UTS UNIVERSITY OF TECHNOLOGY SYDNEY

An *in vitro* study investigating the combined toxicity of the cyanotoxins β-N-methylamino-L-alanine (BMAA) and 2,4-diaminobutyric acid (2,4-DAB)

by Lisa Pu

Thesis submitted in fulfilment of the requirements for the degree of

Masters of Science (Research)

under the supervision of
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Declaration of Original Authorship

I hereby declare the contents described in this thesis are of original research and have not been submitted to any other institute for a higher degree. The contribution to my thesis is wholly my work unless acknowledged otherwise.

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Steele, J. R., Italiano, C. J., Phillips, C. R., Violi, J. P., **Pu, L.**, Rodgers, K. J., & Padula, M. P. (2021) Misincorporation Proteomics Technologies: A Review. *Proteomes*. 9 (1), 2.

- Contributions involved creating several figures and diagrams to illustrate concepts.

Italiano, C. J., **Pu, L.**, Violi, J. P., Duggin, I. G., & Rodgers, K. J. (2021) Tolerance towards β-methylamino-L-alanine in *Escherichia coli* requires cysteine biosynthesis genes. *Research in Microbiology*.

- Contributions involved preparing and running samples for mass spectrometry and the analysis of glutathione levels in *Escherichia coli.*

Pu, L., Castorina, A., & Rodgers, K. J. (2022) Cyanobacterial toxins BMAA and 2,4-DAB perturb the L-serine biosynthesis pathway in SH-SY5Y neuroblastoma cells: a proteomic study. **(Submitted)**

Pu, L., Violi, J. P., Steele, J. R., Padula, M. P., & Rodgers, K. J. (2022) Changes to intracellular amino acid levels in SH-SY5Y cells exposed to the cyanotoxins BMAA and 2,4-DAB. **(Submitted)**

List of Abbreviations

2,4-DAB	L-2,4-diaminobutyric acid
3PG	3-phospho-D-glycerate
AEG	N-(2-aminoethyl) glycine
ALS/PDC	Amyotrophic lateral sclerosis/Parkinson's dementia complex
BAMA	B-amino-N-methylalanine
BMAA	β-N-methylamino-L-alanine
BMI	Body mass index
BOAA	β-N-oxalylamino-L-alanine
CSF	Cerebrospinal fluid
ER	Endoplasmic reticulum
fMND	Familial motor neurone disease
HPLC	High-performance liquid chromatography
IPA	Ingenuity pathway analysis
LOAEL	Lowest observable adverse effect level
MND	Motor neurone disease
NFT	Neurofibrillary tangle
NMDA	N-methyl-D-aspartate
NPAA	Non-protein amino acid
PHGDH	3-phosphoglycerate dehydrogenase
PPP	Pentose phosphate pathway
PSAT1	Phosphoserine aminotransferase 1
PSPH	Phosphoserine phosphatase
sMND	Sporadic motor neurone disease
SOD1	Superoxide dismutase 1
TCA	Tricarboxylic acid cycle
TDP-43	TAR DNA-binding protein 43

Abstract

Sporadic motor neurone disease is a neurodegenerative disease with poorly understood aetiology. It accounts for up to 90 to 95% of motor neurone disease cases, with the remaining 5 to 10% being familial. Development of the sporadic form of the disease may be due to a contribution of several factors such as lifestyle, genetic susceptibility, aging and environment. One of the proposed environmental factors is exposure to cyanobacterial neurotoxins. A link between exposure to cyanobacterial (blue-green algal) toxins and a high incidence of neurodegenerative diseases reported on Guam in the 1940s resulted in the discovery of the novel amino acid, β-N-methylamino-L-alanine (BMAA). BMAA is being investigated as a potential trigger for MND based on *in vitro* and *in vivo* toxicity studies as well as recent epidemiological studies that have linked exposure to cyanobacterial blooms to higher incidences of MND in several locations worldwide. In over 50 years of research, the focus has primarily been on BMAA despite there being several other isomers including L-2,4-diaminobutyric acid (2,4-DAB) which have neurotoxic effects. BMAA and 2,4-DAB are produced concurrently by cyanobacteria, and it is logical to investigate their toxicity together as well as individually. This thesis aims to investigate further the toxic mechanisms of these two isomers and how they might contribute to the development of sporadic neurodegenerative disorders.

