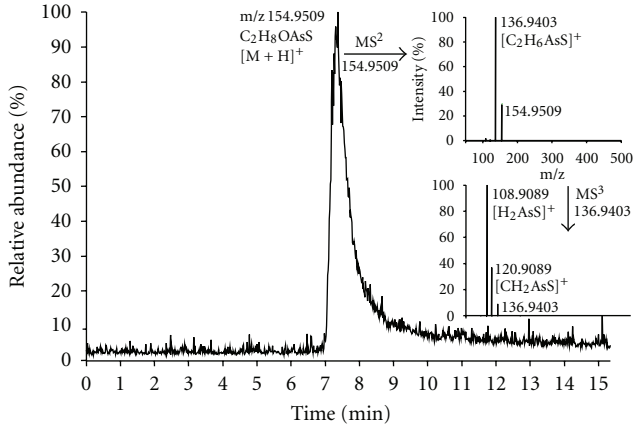
3.3. Cellular Bioavailability of Thio-DMA^V. To assess cellular bioavailability in A549 cells and to correlate cellular toxicity of thio-DMA^V with cellular arsenic content, cellular arsenic concentrations were determined after 24 h incubation by electrothermal atomic absorption spectrometry.

Comparing extracellular and intracellular arsenic concentrations, a 9-10-fold accumulation was observed in cells incubated with up to 15 μM thio-DMA^V (Figure 4). Thio-DMA^V showed no significant effects on cell volumes (Figure 4) at noncytotoxic concentrations, but increased cell volumes in case of cytotoxic concentrations (≥10 μM) by up to 44%. Mean (±SD) volumes of nonincubated control cells were 2.68 (±0.14) $*$ 10^{-12} L.

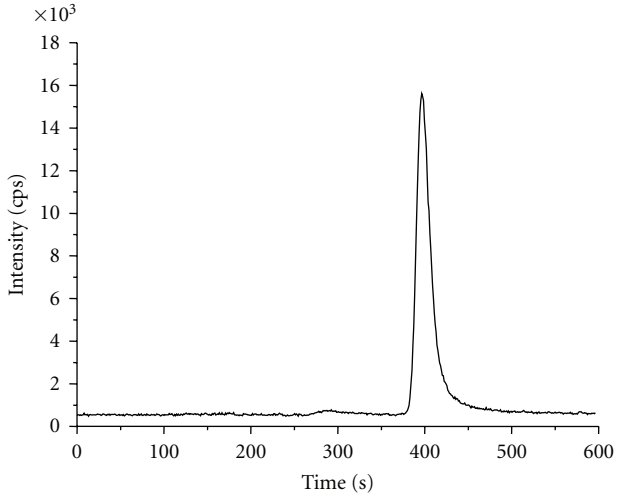
Interestingly, the concentration of cellular arsenic strongly correlated with the cytotoxicity of thio-DMA^V, resulting in a correlation coefficient of −0.986 (cell number) or −0.998 (colony forming ability), respectively.

3.4. Induction of DNA Strand Breaks by Thio-DMA^V. A possible generation of DNA strand breaks by thio-DMA^V was investigated in A549 cells after short-term (1 h) and long-term (24 h) incubation, applying the alkaline unwinding technique. Up to high, already cytotoxic thio-DMA^V concentrations both after 1 h and after 24 h incubation, no significant induction of DNA strand breaks was observed (Figures 5(a) and 5(b)).

3.5. Formation of Micronuclei and Multinucleated Cells by the Arsenicals. The two basic mechanisms leading to the onset of micronuclei are disturbance of the chromosome segregation



(a)



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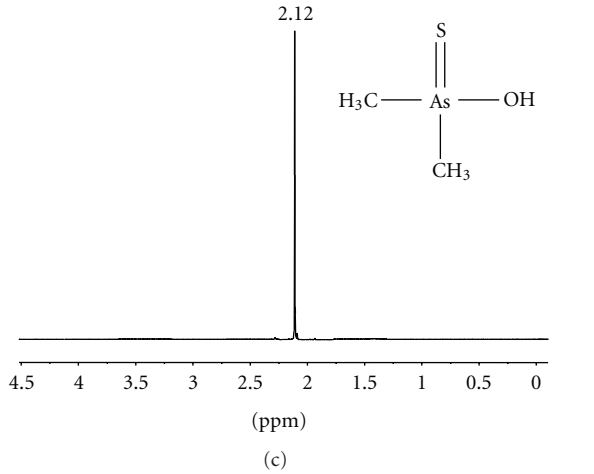

(c)

Figure 2: Analytical characterization of thio-DMA^V. (a): HPLC-FTMS total ion chromatogram including fragmentation spectra of the parent ion m/z 154.9509 $[C_2H_8OAsS]^+$; (b): HPLC-ICPMS chromatogram selectively monitoring m/z 75 (As); (c): ^{1}H-NMR spectrum of thio-DMA^V in D_2O.

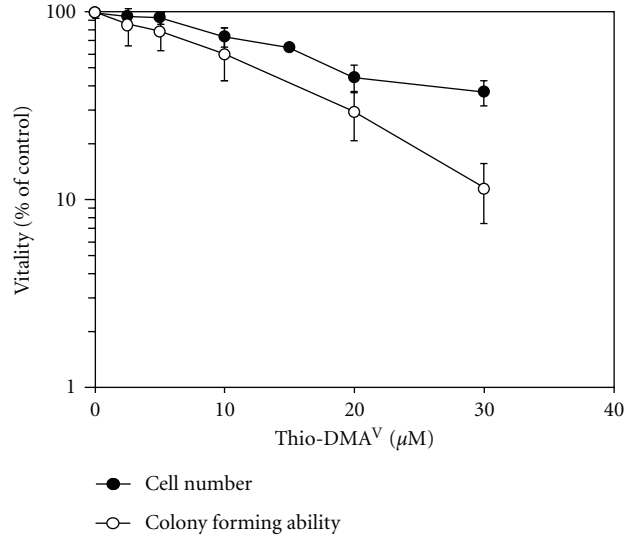

Figure 3: Cytotoxicity of thio-DMA^V in A549 cells after 24 h incubation. Cytotoxicity was determined by a decrease in cell number (closed symbols) and effects on colony forming ability (open symbols). The data represent mean values of at least six determinations ± SD.

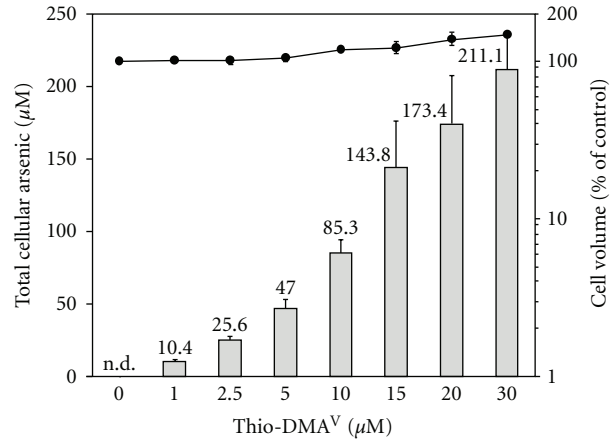

Figure 4: Cellular bioavailability of arsenic in A549 cells after 24 h incubation with thio-DMA^V. Logarithmically growing A549 cells were treated with thio-DMA^V for 24 h, trypsinized, and cell number as well as cell volume was determined. Finally, arsenic was quantified by electrothermal atomic absorption spectroscopy. Shown are mean values of at least six independent determinations +/± SD; n.d.: below detection limit.

machinery and chromosome breakage. Thus, in somatic cells, micronuclei can only occur after mitotic division, and in the cytokinesis-block micronucleus assay (CBMN), which is based on cytokinesis inhibition by cytochalasin B, cell proliferation and thereby mitosis are generally controlled by a scoring of mono- and binucleated cells [35]. However, our first CBMN studies indicated that several arsenicals interact with actin and/or the effect of cytochalasin B (data not shown). To assess the induction of micronuclei by the arsenicals, we omitted the application of cytochalasin B. To

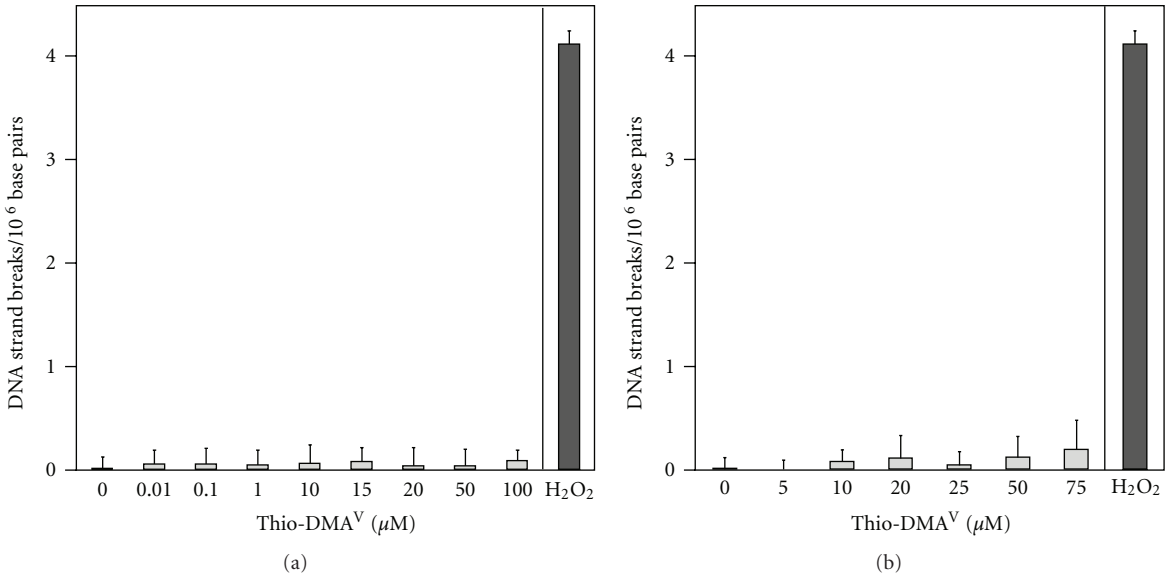

Figure 5: DNA strand break formation after 1 h (a) and 24 h (b) incubation with thio-DMA^V in A549 cells. DNA strand breaks were quantified by alkaline unwinding; 5 min incubation with 75 μM H_2O_2 served as positive control. Shown are mean values of at least six determinations + SD.

ensure mitosis, we controlled cell proliferation by means of cell number quantification and chose an incubation time of 24 h, which is equivalent to 1.25 cell cycles of the A549 lung cells. This incubation time was previously used to examine cellular toxicity of arsenicals in A549 cells [31] and, therefore, opens the possibility to compare results. Furthermore, this protocol allows the proper quantification of the formation of multinucleated cells by the arsenicals at the same time.

At noncytotoxic to beginning slightly cytotoxic concentrations, MMA^{III} (0.5, 1 μM), thio-DMA^V (5 μM), MMA^V (250 μM), and DMA^V (250 μM) induced a small, but significant number of around 20 micronuclei (Figure 6(a)). At higher, already cytotoxic arsenic species concentrations, micronuclei formation increased and became also significant in case of arsenite ($\geq$50 μM). At highly cytotoxic concentrations, 5 μM DMA^{III} and 30 μM thio-DMA^V showed strongest effects, inducing 188 ± 34.5 and 118 ± 4.6 micronuclei, respectively.

Moreover, thio-DMA^V and especially DMA^{III} increased the formation of multinucleated cells and the occurrence of binucleated cells in comparison to untreated control cells (Figure 6(b)). However, significant effects were restricted to cytotoxic concentrations. For all other applied arsenicals, no significant increased occurrence of bi- and multinucleated cells was observed.

4. Discussion

The data presented in this study provide further evidence for the strong cellular toxicity of the recently identified arsenic metabolite thio-DMA^V in human cells.

In the applied human lung cells, cytotoxicity of thio-DMA^V strongly correlates with its cellular bioavailability. For other arsenic species, a similar correlation has been reported in A549 cells [31] as well as in human urothelial (UROtsa) and hepatic (HepG2) cells [39] before.

When comparing the respective arsenic incubation concentrations, thio-DMA^V exerts higher cytotoxicity than arsenite, whereas effects are lower as compared to MMA^{III} and DMA^{III}. When additionally taking into account the cellular bioavailability of the arsenicals, among all arsenicals, applied thio-DMA^V shows the highest cytotoxicity in A549 cells. For instance, 30% reduction in cell number occurred after 24 h incubation with 5 μM DMA^{III}, which is related to 237 ± 38.2 μM cellular arsenic [31]. 12.1 μM thio-DMA^V caused a similar reduction in cell number; however, it corresponds to a cellular arsenic concentration of 115 ± 9.4 μM. Thus, a similar cytotoxic effect is achieved at twofold lower cellular arsenic concentrations. In summary, referring to the extracellular incubation concentrations, in A549 human lung cells the arsenicals follow the cytotoxic order: DMA^{III} > MMA^{III} > thio-DMA^V ≫ arsenite ≫ MMA^V ~ DMA^V. Taking into account the cellular uptake of the arsenic species and thereby referring to the effective cellular arsenic concentrations, the cytotoxic order switches to thio-DMA^V ~ arsenite ~ MMA^{III} > DMA^{III} ≫ MMA^V ~ DMA^V. This is somehow contrary to the study by Naranmandura et al. [26], where at the respective IC50 concentrations, cellular thio-DMA^V uptake was higher as compared to DMA^{III} and arsenite uptake. This different outcome might be due to the different cell systems applied, but most likely results from the different cytotoxicity endpoints investigated. By using the MTT test, Naranmandura et al. used a cellular metabolism-related cytotoxicity endpoint, which quantifies the impact of the arsenicals on the activity of cellular dehydrogenases. In contrast, in this study we quantified cell number, which comprises cell death and proliferation inhibition by the arsenicals. Moreover, colony forming ability, which

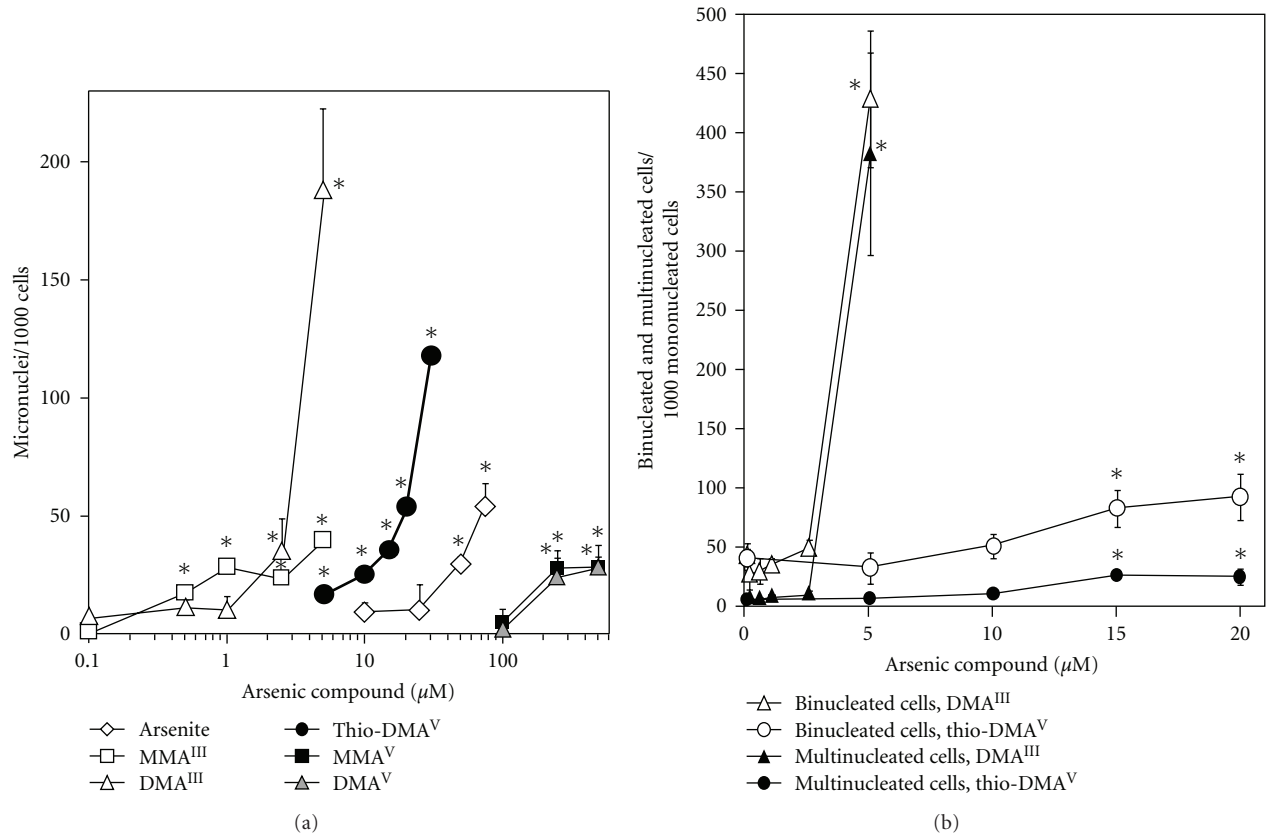

Figure 6: Induction of micronuclei (a), binucleated and multinucleated cells (b) after 24 h incubation of A549 cells. After 24 h in culture, logarithmically growing A549 cells were treated with arsenite, MMA^{III}, DMA^{III}, thio-DMA^{V}, MMA^{V}, or DMA^{V} for 24 h; cells were fixed, stained and finally analysed by fluorescence microscopy as described in Materials and Methods. (a): additionally induced micronuclei, micronuclei level in control was 27 ± 3; (a, b): shown are mean values of at least three independent determinations + SD; statistically significant different from nonarsenic exposed controls: $^{*}P < 0.01$ as determined by Student's t-test.

is generally considered as benchmark long term-cytotoxicity assay for directly not acute cytotoxic compounds, was applied as second cytotoxicity endpoint. Very interestingly, thio-DMA^{V} exerts stronger cytotoxicity regarding the endpoint colony forming ability, which points to an indirect mode of toxic action. This has similarly been shown before for the trivalent methylated metabolites [31].

In contrast to all other methylated arsenic metabolites [18], in A549 cells thio-DMA^{V} showed no generation of DNA strand breaks up to high cytotoxic concentrations. This is also in line with the fact that thio-DMA^{V} did not significantly increase reactive oxygen species level in A549 cells (as assessed by DCFDA fluorescence) up to high cytotoxic concentrations (data not shown). This is in contrast to the postulated, reactive oxygen species-mediated toxic mode of action of thio-DMA^{V} [28, 40]. Accordingly thio-DMA^{V}, as well as DMA^{III} and arsenite, exerted strong genotoxicity on the chromosomal level only at cytotoxic concentrations. Thus, in the present study, MMA^{III} is the only arsenical-inducing micronuclei at noncytotoxic, exposure-relevant concentrations starting at 0.5 μM. Micronuclei formation in A549 cells results at least partly from the earlier observed induction of DNA damage by 0.5 μM MMA^{III} [18].

For DMA^{III}, micronuclei induction has been shown before in CHO cells [38] and is discussed to be due to both aneugenic and clastogenic effects of DMA^{III}. In SHE (Syrian hamster embryo) cells after 24 h incubation, 20 μM thio-DMA^{V} induced chromosome structural aberrations including chromatid gaps, chromatid break and chromatid changes [25]. This fits nicely to earlier data by Kuroda et al. in V79 Chinese hamster lung cells: here, the unknown microbial metabolite of DMA^{V}, which is nowadays strongly discussed to be thio-DMA^{V}, induced chromosomal aberrations as well as sister chromatid exchange, mitotic arrest, and tetraploids [41]. In this study, thio-DMA^{V} and especially DMA^{III} additionally increased the formation of multinucleated and binucleated cells, which most probably results from spindle abnormalities induced by these arsenic species [25, 42]. Furthermore, the increased formation of binucleated cells indicates an inhibitory effect of DMA^{III} and thio-DMA^{V} on cytokinesis. Accordingly, in the same concentration range, both arsenicals caused a G2/M cell cycle phase arrest after 24 h incubation in A549 cells (data not shown). For thio-DMA, this has already been shown before in human HepG2 hepatocarcinoma cells [25] and A431 epidermoid carcinoma cells [26].

When rating the formation of micronuclei, micronucleated and binucleated cells, it once again has to be clearly stated that all these effects were restricted to high concentrations of arsenite, thio-DMA^V, and DMA^{III}. Strong effects were observed exclusively for DMA^{III}, with a sevenfold increase in micronuclei induction, a tenfold increased occurrence of binucleated cells, and an 80-fold increase in multinucleated cells after 24 h incubation with 5 μM DMA^{III}. Thus, in case of DMA^{III}, these effects most likely trigger DMA^{III} cytotoxicity, especially regarding the endpoint colony forming ability. This is unlikely for thio-DMA^V and even more unlikely for arsenite.

