The Role of Non-Protein Amino Acids in Protein Folding Disorders

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Certificate of Authorship and Originality

I, Brendan James Main declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This document has not been submitted for qualifications at any other academic institution.

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Publications & Conference Proceedings

Dunlop R. A., **Main B. J**., Rodgers K. J. (2015) The deleterious effects of non-protein amino acids from desert plants on human and animal health. *Journal of Arid Environments.* 112, 152-158.

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List of Figures

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Abbreviations

2,4-DAB	L-2,4-Diaminobutyric acid
AEG	N-(2-Aminoethyl) glycine
ALS	Amyotrophic lateral sclerosis
ALSFRS-R	ALS functional rating scale
ALS-PDC	ALS – Parkinson's dementia complex
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
AZE	azetidine-2-carboxylic acid
Bcl-2	B-cell lymphoma 2
BMAA	β-methylamino-L-alanine
BOAA	β-N-oxalylamino-L-alanine
CHOP	CCAAT/-enhancer-binding protein homologous protein
CSF	Cerebrospinal fluid
DTT	Dithiothreitol
EIF2α	Eukaryotic Initiation Factor 2 α
ER	Endoplasmic reticulum
fALS	Familial ALS
FDA	Food and Drug Administration
FMOC	Fluorenylmethyloxycarbonyl chloride
GC-MS	Gas chromatography mass spectrometry
GC-TOFMS	Gas chromatography time-of-flight mass spectrometry
Grp-78	78 kDa glucose-regulated protein
H^3	Tritiated
HILIC	Hydrophobic interaction liquid chromatography
HPLC-FD	High performance liquid chromatography - fluorescence detection
LAT1	L-type amino acid transporter 1

LAT2	L-type amino acid transporter 2
LC-MS	Liquid chromatography - mass spectrometry
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
L-DOPA	L-3,4-dihydroxyphenylalanine
LMN	Lower motor neurons
m/z	Mass to charge ratio
MND	Motor neurone disease
MS	Multiple sclerosis
NFT	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NPAA	Non-protein amino acid
ODAP	Oxalyldiaminopropionic acid
PBP	Progressive bulbar palsy
PCF	Propyl-chloroformate
PLS	Primary lateral sclerosis
PMA	Progressive muscular atrophy
RT	Retention time
sALS	Sporadic ALS
SDS	Sodium dodecyl sulfate
SLE	Systemic lupus erythematosus
SOD1	Superoxide dismutase 1
TDP-43	TAR DNA-binding protein 43
UMN	Upper motor neurons
UPR	Unfolded protein response

Abstract

Non-protein amino acids are a group of small molecules with structural similarities to the canonical amino acids used in protein synthesis. Many of these molecules are produced by plants, animals, bacteria, and fungi, and are ubiquitous within our environment. A number of non-protein amino acids have been linked to human pathologies, including a number of neurodegenerative diseases.

β-methylamino-L-alanine (BMAA) is a cyanobacterial-derived non-protein amino acid that has been linked to the development of amyotrophic lateral sclerosis, as well as Parkinson's disease and dementia. Following its discovery in the 1960s, BMAA has been shown to be produced by a number cyanobacteria, and more recently other phytoplankton species including diatoms and dinoflagellates. BMAA is found globally in freshwater, saltwater, and terrestrial environments.

While BMAA has been identified in samples sourced from a huge variety of global ecosystems, its presence in Australian waterways has remained largely unexplored. For this study, sixteen mixed population algal surface bloom samples were collected from a number of sites in urban and rural New South Wales. The presence of BMAA, and its isomers L-2,4-Diaminobutyric acid (2,4-DAB) and N-(2-Aminoethyl) glycine (AEG) was determined using reverse phase liquid chromatography – tandem mass spectrometry. Ten of the samples were found to contain BMAA, while 2,4-DAB was found in all sixteen. The presence of these suspected toxins in urban areas, as well as in waterways critical for agriculture, suggests Australians may be exposed to BMAA and 2,4-DAB regularly.

The ability of BMAA to associate strongly with proteins has been well reported. To investigate this relationship, radio labelled BMAA was incubated with both human neuroblastoma cells and *Escherichia coli*. Protein-bound BMAA increased in a linear fashion over time in neuroblastoma cells but not in *E. coli* suggesting that prokaryotes and eukaryotes may manage the presence of

BMAA differently. Protein bound BMAA was only observed in live cells and not in protein lysates indicating that some form of biological processing is required for protein binding to occur. Protein bound BMAA was also found to distribute across fractionated cell proteins in the same manner as ^{3}H leucine, suggesting both share similar binding properties.

The potential for synergistic toxicity between BMAA and its structural isomers, 2,4-DAB and AEG, was also explored. Cell viability was significantly reduced in cells exposed to BMAA or 2,4-DAB in concentrations as low as 250 µM, and similar toxicity was only observed in AEG treated cells at concentrations of 1000 µM or higher. Cells exposed to BMAA, or combinations of BMAA and other isomers, resulted in increased expression of a number of markers of endoplasmic reticulum (ER) mediated proteotoxic stress, a phenomenon that was not observed in cells exposed to 2,4-DAB or AEG on their own. Significant increases in caspase 3 and cathepsin activity were only observed in cells incubated with a combination of BMAA and 2,4-DAB, suggesting that while 2,4-DAB does not share the same mechanism of toxicity as BMAA, it may contribute to its cytotoxicity.

We observed that neuroblastoma cells exposed to BMAA produced a number of markers of proteotoxic stress, including increases in caspase 3 and cathepsin activity as well as increased expression of the ER stress marker CCAAT/-enhancer-binding protein homologous protein (CHOP). Co-incubation with low concentrations of L-serine resulted in complete inhibition of this toxicity, supporting the hypothesis that BMAA is misincorporated into proteins in place of L-serine or that L-serine can counteract the cytotoxicity associated with BMAA through other mechanisms. These results also suggest that the effects of BMAA exposure may be mitigated through the use of L-serine, providing a possible pharmacological intervention for neurodegenerative disease sufferers affected by BMAA exposure.

The sporadic nature of a number of neurodegenerative diseases strongly indicates the presence of environmental factors in their aetiology. This project has demonstrated that algal non-protein

amino acids are neurotoxic and may play a role as an environmental factor in the onset of disease. The formation of aberrant protein structures is a hallmark of neurodegeneration; the affinity of BMAA to bind to proteins, as well as its ability to induce ER-stress, is a strong indication that BMAA may be misincorporated into proteins. This is supported by the evidence that BMAA toxicity is mitigated through co-exposure to L-serine. Moving forward, robust and ongoing monitoring of these toxins in rural and urban waterways is critical to our understanding of the risk of human exposure, as well as the identification of potential exposure routes.

Chapter One:

Introduction and Overview

Chapter One: Introduction and Overview.

1.1 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), also commonly known as motor neurone disease (MND), is a debilitating neurodegenerative disease that affects approximately 1.9 in 100,000 people globally (Arthur et al. 2016). ALS is characterised by the progressive weakening, and eventual loss of voluntary muscle function, caused by the degradation and death of associated motor neurons. The rapid deterioration of function in ALS patients means that they are typically given a life expectancy of around 3 years from diagnosis (Turner et al. 2003); this number however is highly variable with around 10% surviving ten years or more after diagnosis. While ALS remains a relatively well-known disease, in part due to the public profile of patients such as former baseball player Lou Gehrig and Professor Stephen Hawking, as well as fundraising efforts like the 'ice bucket challenge', there is little understanding of the underlying causes of the disease.

ALS presents with progressive degradation of both the upper and lower motor neurons, however the name ALS can be used to describe a family of motor neurone diseases including progressive muscular atrophy (PMA), primary lateral sclerosis (PLS) and progressive bulbar palsy (PBP) (Rowland and Shneider 2001). Motor neuron degeneration in PMA and PLS is restricted to the lower motor neurons (LMN) and upper motor neurons (UMN) respectively (Cervenakova et al. 2000; Watanabe et al. 1997), while in PBP degradation is seen in the brain-stem and motor neurons responsible for control of facial muscles (Kühnlein et al. 2008). Traditional ALS is by far the most common of these disorders, and patients diagnosed with PMA, PLS and PBP are often re-diagnosed with ALS following progression of their disease into other motor neuron sections (Rowland and Shneider 2001).

Diagnosis of ALS is difficult as patients will present with different symptoms depending on the initial area affected by neuron degradation (Gupta et al. 2012). The majority of patients initially

present with ‘limb onset’ ALS (Kiernan et al. 2011); these patients have a combination of UMN and LMN symptoms, including muscle weakness and wastage, limb stiffness, and loss of fine motor skills. In comparison, ‘bulbar onset’ ALS patients will present with facial weaknesses, difficulty speaking and swallowing, as well as fasciculation of the tongue (Mitchell and Borasio 2007). Despite the initial differences in presentation, ALS patients will typically rapidly progress to full body paralysis, making any delay in initial diagnosis particularly concerning. Currently, the only widely approved pharmaceutical treatment option for ALS patients is the drug Riluzole; a pre-synaptic glutamate release inhibitor that can, in some cases, extend the life span of ALS patients by months (Festoff et al. 2003). The United States of America (USA) Food and Drug Administration (FDA) approved Edaravone, marketed as Radicava, a free radical scavenging drug developed for the treatment of stroke, for the treatment of ALS in 2017. Clinical trials showed that Edaravone decreased disease progression as measured by the ALS Functional Rating Scale (ALSFRS-R) in the treatment group, as well as decreasing the oxidative stress marker 3-nitrotyrosine in cerebrospinal fluid (CSF) (Sawada 2017).

ALS is divided into two distinct categories, sporadic ALS (sALS) and familial ALS (fALS), the defining factor between them being whether the presenting patient has a family history of the disease. The vast majority of patients with ALS are classed as sALS, with fALS only making up 5-10% of known cases (Deng et al. 2011). A number of distinct genetic mutations have been identified in patients with fALS, the most common of which occurs in the superoxide dismutase 1 (SOD1) gene. SOD1 mutations make up 20% of all diagnosed fALS cases, or 2% of all known cases of ALS, and are characterised by the presence of SOD1 protein aggregate build up within affected motor neurons (Ray et al. 2004).

Historically, there has been a division between research into genetic and non-genetic causes for ALS, however with less than 10% of ALS patients having familial history of the disease (Pasinelli and Brown 2006), an environmental cause is being increasingly viewed as a critical factor in

sporadic cases (Pablo et al. 2009). There is increasing evidence that ALS and other related neurodegenerative disorders are caused not by a single factor, but instead by a combination of genetic susceptibilities and environmental exposures (Al-Chalabi and Hardiman 2013).

Analysis of European ALS incidence data using epidemiological modelling found that ALS is a multistage disease, with as many as six distinct molecular steps required for disease onset (Al-Chalabi et al. 2014). It is likely that one or more of these molecular steps are exposure to environmental factors. The shift in hypothesis from a purely genetic to a multi-factorial aetiology has resulted in increased interest in environmental triggers. The non-protein amino acid (NPAA) β-methylamino-L-alanine (BMAA) had drawn interest as one such environmental trigger.

1.2 ALS, BMAA and the Western Pacific

The Western Pacific ALS foci consist of three separate Pacific communities located in Guam, the Kii Peninsula of Japan, and West Papua (Bradley and Mash 2009). These communities have historically shown an unusually high prevalence of neurodegenerative conditions similar to ALS. The first of these neurodegenerative disease foci was observed in 1945 among the native Chamorro people of Guam. Sufferers displayed symptoms consistent with a number of neurodegenerative conditions; thus the disease was characterised as ALS – Parkinson's Dementia Complex (ALS-PDC) (Hirano et al. 1961). Shortly after the initial discovery on Guam, researchers recognised the symptoms in communities on the Kii Peninsula and in West Papuan tribes. Despite being completely geographically and genetically separate, at its peak these groups had prevalence rates of ALS up to 100 times greater than other populations (Kurland and Mulder 1954).

The discovery of these foci led to a search for causes of the disease among the affected populations. One proposed cause was identified when a link was drawn between the diet and neurological symptoms of the Chamorro People and the diet and symptoms of people who suffered the neurological disease neurolathyrism (Bell 2009). Neurolathyrism, a disorder resulting in weakening and irreversible paralysis of muscles of the lower body, is caused by oxalyldiaminopropionic acid (ODAP), also known as β-N-oxalylamino-L-alanine (BOAA), an amino acid and neurotoxin found in various legumes of the species *Lathyrus Sativus* consumed by sufferers (Rao et al. 1964). While legumes are not part of the Chamorro diet, it was hypothesised that cycad seed flour, a staple part of the Chamorro diet, may contain ODAP. Chromatographic testing of cycad flour found no ODAP, however a compound characterised as α-amino-β-methylaminopropionic acid or β-methylamino-L-alanine was identified (**Fig. 1**) (Vega 1967).

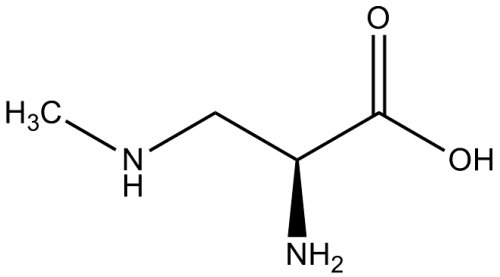

Figure 1: β-methylamino-L-alanine (BMAA)

1.3 β-Methylamino-L-Alanine in the Environment

BMAA shares structural similarity with a number of canonical (protein) amino acids (**Fig. 2)**, including possessing both amine and carboxylic acid functional groups. However, unlike canonical amino acids BMAA is not used in protein synthesis, making it a NPAA. NPAAs are produced by a number of plants, bacteria, and fungi and appear to play roles in defence, storage and as metabolic intermediates (Bell 2003). A number of NPAAs have been shown to impact human

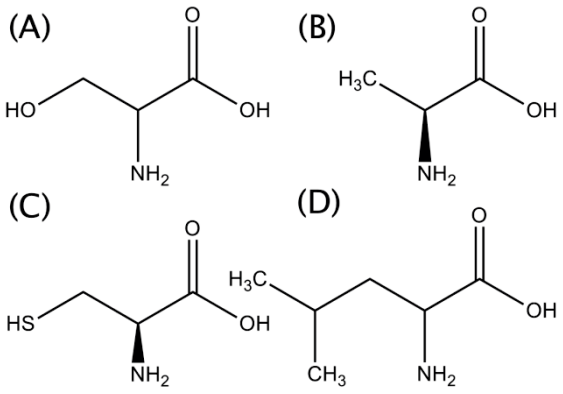

Figure 2: Structural similarity between BMAA and protein amino acids. (A) Serine (0.5), (B) Alanine (0.4884), (C) Cysteine (0.449), (D) Leucine (0.3902). A higher number indicates increased similarity to BMAA.

health through a diverse range of mechanisms including metal chelation, excitotoxicity, nephrotoxicity and metabolic disturbances (Rodgers et al. 2015).

While BMAA was initially believed to be derived from cycads, Cox *et al.* demonstrated that the compound is instead produced by cyanobacteria of the genus *Nostoc,* which reside within the roots of cycads in a symbiotic relationship (Cox et al. 2003). BMAA is produced by the cyanobacteria and distributed throughout its host cycad, accumulating in varying concentrations throughout the plant, with the highest concentrations occurring in the reproductive tissue (Banack and Cox 2003b).

A number of animal studies have concluded that BMAA shows varying levels of toxicity in laboratory animals (Seawright et al. 1990). The most prominent of these is a study in 1987 by Spencer *et al.* that concluded that BMAA induced ALS-PDC like symptoms in primates when supplied to them over a period of 13 weeks by gavage (Spencer et al. 1987). The Spencer study was criticised however, due to the large amount of flour that Chamorro's would need to eat to obtain the BMAA dose that was used to induce ALS-like symptoms in the primate study. It has since been suggested that the high nutrient diet fed to the macaques alongside BMAA may have provided some protection from toxicity (Karamyan and Speth 2008). A recent primate study by Cox *et al.* (2016) showed chronic dietary exposure of BMAA in conjunction with a low protein chow resulted in the formation of neurofibrillary tangles (NFT) and β-amyloid plaques within brain tissue of vervets; both of these structures are hallmarks of neurodegenerative diseases such as Alzheimer's disease.

Further testing of other aspects of the Chamorro diet found that BMAA was present in the tissue of flying foxes, an animal that is considered a delicacy among Chamorro People (Banack and Cox 2003a). Banack *et al.* concluded that while levels of BMAA in cycad flour are lower than that required to produce ALS symptoms, BMAA is biomagnified in animals who consume cycad plant material. This biomagnification resulted in BMAA levels in a single bat that were equivalent to up to 1,014 kg of cycad flour, an amount that had the potential to cause ALS-PDC symptoms (**Fig. 3**).

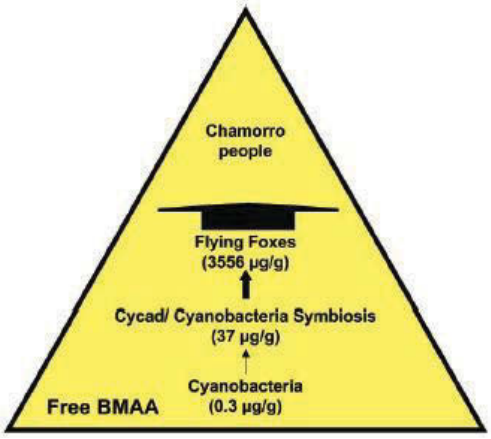

Figure 3: Biomagnification of BMAA in Guam. (Cox et al., 2003)

Analysis of brain tissue from Chamorro ALS-PDC patients found BMAA in both a free amino-acid and a protein bound form, whereas BMAA was not present in the brain tissue of patients with Huntington's or non-neurodegenerative diseases (Murch et al. 2004b; Pablo et al. 2009). BMAA was also found in the brain tissue of Canadians who had died of Alzheimer's disease (Murch et al. 2004b), suggesting that BMAA may be involved in neurodegenerative disease outside of Guam and the Western Pacific.

The role of BMAA in neurodegenerative disease on a global scale is further supported by Cox *et al.* who demonstrated that BMAA is produced by 95% of tested terrestrial and aquatic cyanobacteria species (Cox et al. 2005). BMAA has since been found in environmental samples from Qatar (Cox et al. 2009), Peru (Johnson et al. 2008), Sweden (Jiang et al. 2014b), France (Masseret et al. 2013), North America (Banack et al. 2015; Caller et al. 2009), China (Li et al. 2010), and the Baltic Sea (Jonasson et al. 2010). Interestingly, production of BMAA also does not appear to be limited to cyanobacteria; both diatoms (Jiang et al. 2014a) and dinoflagellates (Lage et al. 2014) produce the NPAA in significant concentrations. The apparent ubiquity of

BMAA in a number of environments combined with its ability to biomagnify dramatically increases the potential of human exposure to significant concentrations.

Cyanobacteria are predominately found in aquatic environments, with large blooms being common in both salt and fresh water ecosystems; it is therefore of little surprise that BMAA is found in a number of aquatic species. BMAA has been identified in organisms ranging from zooplankton to invertebrates and fish, many of which are commonly consumed as food by humans and animals (Jonasson et al. 2010). The biomagnification of BMAA means it is often found in higher concentrations in the upper members of the food chain, including bottom dwelling fish (Jonasson et al. 2010) and sharks (Mondo et al. 2012; Mondo et al. 2014).

While more prominent in aquatic environments, cyanobacteria are also present in terrestrial ecosystems. Large amounts of cyanobacteria are present in desert crusts, such as those found throughout the Persian Gulf. An investigation by Cox *et al.* into the desert crusts of Qatar found that up to 56% of the crust tested contained cyanobacteria (Cox et al. 2009). Cox *et al.* also found these crusts contained significant concentrations of BMAA, which could be a hazard if inhaled. Inhalation of the BMAA containing dust has been proposed as the possible cause for increased incidence of ALS seen in veterans of the first Gulf War (Cox et al. 2009; Horner et al. 2003; Haley 2003).

1.4 β-Methylamino-L-Alanine Detection and Analysis

Detection of BMAA in environmental and patient samples has been carried out using a number of analytical techniques. Early analysis by Cox *et al.* was conducted using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatisation, and high performance liquid chromatography coupled to fluorescence detection (HPLC-FD) as the primary analysis method (Banack and Cox 2003a). HPLC-FD relies on the interaction of the analyte with a chromatographic column stationary phase, causing elution of the target molecule at a specific and reproducible retention time (RT), as well as the level of fluorescence detected, to differentiate between molecules. HPLC-FD has been criticised for overestimating the concentration of BMAA, likely due to the inability to differentiate BMAA from co-occurring and co-eluting structural isomers (Faassen et al. 2012). Alternative techniques using various forms of mass spectrometry have attempted to address these criticisms by allowing more sensitive and specific analyte identification. Initial mass spectrometry techniques largely employed pre-column derivatisation coupled with liquid chromatography – mass spectrometry (LC-MS) (Murch et al. 2004b), or gas chromatography – mass spectrometry (GC-MS) (Guo et al. 2007).

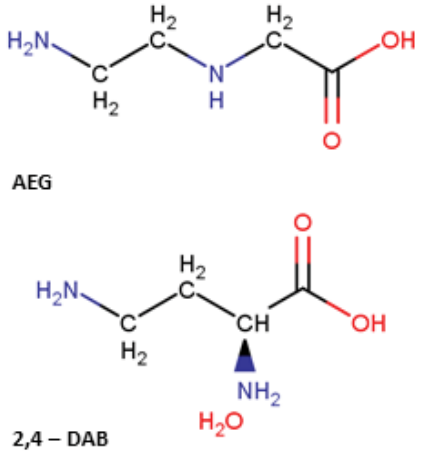

Figure 4: Chemical structure of 2,4 - DAB and AEG

Similar to HPLC-FD, LC-MS and GC-MS rely on RT to differentiate between analytes of the same mass, such as BMAA and its structural isomers, N-(2-Aminoethyl) glycine (AEG) and 2,4-DAB **(Fig. 4)** (Faassen et al. 2012). Both AEG and 2,4-DAB are commonly detected in the same samples as BMAA, making differentiation between the isomers critical for accurate quantification (Jiang et al. 2012). To address the need for accurate differentiation between BMAA, AEG, and 2,4-DAB, the majority of current analytical methods use liquid chromatography – tandem mass spectrometry (LC-

MS/MS). LC-MS/MS allows isomer differentiation using RT, the intact or parent ion mass to charge ratio (*m/z*), the m/z of a fragment or product ion resulting from collision induced dissociation of the parent ion, and the ratio of parent to product *m/z*, significantly improving isomer identification (Faassen et al. 2012).

The vast majority of analytical publications have employed some form of derivatisation of BMAA and other cyanobacterial NPAAs to improve analyte detectability. Post column derivatisation with ninhydrin was favoured in earlier BMAA papers, however this was quickly superseded by HPLC-FD techniques employing pre-column derivatisation with fluorenylmethyloxycarbonyl chloride (FMOC) which proved to be significantly more sensitive (Kisby et al. 1988). Sample derivatisation has been employed by a number of gas chromatography – mass spectrometry (GC-MS) methods; ethyl-chloroformate derivatisation with GC-MS (Snyder et al. 2009), and trimethylsilylation derivatisation with two-dimensional gas chromatography time-of-flight mass spectrometry (GC-TOFMS) (Snyder et al. 2010) have shown limited ability to detect BMAA in samples.

Success in BMAA detection has been seen with protocols using AQC derivatisation coupled with HPLC-FD (Banack and Cox 2003b), LC-MS (Murch et al. 2004b), and LC-MS/MS (Metcalf et al. 2008). AQC derivatisation and LC-MS/MS using a triple quadrupole instrument was employed by Glover *et al.* (2015) in a BMAA isolation and analysis method validated to AOAC International standards; this remains the gold standard for BMAA detection. Propyl-chloroformate (PCF) derivatisation (commonly using the Phenomenex EZ:faast™ free amino acid derivatisation kit) has also seen significant use in existing literature (Esterhuizen and Downing 2008; Esterhuizen-Londt et al. 2011). Analysis of un-derivatised BMAA using hydrophobic interaction liquid chromatography (HILIC) separation has also seen extensive use (Kubo et al. 2008; Rosen and Hellenas 2008; Beach et al. 2015; Faassen et al. 2009). However HILIC-MS/MS has been criticised for an increased possibility of false negatives due to lower column efficiencies and matrix effects

(Banack et al. 2010). A comparison of BMAA analysis methods using AQC, PCF, and HILIC found little difference between the three different methods for the majority of samples analysed. Both PCF and HILIC failed to detect BMAA in at least one known positive, highlighting that lack of detection is not an indication of lack of presence (Baker et al. 2017).

Despite there being a large body of literature on BMAA detection, there is a significant variation in published data on both detection and quantification of BMAA in samples. This variation has been attributed to imprecise mechanisms of detection and a lack of detail in method descriptions (Faassen 2014). The development of a consistent and precise method of detection and quantification of BMAA is required to accurately determine its presence within the environment.