Initially cell viability assays were performed to determine the toxicity of the neurotoxins individually, and to identify the most toxic combination. Equimolar concentrations of BMAA and 2,4-DAB resulted in the highest toxicity to the cells and was used in subsequent studies. Proteomic analysis then revealed significant enrichment in pathways involved with energy production (fatty acid β-oxidation and glycolysis) and L-serine biosynthesis. The proteomic data on the L-serine biosynthesis enzymes were then validated using RT qPCR to determine expression levels of the three enzymes involved, as well as protein levels via Western blotting. 2,4-DAB alone and in combination with BMAA significantly decreased the expression of the first enzyme involved in the L-serine biosynthesis pathway, 3-phosphoglycerate dehydrogenase (PHGDH). Supplementation with the glycolytic metabolite pyruvate before exposure to the neurotoxins was

protective and prevented the impact of the toxins on the PHGDH gene expression. These results highlight the importance of the contribution to energy dysfunction which may parallel those seen in some neurodegenerative diseases. The toxins' ability to interfere with L-serine biosynthesis enzymes may be another route by which BMAA could disrupt homeostasis in cells.

To further understand the ability of the toxins to disrupt cellular metabolism, LC-MS/MS was used to quantify the level of amino acids and antioxidant capacity of cells exposed to BMAA, 2,4-DAB and the combination. 2,4-DAB exposure showed evidence of oxidative stress which was increased when combined with BMAA. Intracellular L-alanine levels were significantly decreased following treatment with BMAA and 2,4-DAB alone. The decreases in L-alanine levels in cells might support existing studies that have demonstrated the affinity of BMAA for alanyl-tRNA synthetase. The impact of the cyanotoxins on L-serine biosynthesis could be important to the *in vivo* toxicity of BMAA since it is known that L-serine is protective, but the mechanism through which it protects against BMAA has not been identified. Since L-serine is an important amino acid in the CNS, damage to its biosynthesis by continuing exposure to these cyanotoxins could result in permanent neuronal damage.

The results of these studies contribute to the ever-growing knowledge of BMAA and its role in neurodegenerative diseases and highlight the importance of studying the toxin in combination with its isomers that are found concurrently in nature.

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Chapter 1: Introduction

1.1 Motor neurone disease

Motor neurone disease (MND) is a progressive neurodegenerative disease affecting cortical and spinal motor neurons resulting in respiratory paralysis with death typically occurring 3 to 5 years after diagnosis (Hu et al., 2009). Approximately 1 to 3 new cases per 100,000 people occur every year in Europe, North America and Asia with the rates being fairly uniform around the globe (Chiò et al., 2013). The incidence of MND increases with age and the cumulative lifetime risk of developing MND is approximately 1 in 400 by the age of 85 (Johnston et al., 2006). Medical costs of MND which burden the health care system are up to 1 billion per year in the United States (Larkindale et al., 2014). Public awareness of MND is increasing through events like the Ice Bucket Challenge and the death of renowned physicist Stephen Hawking. Despite an increased awareness of this debilitating disorder and some scientific advancement, MND remains a terminal disease with a poorly understood aetiology.

As MND progresses, the degeneration of the upper motor neurons results in muscle stiffness and fine movement impairment while lower motor neuron degeneration leads to muscle weakness, wasting and twitching (Al-Chalabi et al., 2016). Interestingly, retinal and bladder motor neurons remain unaffected until late in the disease (Brown & Al-Chalabi, 2017). MND diagnosis and subsequent treatment are dependent on the location of onset. Spinal onset MND initially develops in the limbs while bulbar onset MND involves the lower cranial nerves which leads to muscle weakening in the tongue and vocal apparatus (Al-Chalabi et al., 2016). Regardless of the location of onset, eventual full-body paralysis is an inevitable outcome.

Only two therapeutic drugs have been approved for clinical use despite there being more than 200 MND clinical trials of drugs that have failed to show efficacy (Jaiswal, 2019). The first approved drug was Riluzole, a glutamate neurotransmission inhibitor approved in the early 1990s which was shown to increase the survival of patients by 3 months on average (Bensimon et al., 1994). The second drug, Edaravone, is a free radical scavenger drug that was initially developed for the treatment of stroke but was approved for MND treatment in 2017 and has demonstrated the ability to slow MND

progression during early disease stages (Abe et al., 2017) and decreased oxidative stress markers in cerebrospinal fluid (Sawada, 2017).

There are two defined categories of MND; sporadic MND (sMND) which accounts for around 90 to 95% of all cases and familial MND (fMND) which only accounts for 5 to 10% of cases (Byrne et al., 2011). Several genetic mutations such as the superoxide dismutase 1 (SOD1) mutation, have been identified that are known to result in MND (Table 1) (Zufiría et al., 2016). These genes account for 50% of fMND but only 5% of sMND cases (Zou et al., 2017). sMND may have resulted from a combination of aging, genetic susceptibility and environmental factors in a six-step process (Al-Chalabi et al., 2014; Chiò et al., 2018). The number of steps to reach the MND "threshold" is further reduced if a person carries one of the four major susceptibility genes such that a SOD1 mutation only requires two steps, C9orf72 mutation requires three steps and TAR DNA-binding protein 43 (TDP-43) mutation requires four steps (Chiò et al., 2018). Mutations in these four major genes may result in faulty proteins which may form insoluble aggregates or alter cellular metabolism in terms of protein homeostasis, protein trafficking or RNA metabolism (Table 1).