In summary, thio-DMA^V seems to exert its high cellular toxicity by a different mode of action than arsenite, MMA^{III}, and DMA^{III}. Our data strongly indicate that in human A549 lung cells, thio-DMA^V does not act via a genotoxic mode of action. Nevertheless, to assess the role of thio-DMA^V in inorganic arsenic-induced carcinogenicity, to date still too little is known about thio-DMA^V. This is particularly valid as thio-DMA^V is a human metabolite not only of inorganic arsenic but also of seafood related organic arsenicals, which indicates that further mechanistic studies are urgently needed to identify its toxic mode of action and finally assess the potential implications for human health.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Production of Toxic Volatile Trimethylbismuth by the Intestinal Microbiota of Mice

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The biotransformation of metals and metalloids into their volatile methylated derivatives by microbes growing under anaerobic conditions (e.g., the mammalian intestinal microbiota) plays an important role in spreading these compounds in the environment. In this paper, we could show that the presence of an intact intestinal microbiota of mice provides the *conditio sine qua non* for the production of these mostly toxic derivatives. To document the indispensible role of the intestinal microbiota in methylating metals and metalloids to volatile derivatives under *in vivo* conditions, we compared the methylation capability of conventionally raised (CONV) and germ-free (GF) B6-mice fed with chow containing colloidal bismuth subcitrate (CBS) as the starting material for the formation of volatile methylated metal(loid)s. Permethylated volatile trimethylbismuth ($(CH_3)_3Bi$) was only detected in the blood of the conventionally raised mice. Concomitantly, a higher bismuth concentration was found in organs such as liver, lung, testicles, and brain of the CONV mice as compared to those of GF mice ($P > 0.01$), strongly suggesting a correlation between the intestinal biomethylation of bismuth and its accumulation in mammalian tissues.

1. Introduction

The biotransformation of metals and metalloids into their volatile methylated derivatives raises ecological and health concerns, since this process increases the toxicity and distribution of the respective elements in our environment (e.g., As, Sb, Te, and Bi) [1–7]. Microorganisms, in particular methanoarchaea growing under anaerobic conditions (e.g., in wetlands, lake sediments, sewage sludge, landfills), are responsible for this process [2, 8–12]. Recent reports of our lab indicate that these transformations are also catalyzed by microbiota originating from the mammalian intestine: we detected the *ex situ* production of volatile metal(loid)s in human feces samples and in contents of isolated gut fragments of conventionally raised (but not of germ-free) mice after ingestion of bismuth containing pharmaceuticals [11] or in pure cultures of common gut methanoarchaea and bacteria [13]. As the toxicity of trimethylbismuth (TMBi) was found to be approximately two orders of magnitude higher in comparison to inorganic bismuth both in animal experiments [5] as well as in more recent studies on cytotoxicity [4], the production of methylated bismuth species by intestinal biomethylation is of potential health concern.

However, all studies to date concerning the role of the intestinal microbiota in the methylation of metals and metalloids were performed under *ex situ* conditions, physiological conditions within the living organisms have been disregarded so far. Our present study addresses this deficit by analyzing the role of the intestinal microbiota in transforming metal(loid)s to volatile methylated derivatives *in vivo* by comparative studies of conventionally raised (CONV) and germ-free (GF) mice fed with colloidal bismuth subcitrate (CBS) containing chow. After a feeding period of 14 days, the formation of volatile bismuth compounds was followed by determining their content in the blood of the animals using purge-and-trap gas chromatography hyphenated to

element-specific detection by inductively coupled plasma mass spectrometry (PT-GC/ICP-MS). Moreover, the effect of the intestinal microbiocenosis on the uptake of bismuth was studied by analyzing the total content of bismuth in the organs. Conventionally raised mice, which were not fed with bismuth containing chow, were used as control group.

Bismuth was used as model starting material for metal(loid) methylation in the present study because of its common presence in our environment due to its importance in technological application (e.g., as additive in cosmetics, catalysts, industrial pigments, alloys and ceramics [14]) and in health care (as antimicrobial agent in pharmaceuticals for the treatment of peptic ulcers caused by *H. pylori*). For a long time, bismuth was thought to be a less toxic element but since the 1970s it is well accepted that high-dosed medical treatment with bismuth subgallate and subnitrate can cause severe encephalopathies, renal failure, and genotoxic effects [15–17]. However, the clinical use of CBS does not appear to cause neurotoxicity [18].

2. Materials and Methods

2.1. Handling and Feeding of the Mice. Feeding experiments were conducted with CONV and GF B6-mice (Mus musculus, males, ages 12 to 17 weeks). All experiments were carried out in accordance with the German animal welfare act for animal experiments. Animal husbandry for CONV mice was performed by the Central Animal Laboratory of the University Hospital Essen, and GF mice were bred in the Animal Center of the University of Ulm and kept under sterile conditions in a germ-free isolator.

The animals were housed individually in standard Macrolone cages type III (38 by 22 by 15 cm) with Softwood bedding material (type S 3/4, Sniff GmbH, Soest, Germany) under standard laboratory conditions (12-h light-12-h dark cycle with lights on at 8 a.m.; temperature, 21 +/− 1°C; relative humidity, 55% +/− 10%). Both CONV and GF mice were fed with an autoclaved, standard diet enriched with colloidal bismuth subcitrate (CBS) obtained from De-Noltab (Yamanouchi Europe B.V., The Netherlands) with a final bismuth content of 50 mg kg^{-1} (SNIFF V1534Bi, SNIFF GmbH, Soest, Germany). The diet and bottled tap water were available *ad libitum*. The bismuth uptake of mice during the bismuth-enriched diet was calculated to be approximately 8.3 mg kg^{1} day^{-1}. Conventionally raised mice fed with a autoclaved, commercial standard mouse diet without CBS (SNIFF V1534) served as control animals.

The feeding experiments were conducted for 14 days in three groups:

(i) CONV control mice (three individuals) fed with standard diet,

(ii) CONV mice divided in 8 subgroups with 3 individuals each (24 CONV mice fed with a bismuth-enriched diet),

(iii) GF mice divided in 7 subgroups with 3–5 individuals each (28 GF mice fed with bismuth-containing chow).

2.2. Sampling of Blood and Organ Tissues of the Mice. After 14 days of feeding with standard or CBS-containing diet, the mice were anesthetized with a ketamine/xylazine-narcosis (120 mg kg^{-1} body weight or 13 mg kg^{-1} body weight, resp.; i.p.). The blood was extracted via cardiac puncture under sterile conditions and transferred immediately into autoclaved, helium-filled, and gas-tight-sealed 120 mL glass vials containing A. bidest with 1‰ anti-foaming agent (antifoam 289 Sigma-Aldrich, Taufkirchen, Germany) and heparin (750 IE mL^{-1}, Ratiopharm, Ulm, Germany). To increase the sample volume for a reliable determination of the volatile derivative, the blood of three to five mice was pooled in subgroups with a total volume of 3-4 mL blood. After cervical dislocation, the mice were dissected and the organs (liver, lung, kidneys, testicles, and brain) were removed and stored at −80°C until analysis of total bismuth concentration in these tissues. Speciation analysis was performed within 12 h after sampling.

2.3. Speciation and Quantification of Volatile Methylated Bismuth. The volatile bismuth in the headspace of the blood samples was analyzed using a modified purge-and-trap gas chromatographic system (PT-GC) coupled to an inductively coupled plasma mass spectrometer (ICP-MS) (Fisons VG, Plasma Quad II) as an element-specific detector as described previously [8]. The bismuth species eluting from the GC column were detected online by ICP-MS at a mass/charge ratio of *m/z* 209, and the identity of the detected volatile species was ensured by boiling point chromatographic retention time correlation (boiling point of $(CH_3)_3Bi$ at 760 mmHg = 108.8°C resulting in a retention time of 150 s under the given conditions). The limit of detection (LOD) of the analyte was calculated with the 3σ criterion based on the noise of the baseline, that is 1.5 fmol for bismuth. The whole headspace of the vials (~105 mL depending on the volume of the extracted blood) was purged and trapped for five minutes. The completeness of the $(CH_3)_3Bi$ removal from the blood was verified by repetition of the purge and trap procedure. The content of $(CH_3)_3Bi$ was calculated per g blood wet weight.

2.4. Total Metal Analysis of Organ Tissues. The bismuth content of the organs was determined after homogenizing the tissues using the Microdismembrator S (Sartorius, Göttingen). The homogenates were dried at 110°C to constant weight. Aliquots (0.1 to 0.5 g) of the dried homogenates were suspended in 4 mL HNO_3 (65%, subboiled) and 2 mL H_2O_2 (30%, Suprapur; Merck, Darmstadt, Germany), digested in a microwave-accelerated reaction system (MARS 5; CEM, Kamp Lintfort, Germany) and analyzed by ICP-MS-analysis as described previously [11]. Total metal content was referred to g dry weight. For statistics, the nonparametric Mann-Whitney-Wilcoxon *U* test was used.

3. Results and Discussion

3.1. The Transformation of Bismuth to Its Permethylated Volatile Derivative $(CH_3)_3Bi$ in Mice Requires an Intact Intestinal Microbiota. To evaluate the role of the intestinal microbiota

Table 1: $(CH_3)_3Bi$ content in blood samples of CONV and GF mice fed with CBS containing chow and CONV control mice on standard diet. The absolute limit of detection (LOD) for $(CH_3)_3Bi$ based on the 3σ criterion was 1.5 fmol, whereas the relative LOD was between 4.5 and 6 fmol g^{-1}.

CONV mice		GF mice		CONV control mice	
Subgroup	fmol g^{-1} (w. w.)	Subgroup	fmol g^{-1} (w. w.)	Subgroup	fmol g^{-1} (w. w.)
1	15.1	1	LOD	1	LOD
2	16.8	2	LOD	2	LOD
3	71.4	3	LOD	3	LOD
4	8.4	4	LOD		
5	6.2	5	LOD		
6	35.1	6	LOD		
7	12.3	7	LOD		
8	7.4				

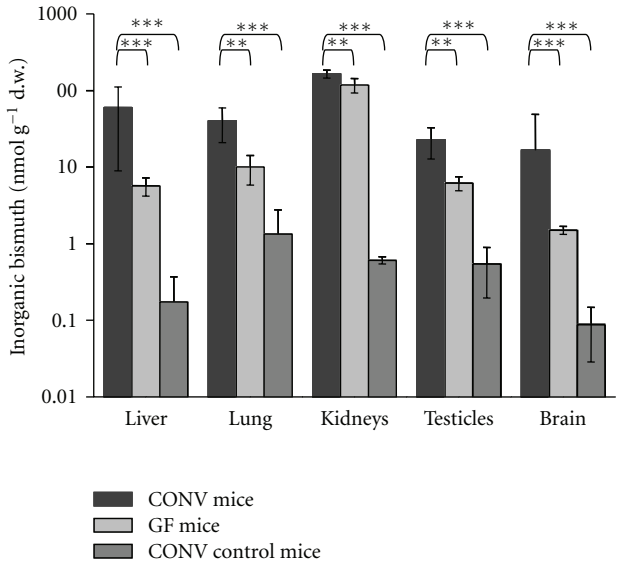

Figure 1: Bismuth content (means ± SD (nmol g^{-1} d. w.)) of organ tissues of CONV mice and GF mice fed with CBS containing chow as well as that of the CONV control mice fed with a standard diet. (Significance levels: $^{***}P < 0.001$, $^{**}P < 0.01$.)

of mice in transforming metal(loid)s into their volatile methylated derivatives under *in vivo* conditions, we determined the content of volatile bismuth compounds in the blood of CONV mice (i.e., mice with intact intestinal microbiota) compared to that of GF mice, both fed with CBS-enriched chow. For that purpose, the blood of the mice was extracted via cardiac puncture and analyzed for volatile bismuth compounds after a feeding period of two weeks. As shown in Table 1, permethylated $(CH_3)_3Bi$ was the only volatile compound and it could exclusively be found in the blood of CONV fed with CBS-enriched chow mice. Neither in the blood of GF mice fed with CBS-enriched chow nor in the blood of CONV mice fed without CBS-enriched chow (CONV control mice) $(CH_3)_3Bi$ could be observed. This result clearly indicates that the presence of the intestinal microbiota is indispensible for the transformation of metal(loid)s into their volatile-methylated derivatives. Most probably, the microbiota itself is responsible for the transformation under *in vivo* conditions as already suggested by various *ex situ* analyses of cultures derived from the intestinal microbiota [11, 13]. We cannot, however, exclude that epithelial and/or mesenchymal cells of the mice partly contribute to these transformation processes under *in vivo* conditions implying, however, that the intact intestinal microbiota induces such an activity.

Previous studies of intestinal biovolatilization of bismuth in human fecal samples and in human breath after ingestion of bismuth have shown an extremely high interindividual variability of 5 [11] and, 3 orders of magnitude [19], respectively. In this study, the variation of the $(CH_3)_3Bi$ content observed in the blood of the different subgroups of the CONV mice is in accordance with previous studies [11] ranging from 6.2 to 71.4 fmol g^{-1} w.w. with a mean value of 21.6 ± 22.1 fmol g^{-1} w.w. ($n = 8$). Considering the highly standardized conditions of animal husbandry applied in this study, this relatively high variability probably reflects differences in the composition of the intestinal microbiota and its metabolic activity across the test population.

3.2. Methylation of Bismuth Is Accompanied with an Accumulation of Bismuth in Organ Tissues. To examine the impact of the methylation on the absorption of bismuth by the mammalian organism, which could cause toxic effects of the bismuth derivatization, the bismuth content of liver, lung, kidneys, testicles, and brain of CONV and GF mice was investigated by microwave digestion and subsequent analysis by ICP-MS. Interestingly, the bismuth content of all organ tissues tested was significantly higher in CONV mice as compared to GF mice (Figure 1), indicating that the intestinal microbiocenosis increases the bioavailability of bismuth from the intestine. While mobilization of bismuth by chelation would presumably play a major role in this process, the formation of methylated bismuth species produced in the CONV mice can significantly contribute. At first, methylated metal(loid) species can more easily permeate through cell membranes due to their higher hydrophobicity—accumulated in the cell by internal complexation with various compounds—and thus permanently

detracted from the blood. Next, the formation of permethylated trimethylbismuth proceeds via partly methylated mono- and dimethyl bismuth, which are not accessible via GC-ICP-MS due to their low volatility, but can also contribute to the increased bioaccessibility of bismuth due to biomethylation.

The comparably high content of bismuth in the kidneys of both CONV mice and GF mice is probably due to the fact that the main part of inorganic bismuth is excreted via the urinary system in mammals [17]. In the organ tissues of the CONV control mice fed with chow without bismuth addition, only low amounts of bismuth close to the LOD were detected as expected for ICP-MS-analysis.

4. Conclusion

The observation that only CONV mice possessing an intact intestinal microbiota but not GF mice are able to transform bismuth to its permethylated derivative $(CH_3)_3Bi$ proves the indispensable role of the intestinal microbiota in methylating metals and metalloids to their volatile derivatives in the living organism.

The detection of volatile $(CH_3)_3Bi$ in blood of CONV mice fed with CBS containing chow could be of some relevance for healthiness. Obviously, the methylation of bismuth to its volatile derivative $(CH_3)_3Bi$ promotes the dispersal of the metal in the mammalian organism via the blood resulting in a significant accumulation of bismuth in organ tissues, presumably due to its increased hydrophobicity caused by methylation, which increases interactions with and penetration of cell membranes. To evaluate the resulting health risk, the bismuth species accumulated in tissues throughout the organism and its inter- and intracellular interactions has to be determined.

Abbreviations

LOD:	Limit of detection
$(CH_3)_3Bi$:	Trimethylbismuth
CBS:	Colloidal bismuth subcitrate
CONV mice:	Conventionally raised mice
GF mice:	Germ-free mice
PT-GC system:	Purge-and-trap gas chromatographic system
ICP-MS:	Inductively coupled plasma mass spectrometer.

Acknowledgment

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Research Article

Toxicity of Volatile Methylated Species of Bismuth, Arsenic, Tin, and Mercury in Mammalian Cells *In Vitro*

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The biochemical transformation of mercury, tin, arsenic and bismuth through formation of volatile alkylated species performs a fundamental role in determining the environmental processing of these elements. While the toxicity of inorganic forms of most of these compounds are well documented (e.g., arsenic, mercury) and some of them are of relatively low toxicity (e.g., tin, bismuth), the more lipid-soluble organometals can be highly toxic. In the present study we investigated the cyto- and genotoxicity of five volatile metal(loid) compounds: trimethylbismuth, dimethylarsenic iodide, trimethylarsine, tetramethyltin, and dimethylmercury. As far as we know, this is the first study investigating the toxicity of volatile metal(loid) compounds *in vitro*. Our results showed that dimethylmercury was most toxic to all three used cell lines (CHO-9 cells, CaCo, Hep-G2) followed by dimethylarsenic iodide. Tetramethyltin was the least toxic compound; however, the toxicity was also dependend upon the cell type. Human colon cells (CaCo) were most susceptible to the toxicity of the volatile compounds compared to the other cell lines. We conclude from our study that volatile metal(loid) compounds can be toxic to mammalian cells already at very low concentrations but the toxicity depends upon the metal(loid) species and the exposed cell type.

1. Introduction

Biomethylation of metals and metalloids by microorganisms is a widespread phenomenon in anaerobic habitats including waste deposits, sewage sludge, and alluvial soils [1–3]. The stepwise methylation results in both partly methylated nonvolatile species as well as fully methylated volatile metal(loid) compounds. Considering the direct exposure to humans, the formation of volatile metal(loid) compounds by the intestinal biocenosis has attained considerable attention in the last years [4]. *In vivo* studies showed that after ingestion of bismuth subcitrate, the metal will be methylated by microbes in the gut and volatile trimethylbismuth (Me_3Bi) can be detected in blood and breath [5]. Furthermore, arsenic, selenium, tellurium, and antimony were volatilized by the microbiocenosis of an *in vitro* model of the human intestinal microbiota [6]. Hollmann et al. have shown that colloidal bismuth subcitrate (CBS) as well as bismuth cysteine is methylated by human liver cells *in vitro* [7].

Whereas for the toxicity of nonvolatile methylated metal(loid) species, research has successively intensified in particular for arsenic [8–12] and mercury [13–15], little conclusive data are available in case of volatile species. The genotoxicity of volatile arsines has been a subject of several studies. Dimethylarsine (Me_2AsH) induced DNA damage in human embryonic cells by formation of a peroxyl radical $(CH_3)_2AsOO_*$ [16]. Furthermore, Kato et al. showed that trimethylarsine (Me_3As) induced micronuclei in the bone marrow of mice after intraperitoneal injections of 8.5 and 14.7 mg/kg [17]. These findings were confirmed by Andrewes et al. [18] who investigated the DNA-damaging potential of Me_2AsH and Me_3As using supercoiled DNA. They concluded that the latter two arsines are about 100 times more potent than the most genotoxic nonvolatile arsenical, dimethylarsinous acid (Me_2AsOH).

In comparison to nonvolatile species, volatile compounds demand a more complex experimental design and careful handling of the substances. Moreover, most studies focus on the toxicity of one compound or several compounds

from one element, which makes a comparison between volatile organometal(loid) species difficult due to the different experimental systems used.

In this study, we aimed to comparatively investigate the cytotoxic and genotoxic effects of the volatile metal(loid) compounds trimethylbismuth (Me_3Bi), dimethylarsenic iodide (Me_2AsI), trimethylarsine (Me_3As), tetramethyltin (Me_4Sn), and dimethylmercury (Me_2Hg).

For our studies, we developed an exposure system dedicated for the exposure to volatile organometal(loid) species. Three different cell types were chosen for toxicity testing: CHO-9 cells—an established cell system for toxicity testing, CaCo cells—human colon cells, and HepG2 cells—human hepatic cells. Same cell types were used in previous studies investigating cellular uptake and toxicity of nonvolatile organic and inorganic metal(loid) compounds [19–23]. To the best of our knowledge, this is the first study testing the toxicity of these volatile metal(loid) species *in vitro*.

2. Material and Methods

2.1. Cell Cultures

2.1.1. Human Hepatoma Cells. (HepG2) (ATCC, HB 8065) were cultured in minimal essential medium (MEM) with Earle's BSS and sodium bicarbonate (CC, PRO, Germany) supplemented with 10% heat-inactivated FCS (Gibco), nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), and 100 IU/mL penicillin/streptomycin (CC, PRO).

2.1.2. Human Colon Cells. (CACO-2) (ATCC 169) were cultured in 75% MEM with 20% FCS, 5% nonessential amino acids (0.1 mM), 1% L-Glutamine, and 0.5% gentamycin.

2.1.3. Chinese Hamster Ovary Cells. (CHO) were purchased from ECACC (UK, Cat. no. 85050302) and grown in Ham's F12 medium (CC, PRO) supplemented with 10% FCS, and 100 IU/mL penicillin/streptomycin (CC, PRO).

All the adherent growing cell lines were kept at 37°C in a 5% CO_2 atmosphere.

Prior to exposure approximately 2×10^6 cells were placed on the membrane of cell culture inserts (ThinCerts, 0.4 μm membrane, transparent; Greiner bio-one, Germany) with 3 mL of their respective medium for 24 h.

2.2. Reagents (Metal(loid) Compounds). All volatile organic metal(loid) compounds were of analytical grade unless stated otherwise and were either synthesized in the Institute of Environmental Analytical Chemistry or purchased from the following suppliers: trimethylbismuth (Me_3Bi) from VeZerf (Idar-Oberstein, Germany), trimethylarsine (Me_3As) from Sigma-Aldrich (Taufkirchen, Germany), tetramethyltin (Me_4Sn) from Strem Chemicals (Kehl, Germany), and dimethylmercury (Me_2Hg) from Acros Organics (Geel, Belgium). Dimethylarsine (Me_2AsI) was synthesized as described in Styblo et al. [24]. Briefly, to 30 mL of an aqueous solution of dimethylarsenic acid ($(CH_3)_2AsO(OH)$) and potassium iodide (KI) concentrated sulphuric acid was added. For the reduction step, SO_2 was bubbled through the mixture and a yellow oil ($(CH_3)_2AsI$) was separated after distillation. Identification was performed by ^{1}H-NMR and GC-MS analysis (data not shown). Boiling points of all used metal(loid) species are given in Table 1.