1.5 Acute Versus Chronic Toxicity

Early theories on the mechanism of BMAA induced neurotoxicity focussed on acute toxicity through interaction with glutamate receptors (Spencer et al. 1987). BMAA is able to form two stable carbamate adducts in the presence of bicarbonate (Zimmerman et al. 2016), the α-carbamate exhibiting little activity on N-methyl-D-aspartate (NMDA) receptors. However, the β-carbamate form has striking structural similarity to glutamate, the physiological ligand of NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Diaz-Parga et al. 2018). The β-carbamate form of BMAA appears to be capable of mimicking glutamate, activating these receptors (Myers and Nelson 1990; Nunn and O'Brien 1989). The carbamate theory gained support when *in vitro* research demonstrated that when exposed to BMAA, cultured murine cortical cells showed no evidence of cell death, however the addition of bicarbonate for 1 hr resulted in substantial cell death within 24 hrs (Weiss et al. 1989).

The role of amino acid analogues in acute pathology is well demonstrated in conditions such as neurolathyrism, where neuroexcitation from ODAP is the direct mechanism of the disease

(Kumar et al. 2011). Prolonged dietary exposure to ODAP from the seeds of *L. sativus* causes the distinct symptoms of lathyrism which include irreversible lower limb paralysis as well as muscle tremors and atrophy. ODAP is structurally similar to L-glutamate, and able to act as an agonist to non-NMDA receptors resulting in excitotoxicity (Warren et al. 2004). Disease progression following exposure is where ODAP and BMAA toxicity differ; while patients with lathyrism often develop symptoms within months of exposure to ODAP, patients with ALS-PDC have a delay of up to 20 years between exposure to BMAA and development of disease symptoms (Murch et al. 2004a).

As early as 1986, Spencer *et al* observed that BMAA elicited two different forms of toxicity in exposed primates, the acute glutamate receptor activation observed previously, and another unidentified chronic mechanism (Spencer et al. 1986). Whilst *in vivo* exposure to high concentrations of BMAA does result in acute ALS-like symptoms such as jerky movement and partial paralysis, the effects quickly decline after BMAA treatment is halted (Rakonczay et al. 1991). This *in vivo* progression is not consistent with the long latency period seen in the onset of ALS-PDC in patients from Guam, suggesting a secondary chronic mechanism may be the cause of ALS-PDC.

1.6 Incorporation of Non-Protein Amino Acids into Proteins

Murch *et al.* noted that BMAA existed in two forms within cycad flour; as a free amino-acid and as a protein associated form that required acid hydrolysis to liberate (Murch et al. 2004a). This observation was mirrored in brain tissue of ALS-PDC and Alzheimer's patients where once again BMAA was present in both forms, suggesting that it is both incorporated into proteins and capable of crossing the blood brain barrier (Murch et al. 2004b). Brain perfusion experiments by Smith *et al.* demonstrated that BMAA is readily transported across the blood brain barrier by the L-type amino acid transporter 1 (LAT1) membrane transport protein (Smith et al. 1992). LAT1

transport proteins are multi-substrate transmembrane transporters that facilitate the movement of a number of amino acids and amino acid like compounds across membranes (Kanai and Endou 2003). LAT1 and LAT2 transporters have also been implicated in the transport of BMAA into milk, presenting another form of dietary exposure (Andersson et al. 2016; Andersson et al. 2013).

While commonly referred to as protein associated or protein bound, the precise nature of the interaction between BMAA and proteins is not clear. Dunlop *et al.* demonstrated BMAA is incorporated into proteins by a mechanism dependent upon protein synthesis (Dunlop et al. 2013). Multiple cell types including fibroblasts, neuroblastoma cells, and primary coronary artery cells, showed increased BMAA-protein association as the concentration of BMAA in the culture media increased. BMAA-protein association was prevented in the presence of cycloheximide, a eukaryotic protein synthesis inhibitor. *In vitro* experiments using tritiated (^{3}H) BMAA showed that ^{3}H BMAA could not be dissociated from proteins with sodium dodecyl sulfate (SDS), dithiothreitol (DTT), or SDS and DTT combined; only proteolysis, or acid hydrolysis, could liberate BMAA from proteins. These results are consistent with the incorporation of the amino acid into proteins during synthesis, opposed to passive binding of BMAA to existing proteins.

The observation that BMAA is protein associated and may be incorporated into proteins is supported by research that shows that L-3,4-dihydroxyphenylalanine (L-DOPA), an oxidative modification of tyrosine (3-hydroxytyrosine) and also a NPAA, is incorporated into human neuroblastoma cells *in vitro* (Ozawa et al. 2005; Rodgers et al. 2006; Rodgers and Shiozawa 2008). This incorporation results in the formation of autofluorescent bodies in the cell, accumulation of degradation resistant aggregates, and eventually cell death via apoptosis (Dunlop et al. 2011; Dunlop et al. 2008). Although DOPA is a more reactive compound, owing to 2 redox active hydroxyl groups at positions 3 and 4 on the phenolic ring, it could be hypothesised that exposure to BMAA may also lead to the accumulation of toxic BMAA-containing aggregates.

The effect of aggregate build up in neurons may be exacerbated by the long life and low turnover of neurological cells, increasing the potential for aggregates to accumulate to a level where they become toxic and induce cells to undergo apoptosis.

Protein misincorporation is not a unique property of BMAA and L-DOPA. Azetidine-2-carboxylic acid (AZE) is a plant derived NPAA that is misincorporated in place of proline into proteins (Bessonov et al. 2010). AZE is commonly found in a number of members of the *Beta vulgaris* plant family, including table and sugar beets, and has been detected in a number of sugar beet by-products used as livestock dietary supplements. There is evidence to suggest that use of AZE containing feed in agriculture may be the primary pathway for human exposure to the toxin (Rubenstein et al. 2009). The misincorporation of AZE into myelin basic protein (MBP) has been proposed to be a trigger for the apparent immunogenicity of the protein in multiple sclerosis (MS) patients (Rubenstein 2008).

A number of other plants also produce amino acid analogues which can be misincorporated into proteins during synthesis. L-canavanine is a NPAA produced by a number of plant species including alfalfa and is structurally closely related to the amino acid L-arginine. Canavanine has been shown to misincorporate in place of arginine following dietary exposure in insects, and is proposed to be produced by plants as an insecticide (Rosenthal et al. 1989a; Rosenthal et al. 1989b). Consumption of alfalfa has also been shown to increase the severity of the symptoms of systemic lupus erythematosus (SLE), and this may be due to canavanine containing proteins interrupting the action of antigen presentation (Akaogi et al. 2006).

1.7 The Fidelity of Protein Synthesis

Protein synthesis has a number of intrinsic error checking mechanisms; however, errors do occur at a rate of approximately 1 incorrect amino acid incorporated during protein synthesis for every 10,000 translated (Yadavalli and Ibba 2013). While the error rate during translation is low,

changes to even a single amino acid during protein synthesis can result in dramatic change in phenotype.

The result of minor errors during protein synthesis is well demonstrated in mice with the 'sticky mouse' mutation. These mice have been shown to have a defective alanyl-tRNA synthetase; this mutation results in the inability of the synthetase to clear mischarged amino acids, causing L-serine to be substituted for alanine approximately once per 500 reactions (Lee et al. 2006). The misincorporation of L-serine into proteins during synthesis results in the formation of misfolded proteins that appear to seed aggregates within the Purkinje neurons of the mice causing the neurons to die. The death of these neurons is believed to cause the distinctive ataxia that characterises the sticky mouse mutation.

The presence of misfolded and aggregated proteins in people suffering from a range of neurodegenerative diseases has been known for some time. These diseases fall into a larger category of conditions known as proteopathies that are characterised by the presence of unusual, misfolded or aggregated proteins (Walker and LeVine 2000). Cognitive proteopathies such as Alzheimer's disease, Parkinson's disease, Huntington's disease and ALS remain some of the most poorly understood and debilitating diseases found in humans. The cause of the protein aggregation seen in these proteopathies can be genetic, as seen in Huntington's disease, or environmental such as in Kuru, however the triggers for protein aggregation in diseases like ALS still remain largely unknown. It is possible that minor mistakes in protein synthesis, such as those demonstrated in the "sticky mouse" mutation, may be the trigger responsible for onset of this disease.

While the presence of misfolded proteins within a cell may seem largely innocuous, post mitotic cells such as motor neurons are particularly susceptible to their presence (Lee et al. 2006). Where rapidly dividing cells are able to spread misfolded proteins amongst daughter cells, post mitotic cells are left with an increasingly large build-up of atypical proteins. The presence of

misfolded proteins within a cell can in turn serve to 'seed' the aggregation of other proteins, resulting in an amplification of the effect of a misfolded or structurally aberrant protein (Wood et al. 1999; Murphy 2002).

Examples of aggregated proteins implicated in neurodegenerative disease include: amyloid-β and tau in Alzheimer's disease, α-Synuclein in Parkinson's disease, Huntingtin in Huntington's disease, and Prion protein in Prion diseases such as Creutzfeldt–Jakob disease and Kuru (Jucker and Walker 2011). Both TAR DNA-binding protein 43 (TDP-43) and SOD1 aggregates have been shown in ALS patients, with SOD1 mutations specifically identified in approximately 20% of people suffering from familial ALS (Ross and Poirier 2004). Interestingly, while TDP-43 aggregates are present in a large number of both sporadic and familial ALS patients, they are not present in patients with SOD1 mutations (Robertson et al. 2007). The involvement of different protein aggregates in different cases of ALS suggests that while protein aggregates play a role in the disease pathway, no single protein is responsible for its onset. The apparent ability for BMAA to induce the formation of aberrant protein structures in primate models is further evidence that it may play a role in the development of neurodegenerative disease (Cox et al. 2016).

Current literature on the role of protein aggregates in neurodegeneration offers little insight beyond stating their presence in patients suffering from a wide range of diseases, however the presence of these aggregates in such a large number of conditions suggests that they may play a significant role in neurodegeneration and warrant further investigation. Studies focusing on the role of aggregated proteins in tauopathies, a category of proteopathy characterised by the presence of tau aggregates, directly implicated the activation of the endoplasmic reticulum (ER) mediated unfolded protein response (UPR) in these disorders (Hoozemans and Scheper 2012).

The UPR plays a key role in ER stress and the cellular response to the presence of misfolded and aggregated proteins. Initially, the UPR is engaged in down regulation of protein translation

through phosphorylation of Eukaryotic Initiation Factor 2 (eIF2α) to ease the burden on the ER (Wek and Cavener 2007). This is complimented by increased expression of folding chaperones in an attempt to refold misfolded proteins (Brown and Naidoo 2012). Under prolonged ER stress, the UPR changes focus away from protecting the cell and towards apoptosis (Haynes and Ron 2010). This appears to be primarily achieved through increased expression of CHOP, which in turn promotes pro-apoptotic proteins and down regulates anti-apoptotic proteins such as B-cell lymphoma 2 (Bcl-2) (Nishitoh 2012). The result of prolonged UPR activation and upregulation of CHOP is the initiation of intrinsic apoptosis pathways (Matus et al. 2008; Chakrabarti et al. 2011).

While there is a large body of evidence to suggest that protein aggregation and activation of the UPR play a role in the progression of neurodegeneration, the majority of that evidence is largely correlative (Matus et al. 2008). Preliminary investigations have suggested that stress due to protein aggregation and activation of the UPR may have a causal role in these disorders, however the identification of the agent behind the protein aggregation in disorders like ALS proves to be the most reliable yet elusive way to demonstrate whether aggregation of proteins is the driving factor in the progression of the disease.

1.8 BMAA & Neurodegeneration

As previously mentioned, the longevity and slow turnover of neuronal cells make them especially vulnerable to protein aggregate build-up, which can result in apoptosis. While previous literature has demonstrated that BMAA can induce ALS-like symptoms in animal models (Cox et al. 2016; Scott and Downing 2017), and that it is present in the brains of people who suffer from neurodegenerative disease (Murch et al. 2004a; Murch et al. 2004b), the mechanism of toxicity for BMAA is largely unknown. The observation that BMAA can be misincorporated into proteins resulting in misfolding and aggregation presents a viable hypothesis that the progressive loss of motor neuron function seen in ALS patients is the result

of cell stress mediated apoptosis of the motor neuron. This hypothesis is supported by findings that demonstrate increased expression of pro-apoptotic UPR related genes following *in vitro* BMAA treatment (Okle et al. 2013). Misincorporation provides a practical explanation for why we find NPAAs such as BMAA so strongly associated with proteins.

Over the past four decades, research into the role of NPAAs in neurodegeneration has suffered from a lack of understanding behind the mechanisms of toxicity, and the methods of detection. BMAA research in particular has been stunted by imprecise techniques and poorly described methods. Improving detection techniques and further development of the mechanism of BMAA toxicity is vital in understanding how it and other NPAAs may play a role in the development of diseases like ALS. The lack of understanding into the mechanism of ALS is one of the key reasons that treatment for the disease has remained elusive for so long. Continued research into the genetics of ALS has implicated a number of genes in the progression of the disease, all of which are associated with misfolded proteins, however this only reveals a partial picture of the pathogenesis of ALS. With 90% of ALS sufferers showing no genetic history or predisposition to ALS, it is highly probable that the majority of sufferers are victims of an aetiological mix of genetics and single or multiple environmental triggers, including BMAA. If these triggers can be identified, and the mechanism of their action characterised, it may unlock new methods to prevent or treat ALS.

1.9 Thesis Aims and Overview

There has been limited analysis for the presence of BMAA and other algal NPAA toxins in the Australian environment prior to this project. Cox *et al.* (2005) remains the only publication to have analysed an Australian cyanobacterial species, where it was reported that a laboratory maintained isolate of *Cylindrospermopsis raciborskii* was positive for the presence of BMAA. The susceptibility of Australian rivers to persistent algal blooms (Davis and Koop 2006; Bowling and Baker 1996), and the reliance on these rivers for agriculture and recreation (ABS 2008) make the absence of analysis of algal NPAA toxins in Australia particularly concerning. Chapter Two of this thesis aims to address these issues through the development of a robust method for NPAA toxin analysis of Australian environmental samples, and to determine if the agriculturally important waterways of south eastern Australia were prone to contamination from BMAA and its isomers.

The presence of a protein bound fraction of BMAA in environmental and patient samples was first described by Murch *et al.* (2004a). Here, Murch suggested that BMAA may build up in a protein bound form within patients, slowly releasing over time causing reoccurring neurological tissue damage. The role of protein bound BMAA was further explored by Dunlop *et al.* (2013), where it was reported that BMAA was misincorporated into proteins during protein synthesis, resulting in the formation of toxic protein aggregates within cells. Alternative studies have suggested that BMAA is not incorporated into proteins, but instead post-translationally bound to them (van Onselen et al. 2017; van Onselen et al. 2015; van Onselen and Downing 2018). Chapter Three explores the relationship between BMAA and proteins, and aims to determine whether BMAA is incorporated during synthesis or superficially bound to proteins. This chapter also explores the differences between BMAA incorporation in prokaryotes and eukaryotes. While a number of studies have previously explored BMAA in eukaryotes and prokaryotes separately, this is the first direct comparison of the two.

The detection of BMAA in environmental and patient samples has been a topic of robust debate for a number of years. One of the major focuses of this debate is whether BMAA is reliably separated from its co-occurring structural isomers, 2,4-DAB and AEG (Faassen et al. 2012). Interestingly, while there is a significant body of literature exploring the analytical implications of the co-occurrence of these three NPAAs, there has been little exploration of the toxicological implications.

AEG has previously been proposed to function as the backbone of ancient peptide nucleic acids (Banack et al. 2012). It has however, only been the subject of preliminary toxicological assessment which found it was toxic to brine shrimp (*Artemia salina*) at high concentrations (Metcalf et al. 2015). 2,4-DAB however, has been the topic of a number of toxicological investigations, in which it was demonstrated that 2,4-DAB produced marked neurotoxicity in rodents (Chen et al. 1972). Chapter Four aims to identify the presence of potential synergistic toxicity between BMAA and its co-occurring isomers, as well as the potential mechanisms underlying any observed toxicity.

A common feature of misincorporation is the ability to outcompete NPAA misincorporation through increased concentration of the canonical amino acid it is being mistaken for (Rodgers and Shiozawa 2008). This effect is likely due to the tRNA synthetase having a higher binding affinity for the canonical amino acid versus the NPAA that it is mistakenly binding. BMAA has previously been shown to upregulate a number of proteins associated with the UPR; this is consistent with the formation of structurally aberrant proteins (Okle et al. 2013). It has been proposed that the formation of these misfolded proteins may be driven by the misincorporation of BMAA in place of L-serine during protein synthesis (Dunlop et al. 2013). Chapter Five aims to determine if the toxic effect of BMAA on human neuroblastoma cells can be reduced or prevented through co-incubation with L-serine.

Chapter Two

Detection of the suspected neurotoxin β-Methylamino-L-alanine (BMAA) in cyanobacterial blooms from multiple water bodies in Eastern Australia

Chapter Two: Detection of the suspected neurotoxin β-Methylamino-L-alanine (BMAA) in cyanobacterial blooms from multiple water bodies in Eastern Australia.

Chapter Overview

The Murray-Darling basin in south eastern Australia has historically struggled with frequent, large, and persistent algal blooms. The warm climate and slow moving water, coupled with agricultural run-off and nutrient rich water from impoundments provides perfect conditions for cyanobacterial species to flourish. While the waterways that form the basin are regularly tested for cyanotoxins such as microcystins and saxitoxins, a significant survey of the emerging cyanobacterial toxins BMAA and 2,4-DAB in Australian water had not been undertaken prior to this study.

In the studies described in this chapter, sixteen surface algal samples collected from rural sites within the Murray-Darling basin and urban sites in Sydney, New South Wales were analysed. BMAA was present in ten of the tested samples, while 2,4-DAB was present in all sixteen samples. The results presented here confirm that BMAA and 2,4-DAB are present in Australian algal blooms, and that blooms within the Murray-Darling basin may be a significant source of human BMAA and 2,4-DAB exposure.

Certificate of authorship and originality

This paper was published in *Harmful Algae* © Elsevier Ltd. I certify that the work presented in this chapter has not previously been submitted as part of the requirements for a degree. I also certify that I carried out the majority of the work presented in this paper.

- Brendan J Main: conducted experiments and wrote the majority (~95%) of the manuscript.
- Lee C. Bowling: wrote ~5% of the manuscript, and proof read the final draft
- Matthew P. Padula, David P. Bishop, Simon M. Mitrovic, and Gilles J. Guillemin: Proof read the manuscript and provided guidance on experiments.
- Kenneth J. Rodgers: Proof-read and edited the manuscript, conceived project idea, and provided guidance on experiments.

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Detection of the suspected neurotoxin β-methylamino-L-alanine (BMAA) in cyanobacterial blooms from multiple water bodies in Eastern Australia

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ABSTRACT

The emerging toxin β-methylamino-L-alanine (BMAA) has been linked to the development of a number of neurodegenerative diseases in humans including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, and Parkinson's disease. BMAA has been found to be produced by a range of cyanobacteria, diatoms, and dinoflagellates worldwide, and is present in freshwater, saltwater, and terrestrial ecosystems. Surface scum samples were collected from waterways in rural and urban New South Wales, Australia and algal species identified. Reverse phase liquid chromatography-tandem mass spectrometry was used to analyse sixteen cyanobacterial scum for the presence of BMAA as well as its toxic structural isomer 2,4-diaminobutyric acid (2,4-DAB). BMAA was detected in ten of the samples analysed, and 2,4-DAB in all sixteen. The presence of these toxins in water used for agriculture raises concerns for public health and food security in Australia.

1. Introduction

Cyanobacteria are an ancient group of microorganisms that have colonised many terrestrial and aquatic environments. They produce a wide range of bioactive molecules, some of which are toxic to humans and animals. Cyanotoxins are a diverse range of molecules with mechanisms that include hepatotoxins such as microcystins, and neurotoxins such as saxitoxins and anatoxins (Aráoz et al., 2010; Funari and Testai, 2008; Rodgers et al., 2017; Van Apeldoorn et al., 2007). There is an increasing body of evidence to suggest that the non-protein amino acid (NPAA) β-Methylamino-L-alanine (BMAA) which is produced by cyanobacteria as well as diatoms and dinoflagellates (Esterhuizen and Downing, 2008; Jiang et al., 2014a; Jiang et al., 2013; Lage et al., 2014; Réveillon et al., 2016a), may also be toxic to humans and could contribute to the onset of neurodegenerative disease (Cox et al., 2017; Nunn, 2017). BMAA has been shown to trigger neurodegenerative processes in primate models; including the formation of β-amyloid plaques neurofibrillary tangles (NTFs) in vervet monkeys, and a number of neurological dysfunctions in macaques (Cox et al., 2016; Spencer et al., 1987).

Pablo et al. (2009) detected BMAA in the brains of neurodegenerative disease patients from North America, as well as the brains of sufferers of amyotrophic lateral sclerosis – Parkinson's dementia complex (ALS-PDC), an unusual neurodegenerative disease which was primarily observed in residents of the island of Guam in the 1960s. It is important to note that the Pablo study employed pre-column derivatisation with high pressure liquid chromatography – fluorescence detection (HPLC-FD) for analysis, post-hoc liquid chromatography tandem mass spectrometry (LC–MS/MS) was only used to confirm BMAA presence in positive samples. HPLC-FD has been shown to overestimate BMAA concentration (Faassen et al., 2012). A number studies employing alternative analysis techniques have not been successful in

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detecting BMAA in multiple matrices (Montine et al., 2005; Snyder et al., 2009), however, BMAA analysis has been shown to be prone to type II errors (Baker et al., 2017; Banack and Murch, 2018).

BMAA has been shown to be acutely toxic to primary neurological cells (Tan et al., 2017a,b), and two mechanisms have risen to prominence as potential causes for BMAA toxicity. In the presence of bicarbonate, BMAA has been shown to form a β-carbamate, which can act as a glutamate receptor agonist resulting in excitotoxicity (Chiu et al., 2015; Chiu et al., 2012, 2013; Zeevalk and Nicklas, 1989). BMAA has also been shown to bind to proteins, requiring hydrolysis to be liberated (Banack et al., 2007; Polsky et al., 1972). Evidence suggests that in eukaryotes this may occur through the misincorporation of BMAA into proteins during synthesis, possibly in place of serine (Dunlop et al., 2013; Glover et al., 2014; Main et al., 2017). The misincorporation of BMAA into proteins results in the formation of misfolded proteins within the lumen of the endoplasmic reticulum (ER), resulting in ER-stress and the activation of pro-apoptotic pathways as part of the unfolded protein response (UPR) (Main et al., 2016; Main and Rodgers, 2017; Okle et al., 2013; Shen et al., 2016). Both these mechanisms can result in a decline in function or death of neurological cells, potentially leading to symptoms observed in a number of neurodegenerative disorders.

Exposure to BMAA is of particular concern both because of its apparent ubiquity and its proposed ability to accumulate within plant and animal products (Andersson et al., 2018; Contardo-Jara et al., 2014,2018; Reveillon et al., 2015). BMAA has been found in numerous environments globally including aquatic and terrestrial ecosystems in North America (Banack et al., 2015b; Banack et al., 2014), Europe (Jiang et al., 2014b; Jonasson et al., 2010; Reveillon et al., 2015), Asia (Li et al., 2010,2016), South America (Johnson et al., 2008), Africa (Esterhuizen and Downing, 2008), and the Middle East (Chatziefthimiou et al., 2018; Cox et al., 2009). high concentrations of BMAA are often found in large fish or filter feeders (Hammerschlag et al., 2016; Reveillon et al., 2015; Réveillon et al., 2016b) increasing the risk of human exposure through diet. BMAA is also believed to be capable of spreading through aerosolisation, increasing the exposure risk for those who live near waterbodies, which have frequent cyanobacterial blooms (Banack et al., 2015b; Stommel et al., 2013). This risk is supported by epidemiological data collected from North America that shows an increased risk of ALS in people who live near water bodies with frequent cyanobacterial blooms (Bradley et al., 2017), as well as geospatial mapping data that shows increased frequency of ALS clusters near lakes with low water quality (Torbick et al., 2014, 2017).

To date, the detection and quantification of BMAA in Australian ecosystems has been limited. BMAA was detected in a single Australian isolate of *Cylindrospermopsis raciborskii* in an early survey of BMAA producing cyanobacteria (Cox et al., 2005). The apparent ubiquity of BMAA in other global ecosystems would suggest it is likely to be present in Australian cyanobacteria. Waterways such as those in the Murray-Darling basin in eastern Australia are of particular concern. The semi-arid environment, proximity of agricultural land, slow moving water, and high risk of eutrophication make these rivers susceptible to frequent large scale blooms (Baker and Humpage, 1994; Davis and Koop, 2006), some of which have exceeded 1000 km in length and have persisted for over a month (Al-Tebrineh et al., 2012; Bowling and Baker, 1996; Bowling et al., 2013). The frequency and scale of cyanobacterial blooms in eastern Australia, coupled with the multiple proposed exposure routes, suggest that people living in proximity to waterways may be regularly exposed to BMAA. Development of a robust testing program for Australian cyanobacterial blooms is critical to understanding the potential risk of exposure to BMAA in Australia.

This study aims to use established liquid chromatography – tandem mass spectrometry (LC–MS/MS) methods to analyse the presence of algal toxin BMAA and its isomer 2,4-Diaminobutanoic acid (2,4-DAB) in surface scum samples collected from waterways with cyanobacterial blooms in eastern Australia.

