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REVIEW ARTICLE

The Cyanotoxin and Non-protein Amino Acid β-Methylamino-L-Alanine (L-BMAA) in the Food Chain: Incorporation into Proteins and Its Impact on Human Health

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Abstract

The size and frequency of cyanobacterial blooms are increasing concomitantly with rising global temperatures and increased eutrophication, and this has implications for human health. Cyanotoxins, including L-BMAA, have been implicated in triggering neurodegenerative diseases such as ALS/PDC and Alzheimer's disease. L-BMAA is a water-soluble non-protein amino acid that can bioaccumulate up the food chain, in a free- and protein-bound form. While some data exists on the degree of environmental enrichment of L-BMAA in water bodies, cyanobacteria-derived supplements, fruit bats, and seafood, virtually nothing is known about the presence of L-BMAA in other foodstuffs. It has now been shown several times in laboratory settings that plants can absorb L-BMAA into their leaves and stems, but data from wild-grown plants is nascent. One of the mechanisms implicated in L-BMAA bioaccumulation is misincorporation into proteins in the place of the canonical amino acid L-serine. We first identified this as a mechanism of action of L-BMAA in 2013, and since then, several groups have replicated these findings, but others have not. Here, we discuss in detail the experimental approaches, why they may have produced negative findings and propose several ways forward for developing consistency within the field. We emphasize the need to standardize cell culture methods, using L-serine-free medium to study misincorporation of BMAA, and urge accurate reporting of the components present in cell culture media.

Keywords L-BMAA · L-serine · Misincorporation · Protein incorporation · Cyanobacteria · Cyanotoxins · Neurodegeneration

Abbreviations

AD	Alzheimer's disease
AEG	*N*-(2-aminoethyl) glycine
ALS	Amyotrophic lateral sclerosis
ALS/PDC	Amyotrophic lateral sclerosis-parkinsonism-dementia type complex
AQC	6-Aminoquinolyl-N-hydroxysccinimidyl carbamate
L-BMAA	β-methylamino-L-alanine
D-BMAA	β-methylamino-D-alanine
CHX	cycloheximide
DAB	2,4-Diaminobutyric acid
DHFR	dihydrofolate reductase
DTT	dithiothreitol
HBSS	Hank's buffered salt solution
HCl	Hydrochloric acid
HILIC	Hydrophilic interaction liquid chromatography
HPLC/MS-MS	High-performance liquid chromatography with tandem mass spectrometry
LC-MS/MS	Liquid chromatography and tandem mass spectrometry
MND	Motor neuron disease
mRNA	messenger RNA
SDS	sodium-dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid

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Introduction

Cyanobacterial blooms represent a growing problem around the globe since as a species, they are well adapted to thrive in fresh, brackish, saltwater, and even in desert crusts (Chatziefthimiou et al. 2018). Climate change and eutrophication are two proposed causes of the increasing size, frequency, and duration of blooms (Huisman et al. 2018; Morabito et al. 2018; Wells et al. 2015). Cyanotoxins, including β-methylamino-L-alanine (L-BMAA), have been identified in water bodies in disparate parts of the world, including South Africa, the United Kingdom, the USA, Canada, Sweden, China, Australia, France, and even Antarctica (Almuhtaram et al. 2018; Bishop et al. 2018; Lage et al. 2015; Main et al. 2018; Scott et al. 2018; Violi et al. 2019; Wiltsie et al. 2018; Jungblut et al. 2018) and reviewed in Lance et al. (2018)).

Enrichment of BMAA in the Food Chain

L-BMAA is water soluble and can also be protein-associated, leading to bioaccumulation and biomagnification through the food chain (Fig. 1). BMAA is a "non-protein amino acid"—simply meaning, not preferentially used in mammalian protein synthesis—but has also been referred to as a "non-encoded amino acid", "non-proteogenic", "unusual amino acid", and "non-typical amino acid" as summarized in Nunn and Codd (2017).

