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Assessment of the mutagenic and genotoxic activity of cyanobacterial toxin beta-N-methyl-amino-L-alanine in *Salmonella typhimurium*

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ABSTRACT

A neurotoxin β-N-methylamino-L-alanine (L-BMAA) is a non-protein amino acid produced by most cyanobacteria ubiquitously present in aquatic and terrestrial environments. Due to its global presence in surface waters, a widespread human exposure is possible and therefore this toxin represents a health risk for humans and animals. L-BMAA has been linked to the development of a variety of neurodegenerative diseases. Its neurotoxic activity has been extensively studied, while nothing is known on its genotoxic properties. In the present study we evaluated for the first time L-BMAA mutagenic potential using Ames assay on several *Salmonella typhimurium* strains (TA97a, TA98, TA100, TA102 and TA1535). The results showed that the toxin (up to 0.9 mg/plate) did not induce mutations without or with S9 metabolic activation. Its genotoxic activity was further studied with the SOS/*umu*C assay on *S. typhimurium* TA1535/pSK1002 and the results showed that it was not cytotoxic nor genotoxic for bacteria. The present study represents the first evidence that L-BMAA is not mutagenic nor genotoxic for bacteria even at concentrations much higher than those typically found in the environment. However, as most of the cyanobacterial toxins are not bacterial mutagens it is very important to further elucidate its genotoxic activity in eukaryotic cells.

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1. Introduction

β-N-methylamino-L-alanine (L-BMAA), a non-protein amino acid, has been described as a potent neurotoxin (Cox et al., 2003, 2005; Banack et al., 2007; Lobner et al., 2007; Johnson et al., 2008; Metcalf et al., 2008; Karlsson et al., 2009b; Nunn, 2009; Jonasson et al., 2010). It has been implicated in the etiology of human diseases as a significant environmental factor leading to neurologic damage in the brain and central nervous system of humans and animals, potentially contributing to one of the several neurodegenerative disorders of aging such as Alzheimer's disease (Cox et al., 2003; Pablo et al., 2009), amyotrophic lateral sclerosis (Bradley and Mash, 2009; Caller et al., 2009; Cox et al., 2009; Banack et al., 2015), and the amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS-PDC) (Borenstein et al., 2007; Holtcamp, 2012). The neurotoxicity of L-BMAA and associated mechanisms of action have been extensively studied in neurons and various neural cell lines *in vitro* (Chiu et al., 2012; Lee and McGeer, 2012; Dunlop et al., 2013; Munoz-Saez et al., 2013) as well as animal test systems (Karamyan and Speth, 2008; Karlsson et al., 2009b, 2014b; de Munck et al., 2015; Munoz-Saez et al., 2015; Tian et al., 2016).

L-BMAA has been originally identified in seeds from the false sago palm (*Cycas micronesica* Hill.) (Vega and Bell, 1967) and has been discovered in the traditional diets of the Chamorro people of Guam (in Western Pacific Ocean); such as flour made from seeds of cycads as well as in animals feeding on cycad seeds (e.g. flying foxes, deer, and feral pigs) (Spencer et al., 1987, 2010). Later on, Cox et al. (Cox et al., 2005) have discovered that L-BMAA is actually produced by cyanobacteria of *Nostoc* genus that live symbiotically in roots of cycad trees. In recent years L-BMAA has gained increased attention as several studies have demonstrated that it is produced by most of the globally widespread cyanobacterial species (Cox et al., 2005; Esterhuizen and Downing, 2008). Moreover, it has been detected also in planktonic diatoms (Jiang et al., 2014a; Reveillon et al., 2016) and dinoflagellates (Lage et al., 2014). As cyanobacteria are found ubiquitously in water bodies they are considered a growing problem in aquatic ecosystems worldwide

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due to the variety of secondary metabolites including toxins they produce.

Although L-BMAA is water soluble its bioaccumulation in higher aquatic organisms such as shellfish and fish (Banack and Cox, 2003; Cox et al., 2003; Brand et al., 2010; Jonasson et al., 2010; Jiang et al., 2014b; Reveillon et al., 2015; Reveillon et al., 2016) has been documented and thus it can biomagnify in food web. Eventually it can enter the human diet, and thereby lead to human exposure and consequently trigger neurodegenerative diseases. Other possible routes of exposure are direct contact during recreational activities, consumption of contaminated water, and even inhalation of aerosols and dusts (Banack et al., 2010; Stommel et al., 2013). Hence, the presence of L-BMAA in the environment may represent a public health concern. Furthermore, in mice model BMAA can be maternally transferred, either directly (Karlsson et al., 2009a) or trough milk during lactation (Andersson et al., 2013), which means that it is likely that exposure to L-BMAA can have transgenerational effects.