2. Experimental

2.1. Reagents

L-BMAA standard and analytical solvents and other reagents were purchased from Sigma Chemical Co. MO, USA unless otherwise stated. L-2,4-DAB and L-AEG was purchased from Toronto Research Chemicals ON, Canada. Δ-2,4-Diaminobutyric-2,3,3,4,4-d_5 (d5-2,4-DAB) was purchased from CDN isotopes, QC, Canada. EZ:faast HPLC free amino acid derivatisation kit was purchased from Phenomenex Inc. CA, USA.

2.2. Environmental samples

Surface scum samples for analysis were collected by dip sampling directly into the scum or by concentration through a 25 μm plankton net in 250 mL polyethylene sample bottles from a number of waterways in New South Wales (NSW) with cyanobacterial blooms. Because such samples are unsuitable for cell counting, addition 250 mL dip samples were collected from nearby clear water areas from 25 cm below the surface and preserved with Lugols iodine solution for quantitative analysis of major taxa present. Cyanobacterial species were identified and counted to species level for known toxigenic species, otherwise to genus level following the methods described by Hötzel and Croome (1999) using a calibrated Lund cell on a Zeiss Axiovert 35 inverted microscope (Carl Zeiss AG, Oberkochen, Germany) at 200× magnification to a minimum counting precision of ±20%. Counts were converted to biovolume using standard cell sizes determined for south-eastern Australia (Victorian Department of Sustainability and Environment, 2007). BMAA samples were stored at −20 °C until required then thawed on ice prior to analysis and pelleted through centrifugation for 20 min at 3000*g* and 4 °C after which water was removed and pellets freeze-dried overnight.

2.3. Sample processing and hydrolysis

Dry pellets were weighed, and a maximum of 50 mg transferred into fresh tubes with 1 mL of 10% trichloroacetic acid (TCA) in water, and probe sonicated at 80% power for one minute three times on ice. Samples were allowed to stand overnight at 4 °C, after which they were centrifuged for 10 min at 10,000*g*. Sample supernatant was transferred to a fresh labelled tube, and the pellet washed by resuspending and pelleting twice; with 300 μL 10% TCA in water, and once in 10% (v/v) TCA in acetone to reduce drying time. Supernatant from each wash was combined with the original supernatant and then placed in a centrifuge vacuum concentrator until dry. Protein pellets were transferred in to glass shell vials and placed in a centrifuge vacuum concentrator until dry. Dried supernatant was resuspended in 100 μL of 20 mM hydrochloric acid (HCl) and stored at −80 °C until needed.

Shell vials containing dried protein pellets were placed in a vacuum vial, and 1 mL of 6 M HCl added to the bottom of the vacuum vial. Air was removed from the vacuum vials using a vacuum pump, and nitrogen used to purge the vial, this was repeated three times. Samples were then placed overnight in a high temperature oven at 110 °C and hydrolysed in the gas phase of HCl. After hydrolysis, protein pellets were removed from the oven and briefly allowed to cool before being resuspended in 100 μL of 20 mM HCl. The supernatant and protein fractions were then

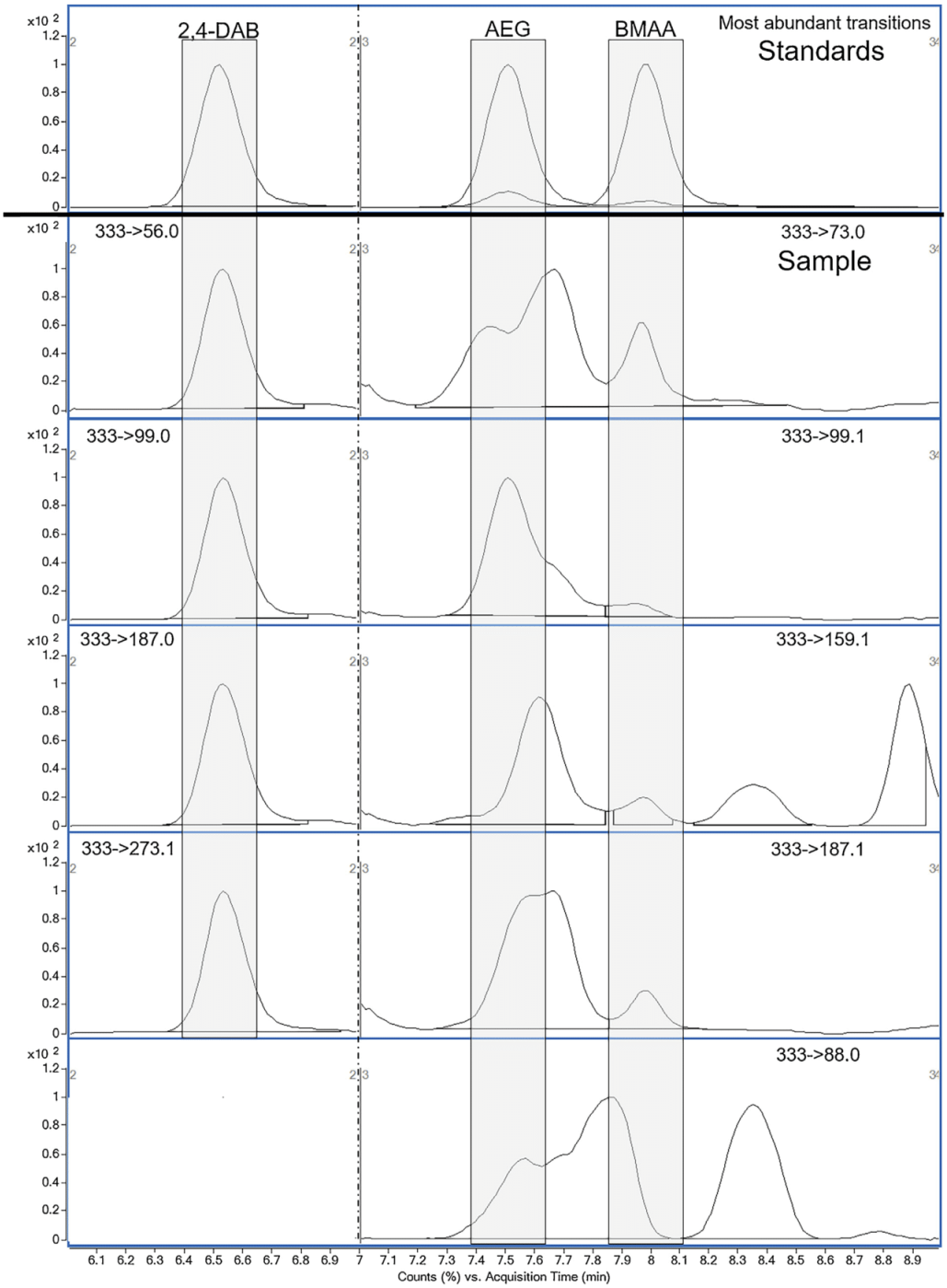

Fig. 1. Scaled chromatograms from UPLC–MS/MS analysis of 2,4-DAB, AEG, and BMAA in a positive sample and standards. Most abundant transition included for standards, all transitions included for sample.

combined and filtered with using 0.22 μM centrifuge filters. Filtered samples were stored at −80 °C until needed.

2.4. Sample derivatisation

100 μL of each sample was diluted 1:2 with 1 ng uL^{-1} d5-2,4-DAB as an internal standard (ITSD) to control for variations in derivatisation efficiency. Sample amino acids were pre-column derivatised with propyl chloroformate using the Phenomenex EZ: faast free amino acid derivatisation kit. Briefly, amino acids are concentrated using dispersive solid phase extraction (dSPE) tips, then eluted and liberated from the dSPE material, amino acids then undergo liquid/liquid extraction and derivatisation before being dried under nitrogen and resuspended in mobile phase.

2.5. Sample analysis

Samples were analysed using an Agilent Technologies 6490 triple quadrupole mass spectrometer coupled with an Agilent 1290 Infinity UHPLC. Chromatography was conducted using a Phenomenex Kinetex 150 × 2.1 mm 1.7 μm C18 100 Å UPLC column by gradient elution, using mobile phases 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in methanol (B) (0.00 min 55% B, 10.00 min 68%B, 10.10 min 100% B, 15.00 min 100% B, 15.10 min 55% B, with a 2 min post run re-equilibration). Flow rate was 0.250 mL min^{-1} and the column temperature was 35 °C. Data were acquired in ESI positive mode, drying gas temperature was 250 °C at 14 L min^{-1}, sheath gas temperature was 250 °C at 11 L min^{-1}, and nebuliser pressure was 20 psi. Capillary voltage was 3000 V and nozzle voltage 1500 V.

Limit of detection (LOD) was defined as a quantification ion signal to noise (S/N) ratio of 3.3, and limit of quantification (LOQ) was defined as a quantification ion S/N ratio of 10 and qualification ion S/N ratio >3. Percentage relative standard deviation (%RSD) was calculated using six repeat injections of 50 pg of BMAA, 2,4-DAB, and AEG spiked into sample matrix. For BMAA calculated LOD was 0.02 pg μL^{-1}, LOQ was 0.05 pg μL^{-1}, and %RSD was 3.48%; for 2,4-DAB calculated LOD was 0.04 pg μL^{-1}, LOQ was 0.13 pg μL^{-1}, and %RSD was 3.00%. Response Linearity was maintained through the tested concentration range of 0.5–2000 pg μL^{-1}. For sample analysis 5 μL of analyte was injected in triplicate, compounds were identified through retention time and scheduled MRM (Fig. 1) using four transitions for each isomer (Fig. 2) (Table 1). Quantification was conducted using the most abundant transition for 2,4-DAB (333.01 > 273.1) and BMAA (333.01 > 187.1). Sample to sample variations in derivatisation efficiency and matrix effects during LC–MS/MS were accounted for by comparing sample ITSD abundance to ITSD abundance in standards.

3. Results & discussion

Sixteen cyanobacterial bloom samples were collected from fourteen different sites in eastern Australia (Fig. 3) from 2015 to 2017. The concentration of BMAA and 2,4-DAB in each sample was determined using LC–MS/MS and expressed as the concentration per dry weight of bloom biomass. BMAA was detected at 9 of the 14

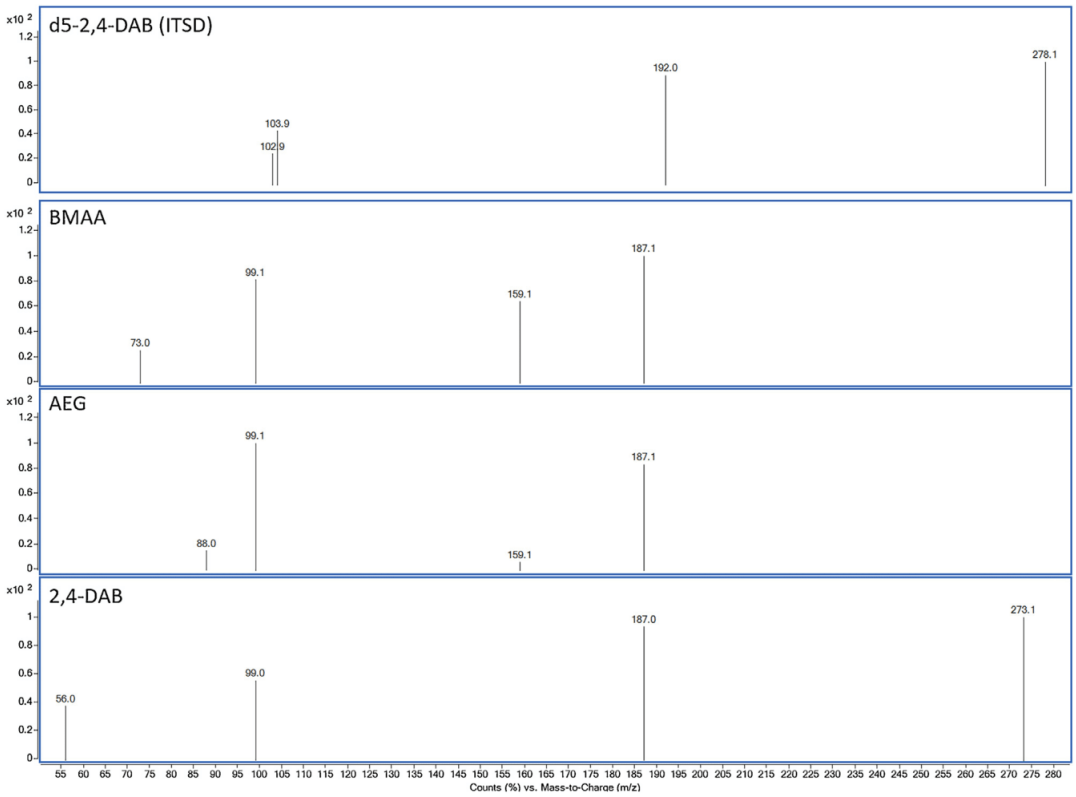

Fig. 2. cyanobacterial bloom sample collection sites from rural New South Wales and Sydney city (inset). Site names and waterways listed in Table 2.

Table 1
Retention times and MRM transitions from UPLC–MS/MS analysis of BMAA, 2,4-DAB, AEG, and internal standard d5-2,4-DAB. 2,4-DAB and BMAA were quantified using most abundant transitions indicated in bold. d5-2,4-DAB ITSD abundance was determined using most abundant transition indicated in bold.

Compound	RT (min)	Transitions			
BMAA	7.98	333.01 > 73 (CE 25)	333.01 > 159.1 (CE 17)	333.01 > 99.1 (CE 29)	**333.01 > 187.1 (CE 9)**
2,4-DAB	6.51	**333.01 > 273.1 (CE 5)**	333.01 > 187 (CE 13)	333.01 > 99 (CE 29)	333.01 > 56 (CE 60)
AEG	7.51	333.01 > 88 (CE 33)	333.01 > 187.1 (CE 9)	333.01 > 99 (CE 29)	333.01 > 56.1 (CE 60)
d5-2,4-DAB	6.48	**338.01 > 278.1 (CE 5)**	333.01 > 192 (CE 17)	333.01 > 103.9 (CE 29)	333.01 > 102.9 (CE 25)

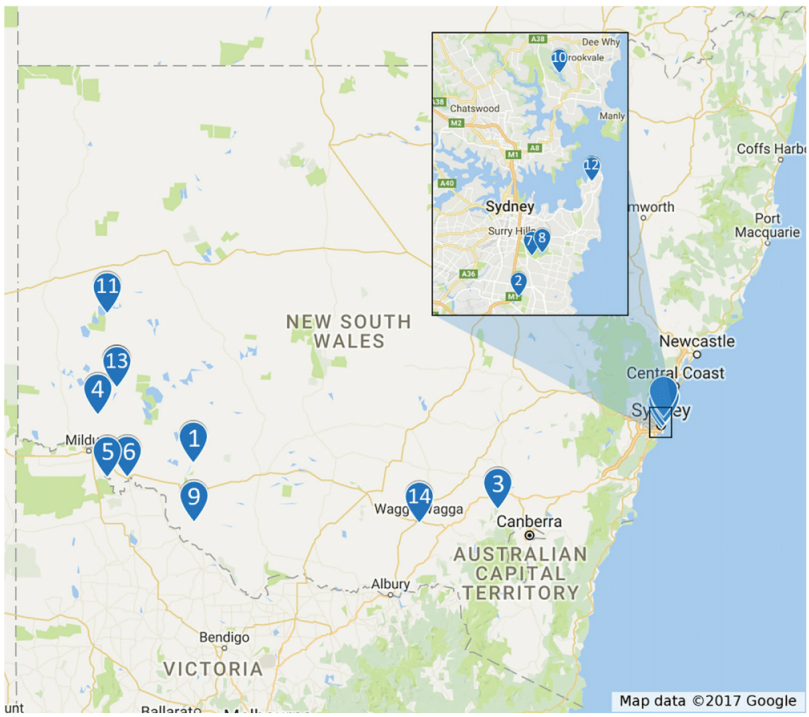

Fig. 3. BMAA and 2,4-DAB concentrations from mixed cyanobacterial samples collected from various sites in New South Wales, Australia. Analysis conducted using LC–MS/MS with scheduled MRM.

sampled sites (Fig. 4). The highest concentration detected was $47.26 \pm 4.32\ \mu g\ g^{-1}$ from Manly Dam, the lowest concentration detected was $12.74 \pm 0.23\ \mu g\ g^{-1}$ from Menindee (Table 2). The average BMAA concentration in the positive samples was $25.39\ \mu g\ g^{-1} \pm 10.32\ \mu g\ g^{-1}$2,4-DAB was detected at all 14 of the sampled sites (Fig. 4). The highest concentration detected was $7.61 \pm 0.05\ mg\ g^{-1}$ from Botany wetlands in Sydney, the lowest concentration detected was $0.28 \pm 0.01\ mg\ g^{-1}$ from Kensington pond in Sydney (Table 2). The average 2,4-DAB concentration across all sites was $1.96\ mg\ g^{-1} \pm 1.91\ \mu g$

This study reports of the presence of known algal neurotoxins BMAA and 2,4-DAB in an Australian ecosystem. Cyanobacterial blooms in Australia are an increasing concern, blooms in rural Australia in particular may be increasing in frequency and severity especially during periods of drought (Bowling et al., 2015). These blooms are often large, persistent, and within close proximity to rural towns (Bowling et al., 2013). While blooms are monitored for the presence of a number of established cyanobacterial toxins, the emerging toxins BMAA and 2,4-DAB have never been reported in Australian cyanobacterial blooms.

The only previous report of BMAA from an Australian species was from a laboratory-grown isolate (CR3) of the freshwater cyanobacterium *Cylindrospermopsis raciborskii* sampled in Queensland (Cox et al., 2005). Cox et al. (2005) employed HPLC-FD as the primary analytical technique, with HPLC–MS used for confirmation.

BMAA was detected in bloom samples collected from nine different sites of the fourteen collected in both rural and urban areas of eastern Australia. BMAA (and 2,4-DAB) was found in a range of cyanobacterial species, several of which are not considered to be otherwise toxigenic in NSW. It should also be noted that concentrated algal scum samples with a mix of species were used for analyses, although generally only one or two species dominated, with remaining species being minor contributors. The high concentrations observed for both BMAA and 2,4-DAB are consistent with other studies that have analysed mixed population field cultures, which are often significantly higher than those observed in axenic lab cultures (Bishop et al., 2018; Faassen, 2014).

Positive BMAA samples from rural areas were collected from sites on the Niemur, Murrumbidgee, and Darling rivers. The

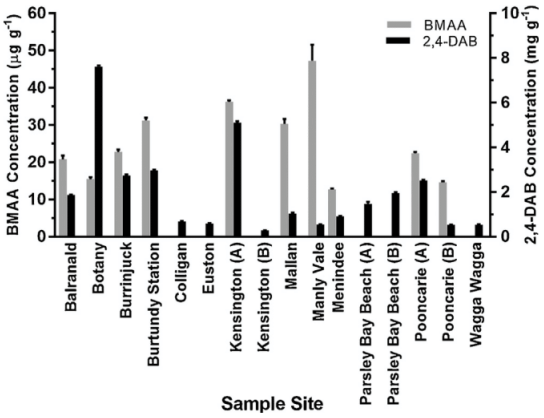

Fig. 4. MS/MS spectra showing relative abundance of all MRM transitions used for analyte identification and quantification.

Murrumbidgee and Darling rivers are major water sources for the Murray-Darling basin (MDB), a significant agricultural region in south west NSW. The MDB accounts for 39% of Australian agriculture and the majority (65%) of the irrigated land in Australia (ABS, 2008). Of the water used for irrigation within the MDB 84% comes from surface water such as the Darling and Murrumbidgee rivers (ABS, 2008). This water is also often used for drinking water supplies for towns along these rivers which can be sometimes impacted by cyanobacterial blooms (Mitrovic et al., 2010; Mitrovic et al., 2003; Sherman et al., 1998).

The presence of BMAA in waterbodies that are used for crop irrigation is concerning. BMAA has previously been shown to be taken up by plants and to become associated with plant proteins (Contardo-Jara et al., 2018; Contardo-Jara et al., 2014). The uptake of BMAA by crop plants therefore increases the risk of human and animal exposure, and has the potential to negatively affect plant health (Esterhuizen-Londt et al., 2011). There is also evidence to suggest BMAA may bioaccumulate, resulting in higher concentrations within animals that feed on plant tissue that contains BMAA (Baptista et al., 2015; Lürling et al., 2011; Murch et al., 2004). BMAA can be transferred as a free amino acid via milk to suckling rodent pups (Andersson et al., 2016; Andersson et al., 2013) raising the possibility of transfer between human mothers and their children, between livestock, and in dairy products. Recent work by Andersson et al. (2018) demonstrating accumulation of BMAA within eggs following administration of ^{14}C BMAA to laying quail hens raises the possibility of human exposure through poultry products.

BMAA positive samples collected from around Sydney were all from freshwater bodies close to residential and recreational areas. These waterbodies are not used for irrigation, and are no longer used for drinking water supply. Manly reservoir is a recreational waterway and is used for a number of activities including kayaking, swimming and water skiing. Frequent participation in water sports has previously been shown to increase the risk of developing ALS, with water skiing in particular having the highest association of all tested risk-factors, above that of smoking or exposure to industrial chemicals (Andrew et al., 2017). Living within 3 km of a water body that has frequent cyanobacterial blooms has also been shown to significantly increase the risk of developing ALS (Andrew et al., 2017); this may be due to exposure to aerosols which have been

Table 2
Concentration of BMAA and 2,4-DAB from mixed cyanobacterial samples collected at various sited in NSW. Collection date, sampling site, water body sampled, and biovolume of dominant species within each bloom are recorded. Map numbers correspond to sites indicated in Fig. 3.

Sample Site	Water body	Map	Collection date	Dominant Species	Biovolume of dominant species ($mm^3 l^{-1}$)	BMAA Concentration ($\mu g g^{-1}$)	2,4-DAB Concentration ($mg g^{-1}$)
Balranald	Murrumbidgee River	1	2016 03 15	*Dolichospermum planktonicum, Oscillatoria* sp.	0.66 0.31	20.8 ± 1.02	1.866 ± 0.02
Botany	Botany Wetlands – Dam 5	2	2015 12 17	*Dolichospermum smithii, Microcystis wesenbergii*	19.0 0.53	15.49 ± 0.54	7.614 ± 0.05
Burrinjuck	Murrumbidgee River – Lake Burrinjuck	3	2016 11 22	*Dolichospermum circinale*	7.77	22.78 ± 0.62	2.741 ± 0.05
Burtundy Station	Darling River	4	2015 05 05	*Microcystis aeruginosa, Anabaenopsis* sp.	0.32 0.31	31.28 ± 0.68	2.974 ± 0.03
Colignan	Murray River	5	2016 05 18	*Chrysosporum ovalisporum*	Unispecific bloom sample only	ND	0.691 ± 0.02
Euston	Murray River	6	2016 05 24	*Chrysosporum ovalisporum*	13.34	ND	0.5783 ± 0.03
Kensington (A)	Kensington Pond	7	2015 04 15	*Dolichospermum planktonicum, Microcystis wesenbergii*	3.47 3.46	ND	0.2819 ± 0.01
Kensington (B)	Duck Pond	8	2015 04 01	*Dolichospermum smithii*	19.89	36.24 ± 0.38	5.103 ± 0.07
Mallan	Niemur River	9	2016 04 19	*Chrysosporum ovalisporum*	845.6	30.31 ± 1.32	1.033 ± 0.04
Manly Vale	Manly Reservoir	10	2017 03 30	*Microcystis aeruginosa*	Unispecific scum sample only	47.26 ± 4.32	0.5428 ± 0.01
Menindee	Darling River	11	2015 12 15	*Anabaenopsis* sp. *Dolichospermum spiroides, Dolichospermum smithii*	2.82 2.19 1.32	12.74 ± 0.23	0.9221 ± 0.01
Parsley Bay Beach (A)	Sydney Harbour	12	2015 03 18	*Trichodesmium erythraeum*	Unispecific scum sample only	ND	1.469 ± 0.09
Parsley Bay Beach (B)	Sydney Harbour	12	2015 03 18	*Trichodesmium erythraeum*	Unispecific scum sample only	ND	1.953 ± 0.04
Pooncarie (A)	Darling River	13	2015 12 15	*Aphanocapsa* sp., *Anabaenopsis* sp.	22.0 2.39	22.41 ± 0.38	2.511 ± 0.03
Pooncarie (B)	Darling River	13	2015 03 11	*Anabaenopsis* sp.	3.24	14.65 ± 0.27	0.5281 ± 0.01
Wagga Wagga	Lake Albert	14	2015 04 11	*Dolichospermum circinale*	11.64	ND	0.5348 ± 0.02

shown to be able to disperse both BMAA and 2,4-DAB (Banack et al., 2015a).

2,4-DAB was detected in significant concentrations in all of the samples tested, there was no apparent relationship between the concentration 2,4-DAB and BMAA in samples suggesting production of both neurotoxins is independent (Table 2). The mechanisms of 2,4-DAB toxicity have been less thoroughly explored than for BMAA. There is evidence that 2,4-DAB is both hepatotoxic resulting in a build-up in ammonia within the brain causing damage (O'Neal et al., 1968), as well as directly neurotoxic through interaction with GABA receptors (Chen et al., 1972; Johnston and Twitchin, 1977). previously it has been shown that combined treatment with 2,4-DAB and BMAA increased cytotoxicity in human neuroblastoma cells relative to treatment with the toxins on their own (Main and Rodgers, 2017). The potential for synergistic toxicity between BMAA and 2,4-DAB has been demonstrated in human motor neurons, where co-treatment with 2,4-DAB significantly reduced the concentration of BMAA required to induce neuronal damage (Tan et al., 2017b). While further research is required to determine if 2,4-DAB has a role in the development of neurodegenerative diseases, it is a known neurotoxin.