The first demonstration of L-BMAA biomagnification being implicated in human disease was reported on the island of Guam where a neurodegenerative disease that manifested as atypical Parkinsonism, dementia, Alzheimer's, and amyotrophic lateral sclerosis (ALS) or motor neuron disease (MND) was reported to be afflicting 25% of the adult population of a small village (Banack and Cox 2003; Bell et al. 1967; Cox et al. 2016; Spencer et al. 1987). Immigrants were also affected, suggesting that this was not exclusively a familial disorder unique to the indigenous Chamorro, who named it Lytico-bodig disease (Garruto et al. 1981). The disease was later renamed by neuroscientists as amyotrophic lateral sclerosis-parkinsonism-dementia type complex, or ALS/PDC (Kurland 1988). In the absence of a clear pattern of inheritance, environmental factors including the Chamorro diet were investigated. It was suspected that the Chamorro people's consumption of cycad (*Cycas micronesica*) flour and fruit bats (Banack et al. 2006) (and to a lesser degree, deer and pigs which fed on the cycad seeds, Banack and Murch 2009) might provide clues to the observed increased levels of neurodegenerative disease. All these species feed on the seeds of the cycad which were later found to contain high levels of protein-bound and free L-BMAA (Banack et al. 2006). The source of this L-BMAA was identified as symbiotic cyanobacteria of the genus *Nostoc* that are harbored in specialized coralloid roots of *Cycas micronesica* trees and bioaccumulate in the terrestrial ecosystem via the proteins (Cox et al. 2007; Cox et al. 2003; Murch et al. 2004). The Chamorro villagers are chronically exposed when they consume flour made from the seed kernels, as well as dine on animals that feed on the seed kernels (Murch et al. 2004). The hypothesis that BMAA triggers Guamanian ALS/PDC

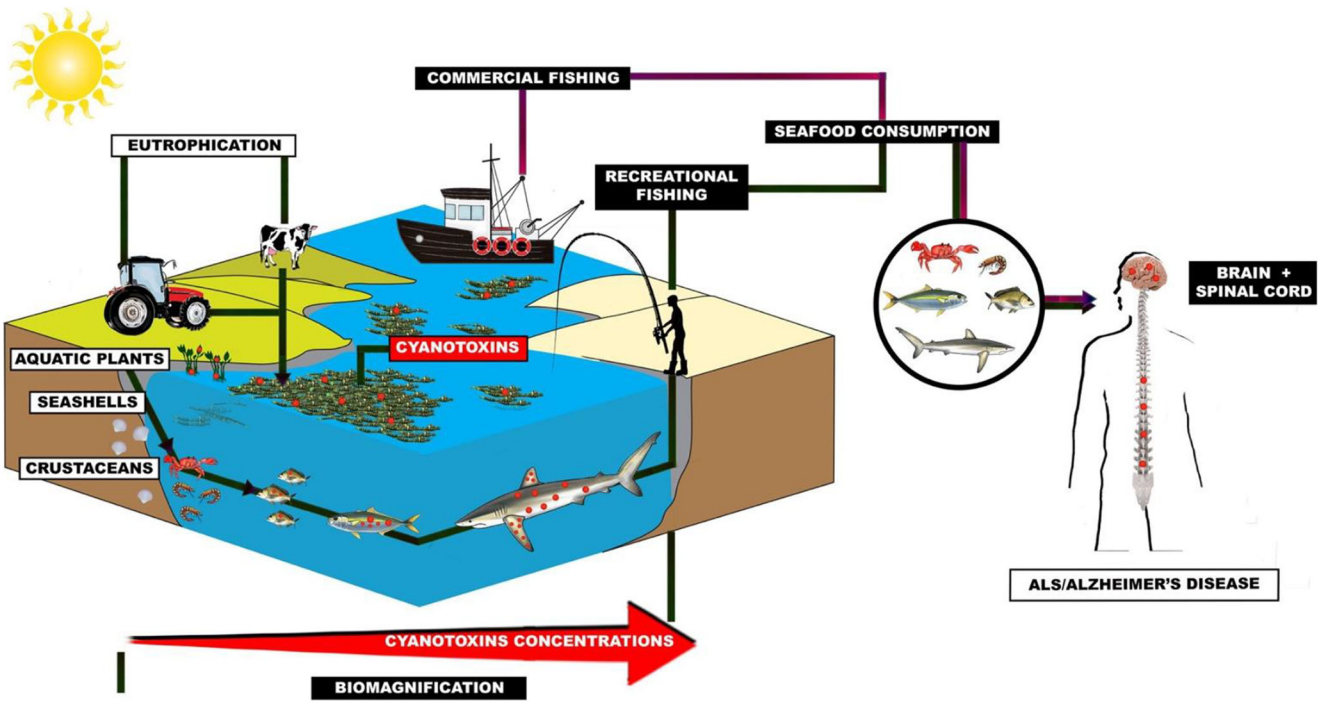

Fig. 1 Bioaccumulation and biomagnification of BMAA in the food chain and impact on human health

has been supported by the finding that chronic dietary exposure to L-BMAA produces neurofibrillary tangles and amyloid plaques the brains of vervets in St. Kitts (Cox et al. 2016).