Figure 1: Modified glass flask for exposure of cells to volatile metal(loid) species. Cells were grown on permeable membranes. Exposure occurred through the membrane.

2.3. Exposure of Cells. For exposure of cells to the volatile organometal(loid) species, the ThinCert cell culture inserts were placed in 1000 mL glass flasks equipped with a Teflon screw cap and two plug valves in order to allow purging of the gas phase. Additionally, a septum screw cap for injection of the volatile test substances was fitted at the lower end of the glass flasks. To fix a ThinCert cell culture insert into the headspace of the exposure glass flask, a suitable glass rack was designed. During exposure, the flasks were stored in an incubator at 37°C (Figure 1). The culture medium was buffered with HEPES (25 mM) (CCPro GmbH, Oberdorla, Germany).

Before exposure, the glass flask was closed and purged with argon for at least 3 minutes to purge oxygen out of the bottle because especially trimethylbismuth is extremely oxygen sensitive. Afterwards different amounts of one metal(loid) were injected through the septa screw cap and cells were exposed for 1 h. This time point was chosen because of results from previous studies which showed that longer exposure times than 1 h caused a high degree of cytotoxicity (data not shown). Exposure concentrations were chosen according to the toxicity of the volatile species. Highly toxic species required lower concentrations than nontoxic species. The concentration range was evaluated in preexperiments (data not shown). After exposure, treated cells were harvested with trypsin (0.05%) (Sigma) for the trypan blue test and the comet assay.

Control experiments with Me_3As verified that the cells are exposed through the membrane and not through the culture medium, as no cytotoxic effect was observable when a nonpermeable cover was placed below the membrane (data not shown).

2.4. Trypan Blue Test. To detect cytotoxicity in exposed cell cultures, cell viability was evaluated with the trypan blue

Table 1: Boiling points of volatile metal(loid) species.

Compound	Abbreviation	Boiling point	Reference
Tetramethyltin	Me_4Sn	78°C	(Hoeppner et al., 1964)
Dimethylarsenic iodide	Me_2AsI	155–160°C	(Lee et al., 1923)
Trimethylarsine	Me_3As	51–53°C	(Dyke and Jones, 1930)
Trimethylbismuth	Me_3Bi	107.1°C	(Bamford et al., 1946)
Dimethylmercury	Me_2Hg	92°C	(Wilde, 1949)

test immediately after exposure of cells. The cell suspension was mixed with an equivalent volume of 0.4% trypan blue solution (Sigma) and subsequently evaluated under the light microscope. The membrane of dead cells is permeable to trypan blue (blue stained cells), whereas living cells remain unstained. Cell viability is expressed as percentage of surviving cells compared to the total number of cells:

$$\%\ \text{viable cells} = \frac{\text{unstained cells}}{\text{unstained} + \text{stained cells}} \times 100. \quad (1)$$

All experiments were repeated at least twice and significance was calculated by the Student's t-test. To compare the toxicity of the different metal(loid) compounds, LC_{50} values (lethal concentration to 50% of the cells) were calculated.

2.5. Alkaline Comet Assay. DNA damage was tested using the Alkaline Comet Assay, first described by Ostling and Johanson [25]. The Comet Assay is a sensitive microgel electrophoresis technique to detect DNA damage in single cells [25]. The assay was performed as described by Singh et al. [26] with minor modifications. In short, microgels were prepared by sticking a chamber slide (Chamber Slides Lab-Tek II, Nalgene Nunc International, Rochester, USA) with eight chambers to a GelBond film (Lonza GmbH, Cologne, Germany). Each chamber was sealed by adding 50 μL of 0.75% low melting point (LMP) agarose (Invitrogen GmbH, Invitrogen GmbH, Germany). 45 μL of LMP agarose were mixed with 20 μL cell suspension containing 8,000 cells. After solidification, cells were lysed overnight at 4°C in freshly prepared lysis solution. Prior to electrophoresis, the slides were incubated in electrophoresis solution for 20 min. Electrophoresis was performed at 300 mA for 20 min and at 4°C. Then the slides were kept in neutralisation solution for 30 min and further transferred to absolute ethanol for 2 h before the gels were left to dry overnight. The DNA was stained for 15 min using SYBR Green and the extent of DNA damage was analysed at a 40x magnification using the Comet Assay IV software (Perceptive Instruments, UK) and a CCD camera attached to a Leica Microscope. Statistical analysis was done using the Mann-Whitney test. The data of three individual experiments have been summarized and are plotted using their mean value and the standard error of mean.

3. Results

3.1. Cyto- and Genotoxicity of Me_2Hg. In comparison to the tested metal(oid) compounds, Me_2Hg was the most cytotoxic and induced 50% cell death (LC_{50}) in CHO-9 cells already at the lowest concentration tested (10.8 μmol/L_{gv}) (Figure 2). The Comet Assay was not applicable in CHO-9 cells because the lowest tested concentration of Me_2Hg was already cytotoxic to the cells. Due to the technical limitation of the minimal applicable droplet size, the applied concentration could not be reduced. Because of its extraordinary toxicity, not all cell lines were exposed to dimethyl mercury. Then, we abstained from exposure of the other cell lines to dimethyl mercury. The LC_{50} value for Me_2Hg in CaCo cells was higher than in CHO-9 cells (40 μmol/L_{gv}), indicating a higher resistance of colon cells to the toxic compound than fibroblasts (Table 2).

Table 2: LC_{50} values of the investigated volatile metal(loid) compounds in different cell lines (exposure time: 1 h). Concentrations are given in $\mu mol/L_{gv}$. n.ct.: not cytotoxic in the tested concentration range, n.t.: not tested.

	CaCo	CHO-9	HepG2
Me_4Sn	170.7	n.ct.	n.ct.
Me_2AsI	334.5	11.2	10.8
Me_3As	128.8	450	85.9
Me_3Bi	110.0	128.0	194.0
Me_2Hg	40.0	10.8	n.t.

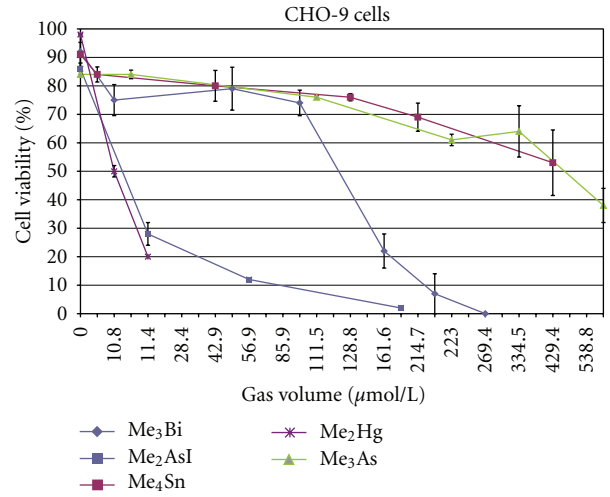

Figure 2: Comparison of cytotoxicity of 5 different metal(loid) compounds (Me_3Bi, Me_4Sn, Me_3As, Me_2AsI, and Me_2Hg) in CHO-9 cells. The experiments were repeated twice.

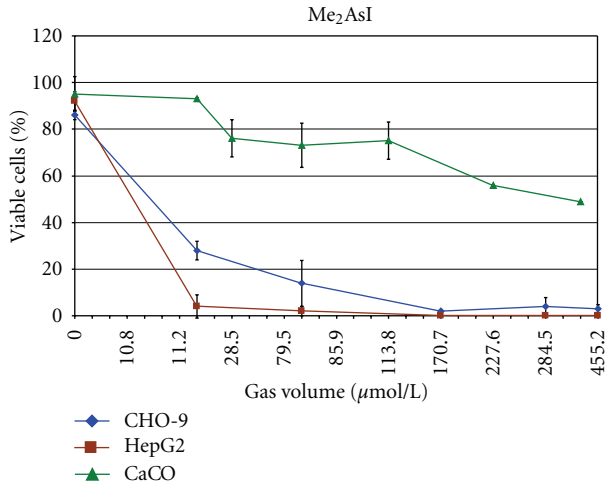

Figure 3: Cytotoxicity of Me_2AsI in CHO-9, HepG2, and CaCo cells. The experiments were repeated three times.

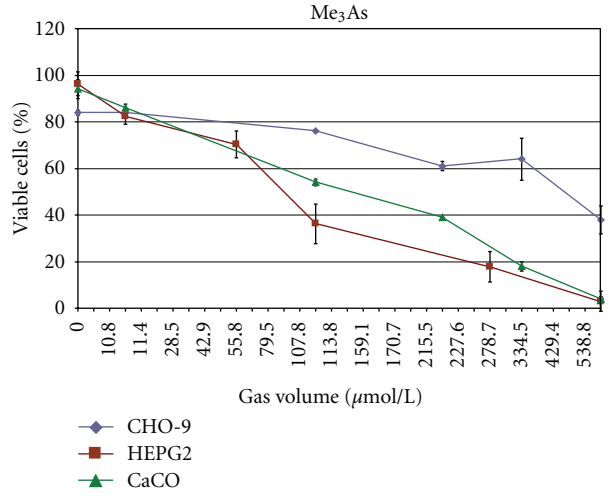

Figure 4: Cytotoxicity of Me_3As in CHO-9, HepG2, and CaCo cells. The experiments were repeated three times.

3.2. Cyto- and Genotoxicity of Me_2AsI and Me_3As. Me_2AsI was highly cytotoxic in HepG2 cells (LC_{50}: 10.8 μmol/L_{gv}) and CHO-9 cells (LC_{50}: 11 μmol/L_{gv}), whereas cytotoxicity in CaCo cells was considerably lower (LC_{50}: 335 μmol/L_{gv}) (Figure 3, Table 2). Similar to Me_2Hg, testing of genotoxicity was not possible because of technical limitations in application of lower concentrations.

Me_3As was cytotoxic in all three cell lines. HepG2 cells were most sensitive (LC_{50}: 86 μmol/L_{gv}) followed by CaCo cells (LC_{50}: 129 μmol/L_{gv}) and CHO-9 cells (LC_{50}: 450 μmol/L_{gv}) (Figure 4, Table 2). There were no significant genotoxic effects in CHO-9 cells detectable up to a concentration of 334 μmol/L_{gv} (Figure 5). The highest tested concentration of 557 μmol/L_{gv} induced significantly elevated tail moments in the comet assay, however, the cytotoxicity was reduced below 50% in these experiments.

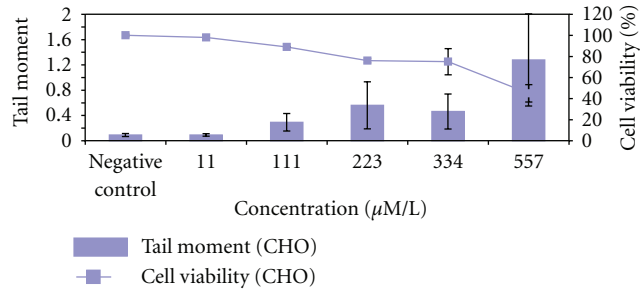

Figure 5: Genotoxicity of Me_3As in CHO-9 cells after 1 h exposure time measured by Comet-Assay. The tests were repeated three times.

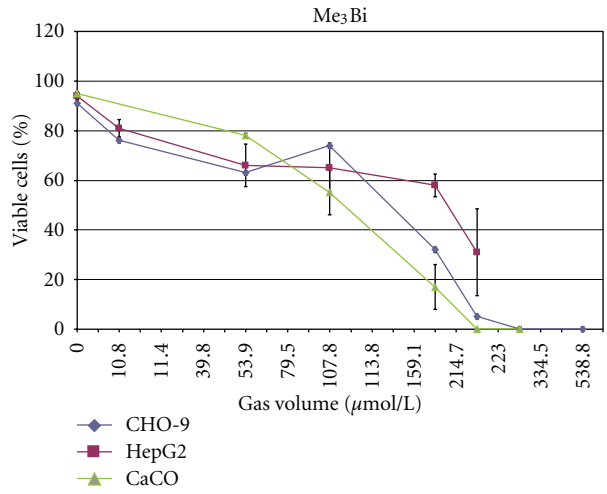

Figure 6: Cytotoxicity of Me_3Bi in CHO-9, HepG2, and CaCo cells. The experiments were repeated three times.

3.3. Cyto- and Genotoxicity of Me_3Bi. The volatile Me_3Bi was cytotoxic in all three tested cell lines (Figure 6).

CaCo cells were the most sensitive cell line (LC_{50}: 110 μmol/L_{gv}), followed by CHO-9 cells (LC_{50}: 128 μmol/L_{gv}) and HepG2 cells (LC_{50}: 194 μmol/L_{gv}) (Table 2). Results of the Comet-Assay revealed that Me_3Bi was genotoxic at concentrations >108 μmol/L_{gv} (Figure 7). However, at higher concentrations (162 and 216 μmol/L_{gv}) Me_3Bi was cytotoxic and thus genotoxic results were not evaluable anymore.

3.4. Cyto- and Genotoxicity of Me_4Sn. Me_4Sn did not show a high level of cytotoxicity and induced 50% cell death (LC_{50}) just in CaCo cells at a concentration of 170.7 μmol/L_{gv}. In CHO-9 and HepG2 cells, the cell viability was not reduced below 50% up to a tested concentration of 429.4 μmol/L_{gv} and 161.7 μmol/L_{gv}, respectively (Figure 8, Table 2).

Genotoxic effects in CHO-9 cells measured by Comet-assay were not significantly elevated after Me_4Sn exposure compared to the untreated control (Figure 9).

4. Discussion

From the metal(loid)s tested in this study, mercury is undoubtedly the most intensively investigated species, but

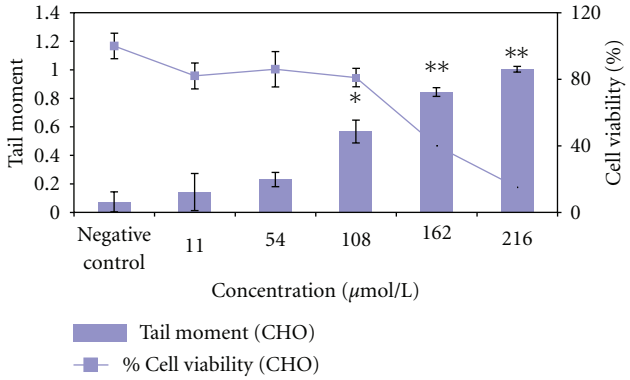

Figure 7: Genotoxicity of Me_3Bi in CHO-9 cells after 1 h exposure time measured by Comet-Assay. The tests were repeated three times. $^*P \leq 0.05$; $^{**}P \leq 0.01$.

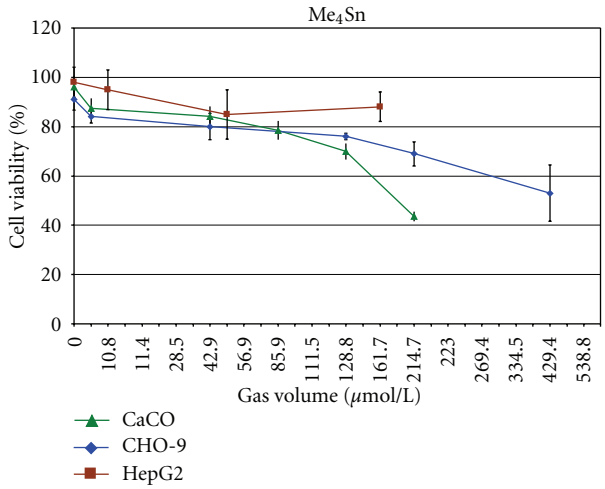

Figure 8: Cytotoxicity of Me_4Sn in CHO-9, HepG2, and CaCo cells. The experiments were repeated three times.

this applies only to elemental and monomethyl mercury but not to the dimethylated species. In our study, Me_2Hg was highly cytotoxic in CHO-9 and CaCo cells. Further studies regarding genotoxicity were not possible because of the high toxicity of Me_2Hg. The extraordinary toxicity of dimethylmercury is at least known since the death of Karen Wetterhahn in 1997, months after spilling no more than a few drops of this compound on her latex-gloved hand [27]. The reason for its extraordinary toxicity is the ability of this lipophilic compound to penetrate the cell membrane. Numerous studies have implicated a molecular mimicry in the uptake of thiol conjugates in selective target cells [28]. Ehrenstein et al. reported a negligible mercury concentration of mercury inside CHO cells after treatment with dimethylmercury [29]. The authors suggest from their study that the volatile mercury species escapes from the treatment solution before it can pass the cell membrane. In our experimental setup, the cells are directly and continuously exposed to the gaseous compound.

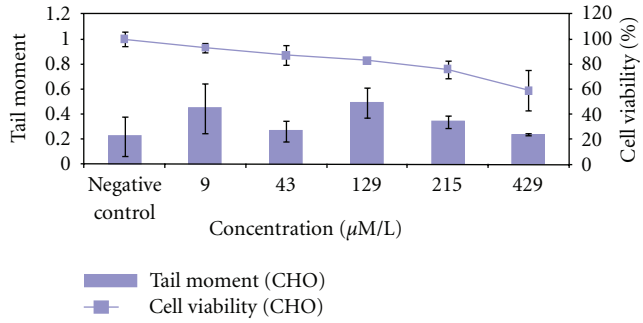

Figure 9: Genotoxicity of Me_4Sn in CHO-9 cells after 1 h exposure time measured by Comet-Assay. The tests were repeated three times.

The toxicity of the volatile arsenic compounds Me_2AsI and Me_3As were studied in the present experiments. In both cell lines (CHO-9 and CaCo-2 cells), Me_2AsI exhibited a very high cytotoxicity similar to Me_2Hg. In comparison to nonvolatile Me_2AsOH, which is among the most toxic arsenic species reported [12], similar levels of toxicity were found when comparing the LC_{50}-concentrations of gas (gv) and liquid (lv) volumes, respectively (Table 3). Furthermore, Me_3As showed a significant cytotoxicity and genotoxic effects in contrast to the nonvolatile pentavalent form, Me_3AsO, which was not cytotoxic at the concentrations tested (Table 3).

Unexpectedly, we found significant differences between the different cell lines used. In particular, the cytotoxicity of CaCo cells towards Me_2AsI (LC_{50}: 335 $\mu mol/L_{gv}$) was a factor of 30 lower than that found in CHO-9 and HepG2 cells. Contrary to Me_2AsI, CHO-9 cells were a factor of 4 to 5 less susceptible to Me_3As than CaCo and HepG2 cells, respectively. The low susceptibility of CaCo towards Me_2AsI could be attributed to the ability of CaCo cell to express MRP2, a multidrug resistance protein capable of catalysing as efflux [30]. The different behaviours of Me_2AsI and Me_3As indicate different mechanisms of their toxicological action.

Methylated arsenic (III) species have been shown to be genotoxic in several test systems [18, 31–33] and are potent clastogens [34]. In the present experiments, we could not evaluate the genotoxicity of Me_2AsI and Me_3As because of its cytotoxicity at minimal applicable concentrations. Me_3As showed significantly elevated tail moments only at cytotoxic concentrations, thus a genotoxicity testing was also not possible.

The nonvolatile bismuth species monomethylbismuth was already tested for cyto- and genotoxicity in human cells in an earlier study [23]. The results showed that the trivalent monomethylbismuth (MeBi(III)) exerted cytotoxicity even in micromolar concentrations in human hepatocytes (LC_{50}: 350 μM) after 1 h exposure. In the present study, the cytotoxic effect of the volatile Me_3Bi in CaCo, CHO, and HepG2 cells confirmed the observation that methylated bismuth compounds are more toxic than inorganic bismuth compounds. The LC_{50} value in HepG2 cells was 194 $\mu mol/L_{gv}$ for Me_3Bi compared to 350 $\mu mol/L_{lv}$ for MeBi(III).

There seems to be a trend to an increased toxicity of methylated Bi compounds with augmented methyl groups

Table 3: Comparison of toxicity of volatile to nonvolatile species (exposure time: 1 h; n.t.: not tested).

Volatile compound	Cell type	LC_{50} μmol/L_{gv}	Nonvolatile compound	Cell type	LC_{50} μmol/L_{lv}	Literature
Me_4Sn	CHO	n.ct. (up to 400 μM)	Me_3SnCl	CHO	n.ct. (up to 5 mM)	[20]
Me_2AsI	CHO	11.2	Me_2AsOH	CHO	10	[19]
Me_2AsI	HepG2	10.8	Me_2AsOH	HepG2	18	[22]
Me_3As	CHO	450	Me_3AsO	CHO	n.ct. (up to 500 μM)	[19]
Me_3As	HepG2	85.9	Me_3AsO	HepG2	n.ct. (up to 5000 μM)	[22]
Me_3Bi	HepG2	194	MeBi(III)	HepG2	350	[23]
Me_2Hg	CHO	10.8	MeHgCl	CHO	n.t.	

in HepG2 cells. Cytotoxicity of a trialkylated bismuth compound has been detected until now only with triphenylbismuth in human embryonic lung fibroblasts [35]. In the experiments of von Recklinghausen et al. [23] with MeBi(III), the authors demonstrated that the compound is able to induce genomic damage in human lymphocytes by induction of a significant number of chromosomal aberrations and sister chromatid exchanges after 24 h exposure time. In the present experiments, we also detected DNA damage after an exposure of CHO-9 cells to Me_3Bi for 1 hour only. Also here, Me_3Bi seems to be more toxic than MeBi(III).

Recent studies with methylated tin compounds *in vitro* revealed a considerable toxicological potential of some organotin species but demonstrated clearly that the toxicity is modulated by the cellular uptake capability [20]. The highly hydrophobic and volatile compound Me_4Sn induced neither cytotoxicity detected by using the trypan blue test nor genotoxicity evaluated with the comet assay in CHO-9 cells up to a tested concentration of 429 μmol/L_{gv}.

