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

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List of Figures

Please note that the Figures listed below include those in Chapter 1 only

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.