There have been numerous media reports of a ALS hot-spot in south-western NSW, similar hot spots have been reported surrounding waterbodies with BMAA producing cyanobacterial blooms in New Hampshire (USA) (Caller et al., 2009; Torbick et al., 2014; Torbick et al., 2017) and the Thau Lagoon (France) (Masseret et al., 2013). The presence of these hot spots or clusters suggests that exposure to BMAA contaminated water, either directly through aerosols or contact with water; or indirectly through consumption of BMAA contaminated food, may contribute to the development of neurodegenerative disease. The relationship between BMAA and neurodegeneration is supported by numerous *in vivo* (Cox et al., 2016; Scott and Downing, 2017) and *in vitro* (Chiu et al., 2015; Chiu et al., 2012, 2013; D'Mello et al., 2017; Dunlop et al., 2013; Main et al., 2016; Tan et al., 2017a) studies that have demonstrated BMAA toxicity. There is now a significant body of evidence implicating BMAA in neurodegeneration, with a number of proposed mechanisms and exposure routes. This study shows that BMAA was frequently detected in the bloom samples examined so may be common in the aquatic environment. This strengthens the argument that ongoing monitoring of BMAA and 2,4-DAB in water used for potable water supply, in primary production, for recreation, or in close proximity to residential areas should be conducted in Australia.

4. Conclusion

- Emerging algal toxins β-methylamino-L-alanine (BMAA) and 2,4-diaminobutyric acid (2,4-DAB) are present in cyanobacterial bloom samples from Australia.
- Positive samples came from both rural and urban areas of New South Wales. Both toxins were present in samples containing species not known to be toxic in Australia.
- The presence of these toxins in waterways used for irrigation presents concerns for public health and food security in Australia.
- Monitoring these emerging toxins in Australian waterways is required to allow informed decisions to be made about the risks associated with their presence.

Ethical statement

This manuscript represents original research, and has not been published in part or in whole elsewhere. This publication is not currently being considered for publication elsewhere. All authors have actively been involved in work leading to this manuscript, and have read and agreed with its publication. This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of interest

The authors declare they have no conflicts of interest.

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Chapter Three

Investigation of the interaction of β-methylamino-L-alanine with eukaryotic and prokaryotic proteins

Chapter Three: Investigation of the interaction of β-methylamino-L-alanine with eukaryotic and prokaryotic proteins.

Chapter Overview

The previous chapter confirmed the presence of BMAA and 2,4-DAB in a number of Australian cyanobacterial blooms, and critically that these non-protein amino acids were present in algal blooms collected from the agriculturally important Murray-Darling basin. The ability of BMAA to bioaccumulate and biomagnify has been well documented, with increasing 'protein associated' BMAA often seen in higher trophic levels. While the presence of protein bound, or associated BMAA is well described, the exact mechanism underlying this binding has been a topic of significant debate.

The studies presented in this chapter aimed to further investigate the interaction between BMAA and both prokaryotic and eukaryotic proteins. E. coli and cultured human neuroblastoma cells, as well as isolated proteins from both, were incubated with radiolabelled BMAA. The rate of binding and distribution of bound BMAA was compared to that of ^{3}H leucine, a protein amino acid. The use of radiolabelled BMAA increased the sensitivity of the system and avoided the extensive sample processing required for mass spectrometric analysis.

Both BMAA and leucine were unable to bind to a range of pure proteins, or to eukaryotic and prokaryotic whole protein lysate. BMAA was found to bind to eukaryotic proteins in live cells in a linear, time dependent fashion, and distribute across cell proteins similar to leucine. In *E. coli*, protein bound BMAA rapidly increased in concentration then decreased after 1 hour of exposure. These results suggest that BMAA or a BMAA derivative may be incorporated into eukaryotic proteins during synthesis.

Certificate of authorship and originality

This paper was published in *Amino Acids* © Springer Ltd. I certify that the work presented in this chapter has not previously been submitted as part of the requirements for a degree. I also certify that I carried out the majority of the work presented in this paper.

- Brendan J Main: Conducted experiments and wrote the manuscript.
- Carly J Italiano: Aided with experiments
- Kenneth J. Rodgers: Proof-read and edited the manuscript, conceived project idea, and provided guidance on experiments.

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ORIGINAL ARTICLE

Investigation of the interaction of β-methylamino-L-alanine with eukaryotic and prokaryotic proteins

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Abstract
There is a strong body of evidence linking the non-protein amino acid (NPAA) β-methylamino-L-alanine (BMAA) to the development of a number of neurodegenerative diseases. BMAA has been found globally, is produced by a number of organisms including cyanobacteria, diatoms, and dinoflagellates; and has been shown to biomagnify through trophic levels. The role of BMAA in neurodegenerative disease is highlighted by its presence in the brains of a number of neurodegenerative disease patients, where it was found in a protein-bound form. We have previously shown that BMAA is bound to cell proteins, and results in the upregulation of the unfolded protein response, an endoplasmic reticulum stress response activated by the presence of misfolded proteins within the cell. Structurally aberrant proteins are features of a number of neurodegenerative diseases, and further investigation of how BMAA interacts with proteins is crucial to our understanding of its toxicity. Here we use radiolabelled BMAA to investigate the interaction and binding of BMAA to eukaryotic and prokaryotic proteins. We found differences in the presence and distribution of protein-bound BMAA between *E. coli* and neuroblastoma cells, with an increase in binding over time only seen in the eukaryotic cells. We also found that BMAA was unable to bind to pure proteins, or cell lysate in native or denaturing conditions, indicating that biological processing is required for BMAA to bind to proteins.

Keywords Non-protein amino acid · β-methylamino-L-alanine · BMAA · Protein synthesis · Misincorporation · Amyotrophic lateral scerosis

Introduction

The non-protein amino acid (NPAA) β-Methylamino-L-alanine (BMAA) has previously been linked to a number of neurodegenerative disorders including amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD) (Pablo et al. 2009). BMAA was first implicated in the onset of amyotrophic lateral sclerosis–Parkinson's dementia complex (ALS–PDC) on the west pacific island of Guam in the 1960s (Vega and Bell 1967; Whiting 1963), and has since been shown to cause ALS-like symptoms in macaques (Spencer et al. 1987), as well as hallmark neurofibrillary tangles (NFT) and β-amyloid plaques in vervets (Cox et al. 2016). BMAA has also been found in the brain tissue of neurodegenerative disease patients in Guam and North America (Murch et al. 2004a, b). Although originally observed on Guam, BMAA has now been identified in both aquatic and terrestrial eco-systems in North America (Banack et al. 2015; Caller et al. 2009), The Baltic Sea (Jonasson et al. 2010) France (Masseret et al. 2013), Sweden (Jiang et al. 2014b), Peru (Johnson et al. 2008), and Qatar (Cox et al. 2009); and is produced by a number of different cyanobacteria (Cox et al. 2005), diatoms (Jiang et al. 2014a), and dinoflagellates (Lage et al. 2014).

The mechanism of BMAA toxicity has been a topic of debate since its discovery, and two different mechanisms of toxicity have been demonstrated. BMAA is known to form a β-carbamate in the presence of bicarbonate (Nunn and O'Brien 1989); this carbamate has demonstrated acute glutamate receptor excitotoxicity in primary human neurons (Weiss et al. 1989; Lobner 2009; Chiu et al. 2012) as well as cultured glial cells (Chiu et al. 2013, 2015). A second chronic form of toxicity has been demonstrated through the interaction of BMAA with proteins, resulting

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in a disturbance of protein folding, and the formation of misfolded and aggregated proteins; a hallmark of a number of neurodegenerative diseases (Dunlop et al. 2013). Murch et al. (2004a) demonstrated that BMAA was present in vivo as both a free amino acid and in a stable protein-bound complex that could only be separated via acid hydrolysis, Murch suggested that the presence of bound BMAA may interrupt the formation of tertiary structure of neuro-proteins thus altering their activity.

Protein–NPAA complexes have been previously demonstrated; L-DOPA has been shown to incorporate into proteins in place of tyrosine during synthesis (Rodgers et al. 2002, 2006) resulting in the formation of degradation resistant aggregates (Dunlop et al. 2008). A similar model of biosynthetic misincorporation into proteins has been demonstrated for BMAA, where it appears to incorporate in place of L-serine (Dunlop et al. 2013), resulting in upregulation of the endoplasmic reticulum (ER) unfolded protein response (UPR) (Okle et al. 2013). The misincorporation of BMAA in place of L-serine is supported through competition experiments, where co-treatment with BMAA and L-serine prevented increases in UPR gene expression, cathepsin activity, and caspase-3 activity (Main et al. 2016).

Evidence suggests that prokaryotes do not incorporate BMAA into protein, despite taking it up from the media (van Onselen et al. 2015). Although incorporation is not seen in prokaryotes, there is still evidence of protein association proposed to be through passive binding of BMAA to proteins. Cyanobacteria treated with BMAA have been shown to have considerable, concentration dependant uptake and protein association of BMAA within 10 min of exposure. This protein associated BMAA then decreases over time and is below detection levels after 72 h (Downing et al. 2012).

The level of passive binding of BMAA to eukaryotic proteins, however, has not yet been fully explored. Treatment of both neuroblastoma cells and *E. coli* with radiolabelled BMAA allows accurate quantitation of low concentrations of protein associated BMAA in both eukaryotic and prokaryotic cells. Determining if any amount of BMAA is passively bound to eukaryotic proteins is critical to our understanding of how this NPAA can trigger disease.

Reagents

^{3}H BMAA (specific activity – 2960 GBq/mmol, concentration 37 MBq/mL), ^{14}C BMAA (specific activity – 2.035 GBq/mmol, concentration 3.7 MBq/mL), and ^{3}H Leucine (specific activity – 5.14 TBq/mmol, concentration 37 MBq/mL) were sourced from American Radiolabeled Chemicals, Inc. MO, USA. Dulbecco's Modified Eagle's Medium (DMEM), Hank's buffered saline solution (HBSS), and foetal bovine serum (FBS) from Sigma Chemical Co., MO, USA. Liquid scintillation counting was done using Ultima Gold LSC-cocktail from PerkinElmer, MA, USA. Micro Bio-Spin P6, 6 kDa size exclusion columns are from Bio-Rad, CA, USA.

All aqueous solutions and buffers were prepared using 18 mΩ water. All other chemicals, solvents and chromatographic materials were of analytical reagent or cell culture grade.

Methods

Mammalian cell culture

SH-SY5Y human neuroblastoma cells (American Tissue Culture Collection, catalogue number CRL-2266) were cultured as follows: DMEM was supplemented with 10% heat-inactivated (HI) FBS, and 2 mM L-glutamine. Cells were maintained at 37 °C with 5% CO_2 in 175-cm^2 flasks until they were plated. For experiments, cells were plated at an approximate density of 2×10^4 cells per well in 6-well plates. For treatments DMEM was substituted with HBSS with 10% HI FBS.

E. coli Culture

Wild-type *E. coli* K-12 strain BW25113 was used for all bacterial experiments. The strain was stored at – 80 °C in 16% glycerol and revived as needed.

E. coli from frozen stocks were grown overnight at 37 °C on Luria–Bertani agar plates (Difco. Becton–Dickinson, NJ, USA.). A single colony was used to inoculate 3 mL of M9 minimal media, supplemented trace-elements solution designed to improve bacterial grow (Duggin et al. 2015). M9 minimal media was prepared as described by Sambrook, Fritsch and Maniatis (Evans 1990). Cultures were then incubated overnight at 37 °C with shaking at 150 rpm.

Following 16–18 h of growth, cultures were transferred to fresh M9 media and adjusted to an optical density (OD600) of 0.1. Cultures were incubated as described previously and monitored spectrophotometrically, until mid-log phase was reached (OD ~ 0.6).

Cultures where then centrifuged at 4000 RCF for 10 min at room temperature. Supernatant was discarded and the bacterial pellet was resuspended in an equal volume of M9 medium containing either 1.25 nM ^{3}H labelled-BMAA or 0.72 nM ^{3}H labelled leucine.

Resuspended cultures were incubated at 37 °C with shaking as described previously. At the indicated time points, cultures were pelleted at 4000 RCF for 10 min and washed in 30 mL of phosphate buffered saline (PBS) three times. Cell pellets were then frozen on dry ice before being stored at – 80 °C.

For 24-h end point assays of BMAA and Leucine protein incorporation, optical density measurements were taken over the incubation period to track the growth of the *E. coli* over time.

Incubation of pure proteins with radiolabelled BMAA in native and denaturing conditions

Bovine serum albumin (BSA), α-casein, and ribonuclease A were made up in HBSS at 1 mg/mL. Protein was diluted 1:2 into either 'native' buffer consisting of HBSS with 2.5 nM ^{3}H BMAA, or 'denaturing' buffer consisting of HBSS with 8 M urea, 6 M thiourea, and 2.5 nM ^{3}H BMAA. The samples were incubated for 24 h at 37 °C with constant agitation. Unbound BMAA was removed from 75 µL sample aliquots using micro bio-spin size exclusion columns, aliquots were then transferred to scintillation vials, 5 mL of LSC-cocktail added, and disintegrations per minute calculated using a PerkinElmer Tri-Carb 2810 TR liquid scintillation counter.

24-h incubation of SH-SY5Y cells and SH-SY5Y lysate with radiolabelled amino acids

For live cell treatment SH-SY5Y cells were plated and left to adhere overnight. Following adherence, cells were washed three times with HBSS prior to treatment. ^{3}H BMAA or ^{3}H leucine was diluted 1:10,000 (1.25 nM BMAA; 0.72 nM leucine) from stock into HBSS 10% FBS and triplicate wells of a 6-well plate were treated for 24 h. Following treatment, cells were washed three times in HBSS then scraped into 100 µL of 0.5% Triton-x100 and the triplicates pooled. Cells were lysed via probe sonication at 40% power for 10 s three times on ice. Samples were then centrifuged for 5 min at 5000 RCF to pellet cell debris, and the supernatant transferred to a fresh tube. Protein concentration was determined using a bicinchoninic acid (BCA) assay (Smith et al. 1985).

For cell lysate incubations SH-SY5Y neuroblastoma cells were removed from a confluent 175 cm^2 flask using TrypLE (Gibco, MD, USA) and pelleted by centrifugation at 900 RCF for 5 min. The cell pellet was then resuspended in HBSS and lysed via probe sonication at 40% power for 10 s three times on ice. Samples were centrifuged for 5 min at 5000 RCF to pellet cell debris, and the supernatant transferred to a fresh tube. Protein concentration was determined using BCA assay and normalised to 1 mg/mL, samples were then diluted 1:2 into HBSS with either 2.50 or 1.44 nM ^{3}H leucine in HBSS and incubated for 24 h at 37 °C with constant agitation.

Unbound BMAA was removed from 75 µL sample aliquots using Bio-Rad Micro Bio-Spin size exclusion centrifuge columns. Following clean-up samples were transferred to a scintillation vial with 5 mL of LSC-cocktail and the concentration of bound amino acid determined using liquid scintillation counting (LSC).

Time course treatment of SH-SY5Y cells and SH-SY5Y lysate with radiolabelled BMAA

SH-SY5Y cells were plated and left to adhere overnight. Following adherence, cells were washed three times with HBSS prior to treatment. Radiolabelled amino acids were diluted from stock into HBSS 10% FBS as follows; ^{14}C BMAA diluted 1:5000 with a treatment concentration of 364 nM, ^{3}H BMAA diluted 1:10,000 with a treatment concentration of 1.25 nM. Triplicate wells of a 6-well plate were treated with either ^{14}C or ^{3}H BMAA media for 0, 3, 6, or 9 h. Following treatment, cells were washed three times in HBSS then scraped into 330 µL of 0.5% Triton-x100 and the triplicates pooled. Cells were lysed via probe sonication at 40% power for 10 s three times on ice, samples were then centrifuged for 5 min at 5000 RCF to pellet cell debris, and the supernatant transferred to a fresh tube. Protein concentration was determined using BCA assay.

For lysate incubations SH-SY5Y neuroblastoma cells were removed from a confluent 175 cm^2 flask using TrypLE and pelleted by centrifugation at 900 RCF for 5 min. The cell pellet was then resuspended in HBSS and lysed via probe sonication at 40% power for 10 s three times on ice. Samples were centrifuged for 5 min at 5000 RCF to pellet cell debris, and the supernatant transferred to a fresh tube. Protein concentration was determined using BCA assay and normalised to 1 mg/mL, samples were then diluted into HBSS with ^{3}H BMAA to a final concentration of 0.5 mg/mL total protein, with 1.25 nM ^{3}H BMAA. Samples were incubated for 0, 3, 6, or 9 h at 37 °C with constant agitation.

Protein was precipitated by adding 1 mL of sample cell lysate to 9 mL of 10% trichloroacetic acid in acetone and leaving the samples overnight at 4 °C. Protein was then pelleted by spinning the samples for 15 min at 3600 RCF, and washed with 10 mL of acetone to remove residual unbound BMAA, this was repeated three times. Following washing, the protein pellet was allowed to dry on the bench before being resuspended in 100 µL of neat formic acid and left to dissolve overnight.

After dissolving in formic acid the protein was added to a scintillation vial with 5 mL of LSC-cocktail and bound amino acid determined using LSC.

FPLC separation of proteins from SH-SY5Y cells incubated with radiolabelled amino acids

SH-SY5Y cells were treated 24 h with either ^{3}H BMAA or ^{3}H leucine diluted 1:5000 from stock (2.5 nM BMAA; 1.44 nM leucine) into HBSS with 10% FBS. Following treatment cells were washed with HBSS and then scraped into

500 µL of fast protein liquid chromatography (FPLC) buffer (50 mM Tris pH 7.2 with 0.15 M NaCI and 0.05% Tween 20) and lysed via probe sonication at 40% power for 1 min, three times on ice. Samples were then centrifuged for 5 min at 5000 RCF to pellet cell debris. Samples were concentrated to 50 µL and unbound amino acids removed using Vivaspin 3000 kDa molecular weight cut off filters (MWCO) (Sartorius AG, Göttingen, Germany).

Proteins were fractionated using an ÄKTAexplorer 100 FPLC and a Superdex 75 HR 10/30 column (GE Healthcare, IL, USA). The following conditions were used; 100% buffer A (50 mM Tris pH 7.2 with 0.15 M NaCI and 0.05% Tween 20), flow rate – 0.500 mL/min, fraction volume – 300 µL, injection volume – 50 µL. Following fractionation, the protein concentration of each fraction was determined via BCA assay, and the concentration of bound labelled amino acid determined via LSC.

24-h incubation of *E. coli* and *E. coli* cell lysate with radiolabelled amino acids

Following treatment, *E. coli* was pelleted by centrifugation for 10 min at 3000 RCF and the media removed. *E. coli* was then washed by resuspending in PBS and re-pelleted, three times. After washing, the pellet was resuspended in 300 µL of 0.5% Triton-x100 and lysed via probe sonication at 40% power for 1 min three times on ice, samples were then centrifuged for 5 min at 5000 RCF to pellet cell debris, and the supernatant transferred to a fresh tube. Protein concentration was determined using a BCA assay.

For lysate incubations *E. coli* was pelleted, washed, and lysed as above. Protein concentration was determined by BCA assay and normalised to 1 mg/mL. Samples were then diluted 1:2 into HBSS containing either 1:5000 (2.5 nM) ^{3}H BMAA or 1:5000 (1.44 nM) ^{3}H leucine and incubated for 24 h at 37 °C with constant agitation.

Unbound BMAA was removed from 75 µL sample aliquots using Bio-Rad Micro Bio-Spin size exclusion centrifuge columns. Following clean-up samples were transferred to a scintillation vial with 5 mL of LSC-cocktail and concentration of bound amino acid determined using LSC.

Time course treatment of *E. coli* cells and lysate with radiolabelled BMAA

Following treatment, *E. coli* was then pelleted by centrifugation for 10 min at 3000 RCF and the media removed. *E. coli* was then washed by resuspending in PBS and re-pelleted three times. After washing, the pellet was resuspended in 300 µL of 0.5% Triton-x100 and lysed via probe sonication at 40% power for 1 min, three times on ice. Samples were then centrifuged for 5 min at 5000 RCF to pellet cell debris, and the supernatant transferred to a fresh tube. Protein concentration was determined using a BCA assay.

For lysate incubations *E. coli* was then pelleted, washed, and lysed as above. Protein concentration was determined by BCA assay and normalised to 1 mg/mL. Samples were then diluted 1:2 into HBSS with either 1:5000 (2.5 nM) ^{3}H BMAA or 1:5000 (1.44 nM) ^{3}H leucine incubated for 0, 1, 2, 3, 6, or 9 h at 37 °C with constant agitation.

Protein was precipitated by adding 1 mL of sample cell lysate to 9 mL of 10% trichloroacetic acid in acetone and leaving the samples overnight at 4 °C. Protein was then pelleted by spinning the samples for 15 min at 3600 RCF, and washed with 10 mL of acetone to remove residual unbound BMAA, this was repeated three times. Following washing, the protein pellet was allowed to dry on the bench before being resuspended in 100 µL of neat formic acid and left to dissolve overnight.

After dissolving in formic acid, the protein was added to a scintillation vial with 5 mL of LSC-cocktail and concentration of bound amino acid determined using LSC.

FPLC separation of proteins from *E. coli* incubated with radiolabelled amino acids

E. coli was treated for 1 or 6 h with either ^{3}H BMAA or ^{3}H leucine diluted 1:5000 from stock (2.5 nM BMAA; 1.44 nM leucine) into culture media. Following treatment, *E. coli* was then pelleted by centrifugation for 10 min at 3000 RCF and the media removed. *E. coli* was then washed by resuspending in PBS and re-pelleted three times. After washing, the pellet was resuspended in 500 µL of FPLC buffer (50 mM Tris pH 7.2 with 0.15 M NaCI and 0.05% Tween 20) and lysed via probe sonication at 40% power for 1 min, three times on ice. Samples were then centrifuged for 5 min at 5000 RCF to pellet cell debris. Samples were concentrated to 50 µL and unbound BMAA removed using Vivaspin 3000 kDa MWCO filters.

Proteins were fractionated using an ÄKTAexplorer 100 FPLC and a Superdex 75 HR 10/30 column. The following conditions were used; 100% buffer A (50 mM Tris pH 7.2 with 0.15 M NaCI and 0.05% Tween 20), flow rate – 0.500 mL/min, fraction volume – 300 µL, injection volume – 50 µL. Following fractionation, the protein concentration of each fraction was determined via BCA assay, and the concentration of labelled amino acid determined via LSC.

Statistical Analysis

Statistical analysis was conducted using Prism version 7.01 (GraphPad software CA, USA) using one-way and two-way ANOVA with Bonferroni multiple comparison post-test or students *t* test to compare replicate means between different

treatments across the samples. Differences were considered significant at $P < 0.05$.

Results

BMAA and leucine do not passively bind to pure proteins under native or denaturing conditions

BSA, α-casein, and ribonuclease-A were incubated with radiolabelled BMAA and radiolabelled leucine in both native and denaturing buffers for 24 h. Unbound amino-acid was removed using size exclusion chromatography and the presence of bound amino acid determined using liquid scintillation counting (LSC). There was no significant binding of ^{3}H BMAA or ^{3}H leucine to pure proteins following 24-h incubation in native or denaturing conditions (Fig. 1). BSA in denaturing conditions had the highest amount of protein association, with 0.3% of supplied BMAA remaining bound to protein. Under the same conditions, the same treatment resulted in 0.1% of supplied leucine remaining remained bound to protein.

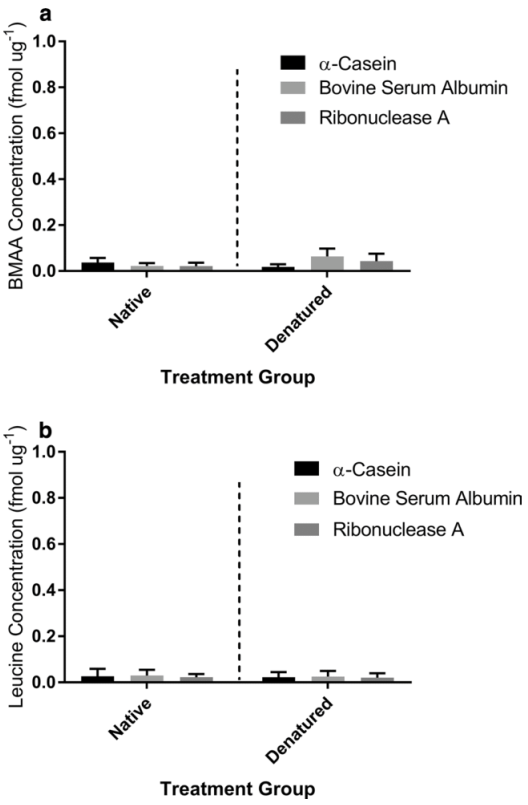

Fig. 1 BMAA and leucine do not passively bind to pure proteins under native or denaturing conditions. α-casein, Bovine Serum Albumin, and Ribonuclease-A were incubated with ^{3}H BMAA (**a**) and ^{3}H Leucine (**b**) in native and denaturing conditions, unbound amino acid was then removed through size exclusion and bound amino acid quantified using liquid scintillation counting ($n = 3$)

Both ^{14}C and ^{3}H BMAA bind to protein in a time dependant manner in live SH-SY5Y cells

SH-SY5Y cells were treated with ^{14}C and ^{3}H BMAA for 0, 3, 6, and 9 h, and the presence of protein bound BMAA was determined by LSC. Both ^{14}C and ^{3}H BMAA showed a linear, time-dependant increase in bound BMAA over the treatment period (Fig. 2). The highest percentage of bound BMAA was seen in the 9 h ^{3}H BMAA treatment, with 8.0% of supplied BMAA remaining bound to protein.