5. Summary

In summary, the present study indicates that some volatile organometal(loid) compounds are able to exhibit a significant toxicity to mammalian cells. While exposure to volatile organometal(loid)s in the environment is relatively rare, the formation of these compounds in the intestine may contribute to the toxicity of ingested metal(loid)s.

In accordance to methylated volatile arsenic species, recent studies of our group indicated that the induction of cyto- and genotoxic effects caused by the nonvolatile trivalent methylated arsenic species is primarily dependent upon their ability to penetrate the cell membrane [12]. Likewise, we assume that the high cyto- and genotoxicity for volatile organometal(loid) compounds found in this study can be attributed to their ability to pass cell membranes.

The observation that the toxicity highly depends both upon the metal(loid) species and the exposed cell type indicates different mechanisms of their toxicological action, which need to be subject of further studies.

Abbreviations

Me_3Bi: Trimethylbismuth
Me_2AsI: Dimethylarsenic iodide
Me_3As: Trimethylarsine
Me_4Sn: Tetramethyltin
Me_2Hg: Dimethylmercury
gv: Gas volume
lv: Liquid volume
LC_{50}: Lethal concentration causing death of 50% of the cells
CaCo: Human colon cells
HepG2: Human hepatoma cells
CHO: Chinese hamster ovary cells
Me: Methyl group.

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Research Article

Toxicity of Methylated Bismuth Compounds Produced by Intestinal Microorganisms to *Bacteroides thetaiotaomicron*, a Member of the Physiological Intestinal Microbiota

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Methanoarchaea have an outstanding capability to methylate numerous metal(loid)s therefore producing toxic and highly mobile derivatives. Here, we report that the production of methylated bismuth species by the methanoarchaeum *Methanobrevibacter smithii*, a common member of the human intestine, impairs the growth of members of the beneficial intestinal microbiota at low concentrations. The bacterium *Bacteroides thetaiotaomicron*, which is of great importance for the welfare of the host due to its versatile digestive abilities and its protective function for the intestine, is highly sensitive against methylated, but not against inorganic, bismuth species. The level of methylated bismuth species produced by the methanoarchaeum *M. smithii* in a coculture experiment causes a reduction of the maximum cell density of *B. thetaiotaomicron*. This observation suggests that the production of methylated organometal(loid) species in the human intestine, caused by the activity of methanoarchaea, may affect the health of the host. The impact of the species to reduce the number of the physiological intestinal microbiota brings an additional focus on the potentially harmful role of methanoarchaea in the intestine of a higher organism.

1. Introduction

Biomethylation of metals and metal(loid)s is an important process which increases the mobility, bioavailability, and toxicity of these elements. Anaerobic microorganisms, in particular methanoarchaea, show the greatest versatility regarding the spectrum of elements that they methylate [1]. Considering human health, the biotransformation of harmless metals, such as bismuth, by the human intestinal microbiota is a highly relevant process. Due to the low toxicity of metallic bismuth and its inorganic salts, bismuth has been classified as a "green element" [2]. Bismuth is therefore widely used in a variety of applications such as cosmetics, catalysts, industrial pigments, and ceramic additives [3]. Bismuth is, however, associated with several adverse reactions such as encephalopathy, renal failure, and even cases of death in the 70s and 80s [4, 5]. It has been suggested that derivatives of this metal may be responsible for these damages.

Our recent studies have shown that, after ingestion of inorganic bismuth, the intestinal microbiota, in particular methanoarchaea, are capable of methylating inorganic bismuth to soluble paritally methylated compounds like monomethyl- (MMBi-) and dimethylbismuth (DMBi) as well as volatile trimethylbismuth (TMBi) [6–10]. TMBi is characterized by a higher volatility and hydrophobicity in comparison to inorganic bismuth and can therefore be easily distributed inside the human body and is able to pass the blood-brain barrier [11]. In mice fed with chow containing colloidal bismuth subcitrate (CBS), elevated concentration of TMBi in their blood and enrichment of Bi in several organs were detected [12, 13]. While the direct impact of the toxic methylated compounds on the physiology of the human body has already been addressed [14], the indirect negative effect of these compounds on the human health by inhibition of the growth of members of the beneficial intestinal microbiota has been little investigated so far.

To overcome this neglect, the influences of methylated bismuth species are investigated for a prominent member of this microbiota, notably for *Bacteroides thetaiotaomicron* [15]. This gram-negative anaerobic bacterium has some advantages for the host. For example, it has some versatile digestive abilities which benefit higher organisms [16]. Additionally, this microbe is important for the defense against the adverse activities of pathogenic microorganisms and guarantees the integrity of the intestine epithelium, thus ranking among the so-called physiological intestinal microbiota [17, 18]. In a previous study, this organism is found to be sensitive to permethylated bismuth, $(CH_3)_3Bi$ [1]. In this study, the toxic effects of partially and fully methylated bismuth derivatives on *B. thetaiotaomicron* were studied in more detail using an *in vivo*-like coculture system with the methanoarchaeum *M. smithii* as the producer of TMBi.

2. Results and Discussion

2.1. Influence of Colloidal Bi Subcitrate (CBS) on Growth of Cultures of B. thetaiotaomicron. In all experiments concerning the toxic effects of bismuth species, we evaluated the reduction of the maximal cell density at the stationary phase upon the application of the various drugs in the late exponential phase. This was taken as a measure for the antimicrobial activity of the compounds. As shown in Figure 1(a), the addition of 250 μM up to 2000 μM CBS to growing cultures of *B. thetaiotaomicron* resulted in a reduction of maximal cell density in the stationary phase. With increasing concentration, stronger growth inhibition is observed with a minimal inhibitory concentration (MIC_{50}) of about 500 μM CBS.

The addition of CBS resulted in a black precipitation of bismuth sulfide (Figure 1 (b)). This is due to the reaction with hydrogen sulfide produced from the anaerobic metabolism of *B. thetaiotaomicron*. The most intensive precipitation was observed at 500 μM similar to the MIC_{50}. Furthermore, the increasing inhibition of CBS on the growth of *B. thetaiotaomicron* resulted in a reduction of the sulfide precipitate.

2.2. Influence of the Methylated Derivatives of Bismuth. Similar growth inhibition effects were observed upon the addition of the methylated bismuth compounds monomethylbismuth, dimethylbismuth, and trimethylbismuth, however, at significantly lower concentrations (Figures 2(a) and 2(b)). The concentrations for the observed growth inhibition were within the nM range (MIC_{50}, 30 nM TMBi) and therefore four orders of magnitude lower compared to bismuth subcitrate (500 μM). In case of TMBi, an addition of up to 30 nM to the headspace resulted in an inhibition of about 50% and therefore at a concentration which is slightly higher than that in a previous study with *B. thetaiotaomicron* (MIC_{50}, 17 nM TMBi) [1]. This is presumably due to the addition of TMBi together with the inoculum. Interestingly, the GC-ICP-MS measurement of the headspace of *B. thetaiotaomicron* after the exposure time indicated that TMBi rapidly degrades in the presence of *B. thetaiotaomicron* cultures. Thus, the question arose whether the toxicity of TMBi is attributed to TMBi itself or to its partly methylated degradation products.

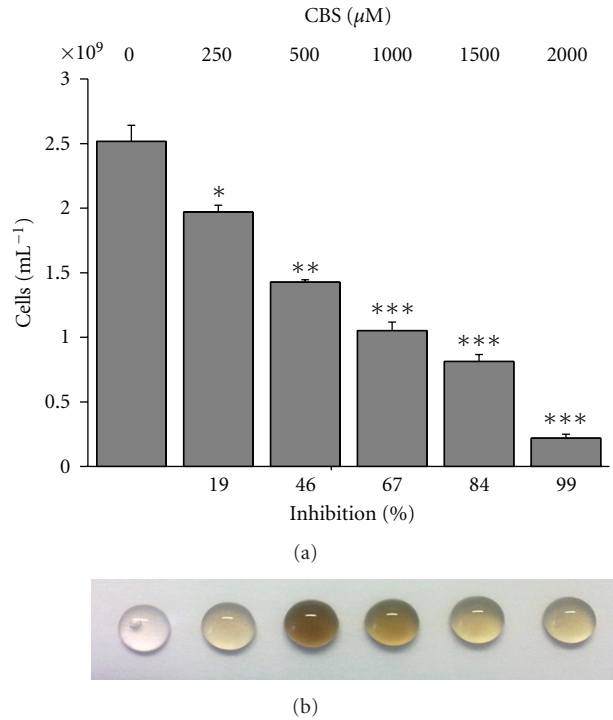

Figure 1: Growth inhibition of *B. thetaiotaomicron* 48 h cultivation after addition of CBS ($n = 3$). The addition of CBS to the growing *B. thetaiotaomicron* cultures (a) resulted in an inhibition of the maximal cell density in the stationary phase at 48 h after the addition of CBS in a concentration-dependent manner. Figure 1(b) shows a black precipitation in 20 μL droplets from the cultures.

Therefore, the toxicity of partially methylated Bi-derivatives on *B. thetaiotaomicron* was assessed.

2.3. Differentiation between the Inhibiting Effects of the Partially Methylated Bi-Derivatives. In experiments with partially methylated, soluble bismuth derivatives monomethylbismuth (MMBi) and dimethylbismuth (DMBi), inhibiting effects on the growth of *B. thetaiotaomicron* were observed at similar levels as those for TMBi (Figure 2(c)). After the addition of these derivatives within the exponential phase, the cell growth was reduced and did not reach the maximum cell density at the stationary phase compared to untreated control cultures. At a concentration of 48 nM, a significant growth inhibition is observed for both MMBi (18% reduction) and DMBi (29% reduction). While the toxicity of partially methylated Bi-derivatives is greater than that of inorganic bismuth, it is in a similar range, but in a lower range than that of TMBi.

2.4. Coculture of B. thetaiotaomicron with M. smithii. To confirm the relevance of the *in vitro* results for the situation *in vivo*, an *in vivo*-like coculture system was constructed (Figure 3). The design presents two separate fluid cultures under a common headspace in order to study the toxic effects of TMBi produced by *M. smithii* on *B. thetaiotaomicron*. CBS was applied to the culture of *M. smithii* in the late exponential phase (80 μM), and the growth behavior of

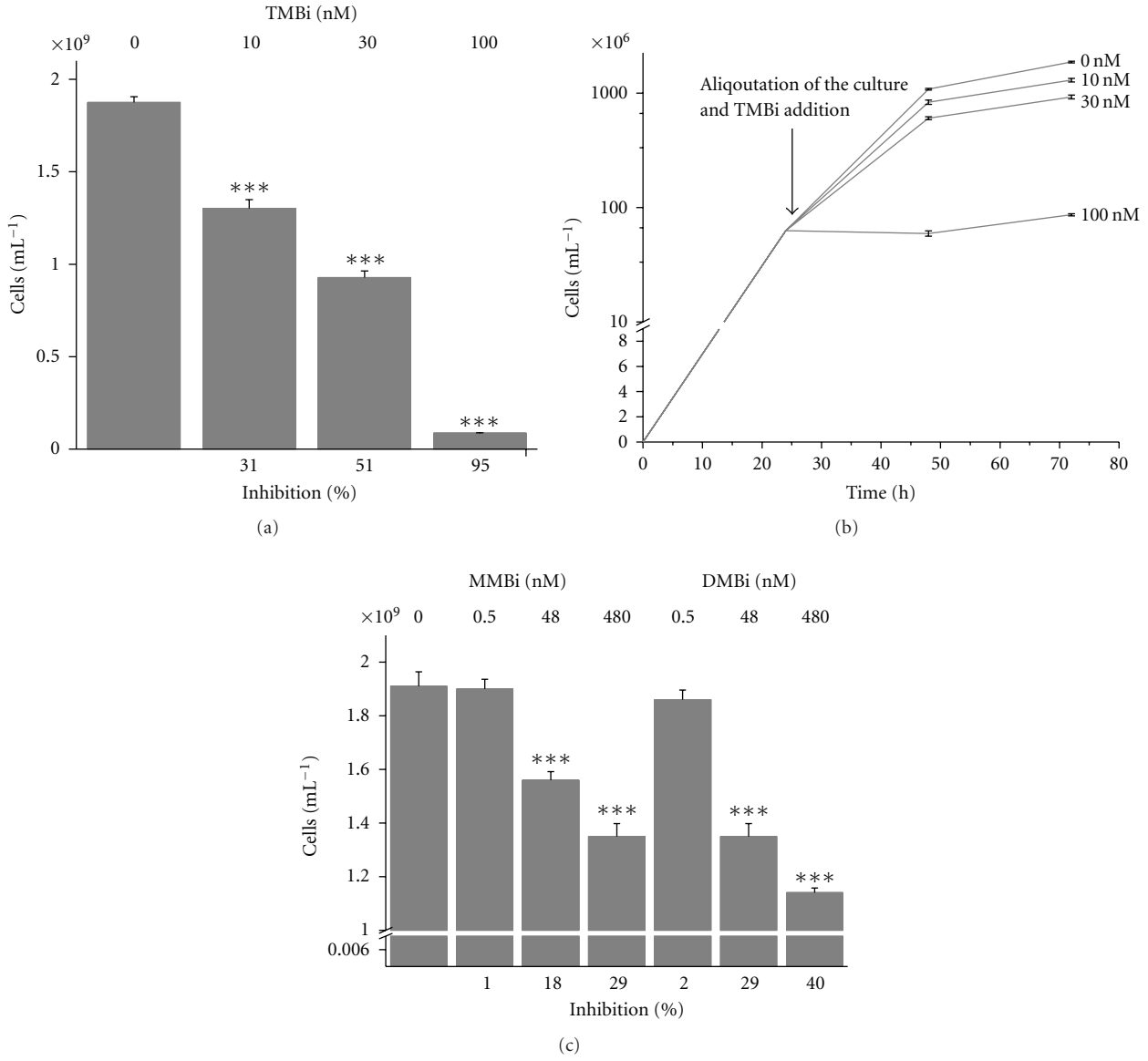

Figure 2: Growth inhibition of *B. thetaiotaomicron* 48 h after exposure of methylated bismuth species ($n = 3$). The addition of TMBi (a) to the headspace or of nonvolatile methylated bismuth species MMBi and DMBi (c) to the liquid phase of growing *B. thetaiotaomicron* cultures resulted in a growth reduction in a concentration-dependent manner. Figure 2(b) shows the growth curve in the presence of TMBi. The culture was separated in aliquots in the late exponential phase and was exposed to different TMBi concentrations. The cell counts were determined after 48 h at the stationary phase.

B. thetaiotaomicron was followed over 48 h. As shown in Figure 4, the growth of *B. thetaiotaomicron* was reduced to approximately half of the density of the untreated control.

In order to verify that the production of TMBi is the reason for the growth reduction, we measured the concentration of the evolved Bi-derivative TMBi in the headspace over 48 h. While in the headspace of isolated *M. smithii* cultures TMBi is found [1], no volatile TMBi was detected in the coculture system due to the rapid degradation of TMBi over the *B. thetaiotaomicron* culture medium as described above. Thus, an alternative approach was developed based on passive TMBi chemotrapping using silver nitrate-coated silica beads analogous to a method recently introduced for the sampling of volatile arsenic and selenium species by Uroic et al. [19]. The *B. thetaiotaomicron* culture located in the inner tube was replaced by the chemotrap and the bismuth content of the chemotrap after 48 h of incubation was analysed by ICP-MS; 1.64 ± 0.04 nmol Bi were trapped by $AgNO_3$-coated silica beads corresponding to 33 nM TMBi in the gas phase, which is quite similar to the MIC_{50} of TMBi.

3. Conclusion

The present work confirms that the methanogens represent members of the intestinal microbiota with the hazardous capability to transform metal(loid)s into toxic methylated

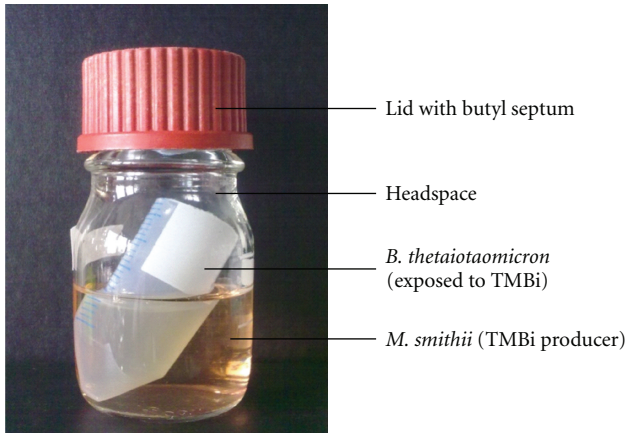

Figure 3: Design of the coculture system. The represented coculture system exhibited two separate liquid cultures under a common headspace. This design allowed the transfer of produced volatile TMBi from the culture of *M. smithii* to the culture of *B. thetaiotaomicron* over the common gas phase.

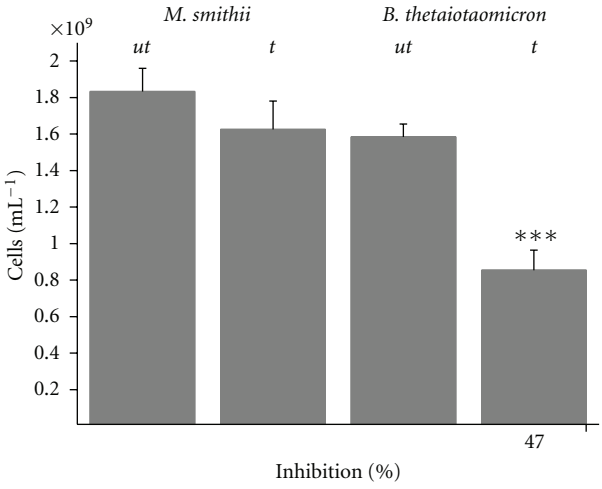

Figure 4: Reduction of the cell counts of *B. thetaiotaomicron* due to the production of volatile TMBi produced by *M. smithii* ($n = 3$). *M. smithii* and *B. thetaiotaomicron* were grown in the coculture system. CBS was applied at a concentration of 80 μM to the culture of *M. smithii* in its late exponential phase. The TMBi production of *M. smithii*, during its 48 h incubation in the presence of CBS, results in a significant cell count reduction of *B. thetaiotaomicron* cultures (*t*) compared to untreated control (*ut*).

derivatives. The volatile organometal(loid) species formed do not only interact directly with the host's organ tissues (e.g., by increasing bioabsorption of bismuth [12] or by intoxification of mammalian cells [20]) but also indirectly. The indirect interaction inhibits the beneficial microbiota in its capacity to help with optimal digestion of complex food and protect the intestinal epithelium. We showed in our research that both volatile TMBi as well as nonvolatile partially methylated derivatives interact with members of the physiological intestinal microbiota. The measurements of the concentration of TMBi in the intestines of mice fed with CBS-containing chow (approximately 8–10 nM, unpublished data) suggest that the produced amount of TMBi is sufficient for this negative interaction, which ends up in a strong inhibition of this microbiota.

Unfortunately, nothing is known about the molecular mechanisms of these phenomena. In addition, the higher hydrophobicity of methylated species will increase the mobility of these compounds and allow them to interact with the cell membrane, enter, and damage the cells. An interaction with inner components of the cell cannot be excluded. Bi^{3+} ions may interact with essential proteins of *B. thetaiotaomicron* and methylated bismuth derivatives could cause methylation reactions with macromolecules, as shown for arsenic [14]. Taking into consideration that methylated bismuth species are by orders of magnitude more toxic than inorganic bismuth and that toxicity decreases with a decreasing degree of methylation together with the instability of TMBi, it seems plausible to assume that the toxicity of TMBi as well as that of the partly methylated species is due to a methyl transfer during degradation of these highly unstable species.

Considering the high potential for negative effects of methanogens on higher organisms, their harmful nature seems to be beyond question. But are the methanogens really examples for true pathogenic archaea? "Pathogenic" means that the organism in question deliberately damages the host for the advantage to reproduce and spread. But does this apply to the methanogens? In a recent study, we have proven that the methylation of metal(loid)s by *Methanosarcina mazei* is not a deliberate detoxification mechanism in analogy to the ArsM system for arsenic. Instead it is a result of the side reaction of methanogenic cofactors (Thomas et al., submitted [21]). Methanogens therefore affect the host merely because of a coincidence of the methanogenesis intermediates and the formation of metal(loid) derivatives. Thus, a special advantage from the production of these harmful metal(loid) derivatives does not seem obvious.

4. Material and Methods

4.1. Standard Cultivation. Growth experiments with *B. thetaiotaomicron* (DSMZ No. 2079) and *M. smithii* (DSMZ No. 861) were performed under strict anaerobic conditions (gas phase: H_2/CO_2 (80 : 20)) at 37°C in serum bottles with a total volume of 120 mL (50 mL fluid, 70 mL headspace). The following complex growth medium was used for both *B. thetaiotaomicron* and *M. smithii*: 0.5 g KH_2PO_4, 0.4 g $MgSO_4 \times 7\ H_2O$, 0.4 g NaCl, 0.4 g NH_4Cl, 0.05 g $CaCl_2 \times 2\ H_2O$, 1 g $C_2H_3KO_2$, 8 g NaCOOH, 4 g $NaHCO_3$, 6 g bacto brain heart infusion, 2 g yeast extract, 6 g peptone, and 10 mL SL-10 (DSMZ) per liter.

4.2. Coculture System. A coculture system with two separate liquid and one headspace phase is constructed by using a 100 mL glass bottle with thick butyl septum (Schott AG, Mainz, Germany) and 30 mL greiner tubes (40 + 10 mL fluid, 50 mL headspace).