Since the discovery of L-BMAA it has been a challenge to clarify its mechanism of action and the link with the pathology of neurodegenerative disorders. Recently, it has been demonstrated that free L-BMAA can bind to the amino acid tRNA synthetase and can be mistakenly incorporated into the protein structure in place of L-serine during *de novo* synthesis (Dunlop et al., 2013). These findings support the proposed mechanism of L-BMAA bioaccumulation (Murch et al., 2004) despite its non-lipophylic and non-protein nature. Moreover, L-BMAA incorporated in proteins can serve as an endogenous toxin reservoir in humans or animals, from which L-BMAA is slowly released back to free form through acid hydrolysis (Murch et al., 2004) and proteolytic cleavage (Dunlop et al., 2013) even for years or decades after its consumption. This fact may explain the long latency period presented by neurodegenerative disorders like ALS-PDC presumably caused by a chronic L-BMAA exposure. Thus L-BMAA by misincorporation into the proteins may represent an environmental factor that can lead to alterations in protein structures, may trigger protein misfunction, misfolding and/or aggregation (Dunlop et al., 2013; Karlsson et al., 2014a) and induce apoptosis (Dunlop et al., 2013; Chiu et al., 2015; Tian et al., 2016) and endoplasmic reticulum stress (Okle et al., 2013) that may lead to neurodegenerative diseases (Walker and Atkin, 2011; Dunlop et al., 2013; Karlsson et al., 2014a; Tian et al., 2016). In addition L-BMAA is structurally very similar to glutamate (Cucchiaroni et al., 2010). It has been shown that the main mechanism by which L-BMAA causes neurotoxicity is by acting as an excitotoxin on glutamate receptors (L-BMAA is mixed glutamate receptor agonist) such as N-methyl-D-aspartic acid (NMDA) and calcium dependent α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)/kainate receptors (Chiu et al., 2012), which is believed to induce activation of calcium-dependent enzyme pathways (Bae et al., 2013) and oxidative stress by depletion of glutathione (Chiu et al., 2012; Engskog et al., 2013; Okle et al., 2013) as well as mitochondrial dysfunction (de Munck et al., 2013). Nevertheless, the exact impact of non-protein amino acid L-BMAA on protein structure and function remains unknown.

The current state of knowledge recognizes that L-BMAA is neurotoxic and most of the research is focused on its potential neurodegenerative effects, while no research has addressed the question whether L-BMAA can induce damage to DNA. Therefore the aim of our study was to investigate the mutagenic and genotoxic potential of this small non-protein amino acid. The mutagenic activity was evaluated with the *Salmonella*/microsomal reverse mutation assay on five different strains of *Salmonella typhimurium* (TA97a, TA98, TA100, TA102 and TA1535) in the absence and presence of metabolic activation. Its genotoxic activity was further studied with the SOS/*umu*C assay on *S. typhimurium* TA1535/pSK1002 in the absence and presence of metabolic activation.

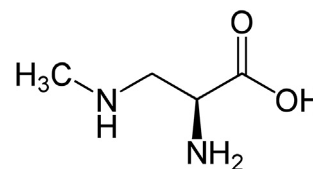

Fig. 1. Structural formula of β-N-methylamino-L-alanine.

2. Materials and methods

2.1. Chemicals

Beta-N-methyl-amino-L-alanine (Fig. 1) (L-BMAA; C4H10N2O2; 154.6 g/mol; CAS number 15920-93-1) was obtained from Sigma-Aldrich (St Louis, MO, USA). A 9 mg/mL (58.21 μM) stock solution of L-BMAA was prepared in sterile bidestilled water, aliquoted and stored at −20 °C. Lyophilized Aroclor 1254 induced male rat liver post-mitochondrial fraction (S9) was obtained from Moltox (Boone, USA), NADPH-Na2 was from Merck (Darmstadt, Germany) and G-6-P was from Sigma-Aldrich (St Louis, MO, USA). All other chemical reagents were of the purest grade available and all solutions were made using Milli-Q water.

2.2. Determination of mutagenicity with Salmonella/ microsomal reverse mutation assay (Ames) assay

Mutagenicity of L-BMAA was tested using the plate incorporation assay with *S. typhimurium* strains TA97a, TA98, TA100, TA102 and TA1535 without and with external metabolic system (Maron and Ames, 1983) according to the *Organisation for Economic Cooperation* and *Development* guideline 471 (OECD TG 471, 1997). L-BMAA is soluble at the concentration of 9 mg/mL; therefore, this concentration was used as the highest tested concentration. The bacterial strains were obtained from Trinova Biochem GmbH (Giessen, Germany). Strains TA97a and TA98 were used for detection of frame-shift mutation and strains TA100, TA102 and TA1535 for base pair substitution. Briefly, 100 μL of L-BMAA (0.11, 0.33, 1, 3 and 9 mg/mL corresponding to the final concentration of 0.011, 0.033, 0.1, 0.3 and 0.9 mg/plate, respectively), 100 μL bacterial culture of *S. typhimurium* (overnight culture at 37 °C) and 500 μL of phosphate buffer (for assays without metabolic activation) or 4% S9 mix (for assays with metabolic activation) were added to 2 mL of molten top agar containing a limited amount of histidine/biotin. Afterwards these were mixed gently and poured onto minimal agar plates. The plates were incubated for 48 h (TA97a, TA100, TA102 and TA1535) and 72 h (TA98) at 37 °C. Subsequently the numbers of spontaneous and L-BMAA induced His^+ revertants were counted. The plates were checked for possible toxic effect of L-BMAA. Three plates were used per experimental point. The number of spontaneous and induced revertant colonies was counted and the Induction Factor (IF) over the control was calculated. IF was determined as the ratio of the mean value from induced revertants for each tested concentration divided by the mean value from spontaneous revertants (control). IF ≥ 2 was taken as the threshold at which the sample was considered as mutagenic. Statistical significance between exposed groups and the control was determined by One-way analysis of variance (ANOVA) and Dunnett's Multiple Comparison Test using GraphPad Prism 6 (GraphPadSoftware). $^*p < 0.05$ was considered as statistically significant; however it has to be pointed out that the statistical significance is not the determining factor for positive result. In addition to IF (Table 1; in

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