BMAA and leucine do not bind to SH-SY5Y proteins passively

Both live and lysed SH-SY5Y cells were incubated with ^{3}H BMAA and ^{3}H leucine for 24 h. There was a significant ($P < 0.0001$) increase in the presence of protein-bound amino acid in the live cell incubations when compared to the lysates. There was also a significant ($P < 0.0001$) difference in protein-bound amino acid present in live treated cells after 24-h when compared to the live 0-hour control (Fig. 3). 24-h incubation of live cells with labelled amino acid resulted in 8.9% of supplied BMAA, and 2.2% of supplied leucine remaining bound to protein. Cell lysate treated for 24 h resulted in 1.4% of supplied BMAA and 0.2% of supplied leucine binding to protein. 0-hour controls resulted in 0.3% of BMAA and 0.05% of leucine binding in live cells; and 0.3% of BMAA and 0.2% of leucine binding in cell lysates.

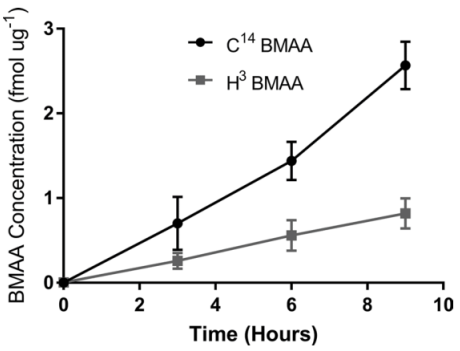

Fig. 2 Both ^{14}C and ^{3}H BMAA bind to protein in a time dependant manner in live SH-SY5Y cells. SH-SY5Y human neuroblastoma cells were incubated with ^{14}C and ^{3}H BMAA for up to 9 h. Protein was isolated using TCA precipitation and the presence of bound BMAA quantified by liquid scintillation counting ($n = 3$)

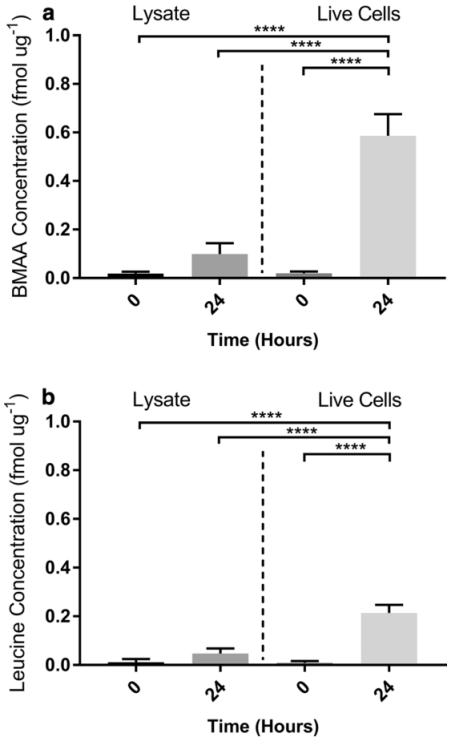

Fig. 3 BMAA and leucine do not bind to SH-SY5Y proteins passively. SH-SY5Y human neuroblastoma cells and neuroblastoma cell lysate were incubated with ^{3}H BMAA (**a**) and ^{3}H leucine (**b**) for 0- and 24-h. Protein was isolated using size exclusion and bound amino acid quantified using liquid scintillation counting. ****$P < 0.0001$ ($n = 3$)

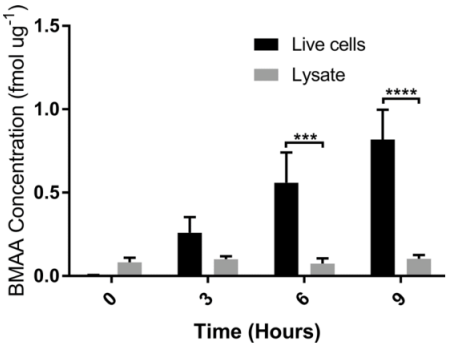

Fig. 4 Protein bound BMAA increases in a time dependant manner in live SH-SY5Y cells but not in SH-SY5Y lysate. SH-SY5Y human neuroblastoma cells were incubated with ^{3}H BMAA for up to 9-hours, protein was isolated using TCA precipitation and the presence of bound BMAA quantified by liquid scintillation counting. ***$P < 0.001$; ****$P < 0.0001$ ($n = 3$)

Protein bound BMAA increases in a time dependant manner in live SH-SY5Y cells but not in SH-SY5Y lysate

Both live and lysed SH-SY5Y cells were incubated with radiolabelled BMAA for 0, 3, 6, and 9 h. The presence of protein-bound BMAA was determined by LSC. There was a time-dependant increase in the presence of bound ^{3}H BMAA in live cells over 9-h; this was not seen in the protein lysate. There was a significant difference in the bound BMAA in the 6-h ($P < 0.001$) and 9-h ($P < 0.0001$) live cell treatment groups when compared to lysates incubated for the same amount of time (Fig. 4). Following 9 h of treatment, 8.0% of supplied BMAA remained bound to protein in the treated live cells, while 0.8% remain bound in the cell lysates.

Bound BMAA and leucine are distributed similarly in SH-SY5Y cell proteins

SH-SY5Y cells were treated for 24 h with ^{3}H BMAA and ^{3}H leucine. Proteins were isolated and fractionated using FPLC, and the presence of radiolabelled amino acid determined with LSC. Both BMAA and leucine were similarly distributed across the protein fractions with higher concentrations of labelled amino acids appearing in larger proteins (Fig. 5).

Low concentrations of BMAA and leucine do not prevent the growth of E. coli

E. coli was grown in the presence of ^{3}H BMAA or ^{3}H leucine for 24 h, with optical density measured regularly to determine growth. There was no difference in the growth curves of *E. coli* in the presence of BMAA when compared to leucine (Fig. 6).

BMAA and leucine do not bind to E. coli proteins passively

Both live and lysed *E. coli* were incubated with ^{3}H BMAA and ^{3}H leucine for 24 h. There was a significant ($P < 0.0001$) increase in the presence of protein bound amino acid in the live cell incubations when compared to the lysates. There was also a significant ($P < 0.0001$) difference in protein-bound amino acid present in live treated cells after 24 h when compared to the live 0-h control (Fig. 7). 24-h incubation of E. coli with labelled amino acid resulted in 0.8% of supplied BMAA, and 0.3% of supplied leucine remaining bound to protein. E. coli lysate treated for 24 h resulted in 0.05% of supplied BMAA and 0.02% of supplied leucine

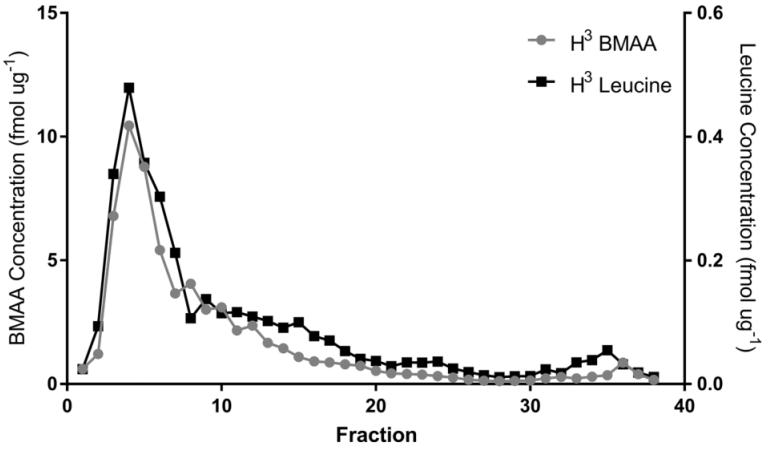

Fig. 5 Bound BMAA and leucine are distributed similarly in SH-SY5Y cell proteins. SH-SY5Y human neuroblastoma cells were incubated with ^{3}H BMAA and ^{3}H leucine for 24-h. Protein was isolated using molecular weight cut off filters before being fractionated using size exclusion FPLC. Bound amino acid was quantified using liquid scintillation counting. (Average of 3 treatments)

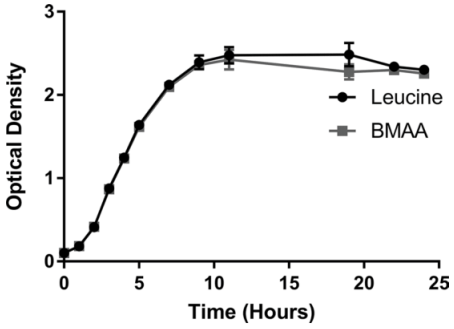

Fig. 6 Low concentrations of BMAA and leucine do not prevent the growth of *E. coli*. *E. coli* was grown in the presence of ^{3}H BMAA or ^{3}H leucine for 24-h, optical density was measured at regular intervals to determine growth

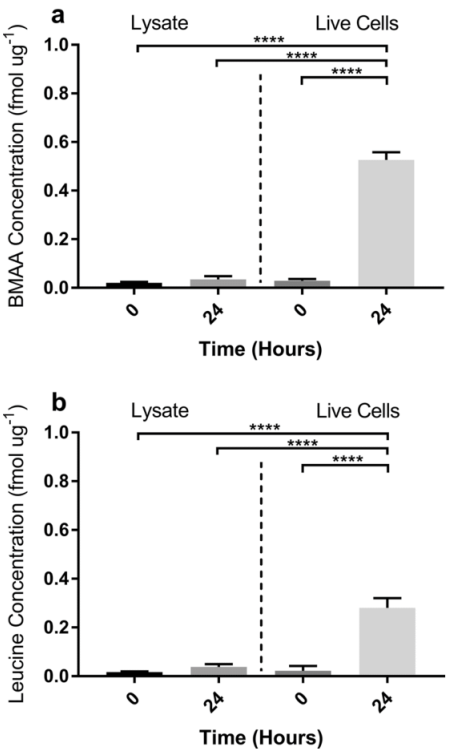

Fig. 7 BMAA and leucine do not bind to *E. coli* proteins passively. *E. coli* and *E. coli* lysate were incubated with ^{3}H BMAA (**a**) and ^{3}H leucine (**b**) for 0- and 24-h. Protein was isolated using size exclusion and bound amino acid quantified using liquid scintillation counting. ****$P < 0.0001$ ($n = 3$)

binding to protein. 0-h controls resulted in 0.04% of BMAA and 0.1% of leucine binding in live cells; and 0.04% of BMAA and 0.02% of leucine binding in cell lysates.

Protein-bound BMAA rapidly increases in live E. coli, then decreases, this does not occur in E. coli lysate

Both live and lysed *E. coli* were incubated with ^{3}H BMAA for 0, 1, 2, 3, 6, and 9 h, and the presence of protein-bound BMAA was determined by LSC. In live *E. coli* there was a rapid increase in protein-bound BMAA within the first hour, after which counts decreased before plateauing between 3 and 9 h. There was a significant difference in the concentration of bound BMAA when compared to the lysate control after 1 h (Fig. 8). (T1 $P < 0.0001$; T2 $P < 0.0001$; T3 $P < 0.0001$; T4 $P < 0.01$; T9 $P < 0.01$). 5.1% of supplied BMAA was bound to protein following a 1-h treatment of

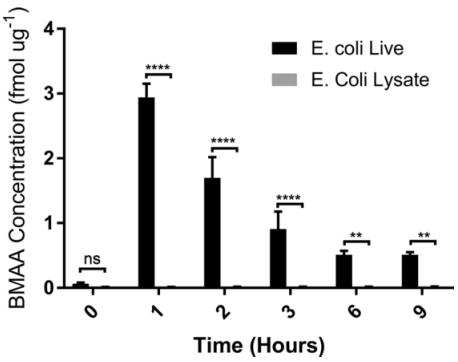

Fig. 8 Protein bound BMAA rapidly increases in live *E. coli*, then decreases, this does not occur in *E. coli* lysate. *E. coli* cultures were incubated with ^{3}H BMAA for up to 9-h, protein was isolated using TCA precipitation and the presence of bound BMAA quantified by liquid scintillation counting. $^{**}P < 0.01$; $^{****}P < 0.0001$ ($n = 3$)

live cells, this decreased to 1.4% after 9 h. BMAA binding was 0.1% across all time points in the treated E. coli lysate.

BMAA does not appear in fractionated E. coli proteins after either 1 or 6 h of treatment

E. coli cultures were treated with ^{3}H BMAA and ^{3}H leucine for 1, or 6 h. Proteins were isolated and fractionated using FPLC, and the presence of radiolabelled amino acid determined with LSC. There was no difference in protein-bound BMAA concentration between the different time points. There was an increase in protein-associated leucine after 6 h of treatment when compared to the 0 and 1 h treatments (Fig. 9).

Discussion

There is now a clear body of evidence to suggest that BMAA interacts with proteins, however, the mechanism of this interaction, and the role this interaction plays in the observed toxicity of BMAA remains a topic of debate. A number of NPAAs have been shown to be capable of misincorporation into proteins during synthesis, often resulting in the development of pathology (Rodgers and Shiozawa 2008; Rodgers et al. 2015). Protein-associated BMAA in cultured cells has previously been shown to increase in proportion to the concentration of BMAA in the culture media; it has also been demonstrated that interruption of peptide bonds through hydrolysis or pronase digestion is required to liberate protein-associated BMAA (Dunlop et al. 2013).

Radiolabel studies using tritiated amino acids have previously been criticised for the possibility of hydrogen exchange falsely skewing the results. Here we show both ^{3}H and ^{14}C BMAA bound to proteins with increasing concentration over time (Fig. 2) indicating that observed increases in bound ^{3}H BMAA was not due to exchange The ^{3}H BMAA used in these experiments features a tritiated methyl side chain, preventing possible label loss through hydrogen exchange or peptide bonding. We also observed no significant difference to the binding of BMAA or leucine to a range of proteins in both native and denaturing conditions (Fig. 1a, b), suggesting that BMAA is incapable of binding to proteins under physiological conditions and protein structure does not affect the binding of BMAA The use of labelled amino acids and liquid scintillation counting opposed to tandem mass spectrometry allows detection of extremely low concentrations of bound amino acid with minimal sample processing. Similar experiments using LC–MS/MS detection require protein hydrolysis and amino acid derivatisation to detect the presence of protein associated BMAA (Downing et al. 2012).

We then directly compared the binding of both ^{3}H BMAA and ^{3}H leucine to mammalian cell proteins in live SH-SY5Y human neuroblastoma cells, and to a SH-SY5Y protein lysate (Fig. 3a, b). Both Leucine and BMAA showed no significant binding to proteins in lysates after 24 h supporting the data using purified proteins. Significant protein binding was, however, observed in live cells after 24 h for both BMAA ($P < 0.0001$) and leucine ($P < 0.0001$) indicating that both amino acids require functional cells for BMAA to be bound to protein, and are unable to form spontaneous bonds to proteins within lysate.

Levels of protein-bound BMAA were significantly higher ($P < 0.001$) in the treated neuroblastoma cells after only 6 h when compared to the lysate (Fig. 4). There was also a clear linear increase ($m = 0.0915$ fmol/h) in protein-bound BMAA over time in live cells, which was not observed in the lysate. This time-dependant linear increase in protein-bound BMAA has previously been observed by Dunlop et al. (2013), and is a hallmark of incorporation of amino acids into proteins in cultured cells where extra and intracellular amino acid concentration rapidly equilibrate (Wheatley 1982).

The distribution of radiolabel in the proteins in the cell lysate following 24-h incubation was investigated using size exclusion FPLC. A Superdex 75 column with an exclusion range between approximately 3 kDa and 70 kDa was used for these experiments, with larger proteins eluting in earlier fractions. Here we observed that the highest concentration of ^{3}H BMAA was present in larger proteins (Fig. 5a). This distribution profile was identical to that observed for ^{3}H leucine (Fig. 5b) suggesting both amino acids shares a similar distribution in neuroblastoma proteins and could be characteristic of translational incorporation into proteins.

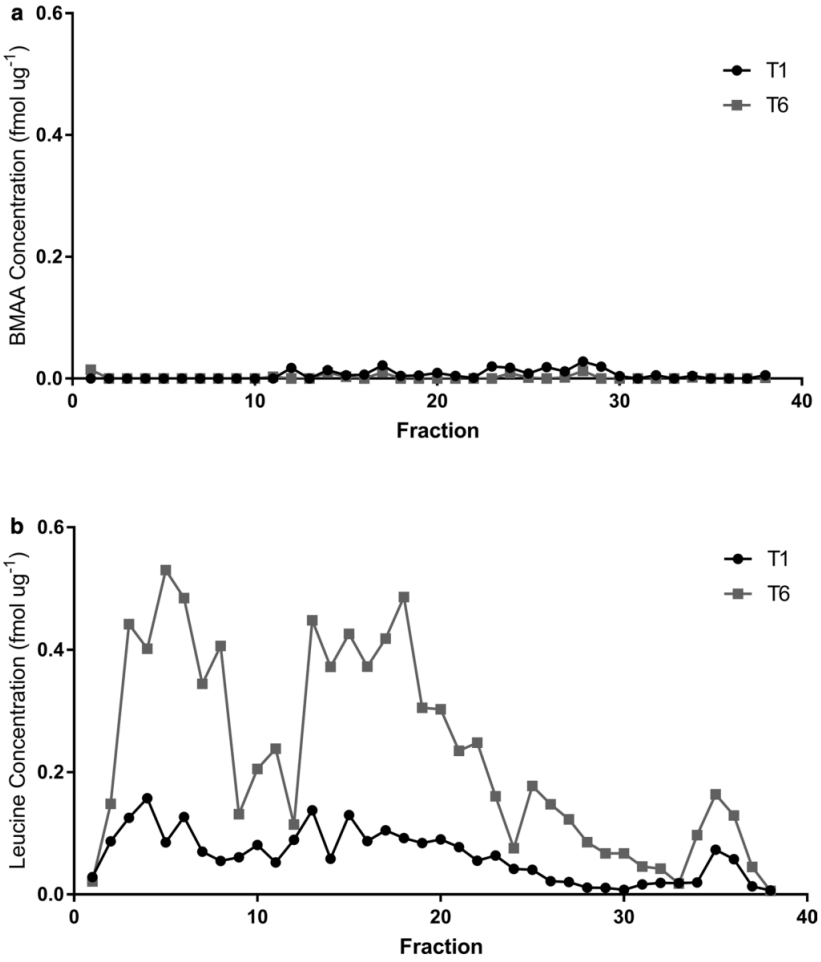

Fig. 9 BMAA does not appear in fractionated E. coli proteins after either 1 or 6 h of treatment. *E. coli* cultures were incubated with ^{3}H BMAA (**a**) and ^{3}H leucine (**b**) for either 1 or 6-h. Protein was isolated using molecular weight cut off filters before being fractionated using size exclusion FPLC. Bound amino acid was quantified using liquid scintillation counting. (Average of 3 treatments)

A number of studies have explored the effect of BMAA on prokaryotes, both Downing et al. (2012) and Berntzon et al. (2013) reported inhibition of growth in cyanobacterial strains supplied with exogenous BMAA. Downing observed light and concentration dependant chlorosis in treated *Synechocystis* PCC6803 consistent with macronutrient depletion; while Berntzon concluded that the observed growth decreases in *Nostoc* sp. PCC 7120 may be due to the formation of reactive oxygen species inhibiting nitrogen fixation. Interestingly, while exogenous BMAA appears to affect the growth of cyanobacteria it does not appear to affect the growth of other prokaryotes (van Onselen et al. 2015). *Escherichia coli*, *Staphylococcus epidermis*, *Lactobacillus casei*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Micrococcus luteus* showed no decrease in growth following BMAA treatment, and no BMAA was detected in proteins from treated bacteria using LC–MS/MS.

At the levels of BMAA and Leucine used our data were consistent with van Onselen et al. and we observed no difference in *E. coli* growth for the ^{3}H BMAA or ^{3}H leucine treated groups (Fig. 6). We did, however, observe a significant increase in protein bound ^{3}H BMAA after a 24-h incubation ($P < 0.0001$) with live *E. coli* when compared to a 0-h control. No binding was seen in *E. coli* protein lysate after the same incubation period (Fig. 7a). We observed the same pattern in cells treated with radiolabelled leucine, with significant binding in live *E. coli* after 24 h ($P < 0.0001$), but no binding in the lysate (Fig. 7b). These results are the same as those observed in the treated neuroblastoma, once again suggesting that *E. coli* proteins are unable to spontaneously bind BMAA.

Protein-bound BMAA in *E. coli* did not increase linearly over time. The highest concentration of bound BMAA in live *E. coli* was observed after only 1 h of exposure, following

which the bound BMAA decreased and plateaued from 6 to 3 h (Fig. 8). This rapid binding of BMAA to protein has previously been observed by Downing et al. in *Synechocystis* PCC6803 where the highest concentration of bound BMAA was detected only 1.2 min post-treatment (Downing et al. 2012). Our findings are not consistent with the uptake and incorporation of physiological amino acids in *E. coli* where a linear increase is typically observed (Britten and McClure 1962).

Size exclusion FPLC was once again employed to determine the distribution of BMAA across treated *E. coli* proteins (Fig. 9a), interestingly no significant difference in binding was seen in any of the fractions in either the 1 or 6-h time points. Total BMAA concentration was lower than expected in both these time points based off the high concentration of protein associated BMAA that was observed in the 1-h time point of the previously conducted time course (Fig. 8). While 3 kDa MWCO filters were used for the FPLC experiments, TCA precipitation of proteins was employed both in the time course section of this study, and the study by Downing et al. where large concentrations of protein bound BMAA were observed in early treatments. This suggests that BMAA may be rapidly bound to a TCA-insoluble form that is less than 3 kDa, a clear difference to neuroblastoma cells where BMAA was primarily bound to larger proteins. Increased bound ^{3}H leucine was observed across most of the collected fractions in the 6-h treatment group when compared to the 0 and 1-h treatment groups (Fig. 9b).

Binding experiments using a range of proteins in both native and denaturing conditions show that BMAA does not spontaneously bind to proteins. The linear increase of bound BMAA in eukaryotic cells is indicative of incorporation, this is supported by the similarity in the distribution of bound BMAA in neuroblastoma proteins to the distribution of bound leucine. This is in stark contrast to BMAA in *E. coli* which appears to be rapidly bound into small, TCA-insoluble complexes before being cleared by the bacteria, with only a low concentration of bound BMAA remaining after 6-h.

This study adds to the strong body of evidence that BMAA is capable of being incorporated into proteins in eukaryotic cells, a potential mechanism of toxicity. Our studies would support the view that this occurs at a low level and might not be detected by analytical techniques that require extensive sample processing. It also highlights significant differences in BMAA-protein association between prokaryotic and eukaryotic organisms. Further investigation of the mechanisms underlying these differences may give us better insight into BMAA toxicity. The differences observed between bound BMAA in *E. coli* and neuroblastoma cells also indicates the importance of using eukaryotic cells, or eukaryotic based cell free expression systems to investigate the role of NPAA misincorporation in human disease.

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Author contributions All experiments were conducted or supervised by BM, CI and KR; manuscript text was written by BM and KR.

Compliance with ethical standards

Conflict of interest The authors declare they have no conflicts of interest.

Ethical statement This manuscript represents original research, and has not been published in part or in whole elsewhere. This publication is not currently being considered for publication elsewhere. All authors have actively been involved in work leading to this manuscript, and have read and agreed with its publication. This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter Four

Assessing the combined toxicity of BMAA and its isomers 2,4-DAB and AEG *in vitro* using human neuroblastoma cells

Chapter Four: Assessing the combined toxicity of BMAA and its isomers 2,4-DAB and AEG *in vitro* using human neuroblastoma cells.

Chapter Overview

Early toxicological assessments of BMAA largely focused on the excitotoxicity of BMAA and its β-carbamate derivative on various glutamate receptors. While this mechanism explains the acute toxicity seen in high doses of BMAA, it does not explain the long latency seen between exposure to lower concentrations of BMAA and onset of neurodegenerative symptoms seen in patients from Guam. As discussed in the previous chapter, the binding of BMAA to proteins in eukaryotes is consistent with incorporation during protein synthesis. The misincorporation of BMAA during protein synthesis raises the possibility of an alternative form of toxicity, mediated by proteotoxic stress.

To investigate the possibility of BMAA induced proteotoxic stress, cultured neuroblastoma cells were treated with BMAA as well as AEG and 2,4-DAB. BMAA treated cells had increased expression of a number of genes associated with ER-stress and ER associated protein degradation, consistent with cells under proteotoxic stress. While AEG and 2,4-DAB treatment did not result in ER stress, 2,4-DAB alone did reduce cell viability, and combined treatments of 2,4-DAB and BMAA resulted in increased expression of the pro-apoptotic protein caspase-3. The results from this chapter suggest that BMAA could cause proteotoxic stress, and that combined exposure to BMAA and 2,4-DAB may have a synergistic or additive toxicity.