It is now well known that L-BMAA can accumulate in seafood and plants exposed to contaminated water or soil (Jiang et al. 2015; Jiao et al. 2013; Jonasson et al. 2010; Lance et al. 2018). L-BMAA has also been detected in aquatic plants, with one group reporting that 42% of aquatic plants collected from Nebraska lakes and dams during 2009–2010 contained free and protein-associated L-BMAA (Al-Sammak et al. 2014). Invertebrates collected from the Kattegat Sea on the west coast of Sweden also contained L-BMAA, including the common mussel (*Mytilus edulis*) and the common oyster (*Ostrea edulis*) (Jonasson et al. 2010). Mussel and scallops purchased in Uppsala, Sweden, but originating from four continents were found to contain BMAA and its structural isomers (Salomonsson et al. 2015). Marine molluscs in China have also been found to contain BMAA (Li et al. 2016). Blue crab and pink shrimp have also been shown to contain L-BMAA (Brand et al. 2010; Christensen et al. 2012; Lance et al. 2018). Several studies have also shown the presence of L-BMAA in different species of fresh and saltwater fish, including alligator gar and sea bream (Brand et al. 2010). Shark flesh and shark-derived products, particularly shark fin, can contain very high concentrations of L-BMAA (Hammerschlag et al. 2016; Holtcamp 2012; Mondo et al. 2014; Mondo et al. 2012). For a more detailed review of BMAA in water and seafoods, see Lance et al. (2018).

While much work has been done investigating BMAA biomagnification in seafood, less is known about plants, and virtually nothing is known about other meats aside from seafood and flying foxes (Banack et al. 2006). One recent study reported the accumulation of BMAA in lettuce (*Lactuca sativa*) and spring onion (*Allium fistulosum*) grown in sterile soil in a laboratory and irrigated with BMAA-containing water (50 μg/L, once a week) for 60 days (Esterhuizen-Londt and Pflugmacher 2019). Watering common wheat (*Triticum aestivum*) with L-BMAA-contaminated water in a laboratory setting resulted in protein-associated BMAA that was liberated by acid hydrolysis (Contardo-Jara et al. 2014). A similar laboratory experiment using Chinese cabbage also showed BMAA uptake into the roots, leaves, and stems after only 1 day of irrigation and at a higher affinity than the wheat. The authors reported that the highest concentrations of BMAA were in the stems and leaves, with the leaves the main site for storage of BMAA (Li et al. 2019). Conversely, there is scant evidence for BMAA accumulation in wild-grown vegetables. From the same study cited above (Esterhuizen-Londt and Pflugmacher 2019), *A. fistulosum* and *B. rapa chinensis* (Pak choi) that had been irrigated with BMAA-contaminated water from Lake Chao in the Anhui province of the People's Republic of China had no detectable BMAA. The authors suggested that the presence of metal ions may have chelated the BMAA or it was sequestered and metabolized by bacteria or other organisms in the soil.

Results for BMAA in cyanobacteria-containing food supplements including *Spirulina* are conflicting. McCarron analyzed ten *Spirulina*-containing samples (six pure *Spirulina* samples from two separate raw material suppliers and four commercially available "green drink" dietary supplements) using both 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatization coupled to liquid chromatography-mass spectrometry and tandem mass spectrometry (LC-MS/MS) as well as hydrophilic interaction chromatography (HILIC) and did not detect any BMAA (McCarron et al. 2014). The authors cautioned, however, that BMAA production in various Cyanobacteria spp. is dependent upon nutrient availability and other environmental conditions (Downing et al. 2011); thus, there may be batch-to-batch variability. Thus, emphasizing the need for each new batch to be tested. A study of five commercially available protein powder supplements containing *Spirulina* purchased in Canada reported low levels of BMAA (mean < 1 μg/g), but high levels (100 μg/g) of the isoforms, *N*-(2-aminoethyl) glycine (AEG), and 2,4-diaminobutyric acid (DAB) (Glover et al. 2015) (see Manolidi for a review (Manolidi et al. 2019). Analysis of samples of Chinese *fa cai* noodle soup containing *Nostoc* also contains BMAA, but it was not detected in counterfeit samples devoid of *Nostoc* (Roney et al. 2009). Finally, a recent study identified BMAA in both milk and eggs (Andersson et al. 2018).

Misincorporation of L-BMAA into Proteins as a Neurotoxic Mechanism

BMAA is considered to be a "non-protein amino acid" since it is not preferentially used in mammalian protein synthesis. However, the presence of an amino and a carboxyl group in the molecule means it could potentially form peptide bonds, and thus become part of the polypeptide chain if it were to be misincorporated (Fig. 2).