A person can be exposed to many environmental factors that could potentially trigger sMND; Vucic applied the MND multi-step process to the Australian population and like Al-Chalabi, found it fitted to a six-step process in males and a seven-step process in females (Vucic et al., 2019). Smoking, head injury, exposure to heavy metals, electromagnetic fields, infectious agents, and toxins have been proposed as potential causative factors (Bozzoni et al., 2016). Another potential risk factor for sMND is exposure to the cyanobacterial neurotoxin BMAA (Banack et al., 2010; Cox et al., 2018). Unlike smoking, and exposure to heavy metals or toxic chemicals, which has been declining over the past few decades, exposure to algal blooms is increasing in line with the incidence of MND (Koreivienė et al., 2014).

Table 1 Major and minor genes involved in the development of MND. The major genes account for 60-80% of fMND while some minor genes may have a residual impact on the development of fMND (Zou et al., 2017; Zufiría et al., 2016).

MAJOR GENES

GENE	LOCATION	PROTEIN	PROTEIN FUNCTION
ALS1/SOD1	21q22.11	Cu/Zn superoxide dismutase	Superoxide radical scavenger, binds and incorporates Cu and Zn molecules
ALS6/FUS	16p11.2	Fused in sarcoma	DNA/RNA binding, DNA repair, regulation of transcription, mRNA splicing, RNA transportation
ALS10/TARDBP	1p36.22	TAR DNA-binding protein 43	DNA/RNA binding, regulation of transcription, mRNA splicing, translation
C9OREJ2/FTDALS1	9p21.2	C9orf72 protein (unnamed)	Abundant in neurons, modulates RNA and protein production, RNA transportation

MINOR GENES

GENE	LOCATION	PROTEIN	PROTEIN FUNCTION
ALS2	2q33.2	alsin	GTPase regulator, possible role in endocytosis, cytoskeleton maintenance, cell signalling and protein and membrane transportation
ALS4/SETX	9q34.13	Probable helicase senataxin	Regulation of transcription, splicing, RNA metabolism, responds to DNA damage by oxidative stress

ALS8/VAPB	20q13.3	Vesicle-associated membrane protein	Targeting and fusion of transport vesicles to membranes
ALS9/ANG	14q11.1	Angiogenin	Ribosomal RNA synthesis, assembles tiRNAs which inhibit protein synthesis and triggers stress granules, induces vascularisation
ALS11/FIG4	6q21	Polyphosphoinositide phosphatase	Biogenesis of endosome carrier vesicles and multivesicular bodies (MVBs), transport of endosome intermediates
ALS12/OPTN	10p15-p14	Optineurin	Golgi complex maintenance, membrane transport, exocytosis, interacts with aggregates such as ubiquitin and TDP-43
ALS13/ATX2	12q23q24.1	Ataxin 2	Endocytic epidermal growth factor receptor trafficking, can form a complex with TDP-43
ALS14/VCP	9p13.3	Valosin-containing protein	Proteasomal degradation, endosomal trafficking, vesicle and aggregate removal
ALS15/UBQLN2	Xp11.21	Ubiquilin 2	Regulation of many protein degradation pathways, assists in removal of misfolded proteins
ALS16/SIGMAR1	9p13.3	Sigma non-opioid intracellular receptor 1	Involved in lipid transport from the ER, regulates ion channels
ALS17/CHMP2B	3p11.2	Charged multivesicular body protein 2b	Formation and sorting of endosomal cargo proteins in MVB
ALS18/PFN1	17p13.3	Profilin 1	Binds to actin, helps structure the cytoskeleton

ALS19/ERBB4	2q34	Receptor tyrosine-protein kinase erbB 4	Tyrosine-protein kinase cell surface receptor for neuregulins
ALS20/HNRNPA1/ A2B1	12q13.13	Heterogeneous nuclear ribonucleoprotein A1 and A2/B1	Involved in pre-mRNA packaging into hnRNP particles, transport of mRNA, possible splice site modulation
ALS21/ MATR3	5q31.2	Matrin 3	Regulation in transcription, retention of defective RNAs and localises in the nuclei of motor neurons by interacting with TDP-43
SQSTM1	5q35.3	Sequestosome-1	Autophagy receptor, transports polyubiquitinated cargo into autophagosomes, regulation of glucose metabolism
NEFH	22q12.2	Neurofilament heavy polypeptide	Maintenance of neuronal calibre, helps form neuronal filamentous networks
GLE1	9q3411	Nucleoporin GLE1	Transportation and metabolism of mRNA
TAF15	17q12	TATA-box binding protein associated factor 15	RNA/ssDNA binding protein, transcription initiation
TBK1	12q14.2	TANK-binding kinase	Regulates inflammatory responses to foreign stimuli, phosphorylates OPTN and SQSTM1 to enhance autophagy