4.3. Quantification of Cells. Cultivated cells are counted with a Thomson counting chamber in moderate dilution.

4.4. Addition of Bismuth. Cultures of *B. thetaiotaomicron* are aliquoted and different bismuth species were added within the late exponential growing phase (1×10^8 cells·mL^{-1}). Inorganic and soluble bismuth species were added to the liquid phase of *B. thetaiotaomicron*. Volatile TMBi was applied to the headspace.

4.5. Analysis of Volatile Metal(loid) Derivatives. Volatile TMBi was analyzed by a modified purge and trap gas chromatograph system [22] coupled to an ELAN 6000 (PerkinElmer) inductively coupled plasma mass spectrometer (P&T-GC-ICP-MS) as described elsewhere. The identification of volatile metal(loid) compounds based on GC-ICP-MS boiling-point retention correlation was verified by using parallel molecular and elemental mass spectrometry (GC-EI-MS/ICP-MS) as described previously [23].

4.6. Chemotrapping of Volatile TMBi. Volatile TMBi was trapped by using silica beads coated with silver nitrate modified from Uroic et al. [19]. These beads were placed over night in a 1% silver nitrate solution and then dried at 40°C. 1 g of these coated silica-beads was placed in the coculture system the inner tube instead of *B. thetaiotaomicron*. After 48 h of incubation, the production of TMBi by *M. smithii* was determined by extraction of the beads with 5% HNO_3, and analysis by ICP-MS after sterile filtration.

4.7. Synthesis of Methylated Bismuth Compounds. The methylated bismuth compounds were synthesized in a two-step process. In a first step, trimethylbismuth was prepared in a method adapted from Marquardt [24]. In brief, a Grignard solution was prepared by adding methyl iodine to magnesium chippings in diethyl ether. Trimethylbismuth was isolated by fractionated distillation. In a second step, trimethylbismuth was solved in diethyl ether and bismuth bromide was added in a molar ratio of 2 : 1 of $BiBr_3$ and $(CH_3)_3Bi$ to yield $(CH_3)BiBr_2$ or in a 1 : 2 ratio to yield $(CH_3)_2BiBr$, respectively. Bismuth cysteine and methyl bismuth cysteine were prepared by adding bismuth(III) bromide ($BiBr_3$) or methylated bismuth bromide ($(CH_3)BiBr_2$), respectively, to a saturated solution of L-cysteine in ultrapure laboratory water. The molar bismuth ratio of the bismuth compound to L-cysteine was 1 : 2. The mixture was stirred at room temperature (20°C) in an inert argon atmosphere. For isolation of these compounds we added small amounts of methanol until precipitation of a yellow solid. The crystalline product was subsequently filtered through a fiberglass filter and dried in vacuum desiccators. Verification of the standards was performed by derivatization by ethylation and analysis by GC-MS as reported previously [25].

4.8. Statistical Evaluation. The significance of the cell numbers were analyzed using a pair-sample *t*-test. At the 0.05 level, the difference of the population means was significantly different with the test difference.

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Review Article

Biochemical Factors Modulating Cellular Neurotoxicity of Methylmercury

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Methylmercury (MeHg), an environmental toxicant primarily found in fish and seafood, poses a dilemma to both consumers and regulatory authorities, given the nutritional benefits of fish consumption versus the possible adverse neurological damage. Several studies have shown that MeHg toxicity is influenced by a number of biochemical factors, such as glutathione (GSH), fatty acids, vitamins, and essential elements, but the cellular mechanisms underlying these complex interactions have not yet been fully elucidated. The objective of this paper is to outline the cellular response to dietary nutrients, as well as to describe the neurotoxic exposures to MeHg. In order to determine the cellular mechanism(s) of toxicity, the effect of pretreatment with biochemical factors (e.g., N-acetyl cysteine, (NAC); diethyl maleate, (DEM); docosahexaenoic acid, (DHA); selenomethionine, SeM; Trolox) and MeHg treatment on intercellular antioxidant status, MeHg content, and other endpoints was evaluated. This paper emphasizes that the protection against oxidative stress offered by these biochemical factors is among one of the major mechanisms responsible for conferring neuroprotection. It is therefore critical to ascertain the cellular mechanisms associated with various dietary nutrients as well as to determine the potential effects of neurotoxic exposures for accurately assessing the risks and benefits associated with fish consumption.

1. Introduction

Methylmercury (MeHg) is a ubiquitous environmental toxicant [1]. Several catastrophic epidemics resulting from the consumption of food contaminated by MeHg have highlighted the potentially disastrous effects of MeHg on living organisms. Important examples include outbreaks in Minamata [2], Niigata [3], and Iraq [4]. MeHg is a potent neurotoxicant which affects both the developing and mature CNS [5, 6]. In infants, MeHg causes widespread and diffuse damage, whereas focal damage is caused in the adult brain. In adults, chronic MeHg poisoning results in the degeneration of the sensory cerebral cortex and the cerebellum, thereby leading to severe neurological disturbances, such as cerebellar ataxia and paresthesia, sensory and speech impairment, and the constriction of the visual field [4, 7, 8]. The pathological changes involve general neuronal degeneration with gliosis in the calcarine, and precentral and postcentral areas of the cerebral cortex, as well as the loss of granular cells in the cerebellar cortex [9]. In biological systems, MeHg exists only at a very low concentration as a free, unbound cation [10] which can bind to sulfhydryl groups (-SH) of amino acids with a very high affinity (log K in the order of 15–23) [10]. This affinity of Hg for sulphur and -SH groups is a major factor underlying the biochemical properties of MeHg, which, consequently, leads to its interference with the enzyme activities of several cellular targets.

In the marine ecosphere, MeHg is sustained [11, 12] and, after bioaccumulation, is introduced into the human population through the dietary intake of fish and seafood products. [13–15]. MeHg toxicity due to the consumption of adulterated fish represents a major public health issue. Greater

fish consumption in many cases is paralleled by increased MeHg intake [16]; however, conversely, lower maternal seafood intake has also been associated with higher risk for a suboptimal developmental outcome [17]. According to the Avon Longitudinal Study of Parents and Children (ALSPAC), the authors reported that maternal seafood intake during pregnancy of less than 340 g per week was associated with an increased likelihood for their children to fall into the lowest quartile for verbal intelligence quotient (IQ) when compared with mothers who consumed more than 340 g of seafood per week. Though Hg consumption was not assessed in this study, it is reasonable to assume that greater fish consumption was paralleled by increased MeHg intake. Moreover, several discrepancies in health outcomes in fish-eating populations have been reported, such as neurodevelopmental impairments in New Zealand [18–20] and the Faroe Islands [21, 22], as opposed to the beneficial effects noted in Canada [23], the Seychelles [24, 25], Peru [26], and the United States [17, 27–30]. Additionally, laboratory studies have also shown that dietary factors, such as selenium, cysteine, protein, fat, fiber, and vitamin contents can modulate the toxicity and excretion of mercury [31, 32]. A previous study [33] has also shown a significantly higher rate of fecal excretion as well as a lower degree of MeHg accumulation in the brains of rats fed naturally contaminated fish as compared to rats fed fish containing chemically added MeHg. The above-mentioned studies indicate that, in addition to intrinsic, genetic factors, the phenotypic responses to MeHg exposure may ultimately depend on a number of complex interactions within biological systems involving both mercury and various dietary factors. It is therefore important to study the effect(s) of confounding dietary factors that occur when fish is consumed on MeHg distribution and neurotoxicity. In this respect, it must be noted that different types of fish accumulate different concentrations of nutrients and contaminants [34–36]. Therefore it is of considerable interest to determine how each component acts individually (as well as with others) and influences the potential risk from MeHg exposure. These cellular and molecular mechanisms of MeHg action, as well as the underlying processes of its interaction with dietary components have yet to be defined, especially in specific central nervous system (CNS) targets. Accordingly, this paper focuses on studies directed toward estimating the effect(s) of dietary modifiers on MeHg neurotoxicity, potentially providing information about critical cellular mechanisms responsible for conferring neuroprotection from a diet that includes MeHg-contaminated fish.

2. MeHg-Induced Oxidative Effects: Reactive Oxygen Species (ROS) Generation and Glutathione (GSH) Depletion

The disruption of redox cellular homeostasis by an excess of ROS formation, which leads to cumulative oxidative stress appears to play a key role in the *in vivo* pathological process of MeHg intoxication [37–42]. Conversely, several studies have demonstrated the partial amelioration of MeHg toxicity in the presence of antioxidants by the inhibition of ROS generation [40, 43, 44]. Although the critical role of oxidative stress in the pathogenesis of MeHg cytotoxicity has been clarified, the molecular mechanisms underlying MeHg-mediated oxidative stress have not yet been fully elucidated. A major source of MeHg-induced increases in ROS generation may be the mitochondrial electron transport chain. The damaged mitochondrion increases oxidative stress, leading to a decrease in defense mechanisms, such as reduced GSH content, which represents one of the principal endogenous antioxidants. In addition, binding to GSH is reported to be responsible for the excretion of MeHg. Therefore, decreased GSH levels usually parallel increased oxidative stress due to MeHg exposure [45–49]. However, two epidemiological studies associating oxidative stress and MeHg exposure [50, 51] have shown both an increase and a decrease in GSH levels with increased total Hg levels. This suggests that MeHg can increase ROS which may either inhibit GSH levels or initiate an adaptive response to oxidative stress by increasing GSH levels. Moreover, studies of human populations, although of direct interest, cannot be controlled for multiple confounding variables. This obstacle can be overcome by conducting studies on laboratory animals; such investigations can identify the mechanisms of action by which neurotoxicants and neuroprotectants interact.

3. Role of GSH Modulators on MeHg-Induced Neurotoxicity

Upregulation [52], or the induction of an increased synthesis of GSH [45], has been reported to provide neuroprotection against MeHg-induced neurotoxicity. A similar alleviation in MeHg-induced cytotoxicity and oxidative stress has been reported with N-acetyl cysteine (NAC) supplementation [39, 53–55]. The mechanisms involved in protection afforded by NAC include increased intracellular GSH [54, 55] as well as a transient increase in the urinary excretion of MeHg, which was shown to cause a decrease in the level of MeHg in both the adult brain and the fetus [53, 56]. In addition, the increased amount of GSH in cortical, as compared to cerebellar, astrocytes has been reported to account for the increased MeHg-induced ROS production in cerebellar astrocytes [55].

Conversely, the depletion of intracellular GSH with diethyl maleate (DEM) has been reported to increase cell-associated MeHg and MeHg-induced ROS [48, 54, 55]. The underlying mechanism of this process involves the conjugation of free sulfhydryl groups of GSH with DEM, which results in the distinct depletion of GSH. Also, gestational exposure to MeHg has been reported to cause the dose-dependent inhibition of cerebral GSH levels, an outcome which could be correlated with increased lipid peroxidation in the pup brain [57]. These biochemical alterations were found to endure even after Hg tissue levels decreased, thus indicating permanent functional deficits observed after prenatal MeHg exposure as well as an additional molecular mechanism by which MeHg induces prooxidative damage in the developing CNS.

In summary, changes in intracellular MeHg content with GSH modulation provide an explanation for the increased

susceptibility of certain cell types towards MeHg-induced oxidative stress [54, 55].

4. Role of DHA in Modulating MeHg-Induced Neurotoxicity

DHA *cis*-4,7,10,13,16,19-docosahexaenoic acid, is one of the most abundant polyunsaturated fatty acids (PUFA) in the phospholipid fractions of the mammalian brain [58, 59]. Both seafood and breast milk serve as major dietary routes of MeHg [60, 61] and DHA [62–65]. The ability of DHA to affect ROS is controversial, as several contrasting studies have documented the ability of DHA to decrease the level of lipid peroxide [66–68] and to cause free-radical-mediated peroxidation in the brain [69–71]. DHA have been reported to modulate MeHg toxicity [33, 72–74]. These studies have demonstrated the beneficial effects of DHA on using a DHA-enriched diet against MeHg-induced decreases in serum albumin levels, changes in mitochondrial membrane potential, and developmental defects. However, other contradictory studies have reported no protection against MeHg-induced behavioral defects [75, 76]. It is therefore important to identify the biochemical mechanisms involved in the DHA protection against MeHg neurotoxicity.

Kaur and colleagues [77, 78] demonstrated that pretreatment with DHA was associated with reduced cell-associated MeHg in neuronal cell lines and primary cells. In addition, decreased ROS and unchanged GSH levels were found in primary cultures, whereas increased ROS and GSH depletion were found in C6 cells [77, 78]. These differences with respect to the effect of DHA on oxidative stress could be due to the fact that the growth of cancerous cells is inhibited by DHA as compared to noncancerous cell types [71, 79]. Indeed, another recent study has shown that fish oil offers significant DNA protection as well as anti-inflammatory effects in the absence of changes in GSH levels [50]. These observations strongly suggest that DHA may neuroprotect against MeHg-induced ROS generation even in the absence of significant changes in GSH levels.

5. Role of Selenomethionine in Modulating MeHg-Induced Neurotoxicity

Selenium (Se) is an essential trace element known to accumulate in significant amounts in numerous species of seafood [80, 81]. The majority of Se in fish is in the organic form, selenomethionine (SeM) [82, 83], and is more bioavailable than are inorganic forms [84]. Selenium has also been detected in human milk [85]. The modulating effect of Se on MeHg toxicity was discovered when researchers observed that marine mammals could accumulate exceptionally high concentrations of Hg and Se compounds without displaying obvious symptoms of intoxication [86, 87]. Several subsequent studies later confirmed that the toxic effects of both organic and inorganic Hg were prevented by Se compounds [88–92]. Treatment with different Se compounds has been shown to effectively protect cells against different toxic effects induced by MeHg exposure, such as cytotoxicity, fetotoxicity, neurotoxicity, and developmental and neurobehavioral toxicity [93–97]. In addition, Se deficiency has been shown to potentiate the adverse effects of MeHg toxicity in rodents [98, 99].

With regard to epidemiological studies and Se content, it is important to note that Faroe Islanders, by virtue of a whale meat diet, are generally exposed to MeHg levels that are in excess of Se levels [100], whereas the Seychellois are largely ocean fish consumers, and Se molar concentrations tend to greatly exceed MeHg concentrations in this seafood source [101]. In addition, the dietary Se status in the New Zealand population was extremely poor at the time of the study [102]. This distinction could be one explanation for the different effects noted in these studies, although additional evidence is needed to support this hypothesis [103]. Therefore, developing a better understanding of the mechanisms associated with the interaction of MeHg and Se is of particular necessity.

Several studies have indicated that the mechanism underlying Se's ability to ameliorate MeHg toxicity is related to an antioxidant effect [104–108], which includes the formation of GSH [109], higher glutathione peroxidase (GPx) activity [85], increased selenoprotein levels [110–112], and the reduction of organic hydroperoxides [113–115]. Additionally, studies have shown that binding of MeHg [116, 117] and the formation of a highly stable organic MeHg-selenocysteine complex [98] also influence the accumulation of MeHg in tissues [118–121] and the uptake of MeHg in cells [114, 122–124]. Furthermore, Se is known to enhance the excretion of MeHg [56, 125], and a recent study has shown [126] that SeM can demethylate MeHg under physiologically and environmentally relevant conditions. Hence, the interactive effects between MeHg and SeM result in reduced cell-associated MeHg and prooxidant response from MeHg.

6. Role of Trolox in Modulating MeHg-Induced Neurotoxicity

Seafood serves as a source of vitamins, with estimates ranging between 4.84 and 17.90 μg vitamin E per gm of fish [127], which makes this vitamin the most significant physiologic membrane-associated antioxidant available from seafood. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble analog of vitamin E [128], serves as a better antioxidant than vitamin E [129, 130] due to its improved access to the hydrophilic compartments of the cells [131], as well as its stoichiometric properties [132]. Trolox scavenges free radicals [59, 133–137] via the H-donating groups [128, 138]. Treatment with Trolox has been reported to protect against MeHg-induced cytotoxicity [139], the decrease in mitochondrial electron transport system enzyme activities, and the increase of mRNAs of antioxidant enzymes [108, 140]. Trolox treatment has also been shown to reverse ROS induction by MeHg in primary astrocyte cultures [44] and to prevent MeHg-induced oxidative stress [141], where the modulating effect of Trolox on cellular ROS levels was not accompanied by changes in cellular MeHg, GSH, or MTT activity [141]. These findings indicate that Trolox affords protection against ROS by

the direct quenching of free radicals and not by MeHg chelation or by the induction of increased levels of GSH or mitochondrial enzymes. In fact, it has been previously shown that *in vivo* protection with Trolox does not affect intracellular GSH [142, 143] or MeHg levels [140]. The recognition of the protective effects of Trolox and the identification of its mechanisms via *in vitro* models establish that vitamin-dependent antioxidant defences are important factors in specific cells for attenuating the neurotoxic effects of a MeHg-contaminated fish diet.

7. Discussion

Fish is not only an excellent nutritional source of protein, vitamins, zinc, and other minerals, but it is also a source of exposure to MeHg [144, 145]. One of the leading controversies in the MeHg literature originates from advisories concerning the consumption of fish [146] and from uncertainties in documentation from various regulatory agencies regarding the effects of MeHg. The Joint FAO/WHO Expert Committee on Food Additives reported in 1978 that "*the fetus may be more susceptible to MeHg toxicity than the adult*" [147]. The United States White House in 1998 convened an international workshop where a variety of possible uncertainties and confounders important to MeHg toxicity evaluation were discussed. Their conclusions stated, "*Even when dietary stresses and co-exposures to other chemicals could plausibly enhance or alter risk, it was still deemed that there are inadequate data on this subject to draw meaningful conclusions at this time*" [148]. Later, in 2000, the National Academy of Sciences committee reported that, "*60,000 children in the United States were at risk as a result of prenatal exposure*" [149]. However, no justification or explanation for that conclusion was provided [16, 150]. The issue that poses a significant dilemma for both consumers and regulatory authorities is whether fish consumption should be encouraged for its nutritional benefits to the developing brain or, conversely, whether fish consumption should be discouraged due to the possible adverse effects of MeHg on the developing CNS. This nutrition versus neurotoxicity controversy can be addressed by estimating the effects of dietary factors on MeHg-induced toxicity as well as by determining the mechanisms behind such effects. A thorough assessment of coexposure from dietary nutrients as well as neurotoxic exposures would offer valuable information for accurately determining the risks and benefits of fish consumption [151, 152].

This paper explores the mechanisms associated with MeHg and dietary nutrients obtained from the consumption of seafood. The toxicity of MeHg has been reported to be caused by a reduction in the amount of intracellular GSH [45, 46, 48], which leads to the augmentation of ROS formation [37, 40–44, 153]. This paper investigates the effects of MeHg on oxidative stress and details the role played by GSH in modifying these effects. It also identifies the biochemical mechanisms underlying exposure to GSH, DHA, Se, Trolox, and MeHg, where these modifiers have been shown to effectively decrease MeHg-induced ROS

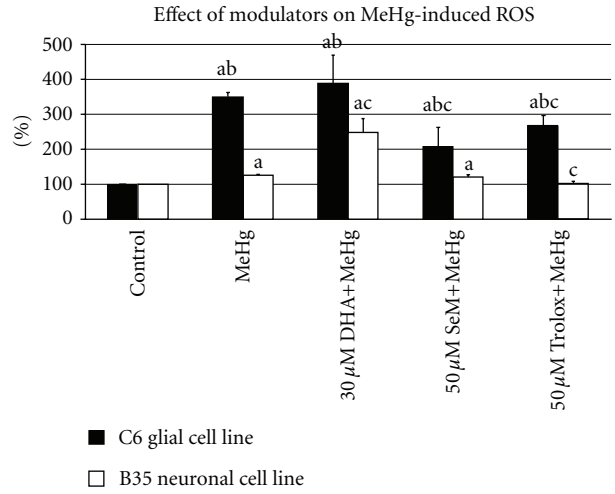

Figure 1: Effect of different modulators on MeHg-induced cellular ROS in C6-glial and B35-neuronal cell lines. Results are expressed as mean ± standard deviation ($n = 8$ replicates for each cell type in two independent experiments). Superscript (a) indicates $P < 0.05$ for control versus each type of treatment; (b) indicates $P < 0.05$ for C6 versus B35 cell line for each type of treatment; (c) indicates MeHg versus DHA/SeM or Trolox+MeHg-treated group. Values represented the percentage of activity relative to control cells.

(Figure 1). In addition, it is important to note that the interaction between these dietary nutrients may have an effect on overall toxicity. For example, the benefits from Se against MeHg toxicity can be influenced by the intake of long-chain, polyunsaturated fatty acids (LCPUFAs) [65, 154]. It has also been shown that the shape of the dose-effect curve for Hg is dependent upon the co-exposure of dietary components such as Se and vitamin E [145]. This paper, concludes that GSH, DHA, Se and Trolox are strong confounders in the association of MeHg toxicity and that the interaction between them may affect the cellular oxidative status. Thus, it is necessary to consider different confounders and the various mechanisms by which they interact with Hg when investigating the potential beneficial effects of fish consumption. Indeed, doing so would provide valuable insight for developing a better understanding of the benefits and risks of fish consumption, acknowledging both the proven beneficial nutrients as well as the potentially dangerous contaminants contained in this important food source. Furthermore, such information would also assist public health authorities as they seek to advise the populace and as they undertake efforts to formulate appropriate dietary recommendations for consumers of fish and seafood products.