In nature, plants synthesize hundreds of non-protein amino acids. Some appear to be adaptive to deter competitors, parasites, and herbivores (Dunlop et al. 2014), and some of these molecules are toxic to humans. For example, Chris McCandless, who was found dead in the Alaskan wilderness in 1992 (Krakauer 2015), likely ingested high levels of L-canavanine

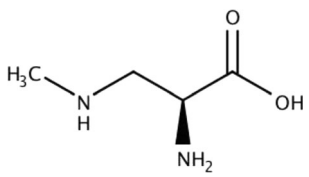

Fig. 2 The structure of L-BMAA showing the carboxyl and amino group that have the potential to form peptide bonds

(Fig. 3) from *Hedysarum alpinum* seeds (the Eskimo potato or "alpine sweetvetch") in the absence of other sources of protein. While it was first thought he starved to death, it was later proposed (Krakauer et al. 2015) that McCandless first became paralyzed from consuming L-canavanine via the potato seeds, and subsequently was unable to forage for food.

Speculation on the nature of the interaction of BMAA with proteins was first presented by Murch et al. (2004) when they proposed a mechanism for the liberation of BMAA from the protein fraction of cycad flour following acid hydrolysis. They were the first to speculate that L-BMAA might substitute for a proteinogenic or canonical amino acid during protein synthesis.

This hypothesis was first examined in 2012 when Okle and colleagues used a human cell-free synthesis system kit (Thermo Fisher, part number 88858 which is no longer listed on the website) consisting of leucine-free medium, ^{14}C-L-BMAA, and human pCFE-GFP mRNA to determine if L-BMAA could be directly incorporated into the protein chain during biosynthesis (Okle et al. 2012). Using liquid scintillation counting, they reported the presence of the ^{14}C radiolabel in the trichloroacetic acid (TCA) precipitated protein fraction from the cell-free system, suggesting L-BMAA had been misincorporated. However, following denaturing SDS-PAGE of the protein fraction and autoradiography, the signal disappeared. The authors offered two explanations for this; (1) the association of L-BMAA with the protein was not covalent in nature, therefore the ^{14}C-label dissociated during electrophoresis, or (2) their method of detection was not sensitive enough.

Notably, this was an indirect measure of L-BMAA incorporation, since movement of the ^{14}C-label was followed, but not the L-BMAA moiety itself.

We took a more targeted approach (Dunlop et al. 2013) when we set out to systematically examine whether L-BMAA could be incorporated into the protein chain via biosynthesis.

To elucidate the mechanisms of L-BMAA protein association, we used a novel cell culture model that enabled us to remove or include individual amino acids as a means for determining if they competed with L-BMAA for incorporation into proteins.

Preliminary studies examining basic structural similarities identified five possible mammalian canonical amino acids as a target for L-BMAA. We then set about screening all 20 canonical amino acids in cell culture, using human cell cultures [two cell lines—MRC-5s (human fibroblasts) and SH-SY5Ys (human neuroblastoma)] and primary cells—human umbilical vein endothelial cells (HUVECs) incubated with ^{3}H-L-BMAA (methyl-^{3}H, tritiated) where L-[4,5-^{3}H] leucine was used as a radio-labeled incorporation control.

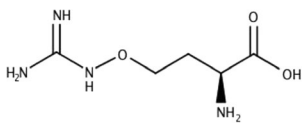

Fig. 3 L-canavanine was reported in high concentrations in *Hedysarum alpinum* seeds found near the site of Chris McCandless' demise

We reported the uptake and incorporation of tritiated L-BMAA into cells and cell proteins, respectively, as a function of time, as determined by liquid scintillation counting of TCA precipitated proteins and supernatant (Dunlop et al. 2013). However, again, we followed the tritiated label, not the BMAA moiety directly, and this will be discussed later in the review.

To determine if the observed interaction of L-BMAA with proteins was covalent in nature, we co-incubated the cells (MRC5s, HUVECs, and SH-SY5Y) with a protein synthesis inhibitor, cycloheximide (CHX). This molecule blocks protein synthesis by interfering with the assembly of the ribosome complex, thus preventing translational protein elongation.

In the absence of CHX, we reported a dose-dependent increase in the tritiated tag into the TCA-precipitated fraction, as measured by liquid scintillation counting. However, when CHX was present in the media, the tritiated label present in the protein fraction was reduced by ~ 75%.