1.2 BMAA and its link to neurodegenerative disease

The connection between BMAA and neurodegenerative disease was first made on the island of Guam in the 1950s (Garruto et al., 1981; Reed et al., 1966). The incidence of several complex neurological disorders was unusually high with a reported rate 50-100 times greater than the general population worldwide (Kurland & Mulder, 1954). The development of the unusual disease, termed amyotrophic lateral sclerosis/Parkinsonism dementia complex (ALS/PDC), was proposed to be linked to the consumption of cycad flour and flying foxes by the native Chamorro people, and residents of the island who adopted the lifestyle of the Chamorros (Banack & Cox, 2003; Banack et al., 2006; Whiting, 1963). Cycad seeds were taken to the United Kingdom for analysis under the assumption that they contained the non-protein amino acid (NPAA) β-N-oxalylamino-L-alanine (BOAA). An extensive investigation by Armando Vega showed that BOAA was not present but he identified the previously undescribed amino acid β-N-methylamino-L-alanine (BMAA) (Vega & Bell, 1967).

It was later discovered that in the coralloid roots of the cycads (*Cycas micronesica)* contained BMAA-producing symbiotic bacteria, *Nostoc* (Vessey et al., 2005). The Chamorro people were aware that the cycad contained many acute toxins and thoroughly washed the flour to remove water-soluble toxins. However, the majority of BMAA was shown to be protein-associated and could not be removed by washing (Cox et al., 2003; Murch et al., 2004a). The ability of BMAA to be in some way bound to, or associated with, proteins might contribute to its bioaccumulation through the food chain (Figure 1). BMAA was detected in the brain tissue of the deceased Chamorro patients but not in the Canadian control patients (Murch et al., 2004b). However, this observation has been questioned by many groups that have failed to detect BMAA in cyanobacterial extracts or brain tissue (Duncan & Marini, 2006; Ince & Codd, 2005). There might have been an initial overestimation of BMAA concentration in cycad seeds and flying foxes due to early detection methods relying on fluorescence for detection following high-performance liquid chromatography (HPLC) separation rather than identification by mass spectrometry. This could have resulted in the misidentification of BMAA instead of one of its isomers (Faassen, 2014; Faassen et al., 2012).

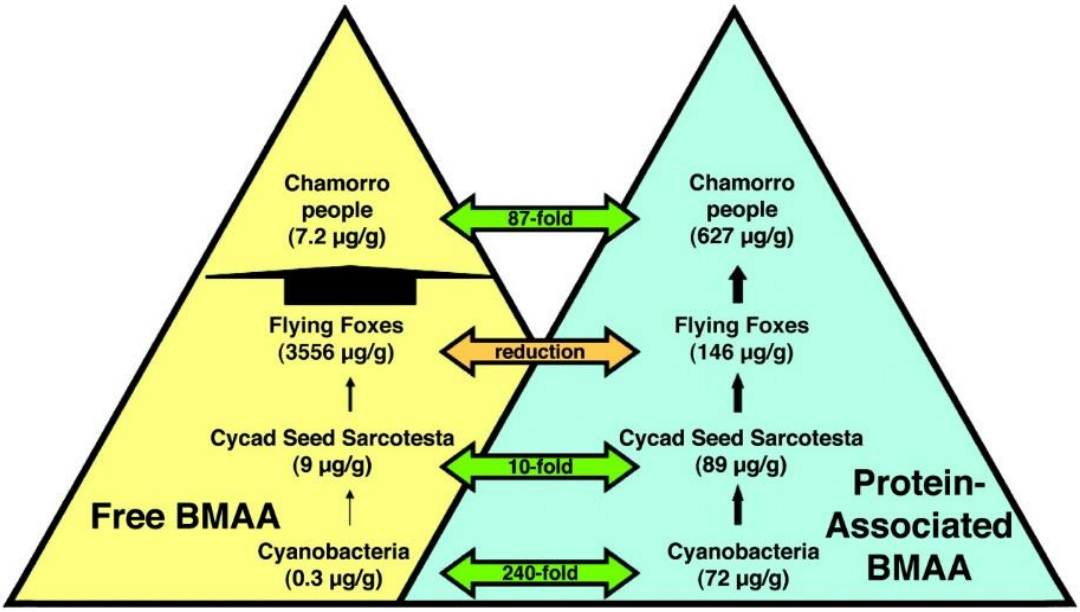

Figure 1 Schematic showing the bioaccumulation of BMAA in Guam with the Chamorro people as the top consumer (Murch et al., 2004a).