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Review Article

Lead-Binding Proteins: A Review

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Lead-binding proteins are a series of low molecular weight proteins, analogous to metallothionein, which segregate lead in a nontoxic form in several organs (kidney, brain, lung, liver, erythrocyte). Whether the lead-binding proteins in every organ are identical or different remains to be determined. In the erythrocyte, delta-aminolevulinic acid dehydratase (ALAD) isoforms have commanded the greatest attention as proteins and enzymes that are both inhibitable and inducible by lead. ALAD-2, although it binds lead to a greater degree than ALAD-1, appears to bind lead in a less toxic form. What may be of greater significance is that a low molecular weight lead-binding protein, approximately 10 kDa, appears in the erythrocyte once blood lead exceeds 39 μg/dL and eventually surpasses the lead-binding capacity of ALAD. In brain and kidney of environmentally exposed humans and animals, a cytoplasmic lead-binding protein has been identified as thymosin β4, a 5 kDa protein. In kidney, but not brain, another lead-binding protein has been identified as acyl-CoA binding protein, a 9 kDa protein. Each of these proteins, when coincubated with liver ALAD and titrated with lead, diminishes the inhibition of ALAD by lead, verifying their ability to segregate lead in a nontoxic form.

1. Introduction

This paper focuses on lead-binding proteins, which, in contrast to the majority of articles in this issue, are not toxic compounds but rather low molecular weight proteins which have the capacity to bind lead and to sequester the lead in a nontoxic form. In the discussion which follows, careful note should be taken as to which lead-binding proteins are inducible, that is, increased in concentration after exposure to lead, and which are constitutive, that is, are present within the organism at all times and may have binding sites saturable by lead but no discernible increase in protein content. The second situation is perhaps most pertinent to enzymes inhibitable by lead, the first to proteins not normally present or present in low quantities in the organism but which increase after lead exposure and offer protection against lead toxicity. The history of lead-binding proteins can be dated back to 1936, when Blackman first described the presence of intranuclear inclusion bodies in the liver and kidney as manifestations of lead poisoning [1]. The next phase in this saga consisted of detailed studies of the composition of renal tubular intranuclear inclusion bodies and the consequent alterations in mitochondrial structure and function which followed, first by Goyer and colleagues, later by Shelton et al. [2–5], and Klann and Shelton [6, 7].

2. Lead-Binding Proteins within Intranuclear Inclusion Bodies in Kidney

Goyer [8] began his studies by examining the renal tubules of rats fed 1% lead acetate for up to 20 weeks, finding that dense, deeply staining intranuclear inclusions were located in the straight portion of the proximal tubules, accompanied by swollen, globular, or ovoid, closely packed mitochondria with many marginated, irregular, or vesicular cristae. Accompanying these mitochondrial changes was the presence of generalized aminoaciduria. In a subsequent paper in the same issue, Goyer and colleagues [9] isolated mitochondria from lead-exposed and control rats and demonstrated that mitochondria from the lead-exposed rats show reduced rates of respiration and oxidative phosphorylation.

Lead within the kidneys in lead-poisoned rats was found to be concentrated in the nuclei and within nuclei in the nuclear inclusion body [10, 11]. Choie and Richter [12] showed further that rapid induction of inclusion bodies by injections of lead salts in the rat resulted in cytoplasmic inclusions, suggesting that they were precursors to the intranuclear inclusions. This was further confirmed by McLachlin et al. [13] who showed in tissue culture studies of rat kidney cells incubated with lead that the cytoplasmic inclusion bodies preceded and disappeared shortly after the appearance of nuclear inclusion bodies.

Lead-containing nuclear inclusions were also found in organs other than the kidney, including liver and glial cells of the central nervous system [14]. Moore et al. [15] dissolved the rat renal intranuclear inclusions in strong denaturing agents and found that the protein in the inclusions is acidic, with high levels of aspartic acid, glutamic acid, glycine and cystine. Moore and Goyer [16] later characterized the protein as a 27.5 kDa protein, which migrates as a single band on acrylamide gel electrophoresis. Repeated intraperitoneal injections of calcium disodium ethylenediamine tetraacetic acid ($CaNa_2EDTA$) resulted in disappearance of the inclusion bodies in lead-exposed rats, together with a marked decrease in kidney lead levels [17].

Shelton and coworkers have also explored the composition of the lead-binding proteins in the nuclear inclusion proteins of lead-exposed rat kidneys. Shelton and Egle [2] first described a 32 kDa protein with an isoelectric point of 6.3, which was isolated from the kidneys of rats treated with 1% lead acetate in rat chow or 0.75% lead acetate in drinking water, given for 13–17 weeks. In contrast to the work of Goyer and coworkers, they employed two-dimensional gel electrophoresis to isolate the protein from the nuclear inclusion bodies and demonstrated that it was present in lead-exposed, but not control kidneys (hence inducible). This protein has been termed p32/6.3. Inhibitor studies with cycloheximide and actinomycin D [13, 18] had indicated earlier that protein synthesis was required for induction of the nuclear and cytoplasmic inclusion bodies.

Egle and Shelton [3] then turned their attention to the brain, where they found unexpectedly that p32/6.3, now characterized by a monoclonal antibody, was constitutively present in cerebral cortex, both in neurons and astrocytes. The protein was concentrated in the insoluble nuclear protein, similar to the lead-exposed kidney. Brain p32/6.3 was detected in rat, mouse, dog, man and chicken. In rat brain, adult levels were achieved in 1-2 weeks after birth whereas only trace amounts were found at 3 days. Brain p32/6.3 increases between postnatal days 10–12 in the guinea pig and days 15–21 in the rat, suggesting that the increase may be related in part to exposure to the external environment [5]. When neuroblastoma cells were cultured after 1 and 3 days exposure to lead, the abundance of p32/6.3 increased. Simultaneous incubation with lead and cycloheximide or actinomycin D also showed an increase in p32/6.3, suggesting that lead selectively retards the degradation of the brain protein [6]. The amino acid composition of partially purified p32/6.3 revealed a high percentage of glycine, aspartic and glutamic acid [4]. Thus Shelton and coworkers have established that an inducible protein, p32/6.3, can be extracted from nuclear inclusion bodies from the lead-exposed rat kidney and a similar or identical protein from adult rat brain. Whether the brain protein is constitutive or inducible by exposure to environmental lead has yet to be determined.

Selvin-Testa et al. [19] and Harry et al. [20] reported that developing rat brain astrocytes exposed to lead developed an elevation in glial fibrillary acidic protein (GFAP), a developmentally regulated protein. Harry et al. [20] consider that the elevated levels of GFAP mRNA during the second postnatal week after lead exposure may reflect the demand on astrocytes to sequester lead.

Oskarsson and Fowler [21] examined the influence of pretreatment with lead by a single i.p. injection of lead acetate (50 mg lead per kg body wt) 1, 3 and 6 days before injecting ^{203}Pb. Rats were sacrificed 24 hours later and the kidneys were examined both microscopically and for the distribution of ^{203}Pb. At 3 days rat, kidneys displayed fibrillar cytoplasmic inclusions, but, at 6 days these inclusions were less prominent and intranuclear inclusions were observed. ^{203}Pb uptake at 6 days was maximal in the purified nuclear fraction and in the nuclear inclusion bodies (7x and 20x control, resp.).

3. Cytoplasmic Lead-Binding Proteins in Kidney and Brain

The remaining studies of nonlead-stimulated cytoplasmic kidney and brain lead-binding proteins have been provided by Fowler and associates.

The first study [22] reported on the lead-binding proteins in kidney postmitochondrial cytosolic fractions. Binding of ^{203}Pb was found in two protein fractions of control kidneys with molecular weights of 11.5 and 63 kDa. Binding was markedly decreased after lead pretreatment. The use of cadmium to stimulate metallothionein synthesis did not increase ^{203}Pb binding to the 11.5 kDa protein. The two binding proteins were also present in brain but not in liver or lung. Subsequently, Mistry et al. [23] demonstrated three lead-binding proteins (11.5 kDa, 63 kDa and >200 kDa) in rat kidney cytosol, which had binding characteristics of high-affinity low-capacity sites with respective *Kd* values of 13, 40, and 123 nM. The 11.5 kDa and possibly the 63 kDa protein were capable of translocating lead into the nucleus as shown by uptake of ^{203}Pb into nuclei incubated with tagged cytosolic proteins.

Goering and Fowler [24] showed that the 11.5 kDa protein, but not the 63 kDa protein, was capable of reversing lead-induced ALAD inhibition in liver homogenates. This effect was mediated both by chelation of lead by the lead-binding protein and by donation of zinc to ALAD [25]. Various divalent metal ions influence the binding of lead to the rat kidney cytosolic binding proteins, with an order of displacement of $Cd^{++} > Zn^{++} > Pb^{++}$. Ca^{++} had no effect, while Fe^{++} had a cooperative effect [26]. These observations may account for the previously demonstrated effect of concomitant lead and cadmium administration in reducing

total kidney lead [27] and preventing the development of intranuclear inclusion bodies [28].

More recent studies by Fowler and DuVal [29] identified the rat renal lead-binding protein as a cleavage product of α2-microglobulin, with a *Kd* of 10^{-8} M lead. There are two forms of the protein in the kidney, differentiated by the cleavage of the first 9 N-terminal residues from the higher molecular weight form. Other studies by Smith and coworkers [30] have found two lead-binding proteins in environmentally exposed human kidneys, identified as acyl-CoA-binding protein (ACBP) or diazepan-binding inhibitor (molecular weight 9 kDa) and thymosin β4 (molecular weight 5 kDa). These polypeptides have a high-affinity for lead (*Kd*~14 nM).

In rat brain, Goering et al. [31] and DuVal and Fowler [32] explored the effect of environmental lead on lead-binding proteins and the ability of rat brain lead-binding proteins to diminish the inhibition of hepatic ALAD by lead. In the first study, a brain protein of 12 kDa was described, in comparison to the kidney lead-binding protein of 9 kDa. Both competitions of lead binding between the brain lead-binding protein and ALAD and donation of zinc by the brain protein (shown by ^{65}Zn uptake) were found to account for the decreased ALAD inhibition. In the second study the rat brain lead-binding protein was described as having a molecular weight of 23 kDa, with significant levels of glutamic acid, aspartic acid, and cysteine. Polyclonal antibody to rat renal lead-binding proteins showed a lack of reactivity with the brain protein, indicating that the proteins are immunologically distinct.

Fowler et al. [33] examined monkey kidney and brain from nonlead treated animals and isolated lead-binding proteins that also had a relatively high content of aspartic and glutamic amino acid residues and were similar in size to the rat lead-binding proteins. Polyclonal antibodies to α2-microglobulin and metallothionein did not cross-react with either monkey kidney or brain proteins. In environmentally lead-exposed humans, Quintanilla-Vega et al. [34] subsequently isolated from brain a thymosin β4 and a second, as yet unidentified, protein with a molecular weight of 20 kDa and a pI of 5.9.

As reported earlier, lead also binds to p32/6.3, a low-abundance, highly conserved nuclear matrix protein that becomes a prominent component of lead-induced intranuclear inclusion bodies [6]. Expression of this protein increases significantly during ontogeny, and was proposed as an indicator of neuronal maturation [7]. Expression also increases markedly in the presence of acute lead exposure *in vitro*, suggesting that lead either structurally alters the protein or inhibits a protease for which p32/6.3 is a substrate [5].

Recently, an astroglial glucose-regulated protein (GRP78) has been identified that acts as a molecular chaperone in endoplasmic reticulum [35, 36]. Intracellular levels of this protein are increased in cultured astroglia during a 1-week exposure to lead. GRP78 depletion significantly increased the sensitivity of cultured glioma cells to lead, as indicated by the generation of reactive oxygen species. This suggests that GRP78 is a component of the intracellular tolerance mechanism that handles high intracellular lead accumulation. Thus it appears that lead directly targets the protein, enabling it to play a protective role in lead neurotoxicity. The generation of reactive oxygen species also has been reported to occur via lead binding to astroglial copper-transporting ATPase, resulting in disruption in copper homeostasis [37].

4. Lead-Binding Proteins in Erythrocytes

Intraerythrocytic lead binding was initially attributed primarily to hemoglobin, molecular weight 64 kDa [38–41], but more recent studies have ascribed the major lead binding to delta-aminolevulinic acid dehydratase (ALAD), molecular weight 240 kDa (vide infra), which is an important step in heme biosynthesis [42]. In contrast to this protein, several studies have focused on an inducible low molecular weight protein which appears in workers chronically exposed to lead and which seems to have a protective effect. The first recognition of this protein was by Raghavan and Gonick [41] who found an approximately 10 kDa protein in lead workers but not in controls following Sephadex G-75 fractionation. Upon subsequent SDS-polyacrylamide gel electrophoresis, the protein split into two bands, only the uppermost of which contained lead. Raghavan et al. [43] then went on to fractionate the erythrocyte lead into a "hemoglobin" fraction, 10 kDa fraction, free lead, and a "residual lead" fraction, thought to be composed of membrane lead and a high molecular weight fraction. Lead workers manifesting toxicity at both high blood lead and relatively low blood lead levels showed high levels of residual lead, attributed in the workers with toxicity at low blood lead levels to a very low quantity of the 10 kDa fraction. In a follow-up paper, Raghavan et al. [44] found elevated levels of lead in the high molecular weight fraction (prehemoglobin) and in the membrane fraction in workers with toxicity at both high and low blood leads. Again, those with toxicity at low blood lead had low levels of the lead bound to the 10 kDa protein. Membrane lead was found to correlate inversely with membrane Na-K-ATPase; no correlation was seen with total blood lead. Gonick et al. [45] partially purified the 10 kDa protein by HPLC using a protein I-125 column, followed by isoelectric focusing on a sucrose gradient column. Three protein peaks resulted, one of 30 kDa, and two of approximately10 kDa. Only one of the latter peaks contained lead. This peak had a pI of 5.3 and a molecular weight, determined by SDS-PAGE, of 12 kDa. The majority of lead was found in this peak, which also contained calcium, zinc, and cadmium. Amino acid analysis showed a very high percentage of glycine (44%), lower quantities of histidine, aspartate, and leucine. The presence of glycine and aspartate corresponds in part to the composition of amino acids in the intranuclear inclusion bodies, as described by Shelton et al. [4] and Moore et al. [15].

Ong and Lee [40] studied the distribution of ^{203}Pb in components of normal human blood. Ninety-four percent

of ^{203}Pb was incorporated into the erythrocyte, and 6% remained in the plasma. SDS-PAGE of plasma showed that 90% was present in the albumin fraction. Within the erythrocyte membrane the most important binding site was the high molecular weight fraction, about 130–230 kDa. Within the erythrocytic cytoplasm the protein band associated with ^{203}Pb had a molecular weight of 67 kDa as shown by the elution characteristics on G-75 chromatography. This was thought to be hemoglobin. Lolin and O'Gorman [39] and Church et al. [46, 47], following the same procedure as Raghavan and Gonick [41], confirmed the findings of a low molecular weight protein in the erythrocytes of lead workers, not found in control patients. Lolin and O'Gorman [39] quantitated the protein, which ranged from 8.2 to 52.2 mg/L RBC in lead workers but none in controls, again implying an inducible protein. They found that the low molecular weight protein first appeared when the blood lead concentration exceeded 39 μg/dL. A positive correlation was seen between the amount of the intraerythrocytic low molecular weight protein and dithiothreitol-activated activity (i.e., "restored" ALAD activity) but not the nonactivated activity. Church et al. [46, 47] also confirmed the findings of Raghavan and Gonick [41]. In the initial paper, they described two patients with high blood lead levels, an asymptomatic worker with a blood lead of 180 μg/dL and a symptomatic worker with a blood lead of 161 μg/dL. In the first patient, approximately 67% of the erythrocyte lead was bound to a low molecular weight protein of approximately 6-7 kDa. In the second patient, the protein only contained 22% of the total erythrocytic lead. In their remaining paper [47], Church et al found that a sample of the low molecular weight protein purified from lead workers, which they termed protein M, had characteristics of metallothionein, such as a molecular weight of 6.5 kDa, a pI between 4.7 and 4.9 and a greater UV absorbance at 254 nm than at 280 nm. Amino acid composition showed 33% cysteine but no aromatic amino acids. This composition differed from that of the low molecular weight protein described by Gonick et al. [45], which had a molecular weight of 12 kDa, a pI of 5.3, and amino acid analysis which showed no cysteine. This discrepancy might be explained by a combined lead and cadmium exposure in the Church et al. study, which may have produced a lead-thionein (vide infra).

Xie et al. [48] used a Biogel A column instead of Sephadex G-75 to separate lead-binding proteins from erythrocyte hemolysates from a control patient and from lead-exposed workers. In this study they showed clearly that the major lead-binding occurred to a large molecular weight protein, consistent with ALAD, in both the controls and lead workers. When they added increasing amounts of lead to the blood of the control patient, a second, low molecular weight, lead-binding peak occurred which became larger than the lead binding to the ALAD peak when the lead concentration exceeded approximately 50 μg/dL. This second peak was also seen in a chronically lead-exposed worker, and was estimated to be less than 30 k Da in molecular weight. Thus these results are consistent with the aforementioned studies.

5. Is ALAD an Inducible Enzyme and Is It the Principal Lead-Binding Protein in the Erythrocyte?

The enzyme ALAD has been found to be the most sensitive indicator of lead exposure and toxicity [49, 50]. In the 1980's, two articles were presented which appear to show that ALAD is inducible after lead exposure in humans. By comparing a nonexposed control population to lead workers, and assaying ALAD by means of immunoassay or as "restored" ALAD activity (i.e., incubation with heat, zinc and dithiothreitol), both articles indicated that the *amount* of ALAD, as contrasted to ALAD *activity*, was increased by lead exposure [51, 52]. Similar findings were reported for the rat [53]. Subsequent studies have focused on the effect of ALAD polymorphism on the susceptibility to lead intoxication. ALAD is a zinc-containing enzyme, which catalyzes the second step of heme synthesis, that is, catalyzes the condensation of two delta-aminolevulinic acid molecules into one molecule of porphobilinogen [51]. It is a polymorphic protein with three isoforms: ALAD-1, ALAD 1-2, and ALAD 2-2. Several studies have shown that, with the same exposure to lead, individuals with the ALAD-2 gene have higher blood lead levels [54–60]. Initially it was thought that these individuals might be more susceptible to lead poisoning [59], but it is now appreciated that the ALAD-2 gene offers protection against lead poisoning by binding lead more securely [61]. It has been shown that ALAD-2 is more electronegative than ALAD-1 and thus the ALAD-2 enzyme may have a higher affinity/stability for lead than ALAD-1 [62]. In support of this statement it can be cited that individuals with the ALAD 1-2/2-2 genotypes in comparison to those with ALAD 1-1 genotypes have not only higher blood lead but also decreased plasma aminolevulinic acid [63], lower zinc protoporphyrin [56], lower cortical bone lead [58], and lower amounts of dimercaptosuccinic-acid (DMSA-) chelatable lead [64].

The significance of erythrocyte ALAD lead-binding was initially confirmed by a study by Bergdahl et al. [55], in which the authors used a FPLC Superdex 200 HR 10/30 chromatographic column coupled to ICP-MS (for determination of lead) to examine erythrocytes from lead workers and controls. They found the principal lead-binding protein peak to be of 240 kDa, rather than the presumed hemoglobin peak reported by Barltrop and Smith [38] and Raghavan and Gonick [41], using Sephadex G-75 chromatography. This was shown to be ALAD by binding to specific ALAD antibodies. Two additional smaller lead-binding peaks of 45 kDa and 10 kDa were also seen, but not identified. Bergdahl et al. [55] attributed the discrepancies in the studies to the fact that Sephadex G-75 separates proteins in the range of 3 to 80 kDa, making the separation of hemoglobin (molecular weight 64 kDa) from ALAD (molecular weight 240–280 kDa) very difficult. In addition, the earlier studies had utilized binding of ^{203}Pb or ^{210}Pb to identify the binding proteins, a technique which may have skewed the findings if ALAD

were already saturated with lead. ALAD-binding capacity for lead has been measured at 85 μg/dL in erythrocytes or 40 μg/dl in whole blood [65], which would permit a greater degree of binding to the low molecular weight component when blood lead exceeded 40 μg/dL. Bergdahl et al. [65] have speculated that the low molecular weight component might be acyl-CoA-binding protein, identical to the kidney lead-binding protein described by Smith et al. [30]. Goering and Fowler [66] had reported earlier that the presence of low molecular weight high-affinity (Kd 10^{-8} M) lead-binding proteins in kidney and brain served as protection against ALAD inhibition in those organs, whereas the absence of these low molecular weight proteins in liver contributed to the greater sensitivity to ALAD inhibition in that organ.

6. Lead-Binding Proteins in Rat Liver

Sabbioni and Marafante [67] explored the distribution of ^{203}Pb in rat whole tissue as well as in subcellular fractions of liver. By far the largest quantity of lead recovered was in the kidney, with lesser amounts in liver, spleen, and blood. Upon subcellular fractionation of the liver, the majority of ^{203}Pb was found in the nuclei, and most of the lead was detected in the nuclear membrane fraction bound exclusively to membrane proteins. The intranuclear lead was associated with histone fractions. As we have seen previously from Oskarsson et al. [22], lead-binding proteins were not found in the cytoplasm of the liver.

7. Lead-Binding Proteins in Intestine

Fullmer et al. [68] have shown in the chick and cow that although lead does not stimulate lead-binding proteins in the intestine directly, lead can displace calcium from calcium-binding proteins, and thus calcium-binding proteins may play a role in intestinal lead transport. Purified calcium-binding protein from chick and cow, as well as calmodulin, troponin C, and oncomodulin, was dialyzed against added labelled and unlabelled lead or calcium. Results disclosed high-affinity binding sites, greater for lead than for calcium. Similar results were obtained with calmodulin, troponin C, and oncomodulin members of the troponin C super family of calcium-binding proteins.