The inclusion of CHX in the culture medium and consequent reduction of radiolabel in the TCA fraction strongly suggested a role for protein biosynthesis in incorporation of the radiolabel. Thus, we concluded that insertion of L-BMAA into the protein chain during protein synthesis plays a role in the observed protein-associated BMAA pool.

Further evidence for a role for protein biosynthesis in the protein association of L-BMAA was provided by incubating SH-SY5Y cells with ^{3}H-L-BMAA, precipitating the proteins with TCA, then treating the pellets with sodium-dodecyl sulfate (SDS), dithiothreitol (DTT), Pronase, or 6 M hydrochloric acid (HCl). These treatments are designed to accomplish several levels of denaturation and/or cleavage of peptide bonds; (1) SDS denatures the secondary structure of the protein; (2) DTT is a reducing agent that breaks disulfide bonds; (3) Pronase is a commercial mixture of enzymes that digests proteins to release individual amino acids; and (4) 6 M HCl, heated to 110 °C for 16 h, breaks peptide bonds to liberate individual amino acids. Radiolabel was only liberated from pellets treated with Pronase or HCl, but not SDS or DTT, providing further evidence that the association of BMAA with proteins is covalent. We identified L-serine as a target for substitution by L-BMAA as we were able to block the incorporation of the radiolabel when L-serine was also present in the culture medium.

In support of a role for protein biosynthesis in L-BMAA protein association, incubation of the enantiomer of L-serine (D-serine) did not prevent the incorporation of the tritiated label. In mammalian cells, L-amino acids are used for protein synthesis, but D-amino acids are not; thus, inhibition of the incorporation of L-BMAA isoform by L-serine (but not D-

serine) suggests a role for protein biosynthesis in L-BMAA incorporation.

Taken together, these observations suggested that competition for incorporation between L-serine and L-BMAA occurs at the level of protein synthesis. However, it is noted that direct interaction between L-BMAA and a tRNA synthetase has not yet been systematically determined. Indeed, all the studies mentioned above are indirect measures of L-BMAA incorporation, since they followed a radiolabel tag and did not measure the BMAA moiety directly.

Direct Measures of L-BMAA Incorporation

We reported (Dunlop et al. 2013) a direct measurement of L-BMAA incorporation, using LC-MS/MS to measure a unique BMAA daughter ion (m/z 258). In these experiments, cells (MRC5s) in culture were incubated in amino acid-free Hank's buffered salt solution (HBSS) supplemented with 250, 500, 750, or 1000 μM L-BMAA for 24 h, and the protein fraction precipitated with 10% TCA, hydrolyzed, then LC/MS/MS used to quantify the amount of BMAA in the protein fraction. In support of our radiolabel studies, we reported the recovery of L-BMAA from MRC5 cell cultures, in a dose-dependent manner ($R^2 = 0.99012$).

Dose-dependent increases in L-BMAA accumulation were also reported in Drosophila fed L-BMAA, in both the free- and protein-bound fractions. Zhou et al. (2009) fed male and female Canton S drosophila 4 mM or 8 mM L-BMAA and measured the L-BMAA pools via fluorescent HPLC with AQC derivatization. They reported more than 2-fold increases in the L-BMAA recovered from the free and protein-bound fractions, except in the males for the 8 mM dose. They did not offer an explanation for the lack of dose dependence in males at 8 mM, instead suggesting it needed further investigation.

Their technique for isolating the protein-bound L-BMAA fraction was the same as other authors cited here where the protein fraction is precipitated with acetone and/or TCA, then the pellet hydrolyzed with hydrochloric acid vapor. Treatment of proteins with 6 M vapor HCl at 110 °C breaks covalent peptide bonds, liberating free amino acids.

Our studies were in whole cells, but other authors have demonstrated BMAA incorporation in cell-free systems. Glover and colleagues used an in vitro cell-free synthesis kit [PURExpress® Δ (aa, tRNA) Kit, New England BioLabs, catalogue number E6840S] using *E. coli*-derived dihydrofolate reductase (DHFR) as a template, as well as human genomic DNA extracted from post-mortem brain tissues of three human patients—one control, one Alzheimer's disease (AD), and one from a patient who had MND or ALS.

When phenylalanine, proline, alanine, glutamate, or serine were omitted from the incubation mix, but L-BMAA was added with *E. coli* DHFR as the template, between 10 and 20% of the supplemented BMAA was found in the protein fraction. This could be increased from 35 to 70% when human genomic DNA (gDNA) was used at the template. Also, interestingly, the gDNA sample from the ALS patient had significantly higher rates of L-BMAA incorporation than the control patient (Glover et al. 2014).