Certificate of authorship and originality

This paper was published in *Neurotoxicity Research* © Springer Ltd. I certify that the work presented in this chapter has not previously been submitted as part of the requirements for a degree. I also certify that I carried out the majority of the work presented in this paper.

- Brendan J Main: Conducted experiments and wrote the manuscript.
- Kenneth J. Rodgers: Proof-read and edited the manuscript, conceived project idea, and provided guidance on experiments.

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ORIGINAL ARTICLE

Assessing the Combined Toxicity of BMAA and Its Isomers 2,4-DAB and AEG In Vitro Using Human Neuroblastoma Cells

Brendan J. Main[1] · **Kenneth J. Rodgers**[1]

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Abstract The non-protein amino acid (NPAA) ß-methylamino-L-alanine (BMAA) is produced by a diverse range of cyanobacteria, diatoms and dinoflagellates, and is present in both aquatic and terrestrial ecosystems globally. Exposure to BMAA has been implicated in the development of neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) and Parkinson's disease (PD). BMAA is often found in nature along with its structural isomers 2,4-diaminobutyric acid (2,4-DAB) and aminoethylglycine (AEG); however, the toxicity of these NPAAs in combination has not been examined. We have previously demonstrated that BMAA induces endoplasmic reticulum (ER) stress and increases caspase and cathepsin activity in human neuroblastoma cells (SH-SY5Y), effects consistent with proteotoxic stress due to disturbances in protein synthesis, folding or turnover. The current study investigates whether 2,4-DAB and AEG share a similar mechanism of toxicity to BMAA, and if simultaneous exposure of cells to BMAA and its isomers results in increased toxicity in vitro. We show that a 48-h treatment with both 500 μM BMAA and 2,4-DAB decreases cell viability in vitro whereas AEG was not cytotoxic under the same conditions. Treatment of SH-SY5Y cells with 2,4-DAB did not increase expression of ER stress markers. Combined treatment of cells with BMAA and 2,4-DAB resulted in increased caspase activity and increased apoptosis above that of BMAA or 2,4-DAB on their own. These results suggest that 2,4-DAB does not share the same mechanism of toxicity as BMAA but the presence of 2,4-DAB increases the toxicity of BMAA to human cells in vitro.

Keywords BMAA · ALS · MND · 2,4-DAB · Non-protein amino acids · Unfolded protein response

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Introduction

A strong link has been drawn between exposure to the non-protein amino acid (NPAA) β-methylamino-L-alanine (BMAA) and the onset of neurodegenerative disorders including amyotrophic lateral sclerosis (ALS) (Murch et al. 2004), Alzheimer's disease (AD) (Cox et al. 2016) and ALS–Parkinson's dementia complex (ALS-PDC) (Banack and Murch 2009). BMAA is known to cause excitotoxicity in primary human neurons, as well as primary rat glial cells (Chiu et al. 2012; Chiu et al. 2013). BMAA has also been shown to cause differential expression of genes associated with mitochondrial function, apoptosis, protein aggregation and cell growth in primary rat glial cells (Chiu et al. 2015). Interestingly, evidence suggests that BMAA is capable of being incorporated into proteins in place of L-serine during protein synthesis, resulting in the formation of misfolded and aggregated proteins within cells leading to apoptosis (Dunlop et al. 2013). The apoptotic effect of 48–72 h exposure to 500 μM BMAA can be prevented in vitro through co-incubation with 50 μM L-serine, further supporting the hypothesis that BMAA is misincorporated into proteins in place of L-serine, resulting in the formation of protein aggregates and activation of the endoplasmic reticulum unfolded protein response (UPR) (Main et al. 2015).

BMAA has been shown to be produced by a wide variety of cyanobacteria (Cox et al. 2005), as well as diatoms (Jiang et al. 2014) and dinoflagellates (Lage et al. 2014), and has

been found globally (Cox et al. 2003; Cox et al. 2009; Jonasson et al. 2010; Masseret et al. 2013; Banack et al. 2015). Interestingly, the positive identification of BMAA is often accompanied with the detection of both 2,4-diaminobutyric acid (2,4-DAB) and aminoethylglycine (AEG)—isomers of BMAA (Craighead et al. 2009; Faassen et al. 2009; Jiang et al. 2012). Further to being detected in the same samples as BMAA, 2,4-DAB is of particular interest as it is also found in a number of legumes (Pilbeam and Bell 1979) and some bacterial cell walls (Schleifer and Kandler 1972). 2,4-DAB has been shown to accumulate within the liver possibly resulting in secondary neurotoxicity due to an increase in ammonia concentration within the body (O'Neal et al. 1968). The co-detection of these NPAAs in environmental samples highlights the importance of investigating the neurotoxicity of BMAA in the context of the other cyanobacterial toxins.

We have previously shown that exposure to BMAA causes increases in lysosomal protease activity and caspase-3 activity, as well as upregulation of genes responsible for the control of the UPR and ER stress in SH-SY5Y neuroblastoma cells (Main et al. 2015). BMAA has also been shown to reduce SH-SY5Y cell viability in low concentrations (Okle et al. 2012). This study aims to investigate if previously observed BMAA toxicity is altered in the presence of 2,4-DAB and AEG, and whether these isomers share similar mechanisms of toxicity in vitro.

Experimental

Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and Eagle's Minimal Essential Medium (EMEM, deficient in serine), TRI reagent, L-BMAA hydrochloride, and caspase-3 reagent Ac-Asp-Glu-Val-Asp-AMC were from Sigma Chemical Co., MO, USA. Z-Arg-Arg-AMC (where Z is benzyloxycarbonyl) and Z-Phe-Arg-AMC were from Bachem AG, Bubendorf, Switzerland. L-2,4-DAB and L-AEG were purchased from Toronto Research Chemicals, ON, Canada. Alamar Blue (resazurin) was purchased from Invitrogen, CA, USA. iScript cDNA synthesis kit and iTaq Universal SYBR Green Supermix were purchased from Bio-Rad, CA, USA.

All aqueous solutions and buffers were prepared using 18 mΩ water. All other chemicals, solvents and chromatographic materials were of analytical reagent or cell culture grade.

Cell Culture

SH-SY5Y human neuroblastoma cells (American Tissue Culture Collection, catalogue number CRL-2266) were cultured as follows: DMEM was supplemented with 10% heat-inactivated foetal bovine serum (FBS), and 2 mM L-glutamine. Cells were maintained at 37 °C with 5% CO_2 in 175-cm^2 flasks until they were plated in 6- and 96-well plates for experiments. During treatment, DMEM culture medium was substituted with serine-free EMEM. Consistent with our previous studies (Dunlop et al. 2013; Main et al. 2015), cell treatments were untreated control, 500 μM BMAA, 500 μM DAB, 500 μM AEG, 500 μM DAB + 500 μM BMAA and 500 μM AEG + 500 μM BMAA in triplicate wells for all experiments. Dose–response curves were conducted using the following concentrations of NPAA for 48 h: untreated, 125, 250, 500, 1000 and 2000 μM.

Cell Viability

SH-SY5Y cells were treated in 96-well plates in 200 μL of media at an approximate density of 2×10^4 cells per well. Following treatment, 20 μL of Alamar Blue reagent was added to each well, and the cells were incubated at 37 °C, 5% CO_2, for 2 h protected from light. After 2 h, cell viability was measured with a Tecan M1000 PRO plate reader by recording both absorbance at 570 nm (with 600 nm reference absorbance) and fluorescence (λex/λem 570 nm/585 nm). Cell viability was expressed as a percentage of control.

RT qPCR

SH-SY5Y cells were treated in triplicate wells of a six-well plate for 24 h; total RNA was then isolated using TRI reagent according to the manufacturer's instructions. Two hundred nanograms of RNA was reverse transcribed in triplicate using an iScript cDNA synthesis kit and then pooled to account for inter-reaction variability. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was conducted in triplicate using the iTaq Universal SYBR Green Supermix, according to the manufacturer's instructions. Predesigned primers were sourced from Sigma (Sigma Chemical Co., St. Louis, MO). Results were quantified using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001) with RAB7A and VCP as housekeeping genes (determined as most stable from a list of common housekeeping genes using NormFinder (Andersen et al. 2004)).

Cathepsin Activity

SH-SY5Y cells were sub-cultured onto six-well plates, allowed to adhere overnight and then treated for 48 h in triplicate. Cultures were washed once in phosphate-buffered saline (PBS) and harvested by scraping into 50 μL of cathepsin assay buffer (5 mM EDTA, 0.005% Brij-35, 0.01% Triton X-100, 0.001 mM pepstatin A, 2.5 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride, 5 mM benzamidine—pH 6 for CatB, pH 5.5 for CatL) and probe-sonicated three

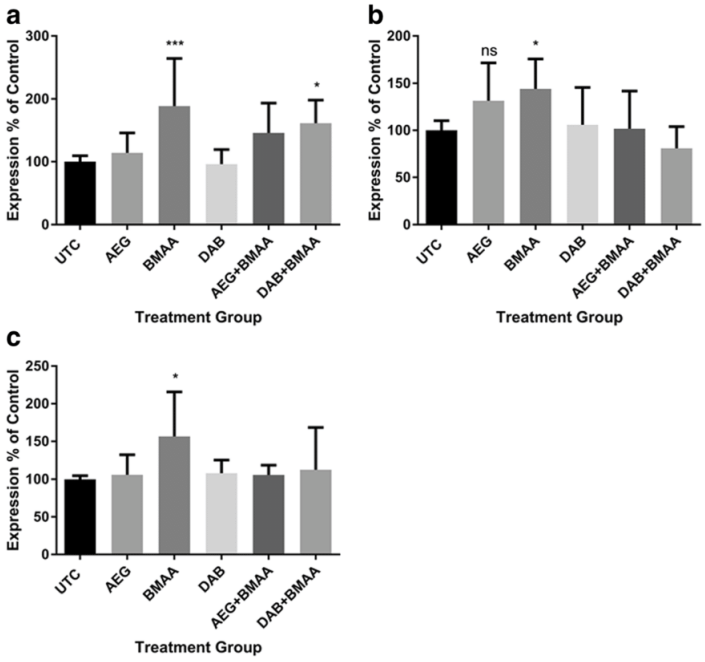

Fig. 5 Gene expression of CHOP (**a**), EDEM1 (**b**) and HERPUD1 (**c**) following 6 h treatment of SH-SY5Y cells with 500 μM NPAAs, measured using RT-qPCR and normalised against the geometric mean of housekeeping genes RAB7A and VCP using the $2^{-(\Delta\Delta Ct)}$ method. Statistical analysis using one-way ANOVA. $^{*}P < 0.05$; $^{***}P < 0.001$; NS = not significant ($n = 3$)

exposed to a combination of BMAA and 2,4-DAB after 48 h (Fig. 3). Caspase-3 is a critical component in the apoptosis cascade, activating a number of downstream pro-apoptotic factors, and indicating the beginning of irreversible apoptosis in cells (Cullen and Martin 2009). Caspase-3 has also been linked to cell death observed in ALS rodent models, with inhibition of caspase activity resulting in delayed disease onset and reduced mortality (Li et al. 2000).

We have previously demonstrated that BMAA interacts with proteins, potentially through misincorporation during synthesis, causing proteins to misfold and aggregate (Dunlop et al. 2013). The formation of misfolded and aggregated proteins from BMAA exposure can result in ER stress-mediated apoptosis (Okle et al. 2012). ER stress occurs when a build-up of misfolded and aggregated proteins is present within the lumen of the ER; in response to this stress, the UPR pathway is mobilised to reduce protein synthesis, promote protein folding and ultimately upregulate apoptosis (Rutkowski and Kaufman 2004). In this investigation, we used the UPR-related gene, C/EBP homologous protein (CHOP) and the ER-associated degradation (ERAD)-related genes: ER degradation-enhancing alpha-mannosidase-like protein 1 (EDEM1) and homocysteine-inducible ER protein with ubiquitin-like domain 1 (HERPUD1) as indicators of ER stress and activation of the UPR and ERAD. CHOP plays a key role in prolonged ER stress by mediating apoptosis through downregulation of anti-apoptotic Bcl-2 and upregulation of pro-apoptotic BiM, BAX, PUMA and DR5 (Yamaguchi and Wang 2004; Nishitoh 2012). EDEM1 is believed to play multiple roles in regulation of misfolded and aggregated protein levels within the ER, first by inhibiting aggregate formation through early identification and isolation of misfolded glycoproteins, and secondly through retro-translocation of these glycoproteins out of the ER lumen for degradation by the ubiquitin proteasome system (Olivari and Molinari 2007). The HERPUD1 protein is upregulated by ER stress and has been shown to play a role in reducing the misfolded protein burden on the ER by shuttling proteins to the 26S proteosome for degradation (Huang et al. 2014; Ho and Chan 2015). We have previously shown that CHOP is upregulated in SH-SY5Y cells, a common human neuron model that is not thought to express functional glutamate receptors unless differentiated (Kritis et al. 2015), following 48 h exposure to BMAA (Main et al. 2015). In the present study, we used the geometric mean of RAB7A and VCP, two housekeeping genes known to be stable, allowing more precise measurement of gene expression at earlier time points (Vandesompele et al. 2002).

Significant increases in gene expression for all three of the observed ER stress proteins were seen as early as 6 h after BMAA treatment, suggesting that BMAA exposure may cause a rapid increase of misfolded proteins within the lumen of the ER (Fig. 5). Gene expression in BMAA-treated cells was still elevated after 24 h (Fig. 6a–c); however, we also observed elevated expression of CHOP in cells treated with 2,4-DAB; CHOP and HERPUD1 in cells co-treated with AEG and

Combined NPAA Treatment Did Not Significantly Decrease Cell Viability When Compared to Single Treatment

Cell viability was measured by quantifying the reduction of resazurin dye to resorufin through both fluorescence (Fig. 2a) and absorbance (Fig. 2b). There was a significant reduction ($P < 0.0001$) in viability in all but the AEG-treated cells when compared to control untreated cells. There was no significant difference in viability between cells treated with single NPAAs and cells treated with a combination of two NPAAs.

48 h Combined Treatment with BMAA + 2,4-DAB Increased the Activity of Caspase-3

Activity of caspase-3 was measured through the cleavage of a fluorescent peptide substrate; there was a significant increase in the activity of caspase-3 in cells treated with 500 μM BMAA + 500 μM 2,4-DAB ($P < 0.05$) when compared to control cells (Fig. 3).

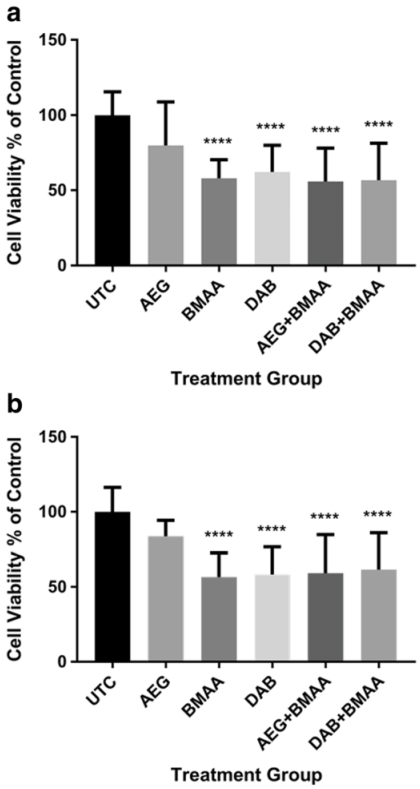

Fig. 2 Viability of SH-SY5Y cells following 48 h treatment with 500 μM NPAAs, measured using an Alamar Blue assay with both fluorescence (**a**) and absorbance (**b**) readings recorded. Statistical analysis using one-way ANOVA. ****$P < 0.0001$ ($n = 3$)

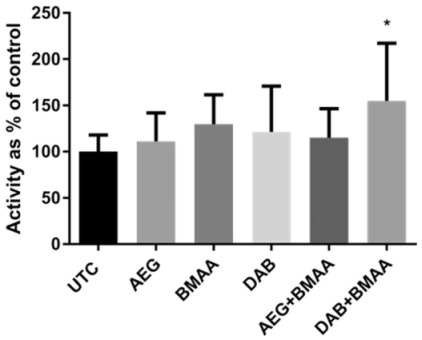

Fig. 3 Caspase-3 activity following 48 h treatment of SH-SY5Y cells with 500 μM NPAAs, measured through the cleavage of a fluorescent substrate peptide. Statistical analysis using one-way ANOVA. *$P < 0.05$ ($n = 3$)

72 h Combined Treatment with BMAA + 2,4-DAB, and BMAA + AEG Increased the Activity of Lysosomal Proteases Cathepsin B and Cathepsin L

Cathepsin B and L activity was measured through the cleavage of a fluorescent substrate peptide; cathepsin B activity was increased in cells treated with 500 μM BMAA + 500 μM 2,4-DAB ($P < 0.01$) for 72 h (Fig. 4a). Cathepsin L activity was also increased in cells treated with 500 μM BMAA + 500 μM 2,4-DAB ($P < 0.01$) when compared to control cells (Fig. 4b).

BMAA Treatment Increased Expression of CHOP, EDEM1 and HERPUD1, Markers of ER Stress and ER-Assisted Degradation After 6 h

SH-SY5Y cells treated with 500 μM BMAA for 6 h had a significant increase in the expression of C/EBP homologous protein (CHOP) ($P < 0.001$) (Fig. 5a), ER degradation-enhancing alpha-mannosidase-like protein 1 (EDEM1) ($P < 0.05$) (Fig. 5b) and homocysteine-inducible ER protein with ubiquitin-like domain 1 (HERPUD1) ($P < 0.05$) (Fig. 5c) when compared to untreated control cells. Treatment with 500 μM BMAA + 500 μM 2,4-DAB also significantly increased expression of CHOP when compared to control cells ($P < 0.05$) (Fig. 5a).

NPAA Treatment Increased Expression of CHOP, EDEM1 and HERPUD1, Markers of ER Stress and ER-Assisted Degradation as Well as Expression of COX6B1 and GPT2 After 24 h

Expression of CHOP was significantly increased in SH-SY5Y cells treated for 24 h with 500 μM BMAA ($P < 0.01$), 500 μM BMAA + 500 μM AEG ($P < 0.001$) and 500 μM BMAA + 500 μM 2,4-DAB ($P < 0.0001$) when compared to untreated

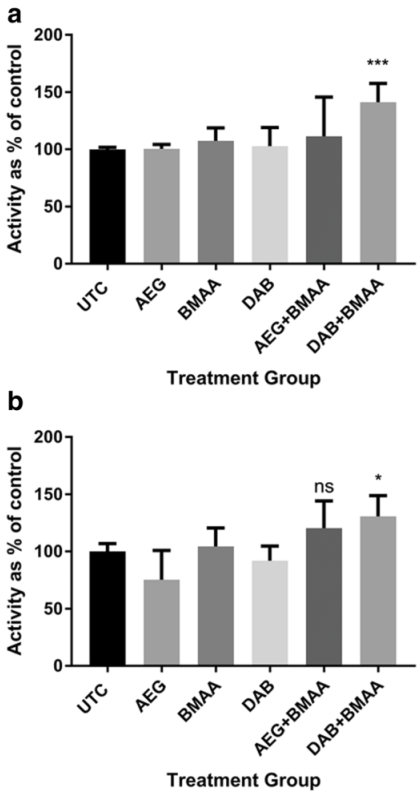

Fig. 4 Cathepsin B (**a**) and cathepsin L (**b**) activity following 72 h treatment of SH-SY5Y cells with 500 μM NPAAs, measured through the cleavage of a fluorescent substrate peptide. Statistical analysis using one-way ANOVA. *$P < 0.05$; ***$P < 0.001$; NS = not significant ($n = 3$)

control cells (Fig. 6a). EDEM1 expression was upregulated in cells treated with 500 μM BMAA ($P < 0.05$), as well as cells treated with 500 μM BMAA + 500 μM 2,4-DAB ($P < 0.05$) (Fig. 6b). HERPUD1 expression was also increased in cells incubated with 500 μM BMAA ($P < 0.01$), 500 μM BMAA + 500 μM AEG ($P < 0.001$) and 500 μM BMAA + 500 μM 2,4-DAB ($P < 0.01$) (Fig. 6c). Expression of the mitochondrial oxidase gene, cytochrome C oxidase subunit VIb1 (COX6B1), was significantly increased in cells treated with 500 μM BMAA + 500 μM 2,4-DAB ($P < 0.01$) (Fig. 6d). The mitochondrial transaminase, glutamic pyruvic transaminase 2 (GPT2), was also increased in cells treated with 500 μM BMAA + 500 μM 2,4-DAB ($P < 0.01$) (Fig. 6e).

Discussion

There is a growing body of evidence to suggest that exposure to cyanobacterial toxins could be a risk factor in the development of neurodegenerative disease (Bradley et al. 2013). While BMAA has been singled out as a likely causative agent for this increased risk, cyanobacteria produce a wide range of toxins including microcystin, anatoxin-a and saxitoxin (Merel et al. 2013) as well as other NPAAs. The NPAA toxin 2,4-DAB is of particular interest; it has previously been shown to be neurotoxic in rats (Chen et al. 1972) and is present in the peptidoglycan cell walls of a number of bacteria (Sasaki et al. 1998), potentially increasing the chances of human exposure. BMAA, DAB and AEG have also been reported in specimens from Lake Mascoma, a lake with a high incidence of ALS in individuals residing in the direct vicinity of the lake highlighting the need for research aimed at investigating the interactions between these amino acid toxins which often co-occur in the same environment (Banack et al. 2015).

Numerous animal studies have shown that BMAA has a pronounced neurotoxic effect. Early experiments on chicks and rodents found that intraperitoneal administration of BMAA resulted in inability to stand, sluggishness and altered gait (Vega and Bell 1967; Polsky et al. 1972). These were followed up by a landmark primate study by Spencer et al. using oral administration of BMAA to macaques; here, Spencer found that BMAA caused significant neurological dysfunction consistent with neurodegenerative diseases, as well as degeneration of motor neurons in the cerebral cortex and spinal cord (Spencer et al. 1987a; Spencer et al. 1987b). Recent work by Cox et al. showed dietary administration of BMAA-produced neurofibrillary tangles (NFT) and β-amyloid plaques, hallmark structures of neurodegenerative disease, in the brains of vervets (Cox et al. 2016). To date, these studies have explored the toxicity of BMAA in isolation, without looking at the possible interaction of BMAA with other cyanotoxins.

In the present in vitro studies, both 500 μM BMAA and 2,4-DAB treatment resulted in a 40% reduction of viability in neuroblastoma cells after a 48-h treatment (Fig. 2), suggesting both NPAAs are toxic to human cells; this toxicity was also shown to be dose-dependant (Fig. 1a–c). AEG toxicity was only observed at concentrations above 1 mM (Fig. 1a). It has previously been thought that 2,4-DAB may cause neurodegeneration indirectly through liver damage, resulting in a build-up of toxic ammonia within the brain (O'Neal et al. 1968). Further research in rodents demonstrated that 2,4-DAB needed to be present within the brains of rodents for there to be any observable neurological changes, indicating that there is a second mechanism for 2,4-DAB neurotoxicity, possibly due to interaction with GABA receptors (Chen et al. 1972; Johnston and Twitchin 1977). Our data supports the theory that 2,4-DAB is directly toxic on neuronal cells, a finding which should be explored further. While there was no significant difference in viability between cells treated with BMAA alone or a combination of BMAA and AEG, or BMAA and 2,4-DAB using the Alamar Blue assay, we did observe a significant increase in caspase-3 activity in cells

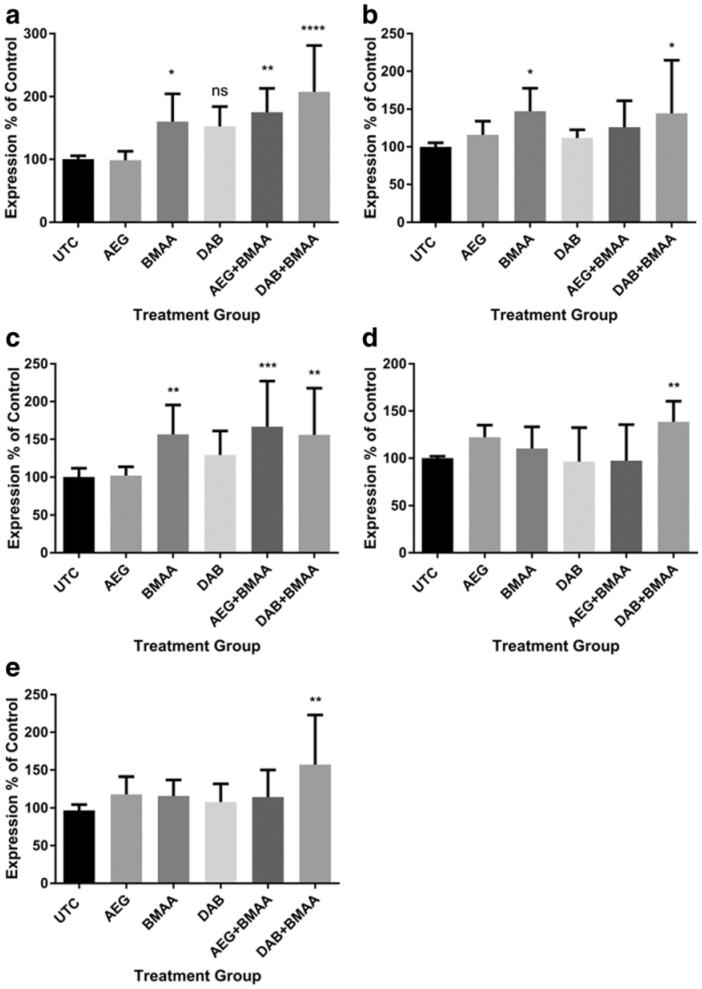

Fig. 6 Gene expression of CHOP (**a**), EDEM1 (**b**), HERPUD1 (**c**), COX6B1 (**d**), GPT2 (**e**) and PHGDH (**f**) following 24 h treatment of SH-SY5Y cells with 500 μM NPAAs, measured using RT-qPCR and normalised against the geometric mean of housekeeping genes RAB7A and VCP using the $2^{-(\Delta\Delta Ct)}$ method. Statistical analysis using one-way ANOVA. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; NS = not significant ($n = 3$)

BMAA; and CHOP, EDEM1 and HERPUD1 in cells co-treated with 2,4-DAB and BMAA. AEG and 2,4-DAB alone did not increase expression of ER stress-related genes, suggesting that in vitro exposure to either AEG or 2,4-DAB may not result in the formation of structurally aberrant proteins.