Numerous noteworthy studies from 1968 to 2007 investigated the toxicity of BMAA in mouse, rat and chick models. The initial *in vivo* studies looked at chicks who were injected intraperitoneally with a single dose of BMAA at 0.2-0.8 g/kg (Polsky et al., 1972; Vega et al., 1968; Whiting, 1988). The chicks demonstrated the inability to stand and extend their legs. Rat and mouse models were studied where the animals ingested 0.4-1.6 g/kg of BMAA and developed muscle weakness, convulsions and a dragging gait (Polsky et al., 1972; Vega et al., 1968). However, the effects were temporary as they disappeared after 12-18 hours. These acute neurotoxic effects did not mimic the long-term chronic effects seen in ALS/PDC or MND patients. In 1991 and 1993, Rakonczay and Matsuoka attempted to investigate the longer-term effects of BMAA by dosing rats intracerebroventricularly with 0.5 mg/day for 10-60 days (Matsuoka et al., 1993; Rakonczay et al., 1991). They recorded abnormal movements such as rigidity, body shakes, side-to-side movement and jerky movement which were again, representative of acute toxicity and lasted only about 10 minutes. The severity of the signs further diminished after days 4-6. They did find a significant decrease in glutamic receptor binding in rats treated with BMAA after 40 days. This was further supported by a study performed by Chang in 1993 who showed a decrease in the number of glutamate

receptors in BMAA-treated rat brains which were protected when co-administered with AP-5 (an NMDA glutamate receptor antagonist) (Chang et al., 1993).

A key study was performed in 1986 where Spencer and colleagues orally administrated BMAA to macaques for up to 3 months. The primates were observed to develop motor neuron dysfunction similar to that seen in MND patients (Spencer et al., 1987). This finding led to BMAA becoming a primary focus of sMND research in the following years. A more recent primate study was performed with vervets that were fed with BMAA-dosed fruit for over 4 months. Neurofibrillary tangles (NFTs) and β-amyloid plaques which are hallmarks of Alzheimer's and ALS/PDC were found in the vervet brains, although no behavioural changes were reported (Cox et al., 2016). The study was further expanded when analysis of the spinal cord of the BMAA-dosed vervets was performed. Neuropathology similar to Guamanian ALS/PDC was seen. Bunina bodies (an MND pathological hallmark), NFTs, neuropathological degeneration of the upper and lower motor neurons, activated microglia and TDP-43-positive inclusions were present in both the vervets chronically exposed to BMAA and a sMND patient who was autopsied parallel to this study (Davis et al., 2020). The neuropathologies were reduced in vervets co-administration with L-serine (Davis et al., 2020).

1.3 Cyanobacterial production of neurotoxins and potential human exposure routes

Cyanobacteria are ancient microorganisms that have colonised many extreme environments including deserts, hot springs and Antarctic regions (Cox et al., 2009; Sompong et al., 2005; Taton et al., 2006). Due to this adaptive nature, it is hardly a surprise that cyanobacteria and their neurotoxins have been detected on every continent (Bishop et al., 2018; Johnson et al., 2008; Li et al., 2016; Magonono et al., 2018; Réveillon et al., 2015; Violi et al., 2019).

Cyanobacteria produce a wide range of toxins including hepatotoxins (microcystins and nodularin), cytotoxins (cylindrospermopsin), dermatoxins (lynbyatoxin) and neurotoxins (anatoxins, saxitoxins and BMAA) (Dittmann et al., 2013). BMAA is universally produced by 97% of cyanobacterial strains (Cox et al., 2005) and possibly by all strains under ideal conditions. BMAA has also been detected in other phytoplankton such as diatoms (Jiang et al., 2014) and dinoflagellates (Lage et al., 2014).

Most analytical studies on cyanobacterial toxins only examine BMAA (Brand et al., 2010; Cox et al., 2009; Johnson et al., 2008). However, cyanobacteria and other phytoplankton often produce BMAA along with its isomers L-2,4-diaminobutyric acid (2,4-DAB) and N-(2-aminoethyl) glycine (AEG) (Bishop et al., 2018). AEG is the most abundantly produced isomer and was reported at a concentration 10,000-fold more than BMAA in desert crusts (Metcalf et al., 2015) and up to 10-fold more in cyanobacterial isolates (Violi et al., 2019). 2,4-DAB is a neurotoxin reported to be up to 10-fold more abundant than BMAA when detected in freshwater organisms or cyanobacteria (Field et al., 2013; Violi et al., 2019).