Similar to Dunlop et al. (2013)) and Okle et al. (2012), Glover and colleagues also sought to differentiate between L-BMAA that was misincorporated into proteins and that which had some other undefined, but non-covalent association with protein. Proteins generated in vitro were precipitated with TCA (25 μL of 0.1 N TCA), and the resulting pellet further denatured with SDS and DTT to produce "free" and "protein-bound" BMAA fractions. By quantifying these free and protein-bound fractions, the authors determined that approximately 50% of the added BMAA was covalently bound and 50% "associated" in a non-covalent fashion. Thus, they confirmed the observations of Dunlop et al. that BMAA is incorporated into human proteins via protein synthesis and additionally made the novel observation that BMAA is also attracted to the three-dimensional structure.

Beri et al. (2017) used the same cell-free kit as Glover to synthesize BMAA-containing proteins, also with DHFR or human mutant superoxide dismutase 1 (SOD1) (hSOD1 G93A) as the template DNA. Any combination of L-serine and L-BMAA, (where the ratio of BMAA:L-serine was 1:1, 10:1 and 100:1) was not able to produce L-BMAA containing peptides. (The authors also conducted an incubation where only L-BMAA was present (and zero L-serine) but found the percent protein coverage was only 12.58% versus 66.67% in the corresponding control reaction). The authors concluded that their in vitro data did not confirm or exclude that BMAA substitutes for L-serine and offered several explanation for these results, 1) they were specifically looking for L-BMAA substituting for L-serine in peptides, where Glover looked for total BMAA following acid hydrolysis, 2) the error rate for the substitution of canonical amino acids with non-protein amino acids is so infrequent that it may fall below the level of detection, or 3) their experimental conditions were not conducive to L-BMAA misincorporation.

Cell culture studies from the Beri group using NSC-34 cells and incubated with 500 μM L-BMAA for 72 h also did not yield positive results for L-BMAA incorporation. Proteomic analysis of cell lysates (protein pellet and supernatant), as well as the exposure media (secreted protein and flow through from a 10,000 molecular weight kDa cut-off filter) using LC-MS/MS did not identify any incorporation of L-BMAA. However, this is not surprising given that the L-BMAA treatment medium contained 0.021 g/L L-serine, which constitutes half the amount present in standard DMEM (the recommended medium for culturing NSC34). Although the culture medium was purchased as L-serine free, the authors supplemented it with

L-serine prior to treating the cells (Beri, J, personal communication, 2018). We have previously shown that ratios as low as 100 times less L-serine can protect cells from apoptosis even when L-BMAA is present in the culture medium (Dunlop et al. 2013), indicating that the affinity of L-serine for the seryl tRNA synthetase is significantly higher than L-BMAA.

It is important to consider that L-serine is not an essential amino acid; thus, it is likely that residual levels of L-serine exist both in the cells and in the culture medium and these may out-compete L-BMAA to prevent misincorporation.

In order the overcome residual L-serine out-competing L-BMAA, in our previous L-BMAA misincorporation studies, we used Hanks Buffered Salt Solution (HBSS, Sigma Aldrich, catalogue number H1837) and supplemented amino acids (250 μM) as required. One could argue the merits of this approach with respect to physiological relevance, but this deliberate approach was used to elucidate whether L-BMAA can be misincorporated at all, using an in vitro model.

The Downing group has also reported negative results for BMAA misincorporation. In one such study (van Onselen et al. 2017), they incubated cell cultures (HepG2, HeLa, Caco-2) with L-BMAA, purified the protein pellets using SDS-PAGE, and quantitated BMAA using acid hydrolysis, AQC derivatization, and high-performance liquid chromatography with tandem mass spectrometry (HPLC/MS-MS). Similar to Okle et al. (discussed above), they did not detect any BMAA in the SDS-PAGE purified protein pellets. The authors speculated that L-BMAA misincorporation either does not occur at all or might occur at a level that is undetectable and therefore is biologically irrelevant. Regarding the latter point, we note that in vivo studies have reported that amino acid substitutions as infrequent as 1/10,000–1/30,000 is sufficient enough to cause motor neuron degeneration in mice (Lee et al. 2006). Okle, who also reported negative results for SDS-PAGE purified TCA-precipitated pellets, suggested that their method may not be sensitive enough to detect any misincorporated BMAA or that other non-covalent interactions of BMAA protein-association may be involved. Indeed, it is very likely that in addition to covalent misincorporation of BMAA into proteins, other mechanisms for BMAA association are in play. Further investigations are needed to systematically characterize these proposed interactions, but it is important to consider that these two distinct mechanisms need not be mutually exclusive.