Mitochondrial function is intrinsically linked to apoptosis, and type II programmed cell death is characterised by a release of cytochrome C from the mitochondria resulting in the activation of the caspase cascade. In the present study, we quantified the expression of two genes related to mitochondrial function, cytochrome C oxidase subunit VIb1 (COX6B1) and glutamic–pyruvic transaminase 2 (GPT2). COX6B1 is a cytochrome C oxidase subunit that is encoded by nuclear DNA and exported to the mitochondria; its upregulation has been linked to increased oxidative stress and has been proposed as a regulator of mitochondrial respiration (Kim et al. 2015). Upregulation of cytochrome c and other COX subunits has also been associated with apoptosis in vitro (Sánchez-Alcázar et al. 2000). GPT2, also known as alanine aminotransferase 2 (ALT2), is a mitochondrial enzyme that catalyses the synthesis of alanine from pyruvate and glutamate and appears to play a role in brain development (Ouyang et al. 2016). GPT2 is upregulated by transcription factor ATF4, a key member of the UPR, during cell stress (Salgado et al. 2014). Expression of COX6B1 (Fig. 6d) and GPT2 (Fig. 6e) was significantly increased in cells co-treated with BMAA and 2,4-DAB possibly indicating an increase in cell stress in this treatment group. This is supported by the increase in caspase-3 activity also seen in the BMAA- and 2,4-DAB-co-treated cells.

Cathepsin B and L are lysosomal cysteine proteases that can be upregulated in response to the presence of protein aggregates within cells, and have previously been observed to be elevated in cells exposed to NPAAs (Dunlop et al. 2013; Main et al. 2015). The lysosome is tasked with the breakdown of large cellular structures and protein aggregates that do not fit within the proteasome, and plays a key role in managing aggregate-induced cell stress (Rashid et al. 2015). Abnormal cathepsin B activity in particular has been linked to ALS, with patients showing increased cathepsin B activity, possibly due to an increase of aggregated proteins within neurons (Kikuchi et al. 2003). Cathepsins have also been shown to play an important role in the early stages of apoptosis, and are believed to help facilitate the release of cytochrome C from the mitochondria (Chwieralski et al. 2006; Droga-Mazovec et al. 2008). After 72 h, we observed significant increases in cathepsin B (Fig. 4a) and L (Fig. 4b) activity in cells co-treated with BMAA and 2,4-DAB. These increases are consistent with the presence of aggregated proteins within the cells, and may also indicate apoptosis.

Our results show that AEG exposure alone had no significant effect on cell viability at 500 μM or lower, suggesting it is not cytotoxic at the same concentrations as BMAA or 2,4-DAB; this is supported by the dose response for AEG where viability was only reduced at the two highest concentrations (Fig. 1a). Co-treatment with AEG and BMAA did not cause any significant difference in cell viability in comparison to treatment with BMAA alone. 2,4-DAB has a similar level of toxicity to BMAA, reducing cell viability by approximately 40% as assessed using the Alamar Blue assay, which measures the activity of both mitochondrial and cytosolic enzymes (Gonzalez and Tarloff 2001). 2,4-DAB, however, does not appear to share the same mechanism of toxicity as BMAA in vitro. 2,4-DAB does not promote increased expression of UPR genes, suggesting that it does not cause an increase in misfolded or aggregated proteins within the cell. Co-treatment with 2,4-DAB and BMAA increased cytotoxicity relative to the toxins used alone resulting in increased mitochondrial stress, caspase-3 activity and cathepsin activity. The increases seen in cells co-treated with 2,4-DAB and BMAA suggest that while 2,4-DAB does not share the same mechanism of toxicity as BMAA, when combined they may have an additive effect.

The ubiquity of NPAAs within cyanobacterial samples means it is improbable that exposure to a single NPAA would occur in nature (Cox et al. 2005; Al-Sammak et al. 2014; Faassen et al. 2009). It appears more likely that exposure to BMAA would also be accompanied by exposure to at least 2,4-DAB and AEG, if not other cyanotoxins. Therefore, it is critical that any potential additive effect be taken into account in future studies investigating cyanotoxins such as BMAA and 2,4-DAB.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare they have no conflicts of interest.

Ethical Statement This manuscript represents original research, and has not been published in part or in whole elsewhere. This publication is not currently being considered for publication elsewhere. All authors have actively been involved in work leading to this manuscript, and have read and agreed with its publication. This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter Five

The use of L-serine to prevent β-methylamino-L-alanine (BMAA)-induced proteotoxic stress *in vitro*

Chapter Five: The use of L-serine to prevent β-methylamino-L-alanine (BMAA)-induced proteotoxic stress *in vitro.*

Chapter Overview

The data presented in chapter four demonstrated that BMAA can induce ER stress, well as other indicators of proteotoxic stress *in vitro*. This proteotoxic stress is consistent with what we would expect from protein aggregates formed from misincorporation, and lends strength to the hypothesis that misincorporation is an underlying mechanism of chronic BMAA toxicity. One of the features commonly observed in non-protein amino acid misincorporation is the ability to outcompete the non-protein amino acid with its canonical amino analogue. This competition mechanism provides a novel treatment possibility for BMAA toxicity through co-treatment with L-serine, which has been proposed to be a structural analogue to BMAA.

In this chapter, neuroblastoma cells were treated with BMAA or co-treated with both BMAA and L-serine. A number of markers of ER-stress, protein aggregation, and apoptosis were measured to determine if L-serine was able to prevent BMAA toxicity. Significant increases in all of the measured markers following BMAA treatment were observed; these effects were prevented when the cells were co-treated with L-serine at one tenth the concentration of BMAA. These results suggest that L-serine may be a novel treatment for BMAA induced toxicity and a potential intervention for neurodegenerative diseases.

Certificate of authorship and originality

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- Brendan J Main: Conducted experiments and wrote the manuscript.
- Rachael A Dunlop: Aided with experiments and provided experimental guidance.
- Kenneth J. Rodgers: Proof-read and edited the manuscript, conceived project idea, and provided guidance on experiments.

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The use of L-serine to prevent β-methylamino-L-alanine (BMAA)-induced proteotoxic stress *in vitro*

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ABSTRACT

β-methylamino-L-alanine (BMAA), a non-protein amino acid synthesised by cyanobacteria, has been linked to a complex neurological disorder on Guam and more recently to other cases of sporadic ALS (sALS), however the mechanisms of BMAA toxicity are not completely understood. We have previously demonstrated that BMAA is misincorporated into newly synthesised proteins by human neuroblastoma cells and fibroblasts, resulting in the formation of autofluorescent material and the induction of apoptotic cell death. In the present study we show that BMAA at low levels does not cause an acute toxicity in neuroblastoma cells but increases the expression of the ER stress marker, C/EBP homologous protein (CHOP) and increases the activity of the pro-apoptotic enzyme caspase-3. We also observed an increase in the activity of the lysosomal cysteine proteases cathepsin B and L, characteristic of the accumulation of proteins in the lysosomal system. We were able to prevent these proteotoxic effects in neuroblastoma cells through co-treatment with L-serine suggesting that they resulted from incorporation of BMAA into proteins. Misincorporation provides a possible mechanism whereby BMAA could initiate misfolding, and the accumulation of aggregate-prone proteins in neurons. This build-up of misfolded proteins could explain the long latency period of the disease previously reported on Guam.

1. Introduction

BMAA, a non-protein amino acid synthesized by all five known morphological groups of cyanobacteria (Cox et al., 2005) has been implicated in a number of neurodegenerative diseases, including sporadic ALS (sALS) (Murch et al., 2004). Only approximately 10–15% of ALS cases can be directly attributed to a known genetic mutation (Kiernan et al., 2011; Navone et al., 2015), the cause(s) of the remaining 85–90% remains unclear. A number of models have been proposed for sALS built around the gene-time-environment hypothesis. In these models genetic susceptibility, age and multiple environmental exposures, each contribute to risk and increase the burden of 'disease causing factors' that can precipitate disease once a threshold level is reached (Al-Chalabi and Hardiman, 2013). Based on observations made on the South Pacific Island of Guam (Cox et al., 2003) and more recent observations made by Stommel and colleagues in New Hampshire (Banack et al., 2015; Caller et al., 2009) and others in France (Masseret et al., 2013) and the Baltic Sea (Jonasson et al., 2010), BMAA is now considered to be a potential environmental risk factor in sALS.

Evidence from Guam suggested that BMAA had a chronic toxicity on the nervous system with disease symptoms manifesting on average 20 years after first exposure (Murch et al., 2004). In the landmark study by Spencer in which cynomolgus macaques fed oral BMAA for up to 12 weeks developed motor-neuron deficits, the authors observed more than one mechanism of toxicity (Spencer et al., 1987). They reported that motor dysfunction developed early after a high dose BMAA whereas signs of extrapyramidal compromise and behavioural change surfaced later in animals receiving smaller doses of BMAA (Spencer et al., 1987). The acute toxicity of BMAA has been well studied *in vitro* and is due in part to overstimulation of glutamate receptors by the β-carbamate of BMAA which forms in the presence of bicarbonate (Lobner, 2009; Weiss et al., 1989). In primary human neurons BMAA exerted an acute toxicity through NMDA receptor excitotoxicity as well as the generation of reactive oxygen species, both of these cytotoxic effects were shown to increase when the bicarbonate concentration in the culture medium was increased (Chiu et al., 2012) suggesting that BMAA carbamates were responsible.

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Much less is known about the chronic mechanisms of BMAA toxicity. We have previously shown that BMAA can exchange for L-serine and become misincorporated into proteins by the synthetic machinery of the cell, resulting in the production of autofluorescent bodies and the induction of apoptosis (Dunlop et al., 2013). ER stress is a common feature of many neurodegenerative diseases and is a response to the synthesis of misfolded and aggregated proteins (Hoozemans and Scheper, 2012). The ER mediated unfolded protein response (UPR) is a complex series of pathways responsible for the recruitment of chaperones to aid with protein refolding, a down-regulation in translation, and finally activation of mitochondria induced apoptosis (Chakrabarti et al., 2011). The intrinsic link between the UPR and neurodegeneration makes it a possible candidate as a mechanism of toxicity for BMAA, further we hypothesise that BMAA will increase the load of misfolded proteins in the cell due to its misincorporation into proteins. In the present study we examine how exposure to BMAA at concentrations lower than those previously used for toxicity studies disturbs protein homeostasis in neuroblastoma cells, we also investigate how these effects can be prevented through co-incubation with L-serine.

2. Experimental

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and Eagle's Minimal Essential Medium (EMEM, deficient in serine), L-BMAA hydrochloride and L-serine were from Sigma Chemical Co., St Louis, MO. *N*-Suc-Leu-Leu-Val-Tyr-AMC (where Suc is succinyl and AMC is 7-amino-4-methylcoumarin) and Boc- Leu-Ser-Thr-Arg-AMC (where Boc is t-butoxycarbonyl) were purchased from Sigma Chemical Co., St Louis, MO. Z-Arg-Arg-AMC (where Z is benzyloxycarbonyl), Z-Phe-Arg-AMC, and Ac-Nle-Pro-Nle-Asp-AMC (where Ac is acetyl and Nle is norleucine) were from Bachem AG, Bubendorf, Switzerland.

All aqueous solutions and buffers were prepared using 18 mΩ water. All other chemicals, solvents and chromatographic materials were of analytical reagent or cell-culture grade.

2.2. Cell culture

SH-SY5Y human neuroblastoma cells (American Tissue Culture Collection, catalogue number CRL-2266) were cultured as follows; DMEM was supplemented with 10% heat-inactivated Foetal Bovine Serum (FBS) (US origin, Gibco Carlsbad, CA, USA), 100 U/mL penicillin and 0.1 mg/mL streptomycin, and 2 mM L-glutamine (Penicillin-Streptomycin-Glutamine, 100X, Gibco, Carlsbad, CA, USA). Cells were maintained at 37 °C with 5% CO_2 in 175 cm^2 flasks until they were plated in 6 well plates for experiments. During treatment, DMEM culture medium was substituted with serine-free EMEM.

2.3. RT qPCR

SH-SY5Y cells were treated with 0 μM or 500 μM BMAA (±50 μM L-serine) in triplicate wells of a 6 well plate for 24 and 48 h, total RNA was then isolated using TRI reagent (Sigma Chemical Co., St Louis, MO) according to manufacturer's instructions. 200 ng of RNA was reverse transcribed in triplicate using a Bio Rad iScript cDNA synthesis kit (Hercules, CA, USA) and then pooled to account for inter-reaction variability. RT-qPCR was conducted in triplicate using the iTaq Universal SYBR Green Supermix, (Bio-Rad) according to manufactures instructions. Custom primers were designed using Beacon Design (version 4.0) and manufactured by GeneWorks, (Hindmarsh, SA, AU) (Table 1). Results were quantified using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001) with beta actin (ActB) as a housekeeping gene, the average coefficient of variation (CV) between repeats was 21%.

Table 1
Custom primer pairs were designed and blasted in NCBI Nucleotide and BLAST, and manufactured by Geneworks (South Australia, Australia). All pairs were checked for efficiency and specificity by running a standard curve and melt curve using untreated control cDNA. Only primers that showed a single melt curve and were close to 100% (±10%) were used in these experiments.

Primer	Sequence
ActB forward	5′–GTCCTCTCCCAAGTCCACACAG–3′
ActB reverse	5′–GGCACGAAGGCTCATCATTCAA–3′
CHOP forward	5′–ACTCTCTGCTTCTCCCTCTGC–3′
CHOP reverse	5′–GTTGCGTCAGTCCCGTGTAC–3′

2.4. Lactate dehydrogenase (LDH) assay

Following treatment, the culture medium was removed from each well, placed in 1.5 ml Eppendorf tubes and centrifuged at 2500 × *g* for 5 min then placed on ice. 1 ml of PBS was placed in each well and the cells detached from the culture plates using a rubber policeman, the cell suspension was then centrifuged at 2500 × *g* for 5 min, and the supernatant removed. The cell pellets were lysed using 3 cycles of freeze thawing in 750 μL of 0.05% Triton X-100 and then placed on ice until required. Aliquots of the media and cell lysate samples were diluted 1:10 into ultra-pure water and 10 μL of each sample added in triplicate to a 96 well culture plate, along with triplicate media and 0.05% Triton X-100 blanks. 200 μL of LDH assay reagent (0.15 mg/ml Nicotinamide adenine dinucleotide (NADH), 2.5 mM sodium pyruvate in PBS) was added to each well, and the plate briefly shaken on a plate shaker.

Change in absorbance at 340 nm was measured every 5 min over 30 min using a Tecan Infinite 200 Pro plate reader and Tecan i-Control software (ver. 1.9.17.0) and percentage viability was calculated.

2.5. Cathepsin activity

SH-SY5Y cells were sub cultured onto 6 well plates, allowed to adhere overnight, then treated in triplicate as follows; untreated control with medium only, medium with 50 μM L-serine, medium with 500 μM BMAA, and media with 500 μM BMAA +50 μM L-serine. Cells were treated for 72 h with medium changes every 24 h. Cells were harvested by scraping into 0.05% Triton X-100, then freeze thawed to lyse. Protein concentration was determined with the BCA assay (Smith et al., 1985), samples were normalised for protein concentration, then 10 μL of normalised lysate added to each plate with 150 μL buffer, and peptidase activity measured by change in fluorescence as described previously (Dunlop et al., 2008). Samples measured in triplicate were averaged and expressed as a percentage of untreated control.

2.6. Caspase 3 activity

SH-SY5Y cells were prepared using the same treatment as those used for cathepsin activity (section 2.5). Cultures were washed once in PBS, pelleted by centrifugation (2500 *g* for 5 min), then snap-frozen in liquid nitrogen and stored at −80 °C until required. The pellets were lysed in lysis buffer [consisting of 100 mM Hepes (pH 7.25), 10% sucrose, 0.1% CHAPS, 0.4% Nonidet P40 and 2 mM DTT (dithiothreitol)] with three freeze–thaw cycles in liquid nitrogen and a water bath at 37 °C. Lysates were centrifuged at 10,000 *g* for 10 min at 4 °C to remove particulates, the supernatant was

collected, and the protein concentration determined using the Bio-Rad Coomassie protein assay method (Bradford, 1976). Lysates were diluted in lysis buffer to allow for a final concentration of 5 μg of protein per well in a 96-well plate. For every part lysate (loaded in triplicate), three parts lysis buffer were added, followed by 5 μl of 1 mM Ac-DEVD-AMC. Changes in fluorescence were read on a CytoFluor fluorescent plate reader (λex − 360/40; λem − 460/40; gain 60). Caspase activity was determined by the change in FU (fluorescence units)/min per mg of protein and expressed as a percentage of the values from control lysates.

2.7. Statistical analysis

Statistical analyses were evaluated using GraphPad software (CA, USA) Prism 4 version 4.03 either using one-way or two-way ANOVA with Bonferroni multiple comparison post-tests or Student T-tests to compare replicate means between different treatments across the samples. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Incubation of SH-SY5Y cells with BMAA induced morphological changes but did not affect viability

Cell morphology (Fig. 1A–D), was assessed in SH-SY5Y cells over a range of BMAA concentrations. Morphology remained largely unchanged except at 1000 μM BMAA where autophagic-like vesicles were observed (Fig. 1D). We then used an LDH assay to assess population viability after exposure to 500 μM BMAA, and 500 μM BMAA +50 μM L-serine for 24 h (Fig. 1E). From these results we determined that 500 μM BMAA was an appropriate concentration to examine changes in cells at sub-lethal doses of BMAA.

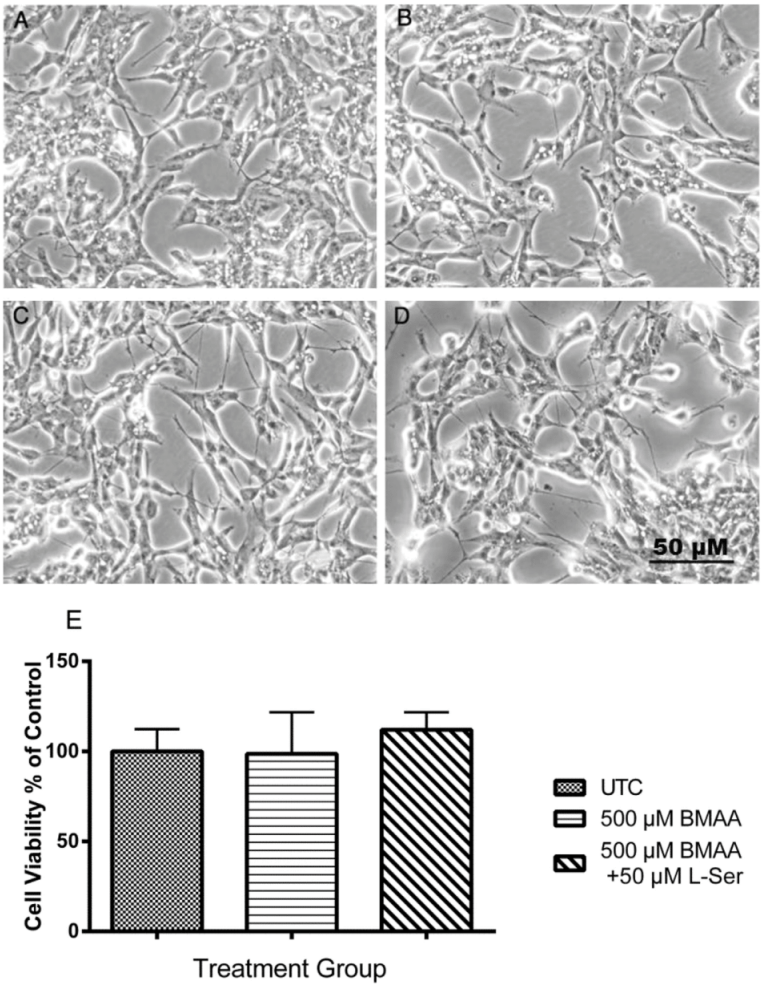

Fig. 1. Acute toxicity of BMAA in SH-SY5Y neuroblastoma cells. **A**–**D**: Morphology of SH-SY5Y neuroblastoma cells after incubation with BMAA, 0 μM (A), 200 μM (B), 500 μM (C) and 1000 μM (D) for 24 h. Phase contrast images were taken at 40× magnification. **E**: Viability of SH-SY5Y cells incubated with BMAA for 24 h as assessed by the LDH assay (UTC, untreated control) (n = 3).

3.2. BMAA-induced expression of CHOP, a marker of ER stress, this was prevented by L-serine

SH-SY5Y cells were incubated with BMAA (500 μM) for 24 and 48 h and CHOP expression measured by RT-qPCR. No change in CHOP expression was seen at 24 h but there was a significant up-regulation of CHOP expression at 48 h, compared to cells treated with medium alone ($P \leq 0.001$) (Fig. 2). Co-treatment of the cells with 50 μM L-serine and 500 μM BMAA prevented the increase in CHOP expression ($P \leq 0.0001$).

3.3. The increased activity of cathepsins B and L induced by BMAA was significantly reduced by co-incubation with L-serine

SH-SY5Y cells were incubated with BMAA in the presence or absence of L-serine, and cathepsin B (CatB) and cathepsin L (CatL) activity measured by cleavage of fluorescently labelled substrates as we have described previously (Dunlop et al., 2008). There was no change in cathepsin activity at 24, or 48 h (not shown) but there was a significant increase in the activities of both cathepsin B (Fig. 3A) and cathepsin L (Fig. 3B) after 72 h when treated with BMAA, when compared to untreated cells ($P < 0.01$). The observed increase was prevented when cells were co-incubated with L-serine 50 μM ($P < 0.01$).

To determine if the observed increase in lysosomal enzyme activity was selective for proteases, and not a result of a global increase in the population of lysosomes, we measured the activity of the lysosomal enzyme arylsuphatase. There was no change in the activity of arylsulphatase, suggesting that protease activity was selectively increased (Fig. 3C).

3.4. BMAA induced activation of caspase-3 was prevented by co-incubation with L-serine

Caspase-3 is an executioner caspase that initiates an irreversible cascade to apoptosis. SH-SY5Y cells were incubated with 500 μM BMAA for 72 h activity of caspase-3 was then assessed by measuring fluorescence resulting from cleavage of the tag AMC, from the peptide Ac-DEVD-AMC over time. There was a significant increase in the activity of caspase-3 in cells treated with BMAA ($P \leq 0.05$) (Fig. 4), this was prevented when cells were co-incubated with L-serine ($P \leq 0.05$).

4. Discussion

BMAA first attracted attention following reports of a high incidence of an unusual neurological disease amongst the people of Guam in the South Pacific (Bradley and Cox, 2009). At one time the disease, termed ALS/Parkinsonism-dementia complex (ALS/PDC), occurred at 50–100 times the normal rate that ALS occurred globally, suggesting a possible environmental link (Hirano et al., 1961; Kurland and Mulder, 1954). Analysis of cycad seeds, which were part of the traditional Chamorro diet, by Bell and colleagues identified the non-protein amino acid BMAA (Vega and Bell, 1967). BMAA was subsequently shown to be biomagnified through fruit bats that foraged on cycad seeds and were eaten in large numbers on Guam (Murch et al., 2004). We provided evidence that BMAA competed with L-serine for protein synthesis in mammalian cells in culture (Dunlop et al., 2013) providing a mechanism to explain why, in some tissues, BMAA was mostly 'protein associated' and could only be released by acid hydrolysis or proteolysis (Dunlop et al., 2013). In addition, since ALS, Parkinsonism and dementia are all disorders characterised by protein misfolding, incorporation of BMAA into proteins provided a mechanism whereby one agent capable of disturbing protein synthesis, folding, and turnover could trigger three separate disorders in susceptible individuals. In the present study we extend these original observations and examine proteotoxic stress in neuroblastoma cells after exposure to BMAA.