8. Lead-Binding Proteins in Lung

Singh et al. [69] described intracellular lead-inclusion bodies in normal human lung small-airway epithelial cells cultured with either lead chromate particles or sodium chromate. Cells exposed to both forms of chromate underwent dose-dependent apoptosis. Lead-inclusion bodies were found in nucleus and cytoplasm of lead chromate, but not sodium chromate, treated cells. Lead, but not chromium, was detected in the inclusion bodies by energy dispersive X-ray analysis. The protein within the inclusion bodies has not been analyzed.

9. Relationship of Lead-Binding Protein to Metallothionein

Similarities of lead-binding protein to metallothionein have been discussed earlier. Maitani et al. [70] commented that hepatic zinc metallothionein could be induced by intravenous intraperitoneal injections of lead into mice, but not by subcutaneous injection. Ikebuchi et al. [71] found that a sublethal dose of lead acetate injected intraperitoneally into rats induced the synthesis of a lead metallothionein in addition to zinc metallothionein. The lead metallothionein contained 28% half cysteine and cross-reacted with an antibody against rat zinc-thionein II.

Goering and Fowler [66, 72] demonstrated that pretreatment of rats with zinc 48 and 24 hrs prior to injection of ^{203}Pb resulted in both zinc and lead coeluting with a zinc-thionein fraction on Sephadex G-75 filtration. In addition, both purified zinc-thionein-I and II bound ^{203}Pb *in vitro*. Gel filtration of incubates containing liver ALAD and ^{203}Pb demonstrated that the presence of zinc-thionein alters the cytosolic-binding pattern of lead, with less bound to ALAD. Zinc-thionein also donates zinc to activate ALAD. In the second paper [66], Goering and Fowler found that pretreatment of rats with either cadmium or zinc affected liver ALAD activity when incubated with lead. Liver and kidney zinc-thioneins, and to a lesser extent, cadmium-zinc-thionein decreased the free pool of lead available to interact with ALAD, resulting in attenuated ALAD inhibition. Liu et al. [73] further showed that zinc-induced metallothionein in primary hepatocyte cultures protects against lead-induced cytotoxicity, as assessed by enzyme leakage and loss of intracellular potassium.

Qu et al. [74] and Waalkes et al. [75] have shown that metallothionein-null phenotypic mice are more susceptible to lead injury over a 20-week period than wild-type mice. Unlike the wild-type mice, lead-treated metallothionein-null mice showed nephromegaly and significantly decreased renal function after exposure to lead. The metallothionein-null mice accumulated less renal lead than wild-type and formed no inclusion bodies. When the observations were extended to 104 weeks, renal proliferative lesions (adenoma and cystic tubular atypical hyperplasia) were more common and severe in metallothionein-null than in wild-type mice. A metastatic renal cell carcinoma occurred in a metallothionein-null mouse whereas none occurred in wild-type mice. Such studies lend credence to the view that metallothionein or a closely related gene is involved in the formation of lead-binding proteins in the kidney.

A more recent study [76] further supports the involvement of metallothionein in lead-induced inclusion body formation. Immunochemical studies revealed that metallothionein could be detected on the outer surface of lead-induced renal inclusion bodies in wild-type mice. Cell lines from metallothionein-null and wild-type mice were exposed to lead, resulting in inclusion body formation in the wild-type, but not the metallothionein-null cells. When metallothionein was transfected into the metallothionein-null cells, inclusion bodies were seen. Further studies revealed that α-Synuclein, an aggresomal protein, showed

poor basal expression in metallothionein-null cells and failed to increase after lead exposure, but increased rapidly in wild-type cells, then decreased after inclusion bodies were formed. An antibody pulldown assay confirmed a direct interaction between α-Synuclein and metallothionein as the proteins coprecipitated with an antibody to metallothionein.

10. Biochemical Studies of Metal Binding to Proteins

Three important papers have appeared concerning the mechanism by which a metal ligand binds to proteins. Lead is known to displace physiologically relative metal ions, such as calcium and zinc, in proteins. Kirberger and Yang [77] reported that approximately 1/3 of the lead binding sites were identified as due to zinc or calcium ionic displacement, whereas two-thirds were opportunistic. Oxygen atoms from amino acids or water represent the major ligand for lead, followed by sulfur and nitrogen. Sulfur acts as the ligand in the case of displacement of zinc by lead at the zinc-binding sites in delta-aminolevulinic acid dehydratase (ALAD). Studies of calmodulin, a calcium binding protein with a propensity for displacement of calcium by lead, showed an initial activation followed by inhibition in response to increasing concentrations of lead. The latter was thought to result from more pronounced conformational changes resulting from additional opportunistic binding. Hanas et al. [78] explored whether the association of lead with chromatin might suggest that the deleterious effects may in part be mediated through alterations in gene function. They specifically examined whether lead altered DNA binding of cysteine-rich zinc finger proteins. It was found that inhibition of Cys_2His_2 zinc finger transcription factors by lead ions at concentrations near those known to have deleterious physiological effects was suggestive for a new molecular mechanism for lead toxicity. However, the changes were seen predominantly at lead concentrations varying from 100 to 400 ug/dL, above the industrial or pathophysiological range. Becker et al. [79] analyzed naturally occurring metal-binding proteins in rat liver and kidney, utilizing nondenaturing gel electrophoresis together with laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). The mass range of proteins separated was from 45 to 120 kDa. Lead was found only in small quantities in the 65 kDa protein band, whereas zinc and copper were scattered throughout the protein bands. If this technique could be extended to the study of lead-treated as well as native rats and the protein mass separated down to 5 kDa, confirmation (or lack thereof) of the results reported earlier by Fowler and associates [22–24, 29, 33] might be anticipated.

11. Discussion

There appears to be a consensus that the enzyme, ALAD, a 240–280 kDa protein, is moderately inducible and is the major lead-binding protein within the erythrocyte. ALAD polymorphism influences the degree of lead binding as the ALAD-2 phenotype binds more lead in a nontoxic fashion than ALAD-1. What is more confusing is the nature and importance of the low molecular weight erythrocytic lead-binding protein. There is no doubt that it appears in lead-exposed workers once blood lead exceeds 40 μg/dL but does not appear in controls. An intriguing observation was made by Xie et al. [48], who noted that the *in vitro* addition of lead to erythrocytes of controls resulted in the appearance of a low molecular weight lead-binding peak migrating in the same position as the low molecular weight peak from lead workers. This suggests that lead can effect a conformational change in a preexisting protein. The nature of the low molecular weight protein is also questionable as Gonick and colleagues [45] identified it as a 12 kDa protein with a high percentage of glycine, plus histidine, aspartic acid and leucine while Church and coworkers [46, 47] identified the protein as a 6.5 kDa molecule, with a large percentage of cysteine and a greater UV absorbance at 254 than 280 nm. These findings suggested to the latter authors that the protein might be a metallothionein.

Metallothionein is a protein that is mildly inducible by lead, but to a much greater degree by zinc and cadmium. What is more significant is that lead binds to preformed metallothionein, stimulated by zinc or cadmium, so that under these conditions a lead-thionein is formed. If concomitant lead and cadmium exposure occurred in the lead workers described by Church et al. [46, 47], that could reasonably account for the finding of metallothionein in these workers. However the possible role of metallothionein as a component of the renal lead-binding protein seems more likely as metallothionein-null mice failed to respond to lead exposure by developing intranuclear lead inclusion bodies, whereas metallothionein-null cells transfected with metallothionein were capable of responding to lead.

Extensive studies of cytoplasmic lead-binding proteins in nonlead-treated rats, human, and monkeys have been reported. Whether these proteins have formed as a result of environmental exposure to lead or are preformed is at present unclear. The lead-binding protein in rat kidney has been identified as a cleavage product of α2- microglobulin. The low molecular weight lead-binding proteins in human kidney have been identified as thymosin β4 (molecular weight 5 kDa) and acyl-CoA-binding protein (molecular weight 9 kDa). In human brain, the lead-binding proteins were thymosin β4 and an unidentified protein of 23 kDa. The principal remaining question is whether the p32/6.3 intranuclear lead-binding protein initially described by Shelton and Egle [2] represents a condensation product from these low molecular weight proteins.

12. Conclusions

Lead-binding proteins occur in many organs in the body, including kidney, brain, erythrocyte, liver, and lung. In most instances, they appear to be of low molecular weight, inducible by lead exposure, and to afford some protection against lead toxicity. The exception is the enzyme delta-aminolevulinic acid dehydratase (ALAD), which is present in high concentration in the erythrocyte, and although

further inducible by lead exposure, is exquisitely sensitive to inhibition by lead.

The relationships between the intranuclear lead-binding protein of the kidney, the cytoplasmic lead-binding proteins, and the lead-binding proteins in erythrocytes remain to be determined. What is clear is that the inducible lead-binding proteins afford protection against lead toxicity.

Abbreviations

ALAD:	Delta-aminolevulinic acid dehydratase
ATPase:	Adenosine triphosphatase
Ca^{++}:	Calcium ion
$CaNa_2EDTA$:	Calcium disodium ethylenediaminetetraacetic acid
Cd^{++}:	Cadmium ion
DMSA:	Dimercaptosuccinic acid
Fe^{++}:	Iron ion
FPLC:	Fast protein liquid chromatography
GFAP:	Glial fibrillary acidic protein
GRP78:	Glucose-regulated protein
ICP-MS:	Inductively coupled plasma mass spectroscopy
i.p.:	Intraperitoneal
Kd:	Dissociation constant
kDa:	Kilodalton
kg:	Kilogram
M:	Molar
mRNA:	Messenger ribonucleic acid
Na-K-ATPase:	Sodium-potassium-activated adenosine triphosphatase
Pb:	Lead
SDS-PAGE:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
UV:	Ultraviolet
Zn^{++}:	Zinc ion.

Disclosure

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Review Article

Revelations from the Nematode *Caenorhabditis elegans* on the Complex Interplay of Metal Toxicological Mechanisms

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Metals have been definitively linked to a number of disease states. Due to the widespread existence of metals in our environment from both natural and anthropogenic sources, understanding the mechanisms of their cellular detoxification is of upmost importance. Organisms have evolved cellular detoxification systems including glutathione, metallothioneins, pumps and transporters, and heat shock proteins to regulate intracellular metal levels. The model organism, *Caenorhabditis elegans* (*C. elegans*), contains these systems and provides several advantages for deciphering the mechanisms of metal detoxification. This review provides a brief summary of contemporary literature on the various mechanisms involved in the cellular detoxification of metals, specifically, antimony, arsenic, cadmium, copper, manganese, mercury, and depleted uranium using the *C. elegans* model system for investigation and analysis.

1. Introduction

Exposure to metals remains a persistent toxicological concern. The accumulation of metals in the environment, stemming from their origination in the earth's crust, as well as from anthropogenic sources, creates the potential for significant human exposures and subsequent health hazards. Deleterious metal-induced health effects, including carcinogenesis and neurodegeneration, have been reported in all body systems, with exposure stemming from multiple sources, including contact with contaminated food, water, air, or soil. The particular metals considered in this review, antimony, arsenic, cadmium, manganese, mercury, silver, and uranium, are among the classes of essential nutrients, as is the case with copper and manganese, as well as the nonessential, naturally occurring metals, such as arsenic, cadmium, and mercury, all of which can induce toxicity depending on the concentration level and exposure duration. Over time, organisms have developed protective mechanisms to deal with metal exposure, most of which function in one of three ways: (1) decreasing the uptake of the metal, (2) stimulating the expulsion of the metal, or (3) activating the organism's general stress response mechanisms. Metals can disrupt homeostasis by generating oxidative stress, inhibiting enzyme activity, impairing DNA repair, and disrupting protein binding and normal cellular function, including proliferation, cell cycle progression, and apoptosis [1–4].

Elucidating the mechanism(s) of metal detoxification has been difficult due to the complexity of mammalian systems and the reductionist approach inherent to cell culture systems. The model organism, *Caenorhabditis elegans (C. elegans),* offers the advantage of an *in vivo* system that is less complex than the mammalian system while still sharing high homology. *C. elegans* possess ~60%–80% of human genes [5] and contain conserved regulatory proteins [6–8].

This soil nematode has been used in a number of toxicity studies due to its well-characterized genetic, physiological, molecular, and developmental stages. Some of the advantages

Table 1: Points to consider when using *C. elegans*.

(i) *Metal concentrations*: Must be measured in worms because of potential differences in uptake due to the worm cuticle (versus ingestion through the pharynx). In addition to concentrations, attention should be paid to the specific metallic salt under consideration as well as speciation that may occur following exposure.

(ii) *Age matching:* May be necessary depending on the toxic endpoint measured due to potential for developmental delay in knockout strains.

(iii) *Dauer stage:* An alternative developmental stage when the larva goes into a type of stasis and becomes lethargic, ceases pharyngeal pumping, synthesizes a new cuticle under the old, and can survive harsh conditions [16].

(iv) *Source of exposure*: *C. elegans* exhibit avoidance behavior and have been shown to avoid certain volatile compounds [29] as well as high concentrations of salts and sugars [30, 31].

(v) *Medium considerations*: *C. elegans* exhibit a wide pH range tolerance and thus can be exploited to measure alterations in toxicity of metals following pH elevation [32]. Differential effects of soil versus aquatic medium have also been documented [33].

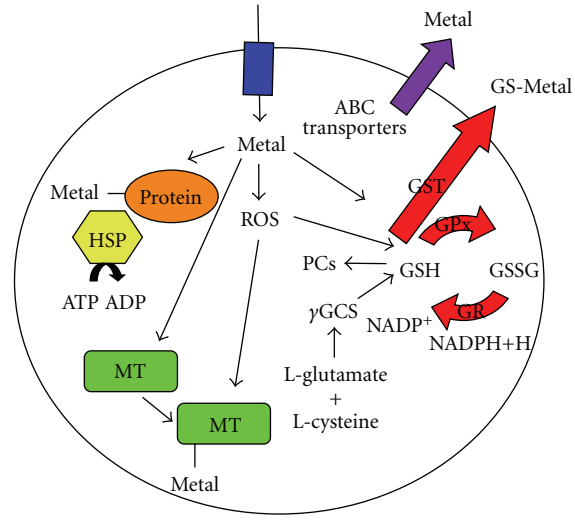

Figure 1: Metal detoxification systems. GSH is converted to GSSG upon exposure to ROS. GR converts GSSG back to GSH while converting NADPH to NADP+. γGCS is the rate-limiting enzyme in GSH synthesis. GSTs assist with the conjugation of GSH to the metal for excretion from the system. Additionally, GSH is known to be protective against metal-generated ROS by binding free radicals. PCs are thiol-rich peptides that can complex with metals and act as chelators. MTs can directly bind and sequester the toxicant and act as antioxidants. ATP-binding cassette (ABC) transporters contribute to heavy metal tolerance by facilitating the excretion of metals, including metals that are conjugated to GS−. HSP70s are ATP-binding proteins that convert ATP to ADP and bind to metals and other proteins, thereby inactivating them and preventing aggregation.

afforded by the *C. elegans* model system are small size (~1.5 mm adult), short lifespan (~3 weeks), and rapid lifecycle (~3 days) [8–10]. At adulthood, a single *C. elegans* hermaphrodite is capable of producing ~300 progeny. *C. elegans* are hermaphrodites, but approximately 1% of *C. elegans* are male, allowing for genetic experimentation [11]. The nematode's small genome and relative anatomical simplicity (less than 1000 cells) contribute to the appeal of this model system for genetic manipulation [11]. In addition, the use of RNA interference (RNAi) and chromosomal deletion in worms has provided valuable information regarding the increased sensitivity of mutant strains to metal toxicity [12–14]. Maintenance of nematode strains is relatively simple; they grow on bacteria-seeded plates and can be maintained at 20°C [15]. Strains can also be frozen indefinitely, easily allowing for the accumulation of large stocks of worms [11]. *C. elegans* provide the researcher with a uniquely powerful model, as the worm's translucent body allows for the *in vivo* visualization of fluorescently labeled individual cells and proteins [16]. Accordingly, the *in vivo C. elegans* model system is especially valuable for the investigation of metal detoxification and is particularly amenable for examining gene-environment interactions, albeit with a few considerations to take into account (Table 1). Several toxicity endpoints are readily detected and well documented in the nematode, including mortality, lifespan, reproduction, and feeding [17–19]. Acute toxicity can also be assessed in the nematode through behavioral endpoints, such as locomotive behavior, head thrashing, body bending, and other basic movements [20–24]. Recently, the role of *C. elegans* as a biomonitor in environmental risk assessment has also been explored [25–28].

Several cellular systems such as the glutathione (GSH), metallothioneins (MTs), heat shock proteins (HSPs), as well as various pumps and transporters work in concert to detoxify and excrete metals. It remains to be established whether the knockdown or overload of one detoxifying system upregulates other compensatory mechanisms. In this review, we offer a brief summary of the ways in which the *C. elegans* model has shed novel insights on the various mechanisms of metal detoxification. Metals considered herein are antimony (Sb), arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg), silver (Ag), and uranium (U).

2. Glutathione

Glutathione (GSH) is a cysteine-containing tripeptide, consisting of glutamic acid, cysteine, and glycine and is found in most life forms. GSH possesses antioxidant properties, since the thiol group of cysteine is a reducing agent and can be reversibly oxidized (GSSG) and reduced (GSH). GSH is maintained in the reduced form by the enzyme, glutathione reductase (GR), and functions by reducing other metabolites and enzyme systems. Glutathione peroxidase (GPx) catalyzes the oxidation of GSH to GSSG in the presence of ROS (Figure 1). GSSG can be converted back into GSH via GR and the conversion of NADPH to NADP+ [51]. Proton-translocating mitochondrial nicotinamide nucleotide transhydrogenase (NNT) catalyzes the reduction of NADP+ by NADH and is an important source of NADPH, as has been demonstrated using *nnt-1* deletion

mutants [52]. Glutathione S-transferases (GSTs) can catalyze the conversion of GSH to GS−, which can then form complexes with various xenobiotics to facilitate excretion [53]. These enzymes are found at particularly high levels in the liver, and GSH is typically the most abundant sulfhydryl-containing compound in cells. Additionally, GSH is known to offer protection against metal-generated ROS by binding free radicals [54]. GSHs have been reported to increase, decrease, or remain constant after exposure to metals [55–57]. The GSH system is found in animals, plants, and microorganisms. *C. elegans* express approximately 50 GSTs [58]. Additionally, phytochelatins (PCs), a family of metal-inducible peptides synthesized enzymatically from GSH by PC synthase (PCS) in the presence of heavy metal ions, have been identified in *C. elegans* [34]. Although PCs are synthesized from GSH, they are broadly classified as class III metallothioneins and have been shown to be important in the detoxification of heavy metals [34].

2.1. Characterization of C. elegans GSH and Interaction with Metals. Liao and Yu [35] investigated the involvement of the GSH system in response to inorganic arsenic exposure. Results confirmed that oxidative stress plays a role in arsenic-induced toxicity by mutating glutamylcysteine synthetase (GCS) (gcs-1), the rate-limiting enzyme in GSH synthesis, in worms. The *gcs-1* loss-of-function strain demonstrated hypersensitivity to arsenic exposure in lethality testing, as compared to wild-type animals, an effect that was rescued by the addition of GSH to the medium, indicating that these enzymes are crucial for mediating arsenic-induced toxicity [35].

Furthermore, Helmcke and Aschner [39] reported a significant increase in fluorescence in a *gst-4*::GFP strain following both acute and chronic exposure to MeHg [39]. It was also demonstrated that knockout *gst-4* worms did not display greater sensitivity to MeHg than did N2 wild-type worms. GSH levels were found to be increased in worms subjected to acute exposure, whereas worms subjected to chronic exposure exhibited depleted levels of GSH. In their hormetic model, Helmcke and Aschner demonstrated an increase in *gst-4*::GFP expression after low concentration, acute exposure, a finding which indicates that *gst-4* may be involved in the hormetic response to MeHg [39]. Taken together, the data from these studies suggest that *gst-4* contributes to the response to MeHg exposure, but that knockdown of this gene does not affect with overall lethality.

In a study examining the role of PCS in the elimination of cadmium, Vatamaniuk and colleagues [36] reported that the *C. elegans pcs-1* gene encodes a functional PC synthase critical for heavy metal tolerance. Using double stranded RNAi against *pcs-1,* this group showed that, although the progeny of worms injected with the dsRNAi and exposed to cadmium (5–25 μM) managed to reach adulthood, these worms were small, necrotic, sterile, and had a much shorter lifespan than did the wild-type controls [36]. After being exposed to 50 and 100 μM concentrations of cadmium, the *pcs-1* worms arrested at the L2–L4 stage were necrotic and died by day 6. The results were definitively dependent on the presence of cadmium, because *pcs-1*-deficient worms, in the absence of cadmium exposure, responded identically to the wild-type controls [36].

Hughes and colleagues [37] examined metabolic profiles following cadmium exposure in phytochelation synthase-1 (*pcs-1*) mutants. Results from these studies showed that the primary response to low levels of cadmium is the regulation of the transsulfuration pathway, due to the fact that cadmium exposure caused a decrease in cystathionine concentrations and an increase in phytochelation-2 and -3 [37]. These results were corroborated by additional studies which demonstrated that *pcs-1* mutants were an order of magnitude more sensitive to cadmium than were the metallothionein mutants. Furthermore, the MT-*pcs-1* triple mutant was found to display an additive sensitivity toward cadmium [37]. Significant findings are summarized in Table 2.