Other labs have also reported negative data in non-neural human cell lines despite using what can be best determined as L-serine-free culture medium. We say "best determined" since although the name of the culture medium is cited, the product number is not and named culture media has many different formulations. For example, Downing and colleagues indicate that they use "nutrient-free medium" but the lack of a catalog number makes it impossible to be certain of the precise medium composition. Scrutiny of three of their manuscripts suggests that their treatment media is "Hyclone" which is L-serine free, but this information was difficult to find. Beri and colleagues also did not publish the precise composition of their culture media, but it was confirmed by a personal communication where it was revealed supplemental L-serine was added.

The issue of incomplete reporting of cell culture medium composition is addressed later in this review.

Autoradiography

Further evidence for BMAA being peptide bound into proteins was presented by Karlsson et al. (2014) using two 10-day-old Wistar rat as an in vivo model for BMAA distribution and autoradiographic imaging. Following subcutaneous injections of carbon-14 labeled L-BMAA (^{14}C-L-BMAA, methyl-^{14}C), the animals were sacrificed at $t = 24$ and 72 h then autoradiography for the ^{14}C tag measured. Following TCA extraction of tissue sections at $t = 24$ h, levels of radioactivity in muscles and liver were reduced, compared to the freeze-dried sections, but were retained in the spleen, bone marrow, and gastrointestinal mucosa. Radioactivity was retained in all tissues at 72 h.

In the same study, the authors quantitated BMAA in eight Wistar pups, 24 h after the administration of two subcutaneous injections of ^{14}C-L-BMAA (methyl-^{14}C). AQC derivatization and LC-MS/MS analysis of the liver, hypothalamus, and pituitary gland revealed a dose-dependent increase in the amount of free and protein-bound BMAA in the tissues. The "free" fraction is defined as acid soluble and the "protein-bound" as the TCA-precipitated fraction. The TCA-precipitated pellet was thoroughly washed prior to hydrolysis to eliminate any residual "free" BMAA that may have associated with the pellet, according to the following protocol; washed with 200 μL cold acetone, then two cycles of precipitation for 30 minutes on ice with 1.8 mL of 10% TCA, then reconstitution.

These data support the hypothesis that L-BMAA is incorporated via protein synthesis since radioactivity was retained in the TCA-extracted tissue slices. Further evidence for incorporation was the observation of a distinct uptake of radioactivity into tissues with high protein synthesis, including the liver, spleen, thymus, bone marrow, and gastrointestinal mucosa. We have also observed a time-dependent increase in the accumulation of radiolabeled BMAA (but as a ^{3}H, methyl-^{3}H) into proteins, consistent with the time required to synthesize proteins (Dunlop et al. 2013). Karlsson et al. were cautious to state, however, that while misincorporation of the ^{14}C-L-BMAA (methyl-^{14}C) does adequately explain this accumulation, they could not exclude that BMAA may become associated with proteins after their synthesis via some other mechanism.

In another study from Andersson et al. (2013), using C57BL/6 mice dams and pups, and mammary gland cell culture (HC11), they reported significantly higher uptake and association of ^{14}C-L-BMAA (methyl-^{14}C) than the enantiomer, ^{14}C-D-BMAA (methyl-^{14}C). Whole-body autoradiography studies showed a pattern of distribution of the radiolabel that was localized to tissues where high levels of protein synthesis take place, for example, the spleen, gastrointestinal mucosa, bone marrow, and the pancreas. In accompanying mammary gland cell culture studies, the authors reported a 7-fold difference in the uptake of L-BMAA when the cells were differentiated, compared to D-BMAA. While this is consistent with L-BMAA being used in protein synthesis, and therefore incorporated into proteins, they could not rule out the upregulation of specific L-amino acid transport mechanisms also being a contributing factor.

Xie et al. (2013) also conducted ex vivo autoradiography using male C57/BL6 rats given an intravenous bolus injection of ^{14}C-L-BMAA (position of the radiolabel not known) and quantitated uptake and accumulation of the radiolabel with liquid scintillation counting. While ^{14}C-L-BMAA was rapidly cleared from the plasma, analysis of the brain tissue showed protein-bound radioactivity peaked at $t = 4$ h, then plateaued by $t = 8$ h. Free BMAA in the brain also decreased over time, as higher percentages were associated with the protein-bound fractions. Dense labeling in sagittal and coronal brain sections was evident in ex vivo autoradiography slices. The authors concluded that BMAA was likely misincorporated into the cerebral proteins, where it may move between free- and protein-bound pools contributing to the proteins having a long half-life.