Exposure to BMAA and its isomers may occur from consuming contaminated food or water, inhalation of aerosolised toxins or participating in recreational water activities such as swimming (Banack et al., 2015; Caller et al., 2009; Stommel et al., 2013). Dietary intake of fish, shellfish and other aquatic animals exposed to contaminated water has been proposed to account for the majority of exposure incidents related to BMAA (Jonasson et al., 2010; Réveillon et al., 2015) but the routes of exposure are not well understood. Organisms are exposed to BMAA in lakes or water bodies with frequent cyanobacterial blooms and can biomagnify BMAA up to 7000 μg/g of BMAA in animals, acting as a potential long-term health hazard for humans (Brand et al., 2010).

Cyanobacterial blooms have become an ever-increasing threat to freshwater ecosystems due to global warming, eutrophication and several urban and industrial activities (Anderson et al., 2002; Glibert et al., 2005; Richardson et al., 2019). Multiple reports of increased sMND cases have occurred near water bodies with frequent blooms (Caller et al., 2009; Field et al., 2013; Masseret et al., 2013; Sabel et al., 2003; Sienko et al., 1990).

1.4.1 BMAA and its isomers; mechanisms of toxicity

2,4-DAB, AEG and β-amino-N-methylalanine (BAMA) are all naturally occurring isomers of BMAA (Figure 2) and may have different mechanisms of toxicity as summarised in Table 2. While BMAA is potentially the most toxic isomer, it is the least abundant compared to 2,4-DAB and AEG (Schneider et al., 2020). Both BMAA and 2,4-DAB are capable of binding to glutamate receptors and causing neuroexcitation-induced toxicity

(Weiss et al., 1989). BMAA is reported to be able to misincorporate into proteins in place of L-serine resulting in protein aggregation (Dunlop et al., 2013), and to cause oxidative stress (Liu et al., 2009) and breaking DNA strands with nitrosation-derived products from BMAA (Potjewyd et al., 2017). Three of the aforementioned biochemical changes are also hallmarks of neurodegenerative diseases (Table 3). *In vitro* research into the toxicology of BMAA has revealed several potential implications in the development of sMND. Firstly, it is capable of inducing protein aggregation and cause the accumulation of ubiquitin-positive proteins (Quinn et al., 2021). Secondly, treatment of human neuroblastoma cells (SH-SY5Y) with BMAA resulted in a significant increase in three ER stress markers (CHOP, EDEM1 and HERPUD1) (Main & Rodgers, 2018; Okle et al., 2013). Lastly, proteomic analysis of BMAA-treated murine neuroblastoma/spinal motor neuron fusion cells (NSC-34) showed several enriched pathways including mitochondrial dysfunction and inflammation response amongst the previously mentioned MND hallmarks (endoplasmic reticulum [ER] stress, protein ubiquitination and oxidative). In summary, BMAA could possibly contribute to all six identified biochemical hallmarks as well as several pathological hallmarks (Table 3) as seen in the vervet study in Section 1.2.

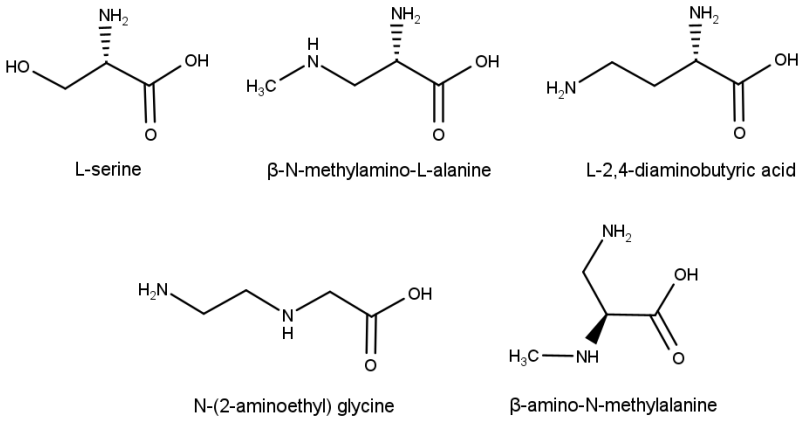

Figure 2 Structure of L-serine, BMAA, 2,4-DAB, AEG and BAMA.

Table 2 Summary of the distribution of BMAA and its isomers and their mechanisms of toxicity.