3. Metallothioneins

Metallothioneins (MTs) belong to a family of cysteine-rich low-molecular-weight metal-binding proteins (MW 3,500–14,000 Da) involved in metal detoxification and homeostasis [59]. MTs bind both metals of physiological importance such as copper and zinc, as well as xenobiotics including arsenic, cadmium, mercury, and silver. The binding of these metals occurs via the interaction of the cysteine residues with thiol groups. Cysteine residues represent approximately 30% of the amino acid content of metallothioneins. Binding of metals by MTs may be transient, as MTs are capable of rapidly releasing metal ions [60]. The protective roles of MTs can be ascribed to their three primary functions: (1) metal homeostasis, (2) heavy metal detoxification, and (3) protection from oxidative stress. Additionally, these proteins have been identified as contributors to the hormetic response [39, 61].

Mammals express four known metallothionein isoforms (MT-I, MT-II, MT-III, MT-IV) [62]. MT-I and MT-II are expressed in almost all tissues and have been best characterized with regard to their protection of the brain [62]. MT-III is especially enriched in the central nervous system, although its role has not yet been clearly defined [63]. MT-IV is most abundantly expressed in the stratified squamous epithelia [64–66]. MT expression has been shown to be induced under stressful cellular conditions such as exposure to cytokines, glucocorticoids, reactive oxygen species (ROS), and metal ions [67]. MTs can bind directly and sequester the toxicant; they also can provide protection by acting as antioxidants [59] (Figure 1). Further, MTs can limit apoptosis and promote the survival of mitochondrial dysfunctional cells by serving as highly efficient reducing elements against reactive oxygen species (ROS) [68]. *C. elegans* contain two distinct isoforms of MTs, known as *mtl-1* and *mtl-2,* which can be induced in response to exposure to various metals [69].

3.1. Characterization of MTs and Their Interaction with Metals. Jiang and colleagues examined the effects of MTs on depleted uranium (DU) in *C. elegans* [12]. This group demonstrated concentration-dependent DU toxicity and

Table 2: Summary of significant findings.

Metals	Effects Observed	Reference
	Glutatione	
Arsenic	Nematodes with loss of glutamylcysteine synthetase (*gsc-1*) demonstrated hypersensitivity to arsenic exposure in lethality testing. Effect was rescued by the addition of GSH to medium.	[34]
Methylmercury	Significant increase in fluorescence of gst-4::GFP following MeHg exposure. Knockout *gst-4* worms not more sensitive than wildtype. GSH levels increased following acute exposure; chronic exposure depleted levels of GSH. GSH found to regulate the hormetic response.	[35]
Cadmium	Low Cd exposure in phytochelation synthase-1 (*pcs-1*) RNAi worms resulted in worms that were small, necrotic, sterile, and had a shorter lifespan. Following higher concentrations of Cd, *pcs-1* worms arrested at L2–L4 stage were necrotic and died.	[36]
Cadmium	Primary response to low levels of cadmium is the regulation of the transsulfuration pathway due to decreases in cystathionine concentrations and increases in phytochelation-2 and -3. MT-pcs-1 triple mutants showed added sensitivity.	[37]
	Metallothioneins	
Depleted uranium	Concentration-dependent DU toxicity and protection by MTs. Mtl-1 knockouts displayed increased cellular accumulation of DU.	[12]
Lead and methylmercury	Pretreatment of larva with heat shock prevented the neurobehavioral deficits and the stress response at lower concentrations (50–100 μM) but not at higher concentrations (200 μM). Mild heat shock and low concentration of either metal found to induce *mtl-1* and *mtl-2* promoter activity and GFP expression. Overexpression of *mtl-1* or *mtl-2* at L2 stage significantly repressed neurobehavioral toxicity.	[38]
Methylmercury	Mtl knockouts displayed increased lethality upon exposure to MeHg. Increases in *mtl-1* following acute MeHg exposure at L1 stage but no change following chronic exposure.	[39]
Silver Nanoparticles	Mtl-2 strain displayed greater AgNP sensitivity than wildtype. Toxicity mediated by ionic silver.	[40]
Cadmium	MT isoforms found to be independent and not synergistic. Cadmium but not copper or zinc was able to influence a concentration-dependent, temporal transcription response.	[41]
Cadmium	Metallothionein status did not influence the metabolic profile in cadmium-exposed or -unexposed worms. Primary response was the regulation of the transsulfuration pathway.	[37]
Zinc and cadmium	Differential metal binding behavior for MT-1 compared to MT-2. MT-1 had optimal behavior when binding Zn, MT-2 optimal behavior when binding Cd.	[42]
Zinc and cadmium	Zinc levels significantly increased in *mtl-1*, *mtl-2,* and double knockouts, *mtl-1* knockout worms demonstrating the most acute level of sensitivity. Cd accumulation found to be highest in *mtl-2* and double mutant strains.	[43]
Cadmium and copper	MT-1 mRNA levels significantly higher in *daf-2* mutants compared to *age-1* mutants and wild-type worms under basal conditions. Cd treatment resulted in 3-fold induction of MT-1 and 2-fold induction of MT-2 mRNA in *daf-2* mutants compared to wild-type controls. Copper did not induce expression in any of the strains tested.	[44]
	Pumps and Transporters	
Arsenite and antimonite	ArsA ATPase (*asna-1*) gene stimulated by As (III) and Sb (III) crucial for establishing tolerance.	[45]
Arsenite and Cadmium Antimony	Inactivation of *mrp-1* rendered As and Cd exposed worms incapable of recovering from temporary exposure to high As and Cd, wildtype worms were able to recover. Worms were hypersensitive to As and Cd exposures when both *mrp-1* and *pgp-1* were deleted. No increased sensitivity in response to antimony observed in *mrp-1* deletion mutants compared to wild types.	[46]

Table 2: Continued.

Metals	Effects Observed	Reference
Cadmium	Suppression of *hmt-1* (half-molecule ABC transporter of the heavy metal tolerance family-1) by RNAi shown to produce inclusions within the nucleus of the intestinal epithelial cells upon exposure to toxic levels of Cd.	[47]
Arsenic, copper, and cadmium	HMT1—conferred tolerance in response to exposure to all three metals revealed through lethality testing following knockdown of *hmt-1*.	[48]
Cadmium	Three-fold induction of *pgp-5* following Cd exposure. Copper and zinc also found to be capable of inducing *pgp-5* expression. Mutant *pgp-5* worms showed developmental delay following Cd and Cu exposure.	[49]
Manganese	Deletion of the three DMT-1-like (divalent-metal transporter) genes resulted in differential effects. *smf-1* and *-3* increased Mn tolerance, and *smf-2* increased Mn sensitivity.	[50]
	Heat Shock Proteins	
Methylmercury	Following 30 minute exposure to acute MeHg, *hsp-4* was unaltered. Hsp-4 induced in L4 worms chronically exposed to MeHg for 15 hours.	[39]
Cadmium and mercury	Cd-inhibited feeding behavior significantly but not completely. Exposure to 1 ppm Cd induced hsp16 genes. Hg also did not entirely inhibit feeding behavior and was shown to inhibit feeding at concentrations similar to those necessary for the induction of a stress response.	[18]

protection by MTs. Results from their study showed that *mtl-1* was an important factor in uranium accumulation in *C. elegans* as knockouts displayed increased cellular accumulation [12].

In a study investigating lead and methylmercury toxicity, Ye and colleagues [38] demonstrated the involvement of MTs in affording a protective cross-adaptation response to neurobehavioral toxicity. This endpoint was assessed by observing behavioral alterations (head thrashing and body bending) in worms that were exposed during the L2 phase to either Pb or MeHg [38]. The study was conducted in conjunction with mild heat shock, wherein pretreatment of the larva with heat shock prevented the neurobehavioral deficits and the stress response at lower concentrations (50–100 μM) but not at higher concentrations (200 μM). Additionally, mild heat shock coupled with exposure to a low concentration of either metal was found to induce *mtl-1* and *mtl-2* promoter activity and GFP gene expression, results that were not observed in either the metal-exposed or heat-shocked cohort alone. Finally, the overexpression of *mtl-1* or *mtl-2* at the L2 stage was shown to significantly repress neurobehavioral toxicity, suggesting that the accumulation of MT protein is necessary to confer the protective response to the toxicant.

Similarly, Helmcke and Aschner [39] reported that *mtl* knockouts displayed increased lethality upon exposure to MeHg. This group also demonstrated increases in *mtl-1*::GFP fluorescence in response to acute MeHg exposure at the L1 stage; however, chronic MeHg exposure produced no change in florescence [39]. Their results indicate that *mtl-1* is important in mediating MeHg toxicity and that the effects occur in a concentration- and time-dependent manner.

Meyer and colleagues [40] examined the aggregation of silver nanoparticles (AgNPs) in wild type and *mtl-2 C. elegans*. Results from these studies showed that the *mtl-2* strain displayed greater AgNP sensitivity than did the wild-type controls. AgNPs were internalized, and the observed toxicity was mediated by ionic silver [40]. These data indicate that there may be a differential preference for *mtl-1* over *mtl-2* depending on the particular metal to which an organism is exposed.

In a 2004 study by Swain and colleagues [41], MTs were shown to play an important role in cadmium trafficking. Using GFP-expressing transgenes, MT-null alleles, and the RNAi knockdown of MTs, this group demonstrated that cadmium but not copper or zinc was able to influence a concentration-dependent, temporal transcription response. Both MT isoforms were found to be independent and not synergistic. Cadmium exposure caused a reduction in body size, generation time, brood size, and lifespan, effects that were magnified in the MT knockdown worms [41].

Hughes and colleagues [37] studied metabolic profiles using proton NMR spectroscopy and UPLC-MS following cadmium exposure in single and double *mtl* knockouts. Results showed that the metallothionein status did not influence the metabolic profile in cadmium-exposed or unexposed worms. The primary response to low levels of cadmium was the regulation of the transsulfuration pathway, due to the fact that cadmium exposure resulted in a decrease in cystathionine concentrations and an increase in phytochelation-2 and -3 [37]. These results were corroborated by data showing that *pcs-1* mutants (phytochelation synthase-1) were an order of magnitude more sensitive to cadmium than were MT mutants. Further, an additive sensitivity toward cadmium was observed in the MT-*pcs-1* triple mutant [37].

A study by Bofill and colleagues [42] examined zinc and cadmium toxicity; results indicated differential metal binding behavior for MT-1 as compared to MT-2. Specifically, the MT-1 isoform showed optimal behavior when binding Zn, and MT-2 showed optimal behavior when binding Cd. Accordingly, it was hypothesized that, due to its induction following Cd exposure, MT2 is primarily responsible for detoxification, whereas MT1 possesses some degree of

constitutive expression and is, therefore, primarily involved in physiological metal metabolism (e.g. zinc) [42]. These findings were corroborated by additional studies which showed that MT-knockout worms exhibited significantly decreased levels of overall fitness after the knockout of MT1 than after MT2 knockout. Further, both MT isoforms displayed a clear preference for divalent metal ion binding as opposed to copper coordination, likely due to the presence of histidines in the MTs [42].

Using both *in vitro* and *in vivo* models, Zeitoun-Ghandour and colleagues [43] examined zinc and cadmium exposures and showed different roles for *mtl-1* and *mtl-2*. Both isoforms were expressed *in vitro* and were exposed to either Zn(II) or Cd(II). Their affinities and stoichiometries were measured, and both isoforms displayed equal zinc-binding ability; however, *mtl-2* had a higher affinity for Cd than did *mtl-1*. These experiments were repeated *in vivo* in *mtl-1*, *mtl-2,* and double knockouts following exposure to 340 μM Zn or 25 μM Cd. Zinc levels were found to be significantly increased in all knockout strains, but *mtl-1* knockout worms demonstrated the most acute level of sensitivity. However, cadmium accumulation was found to be the highest in the *mtl-2* knockout and double mutant strains. Additional studies assessed metal speciation, and results indicated that O-donating ligands play an important role in maintaining zinc levels, independent of metallothioneins status. Further, cadmium was shown to interact with thiol groups, and Cd speciation was significantly different in the *mtl-1* strain when compared with both the *mtl-2* strain and the double knockout strain, suggesting that the two MT isoforms have distinct *in vivo* roles [43]. The authors suggested that MTs are not functioning as metal storage proteins but, rather, are mediating the accumulation and excretion of metals. A follow-up study, showed *in vitro* evidence for the partitioning of zinc and cadmium with different metallothionein isoforms [70]. Employing electrospray ionization mass spectrometry (ESI-MS) to directly observe zinc and cadmium binding preferences, more cadmium ions were found to be preferentially bound to MT-2 than to MT-1; however, Cd^{2+} was shown to be capable of inducing both isoforms. Finally, partitioning was also demonstrated to be more effective at lower Cd : Zn ratios [70].

Using *daf-2* (insulin receptor-like protein) and *age-1* (phosphatidylinositol-3-OH kinase catalytic subunit) mutants, Barsyte et al. [44] examined the expression of MT genes under noninducing conditions and after exposure to cadmium and copper. They reported that MT-1 mRNA levels were significantly higher in *daf-2* mutants compared to both *age-1* mutants and wild-type worms under basal conditions. This study also assessed constitutive MT-1 expression and inducible MT-2 expression. Exposure to cadmium treatment resulted in a three-fold induction of MT-1 and a two-fold induction of MT-2 mRNA in *daf-2* mutants as compared to wild-type controls. Copper did not induce MT-1 or MT-2 mRNA expression in any of the strains tested [44].

Collectively, these studies show differential metal preferences for one MT isoform over another depending on the metal to which an organism is exposed. Most significantly, these studies indicate that the MTs play crucial roles in metal detoxification (Table 2). Indeed, MTs have been associated with a protective effect in cells under numerous states of disease and stress.

Interestingly, serum MT levels of cancer patients are three times higher than those of control patients [71]. A study conducted in Denmark revealed the increased expression of MT-1 and MT-2 mRNA and protein in many human cancers such as breast, kidney, lung, nasopharynx, ovary, prostate, salivary gland, testes, urinary bladder, cervical endometrial skin carcinoma, melanoma, acute lymphoblastic leukemia, and pancreatic cancers [72]. This information is of particular import given the use of metals for the treatment of certain cancers, for example, arsenic as treatment for promyelocytic leukemia. It is interesting to postulate that higher levels of MTs may enhance the efficacy of metal therapeutic agents or, conversely, may lead to resistance to such therapies. Understanding the factors that modulate MT expression will allow for the improved understanding of metalloid toxicity and will provide more effective therapeutic approaches to metalloid-based chemotherapy.

4. Pumps and Transporters

There are a number of pumps and transporters that have been implicated in metal detoxification. These include ATP-binding cassette (ABC) transporters, such as the multidrug resistance-associated protein (MRP) as well as two members of the P-glycoprotein subfamily (PGP-1 and PGP-3), which have been shown to contribute to heavy metal tolerance through the use of *C. elegans* deletion mutants. In *C. elegans*, there are approximately 60 genes encoding ABC transporters, and these genes make up the largest family of transporters [73]. *C. elegans* have four MRP homologues [46] and fifteen Pgp homologues [73]. The Pgps are ubiquitously expressed and are most abundantly found in the apical membranes of the gut and in the excretory organs of the worm [74]. The specific functions of three of the nematode *pgps* have been identified. *pgp-2* is expressed in the intestine and is required for the acidification of lysosomes and lipid storage; *pgp-1* and *-3* contribute to heavy metal and drug resistance [46].

4.1. Characterization of Pumps and Transporters and Their Interaction with Metals. Tseng and colleagues [45] investigated the ArsA protein-mediated detoxification of the metalloids, As(III) and Sb(III). Bacterial ArsA ATPase is the catalytic component of an oxyanion pump that is responsible for resistance to arsenite and antimonite. In this study, wild-type and *asna-1*-mutant nematodes were evaluated for As and Sb response and toxicity. The *asna-1* gene of *C. elegans* was found to be stimulated by As(III); further, Sb(III) was determined to be crucial for establishing tolerance. Although these results occurred in response to As and Sb exposure, the ubiquity of the ArsA ATPase-dependent pathway has not been observed in other species or in response to other metals [45]. Moreover, the exact mechanism(s) of protection has not yet been elucidated.

The role of multidrug resistance-associated protein (MRP) in arsenite and cadmium toxicity was explored in a study by Broeks and colleagues [46]. The targeted

inactivation of *mrp-1* rendered the arsenite—and Cd^{2+}—exposed worms incapable of recovering from temporary exposure to high arsenic and cadmium, whereas the wild-type controls were able to recover. Additionally, worms were also shown to be hypersensitive to arsenite and Cd^{2+} exposures when both *mrp-1* and *pgp-1* (P-glycoprotein-1) were deleted [46]. Lastly, no increased sensitivity in response to exposure to antimony was observed in *mrp-1*-deletion mutants as compared to wild-type controls [46].

Vatamaniuk and colleagues [47] characterized the half-molecule ABC transporter of the heavy metal tolerance family-1 (HMT-1) subfamily in response to cadmium exposure. The suppression of *hmt-1* expression by RNAi was shown to produce punctuate refractive inclusions within the vicinity of the nucleus of the intestinal epithelial cells upon exposure to toxic levels of cadmium [47]. Similarly, Schwartz and colleagues described the *C. elegans* HMT-1 following exposure to arsenic, copper, and cadmium. HMT-1 conferred tolerance in response to exposure to all three metals as shown by lethality testing following the knockdown of *hmt-1* [48].

Kurz and colleagues [49] demonstrated the three-fold induction of *pgp-5* following cadmium exposure. Results of this study showed that strong fluorescence was induced in the intestinal cells of *pgp-5*::GFP worms, where the GFP-encoding gene is under the control of the upstream *pgp-5* promoter [49]. Copper and zinc were also found to be capable of inducing *pgp-5* expression in these worms. Mutant *pgp-5* worms exhibited a developmental delay upon exposure to cadmium and copper. Accordingly, it was concluded that *pgp-5* is required for establishing full resistance to cadmium and copper. In addition, the RNAi knockdown of *tir-1*, an upstream component of the p38 MAPK pathway in the *pgp-5* transgenic reporter strain, was shown to significantly reduce *pgp-5* induction following exposure to cadmium. However, the double-stranded RNA knockdown of ERK (*mpk-1*) and JNK (*med-1* and *kgb-1*) did not affect the induction of *pgp-5* in response to cadmium exposure [49].

Au and colleagues [50] studied the divalent-metal transporter (DMT1) following exposure to manganese. The DMT1-like family of proteins has been shown to regulate manganese and iron in the cell. The deletion of the three worm DMT1-like genes resulted in differential effects on manganese toxicity. The deletion of *smf-1* and *smf-3* increased Mn tolerance, whereas the deletion of *smf-2* increased Mn sensitivity [50]. Significant findings are summarized in Table 2.

5. Heat Shock Proteins

Heat shock proteins (HSPs) are cytosolic molecular chaperones. HSPs promote the refolding and repair of denatured proteins and facilitate protein synthesis upon activation by cellular stress [75, 76]. HSPs, particularly those in the HSP70 family, have also been shown to participate in the hormetic response [39]. HSP70s are ATP-binding proteins that convert ATP to ADP and bind to peptides, thereby, inactivating them and preventing aggregation (Figure 1). Oxidative stress can cause a reduction in cellular ATP levels [77]. Decreased levels of ATP result in the continued prevention of the aggregation of damaged proteins [78]. The functions of the HSP70 products are mediated by the conserved N-terminal ATPase and the C-terminal peptide-binding region [79]. The human and *C. elegans* HSP70 genes have a high degree of homology and share a conserved core "ATPase" structure [79]. The Hsp16 family of stress proteins is produced in *C. elegans* only under stress conditions [80–82].

5.1. Characterization of HSPs and Their Interaction with Metals. In a study examining the effects of MeHg exposure, hsp-4::GFP was measured immediately following the treatment of L1 worms for 30 minutes and L4 worms for 15 hours with this toxicant [39]. After 30 minutes of acute exposure to MeHg, the fluorescence of *hsp*-4::GFP remained unaltered. However, in L4 worms chronically exposed to MeHg for 15 hours, *hsp-4*::GFP was induced. At the same time point in the chronic treatment paradigm, a four-fold increase in *gst-4* fluorescence was detected, but there were no changes in either *mtl-1* or *mtl-2::*GFP expression [39].

Jones and Candido [18] exposed nematodes to cadmium or mercury and measured feeding behavior. For these studies, transgenic lines containing the promoter sequence for hsp16 genes which regulate the production of *E. coli* β-galactosidase were used. Accordingly, to measure stress, levels of this protein were assessed. Results showed that cadmium inhibited feeding behavior significantly but not completely, as a minimal rate of feeding continued at high cadmium concentrations. Further, exposure to cadmium (1 ppm) induced a detectable production of β-galactosidase without inhibiting feeding behavior. The stress response was induced at a concentration of cadmium that was ten times lower than the LC50. Mercury also was shown to inhibit feeding at concentrations similar to those necessary for the induction of a stress response; however, the difference in this instance was less than two fold. Mercury also did not entirely inhibit feeding behavior [18]. Significant findings are summarized in Table 2.

6. Conclusion

The use of *C. elegans* as an experimental model has produced considerable insight and valuable information regarding the multiple and varied processes of metal detoxification. Conclusive biochemical evidence has indicated that different metals are not handled in the same capacity. Many metalloregulatory proteins exhibit selectivity toward their target metal ions. The selectivity and sensitivity of each of these proteins is highlighted in the large body of accumulated research on different metal toxicities as well as various systems of metal detoxification. However, the overall mechanisms, temporal activation, and interplay between different cell detoxification systems remain elusive. Future studies are necessary in order to enhance our understanding of the complex interplay of multiple-cell detoxification systems in response to exposure to different metals. The *C. elegans* model system will be critical for these investigations, as knockouts are easily generated and provide a wealth

of information about metal detoxification in a genetically retractable, inexpensive, and *in vivo* model.

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