Limitations of Autoradiography Studies

It is important to consider the position of the radiolabel within the BMAA molecule when interpreting results from radiolabel and autoradiography studies. In the studies cited above, Dunlop et al. (2013), Andersson et al. (2013) and Karlsson et al. (2014) state that the radiolabel is on the methyl group of BMAA, but the Xie group (Xie et al. 2013) do not provide this information. The position of the label within the BMAA molecule is important to know, since the methyl group on BMAA is potentially labile and may not remain on the BMAA moiety. Thus, recovering and measuring the radiolabel should be considered an indirect measure of BMAA incorporation, unless the radioactive fraction is also analyzed for BMAA. It should be noted that there is at least one form of commercially available radioactive L- and D-BMAA that is labeled on the carboxyl group and termed "metabolically stable" (β-Methylamino-*L*-alanine hydrochloride, [1–^{14}C], catalog number, N C1001, Novandi Chemistry AB, Södertälje, Sweden). It is therefore recommended that researchers consider the position of the radiolabel when choosing a radioactive BMAA, and also ensure they specify the position of the tag in their material and methods.

Perspectives

In the preceding sections, we have summarized published data pertaining to the misincorporation of L-BMAA as a mechanism for neurotoxicity. Some labs, such as our own, report positive results while others insist that L-BMAA cannot be incorporated via protein synthesis. As described above, it is difficult to compare in vitro results, primarily because of a lack of detailed reporting of cell culture medium used. It is therefore suggested, that going forward, a standard protocol for cell culture be established such that future studies can accurately be compared.

We have already reported that L-serine at a ratio ten times lower than L-BMAA in the culture medium is sufficient to protect cells (SH-SY5Y) from apoptosis (Dunlop et al. 2013), probably as a result of inhibition of incorporation. Thus, having L-serine present in the culture medium, even if it is ten times less than the L-BMAA, may be sufficient to inhibit detectable incorporation of BMAA. This has implications for washing cells following seeding and prior to adding treatment media—which is particularly important since L-serine is not an essential amino acid—thereby reducing residual L-serine in the media and cytoplasm. Relevant to this is the need to cite the product number of all culture mediums to facilitate comparison of results. In addition, to our knowledge, no one has yet published a repeat of our studies using the protein synthesis inhibitor CHX. These data provided critical information on the mechanisms of L-BMAA protein association in our initial studies and would be useful if independently repeated.

Regarding the analysis of BMAA via LC-MS/MS or similar, we agree with the assessment of Lance et al. (2018) who state that it is not enough to analyze only the free and protein-bound fractions of samples. Instead, the protein-bound BMAA should be further characterized as "precipitated bound" (BMAA associated with proteins of high molecular weight), and "soluble bound" (BMAA associated with peptides and proteins of low molecular weight). Thus, the total BMAA in the system (which equals soluble bound + soluble free + precipitated bound) and each corresponding fraction should be quantitated when reporting on BMAA accumulation.

We acknowledge that to unequivocally demonstrate that L-BMAA is inserted into the peptide chain at the time of protein synthesis, binding studies with L-serine, L-BMAA, and seryl-tRNA synthetase need to be undertaken. This has previously been reported for tyrosyl-tRNA synthetase and 3,4-dihydroxyphenylalanine (DOPA)—an oxidation product of tyrosine—where electrospray ionization mass spectrometry (ESI-MS) showed 90% L-DOPA incorporation at each

tyrosine site in an *Escherichia coli* cell-free transcription/translation system (Ozawa et al. 2005).

Finally, several other mechanisms of L-BMAA-induced toxicity have been described [and reviewed elsewhere (Delcourt et al. 2018)] and there are probably more as yet unreported.

Conclusion

Increasing evidence suggests that L-BMAA plays a role in triggering neurodegenerative disease in susceptible individuals. While data for L-BMAA bioaccumulation in seafood, fruit bats, and some cyanobacterial-derived supplements is accumulating, for many other foods such as farmed meat, we know virtually nothing. Several mechanisms of action of L-BMAA have been characterized, including our observation that it replaces L-serine in the protein chain during protein synthesis. Replication of this finding has not been consistent, however, and we propose this may be a result of differing methodologies. In a time when the size and frequency of cyanobacterial blooms are increasing, it is essential to minimize these inconsistencies so that we can devise ways to address the potentially increasing public health issue of L-BMAA exposure and its role in neurodegeneration.

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