TOXIN	DISTRIBUTION	DEMONSTRATION OF TOXICITY MECHANISMS
BMAA	Produced ubiquitously by cyanobacteria (Cox et al., 2005), diatoms (Jiang et al., 2014) and dinoflagellates (Lage et al., 2014)	Neuroexcitation via binding of glutamate receptors (Weiss et al., 1989) Potentially causes protein misincorporation, misfolding and proteotoxic stress (Dunlop et al., 2013) Nitrosation of BMAA generates toxic DNA strand-breaking agents in SH-SY5Ys (Potjewyd et al., 2017) Causes oxidative stress (Liu et al., 2009) Increased ER stress markers (Main & Rodgers, 2018)
2,4-DAB	Produced as a component in bacterial cell walls (Schleifer & Kandler, 1972) Found in legumes (Pilbeam & Bell, 1979)	Demonstrated to cause lathyrism in rats (Ressler et al., 1961) Causes secondary ammonia toxicity in the brain of rats (O'Neal et al., 1968) Neuroexcitation via binding of glutamate receptors (Weiss et al., 1989) Direct cytotoxicity of SH-SY5Y cells in vitro (Main & Rodgers, 2018)
AEG	Polypeptide backbone for nucleic acids in ancient cyanobacterial lines (Banack et al., 2012)	Paralytic effect in brine shrimp in low concentrations (Metcalf et al., 2015)
BAMA	Produced by cyanobacteria (Bishop et al., 2018)	No toxicological reports to date

Table 3 Identified pathological and biochemical hallmarks of MND.

PATHOLOGICAL HALLMARKS	
TDP-43 MISLOCALISATION	Mislocalisation of TDP-43 proteins from the nucleus to the cytoplasm (Shan et al., 2009)
UBIQUITIN POSITIVE INCLUSIONS	These inclusions appear similar to the Lewy bodies seen in PD. The major protein component of these inclusions is TDP-43. Found in up to 95% of cases of MND (Wijesekera & Nigel Leigh, 2009)
HYALINE CONGLOMERATE INCLUSIONS (HCI)	These are argyrophilic inclusions seen in spinal motor neurons and are positive for phosphorylated and non-phosphorylated neurofilament stains. Common in fMND, rarely in sMND and are also mentioned in other neurodegenerative diseases (Wijesekera & Nigel Leigh, 2009)
BUNINA BODIES	Eosinophilic hyaline inclusions that stain positive for cystatin and transferrin and are seen inside the cytoplasm of lower motor neurons. Only seen in MND (Wijesekera & Nigel Leigh, 2009)
BIOCHEMICAL HALLMARKS	
INFLAMMATION	Inflammation is a key hallmark in many other neurodegenerative diseases and has also been implicated in MND. Increased cytokine markers correlate with disease severity and degenerating motor neurons are marked by the presence of immune cells such as activated microglia (Frank-Cannon et al., 2009)
OXIDATIVE STRESS	Accumulation of reactive oxygen species (ROS) is a well-established link to neurodegeneration and there is evidence of biochemical changes linked to oxidative stress in MND. Free radical damage and abnormal metabolism changes have been found in the tissue samples of deceased MND patients (Wijesekera & Nigel Leigh, 2009)

PROTEIN MISFOLDING AND AGGREGATION	It is possible for proteins encoded by MND-associated genes to undergo protein misfolding and aggregation. TDP-43, FUS and SOD1 can all form aggregates with themselves or with other proteins and form deposits inside the cell seen as cellular inclusions (Farrawell et al., 2015)
ENDOPLASMIC RETICULUM STRESS	Abnormal enlargement, reduced ribosomes, destruction and fragmentation of Nissl substance (rough ER) have been shown in MND autopsy cases which suggests the involvement of ER stress in impaired protein production (Kanekura et al., 2009)
EXCITOTOXICITY	Excess glutamate can induce neuro excitotoxicity through the NMDA and AMPA receptors which then leads to the increased generation of free radicals and neuronal death. Glutamate levels in cerebrospinal fluid (CSF) have been reported to be elevated in some MND patients (Wijesekera & Nigel Leigh, 2009)
MITOCHONDRIAL DYSFUNCTION	Defective energy metabolism in the mitochondria has been implicated in MND patients. Respiratory chain complexes I and IV have decreased activity and patients showed elevated calcium levels. Mutations in mitochondrial DNA have been reported as well (Wijesekera & Nigel Leigh, 2009)

2,4-DAB, on the other hand, has previously been shown to be directly neurotoxic in rats (Ressler et al., 1961) and SH-SY5Y cells (Main & Rodgers, 2018) and is present in much higher quantities than BMAA in cyanobacteria (Main et al., 2018; Violi et al., 2019). Furthermore, it has been found in the cell wall of 65 strains of gram-positive bacteria (Schleifer & Kandler, 1972). It has also been shown to mediate toxicity via NMDA receptors at 300 µM and inhibit L-cystine uptake at 1000 µM (Schneider et al., 2020). AEG is the most abundant of all four isomers as it is used as a polypeptide backbone for nucleic acids in cyanobacteria (Banack et al., 2012). AEG toxicity has been reported by only one group, which demonstrated the toxicity is mediated by the induction of free radicals and activation of mGluR5 receptors at 10 µM and 30 µM respectively (Schneider et al., 2020). The study suggested AEG to be the most potent neurotoxic isomer of BMAA, more so than BMAA itself (Schneider et al., 2020). Our previous studies have shown AEG to be the least toxic (Main & Rodgers, 2018), however studies performed by Schneider et al., 2020 could have more relevance to the *in vivo* situation as they were performed on primary cell cultures rather than on a transformed cell. BAMA has no toxicological reports to date.