The accumulation of misfolded proteins in the endoplasmic reticulum causes ER stress within cells, the mechanisms and pathways that are employed to deal with this stress are collectively known as the UPR (Chakrabarti et al., 2011). The UPR enhances the expression of genes involved in protein folding, processing, and transport, as well as clearance of terminally misfolded proteins through the ER-associated degradation (ERAD) system, which can collectively alleviate the underlying stress and return the ER to homeostasis (Brown and Naidoo, 2012). In the present study we demonstrated that a concentration of BMAA that did not cause acute necrosis (500 μM) resulted in a significant increase in ER stress in cells after 48 h, as indicated by up regulation of the ER stress sensor C/EBP homologous protein (CHOP). This is consistent with the studies of Okle et al. (Okle et al., 2012) that showed an increase in ubiquinated proteins and CHOP expression in SH-SY5Y cells after a 48 h incubation with BMAA (1 mM); changes that were unrelated to oxidative stress.

ER Stress induced apoptosis results from prolonged activation of the UPR leading to the recruitment of the pro-apoptotic factor

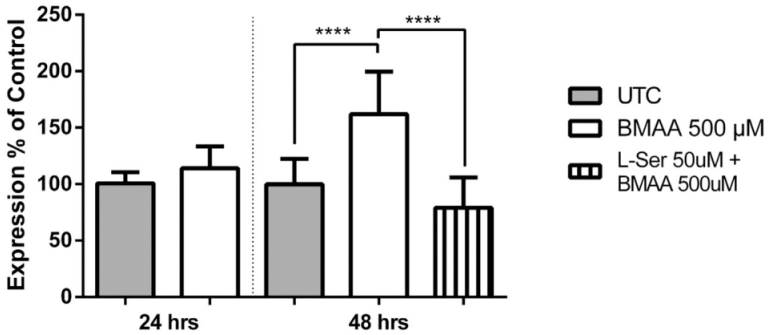

Fig. 2. CHOP gene expression levels after incubation of SH-SY5Y cells with BMAA. SH-SY5Y cells were incubated in 500 μM BMAA for 24 and 48 h. A significant increase in CHOP expression was demonstrated after 48 h as measured by RT-qPCR relative to cells incubated in medium alone (UTC, untreated control). In cells co-incubated with 50 mM L-serine and 500 μM BMAA there was no increase in CHOP expression after 48 h. CHOP expression was normalised to ActB using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Statistical analysis was by one way ANOVA and Tukey's multiple comparison test., ****$P < 0.0001$, for three independent experiments.

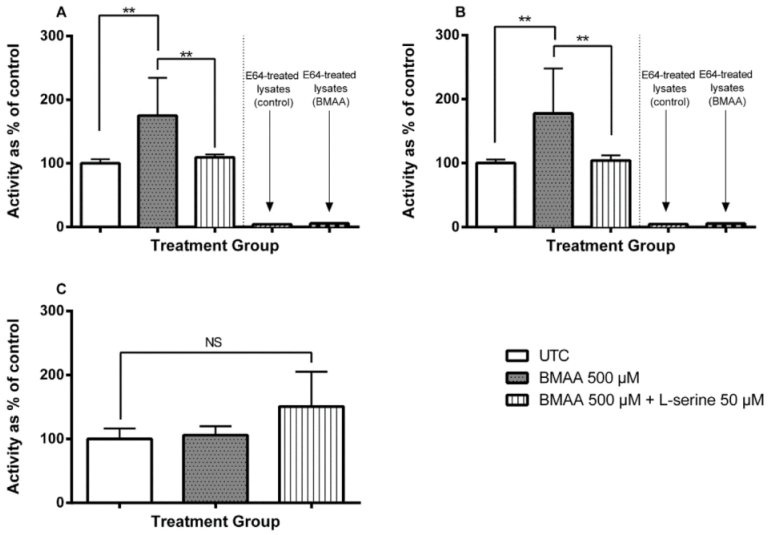

Fig. 3. Lysosomal enzyme activity following incubation of SH-SY5Y cells with BMAA. The activity of the lysosomal cysteine proteinases cathepsin B and cathepsin L, and the sulphatase enzyme, arylsulphatase were measured in SH-SY5Y cell lysates after incubation of cells with 500 μM BMAA for 72 h. Cathepsin B and cathepsin L activity was increased by more than 80%. There was no increase in cathepsin activity when cells were co-incubated with 500 μM BMAA and 50 μM L-serine (A & B). Negative control samples were cell lysates incubated with the irreversible cysteine proteinase inhibitor E-64 (1 μM) for 30 min prior to measuring activity. The activity of arylsulphatase was unchanged following treatment with BMAA (C) suggesting the increase in cathepsin activity did not represent a global increase in the number of lysosomes, n = 3 separate incubations, Statistical analysis was by one way ANOVA and Tukey's multiple comparison test, $^{**}P < 0.01$.

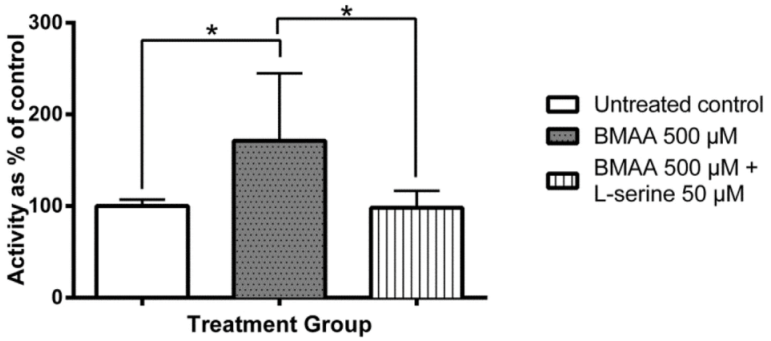

Fig. 4. BMAA increased the activity of caspase-3 in SH-SY5Y cells. Caspase-3 activity in SH-SY5Y cell lysates was increased following a 72 h incubation of cells with 500 μM BMAA. Co-incubation with 500 μM BMAA and 50 μM L-serine prevented the increase in caspase activity, n = 3 separate incubations, Statistical analysis was by one way ANOVA and Tukey's multiple comparison test. $^{*}P < 0.05$.

(CHOP), and downstream activation of effector caspases. We have found that co-incubation with L-serine prevented an increase in CHOP expression suggesting that incorporation of BMAA into protein was the underlying mechanism. After additional 24 h incubation with BMAA we observed a significant increase in the activity of the lysosomal proteases cathepsins B and L (Fig. 3A and B). We have previously shown that cathepsin activity in cells increases following the delivery of non-degradable protein aggregates to lysosomes (Dunlop et al., 2008). As in our previous studies, this occurred without an increase in lysosomal numbers as indicated by no change in activity of the lysosomal enzyme arylsulphatase (Fig. 3C). After a 72 h incubation with BMAA there was also a significant increase in activity of caspase-3 in cells, an important member of the caspase apoptosis cascade (Fig. 4). Prolonged UPR activation has previously been linked to caspase-12 and caspase-3 up-regulation, a hallmark of cells undergoing apoptosis (Reimertz et al., 2003). Again co-incubation with L-serine prevented changes in cathepsin activity and caspase up regulation suggesting that misincorporation of BMAA into protein and protein aggregation was the primary mechanism of toxicity. Damaged proteins are generally ubiquinated and degraded by the proteasome however if the proteins cannot be unfolded they are then re-directed to lysosomes (Rodgers et al., 2002) causing a specific up regulation in lysosomal protease activity as was the case in the present studies. Only 50 μM of L-serine was required to protect against the toxicity resulting from 500 μM BMAA.

5. Conclusions and implications

Taken together, these findings provide a mechanism whereby BMAA could initiate misfolding and aggregation of aggregate-prone proteins in neurons *in vivo*, this could contribute to an increase in

the load of damaged proteins permanently deposited in neurons (Rodgers and Shiozawa, 2008; Rodgers, 2013). Based on the data available, the level of exposure to BMAA from environmental sources would be very small (Jonasson et al., 2010) so it would seem likely that the process of protein misfolding and accumulation would occur over many decades. This would be consistent with the "gene-time-environment" hypothesis for sporadic ALS. BMAA (environmental) could accelerate the rate of misfolding of vulnerable proteins (genetic) or affect individuals more susceptible to BMAA incorporation (genetic); pathological changes might then only develop as the natural defences against protein misfolding and aggregation decline with ageing. Importantly, this is the first demonstration of the protective effect of L-serine against BMAA-induced proteotoxic stress. The high level of BMAA toxicity on Guam could have been due to the high consumption of BMAA-containing foods during times of starvation (low amino acids). In addition it could provide one explanation as to why animal studies in which BMAA is given with food have invariably been negative while other routes of administration have generally produced pathological changes (Karamyan and Speth, 2008).

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Transparency document

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Ethical statement

This manuscript represents original research, and has not been published in part or in whole elsewhere. This publication is not currently being considered for publication elsewhere. All authors have actively been involved in work leading to this manuscript, and have read and agree with its publication.

Conflicts of interest

Authors RD and KR report a patent relating to the use of L-serine in the treatment of neurodegenerative disease has been submitted (PCT/US2012/066373). Author BM reports no conflicts of interest.

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Chapter Six

Concluding Remarks and Future Directions

Chapter Six: Concluding Remarks and Future Directions

6.1 Concluding Remarks

In 1967, the isolation of a novel non-protein amino acid, later characterised as BMAA, was reported by Vega and Bell (1967). The five decades of research that followed its characterisation have built a compelling body of evidence implicating BMAA exposure in the development of neurodegenerative diseases. This project aimed to address a broad set of questions regarding the presence of BMAA in Australia, the interaction of BMAA with proteins, and the mechanisms underlying chronic BMAA toxicity. These aims represented significant gaps in existing literature critical for progressing our understanding of the role cyanobacterial non-protein amino acids play in neurodegenerative diseases both in Australia and globally.

The discovery that BMAA was produced by cyanobacteria in 2003 (Cox et al. 2003) was arguably the pivotal moment in BMAA research. Up to that point, BMAA was seen as a possible causative agent in Guamanian ALS-PDC, with little relevance to global neurodegenerative disorders. The presence of BMAA in a number of cyanobacterial symbionts vastly expanded the potential reach of BMAA as a toxin and was followed by an outbreak of research looking for BMAA in cyanobacterial, environmental, and patient samples across the globe (Nunn 2017). Prior to the studies presented in this thesis, Australian samples remained a conspicuous omission from the growing list of publications analysing cyanobacterial non-protein amino acids worldwide, and thus one of the key aims of this project was to develop a pipeline for LC-MS/MS analysis of local samples; the results of which are presented in Chapter Two.

Before this project commenced, the only Australian sample tested for BMAA in the published literature was a single laboratory maintained isolate of *Cylindrospermopsis raciborskii*. This was one of a number of cyanobacterial isolates tested by Cox et al. (2005) in a wide ranging survey that employed HPLC-FD to quantify BMAA concentrations. The absence of tested samples

directly collected from Australian waters made it difficult to draw conclusions about the concentration of BMAA and its isomers within the local environment, or the likelihood of human exposure, either directly through contaminated water or indirectly through contaminated food.

As part of this project, a sample preparation pipeline and LC-MS/MS method was developed and validated (Chapter Two). Sixteen algal scum samples collected from fourteen sites around eastern Australia were tested for the presence of BMAA and its isomers, 2,4-DAB and AEG. Ten of the tested samples were positive for BMAA, while 2,4-DAB was found in all sixteen, representing the first reported identification of either toxin in samples collected directly from Australia. Critically, the majority of the samples tested were collected from sites within the agriculturally important Murray-Darling Basin region of New South Wales. The presence of BMAA and 2,4-DAB in waterways used for the irrigation of approximately 40% of Australian agricultural land has potential implications for public health and food security in Australia.

The 1967 Vega and Bell paper represented not only the first isolation of BMAA, but also the first assessment of toxicity of the compound; the authors concluding that the synthetic BMAA was "markedly" neurotoxic when administered to chicks. Publications following this one largely focused on the excitotoxic nature of BMAA, with a particular interest in the β-carbamate formed from BMAA in the presence of bicarbonate (Weiss et al. 1988). However, the presence of a significant concentration of protein-bound BMAA in multiple trophic levels of the Guam environment, as well as in the brains of neurodegenerative disease sufferers (Murch et al. 2004a; Murch et al. 2004b) raised the possibility of an alternative form of BMAA toxicity. This was explored further by Dunlop *et al.* (2013) who concluded that BMAA was misincorporated into proteins during synthesis, possibly in place of L-serine, resulting in the formation of structurally aberrant proteins.

The exact nature of BMAA 'binding' has remained controversial, with some groups suggesting that BMAA is post-translationally bound to proteins as opposed to being misincorporated during

synthesis. van Onselen *et al.* (2015) was unable to detect bound BMAA in proteins produced by an *E. coli* based cell free expression system spiked with BMAA after both sodium dodecyl sulphate polyacrylamide gel electrophoresis, and immobilised metal affinity chromatography purification. This lead to the conclusion that prokaryotes do not incorporate BMAA into proteins and therefore previously observed protein bound BMAA was superficially associated with the protein.

The present project sought to clarify the interactions between BMAA and both prokaryotic and eukaryotic proteins by exposing purified proteins, *E. coli* and human neuroblastoma cell lysates, and live *E. coli* and human neuroblastoma cells, to both ^{3}H and ^{14}C enriched BMAA, and quantifying free and protein bound BMAA (Chapter Three). Using radiolabelled amino acids has a number of benefits over analytical mass spectrometry; lower treatment concentrations can be used, reducing the amount of washing required to remove unbound amino acid, and significantly less sample preparation is required prior to analysis. In neuroblastoma cells, a linear increase in BMAA concentration was observed, as well as a distribution of BMAA across cellular proteins consistent with an incorporated amino acid. Interestingly, this was not observed in *E. coli*, where instead the concentration of bound BMAA peaked within an hour of treatment before decreasing over the next eight hours. We also observed no binding to cell lysate or to pure proteins, suggesting that binding may require biological intervention.

This project's findings strengthen the argument for misincorporation playing a significant role in the interaction between BMAA and eukaryotic proteins, as well as the possibility that chronic BMAA toxicity could be mediated by this misincorporation. The differences observed between eukaryotic and prokaryotic protein binding could have a number of important implications as well. The rapid uptake of BMAA into a protein associated form and its subsequent clearing by *E. coli* warrants further investigation, and may provide insights into the role BMAA plays in cyanobacteria and other prokaryotes. From a technical standpoint the differences observed

between prokaryotes and eukaryotes highlight the importance of using mammalian cells and mammalian cell free protein expression systems for BMAA, and potentially other, NPAA research. The non-linear incorporation observed in *E. coli* suggests that using a prokaryotic model is unlikely to yield results relevant to eukaryotic exposure.

The key hypothesis underlying this project was that misincorporation of BMAA into proteins during synthesis could result in the formation and subsequent build-up of misfolded proteins. Aggregated proteins are hallmark features of a number of neurodegenerative diseases, and the presence of these aberrant protein structures can be acutely toxic. The formation of misfolded and aggregated proteins within the cell is closely monitored, a process largely focused around the ER unfolded protein response. In reaction to the presence of misfolded proteins within the lumen of the ER, the UPR upregulates protein folding chaperones, decreases proteins synthesis, and finally initiates mitochondrial mediated apoptosis (Chakrabarti et al. 2011).

The relationship between ER stress and ALS has been explored using cell culture, as well as human pathological samples, and genetically modified mouse models such as various superoxide dismutase-1 (SOD1) mutant mice. Nishitoh et al. (2008) observed increased induction of CHOP and 78 kDa glucose-regulated protein (Grp78) in NSC34 motor neuron like cells expressing mutant SOD1. Overexpressed Grp78 has been reported in spinal cord motor neurons of SOD1 mutant transgenic mice when compared to wild type motor neurons (Tobisawa et al. 2003), indicating that ER-stress undergoes significant activation in SOD1 related ALS. ER-stress and UPR activation has also been shown to be present in patients with sporadic ALS, with significant up-regulation of CHOP observed in spinal sections from sporadic ALS patients compared to sections from controls (Ito et al. 2009).

Okle *et al.* (2013) was the first to report increased ER-stress and UPR mediated apoptosis in human neuroblastoma cells in response to *in vitro* exposure to BMAA. ER stress was measured via increased expression of the pro-apoptotic protein CHOP, and apoptosis measured using

caspase 3/7. Okle and colleagues concluded that low doses of BMAA interacted with proteins resulting in protein homeostasis dysregulation and ER-stress.

A similar increase in CHOP gene expression was observed in this work, as well as an increase in the expression of two ER-associated degradation (ERAD) genes in SH-SY5Y cells treated with BMAA. Increased CHOP and ERAD gene expression was also seen in cells co-treated with BMAA and 2,4-DAB, as well as BMAA and AEG. While 2,4-DAB treatment did decrease cell viability as measured by the alamarBlue assay, BMAA was the only cyanobacterial NPAA tested that caused UPR and ERAD gene upregulation on its own (Chapter Four). The large number and diversity of toxins produced by cyanobacteria underscores the importance of investigating their mechanisms in the presence of each other. It is possible that BMAA undergoes synergistic or additive toxicity when combined with other NPAAs or cyanotoxins. These results suggest that while 2,4-DAB may contribute to cyanobacterial toxicity *in vitro,* it does not share the same mechanism of toxicity as BMAA.

A number of non-protein amino acids have been shown to act as amino acid analogues. Canavanine, AZE, L-DOPA, *o*-tyrosine, and *m*-tyrosine have all been shown to misincorporate in place of various protein amino acids (Rodgers and Shiozawa 2008). While these amino acid analogues have enough similarity to a canonical amino acid to be mischarged by the corresponding aminoacyl-tRNA synthetase, they are often out competed by their respective protein amino acids due to their lower binding efficiency (Rodgers and Shiozawa 2008). Dunlop *et al.* (2013) hypothesised that BMAA was misincorporated in place of L-serine during protein synthesis, and demonstrated that increasing doses of L-serine blocked radiolabelled BMAA incorporation proportionally.

This project expanded on the findings of Dunlop *et al.*, demonstrating that treatment of SH-SY5Y cells with 500 µM BMAA resulted in increased CHOP expression, as well as increased cathepsin and caspase 3 activity. Co-incubation of cells with 50 µM L-serine as well as BMAA negated the

toxicity of the BMAA resulting in no significant difference between the co-treated cells and untreated controls (Chapter Five). A primate study by Cox *et al.* (2016) supported our finding that L-serine co-treatment reduced BMAA toxicity, showing that neurofibrillary tangles present in vervets treated with dietary BMAA were not present in those co-treated with L-serine. It is also important to note that a recent phase I clinical trial investigating the use of L-serine for the treatment of ALS has had promising results, with a dose dependent decrease in disease progression in those individuals in the high dose L-serine treatment group (Bradley et al. 2017).

The aims of this project were to address shortfalls in our knowledge in regards to the presence of BMAA within the Australian environment, as well as to explore the interaction of BMAA with proteins, and the possible mechanisms underlying BMAA toxicity. This project was successful in the development and validation of a method for BMAA analysis, and identification of BMAA and its isomers from a number of blooms in key waterways within Eastern Australia. In addition, this work provides evidence to show that BMAA is incorporated into eukaryotic proteins during protein synthesis and is not just superficially bound to them. We have identified a mechanism by which misincorporated BMAA may result in significant ER-stress leading to apoptosis through activation of the unfolded protein response. Finally, we demonstrated that BMAA toxicity could be negated through co-incubation with L-serine, providing a promising future treatment for ALS.

6.2 Future Directions

While the time dependent increases in BMAA binding that were observed in this project are indicative of incorporation, it is still only circumstantial evidence. The most convincing evidence for misincorporation of NPAAs into proteins remains proteomic identification of single amino acid substitutions. Currently the low incorporation rate, and likely random nature of misincorporation make identifying misincorporated BMAA in peptides a difficult proposition. Proteomic detection using mass spectrometry requires relatively high amounts of NPAA incorporation at the same site within a peptide. A cell free expression system over producing a single protein is therefore the most reliable option for detection. It should be noted that the evidence from this project suggests that a eukaryotic expression system would be the most appropriate option due to the non-linear incorporation of BMAA observed in prokaryotes.

Targeted proteomic analysis of protein aggregates that play key roles in neurodegenerative diseases may also provide key insights into BMAA misincorporation. Aggregates and plaques of proteins such as α-synuclein, amyloid precursor protein, and tau protein are hallmark features of neurodegeneration, and have been found in brain tissue from primates exposed to BMAA (Cox et al. 2016). It is possible that these proteins are susceptible to amino acid misincorporation, or misfolding and aggregation following misincorporation. Using high resolution accurate mass (HRAM) analysis to quantify peptides with predicted mass shifts due to misincorporation would allow us to confirm the presence of BMAA in these aggregated proteins.

With the growing interest in L-serine as a treatment for ALS, a more focused study on the effect of BMAA on the ER unfolded protein response, and the mechanism behind the ability of L-serine to prevent BMAA toxicity would be appropriate. The UPR in particular is a complex system, with three separate pathways and multiple effects (Chakrabarti et al. 2011). One key area for future interest would be the effect of BMAA on the downregulation of protein synthesis, a side effect

of UPR activation. It is possible that long term exposure to BMAA may result in the cell having chronically depressed protein production resulting in cell death.

While these studies explored the effect of BMAA and its isomers on the ER UPR, the cytosol and mitochondria also have independent mechanisms to rectify interruptions to proteostasis. Proteotoxic stress within the cytosol is managed by the heat shock response (HSR) and regulated by the transcription factor heat shock factor 1 (HSF1). When activated, HSF1 up-regulates transcription of a number of chaperones called heat shock proteins (HSPs) in an attempt to refold misfolded proteins (Vabulas et al. 2010). If misfolded cytosolic proteins are unable to be refolded they are ubiquitinated, resulting in proteasomal degradation (Houck et al. 2012). The ubiquitin-proteasomal degradation and cytosolic protein stress pathways remain an under explored area of research that should be further pursued.

The unique evolution of mitochondria, from bacterial endosymbionts to eukaryotic organelles, means that mitochondrial proteins derive from both the mitochondrial and nuclear genomes (Calvo and Mootha 2010). This unique evolution also means mitochondria has a distinct unfolded protein response. This response manages misfolded proteins that are produced within the mitochondria, as well as those imported from the cytosol (Jovaisaite et al. 2014). Parallel to the ER UPR the mitochondrial UPR also upregulates the pro-apoptotic transcription factor CHOP in times of proteotoxic stress (Zhao et al. 2002). The mitochondrial UPR is also able to attenuate global cellular protein synthesis through a similar pathway to the ER UPR (Pellegrino et al. 2013).

The reported lack of editing activity in a number of mitochondrial tRNA-synthetases may mean that mitochondrial proteins are particularly susceptible to BMAA misincorporation (Hilander et al. 2018). This lack of editing combined with the parallels between the mitochondrial and ER responses to misfolded proteins, means that the mitochondrial UPR is of particular interest in BMAA toxicity. Both the cytosolic and mitochondrial stress responses are key mechanisms in

maintaining cellular proteostasis. These responses should be further investigated in the context of BMAA exposure to elucidate whether BMAA toxicity is unique to ER-regulated proteins.

One of the aims of this project was to determine if BMAA and its isomers are indeed in the Australian environment, and this was achieved through analysis of surface scum samples that contained multiple species of cyanobacteria. Moving forward, analysis of agricultural products produced in areas shown to have high BMAA contamination, as well as direct monitoring of contaminated water, would provide a more accurate assessment of the current level of BMAA exposure to the Australian population. Long-term monitoring of BMAA and 2,4-DAB in waterways chronically contaminated with cyanobacteria would also provide vital understanding of how concentrations vary overtime and in different environmental conditions.

Australia has a number of suspected ALS patient clusters, the most prominent of which is in the Riverina region of south-west NSW. This region is largely agricultural land, served by a series of irrigation canals and rivers which are often contaminated with algal blooms. Analysis of patient clusters in the United States has found that they were often located in close proximity to waterbodies with poor water quality, including persistent algal blooms (Caller et al. 2009b; Caller et al. 2012; Torbick et al. 2014). Replicating this spatial clustering analysis would allow us to identify if a similar correlation between ALS patient location and proximity to low water quality exists in Australia.

Detection of BMAA and other NPAAs remains a developing field; small analyte mass and inherently complex matrices makes analysis difficult. Analysis of four different sample matrices by Baker *et al*. (2017) highlights the differences in analyte detection between different amino acid derivatisation and detection techniques. Baker found that HILIC preparation was unable to detect BMAA in blue-crab samples that were strongly positive using PCF or AQC derivatisation. Similarly, PCF was unable to detect BMAA in Spirulina samples that HILIC and AQC reported as

positive. All three sample preparations agreed with the presence of BMAA in cyanobacteria, and its absence in human brain samples.

The variation in detection reliability between methods highlights that a failure to detect a NPAA via a single method is not necessarily an indication that the NPAA is not present in the sample. Assuming mass spectrometry methods are correctly designed and validated, variations in analyte detection are likely due to matrix effects on either derivatisation or detection. Employing either AQC or PCF derivatisation alongside HILIC would reduce the chance of type II errors as well as the variability in BMAA detection currently seen between different laboratories. Current inconsistencies in BMAA detection between laboratories using different techniques has often been one of the fundamental complaints for critics of BMAA research. Reducing this variability is vital to instituting long term monitoring of BMAA and other algal NPAAs in the environment.

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