1.4.2 BMAA misincorporation into proteins in place of L-serine

Misincorporation of BMAA into proteins and protein misfolding have been suggested as a mechanism of toxicity that could contribute to the development of neurodegenerative disease. BMAA has been proposed to misincorporate into proteins in place of the protein amino acid L-serine due to its similarity in structure. This was based mainly on competition studies that showed that levels of radiolabelled BMAA in proteins decreased in the presence of excess L-serine (Dunlop et al., 2013). Misincorporation of NPAAs are known to lead to the formation of abnormal aggregate-prone proteins which is a hallmark of neurodegenerative diseases (Dunlop et al., 2013). Even low misincorporation rates of 1 per 10,000 amino acids can cause the development of neurodegeneration in mice (Lee et al., 2006). In addition, BMAA treatment of cells *in vitro* induced proteotoxic stress and was prevented by co-incubation with L-serine (Main et al., 2016). This supported the hypothesis that L-serine's protective effects were related to an ability to outcompete BMAA for incorporation into proteins. Although this observation demonstrates an interaction between BMAA and L-serine, other

mechanisms could be involved. For example, biosynthetic pathways generally exhibit regulation through negative feedback of the product on the first step of the synthetic pathway (Monod et al., 1963). Whether BMAA could cause inhibition of L-serine biosynthesis is not known. It is worth noting that in the vervet study L-serine was protective in BMAA-treated animals and significantly reduced NFTs and amyloid plaques but did not reduce the amount of BMAA in proteins in the vervet brain (Cox et al., 2016).

1.5 Importance of L-serine in biological processes

L-serine is derived from four possible sources; dietary intake, biosynthesis, recycling of proteins and phospholipids, and from glycine. The main source from which L-serine is derived will differ depending on the type of tissue (de Koning et al., 2003). However, *de novo* biosynthesis of L-serine is crucial in maintaining whole-body homeostasis as therapeutic studies have demonstrated that dietary L-serine alone was insufficient to supplement those with L-serine deficiency syndromes (Tabatabaie et al., 2010). The phosphorylated pathway is the primary route by which L-serine is biosynthesised. It is linked to the glycolytic pathway in which the breakdown of glucose to pyruvate occurs in a 10-step process. An intermediate in the glycolytic pathway, 3-phospho-D-glycerate (3P-glycerate or 3PG), is a substrate for L-serine biosynthesis (Figure 3). The glycolytic intermediate 3PG is converted into L-serine by the action of three enzymes; 3-phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1) and phosphoserine phosphatase (PSPH) (Tabatabaie et al., 2010) (Figure 3). Unlike most negative feedback inhibition pathways where the first step is the rate-limiting step, in L-serine biosynthesis, the pathway that is regulated is the last step, and thus it is controlled by cellular demand for the product (de Koning et al., 2003). L-serine can then be used to synthesise L-glycine and L-cysteine, both of which are precursors in the formation of the antioxidant radical scavenger, glutathione (Amelio et al., 2014; Bannai & Kitamura, 1980). The L-serine, L-glycine and one-carbon network generate carbon units that satisfy many metabolic demands including nucleotide precursors (purines) for anabolic metabolism, redox maintenance and substrates for methylation reactions that shape the epigenetic landscape (de Koning et al., 2003). L-serine is required to synthesise membrane lipids such as phosphatidylserine and sphingolipids (Esaki et al., 2015).

L-serine has been demonstrated to alleviate oxidative stress by increasing glutathione synthesis by producing additional L-cysteine (Zhou et al., 2017). On the other hand, the L-serine 'mimic' BMAA has been reported to inhibit L-cystine (cysteine dimer) uptake leading to glutathione depletion and elevated oxidative stress (Liu et al., 2009; Schneider et al., 2020). The same author reported L-cystine uptake inhibition *in vitro* by 2,4-DAB at 1000 µM concentrations and 300 µM BMAA (Schneider et al., 2020). However, no study has investigated whether the effects of L-serine could protect against BMAA or 2,4-DAB-induced oxidative stress. A downregulation of L-serine production may modulate decreases in levels of other amino acids and antioxidant capacity.

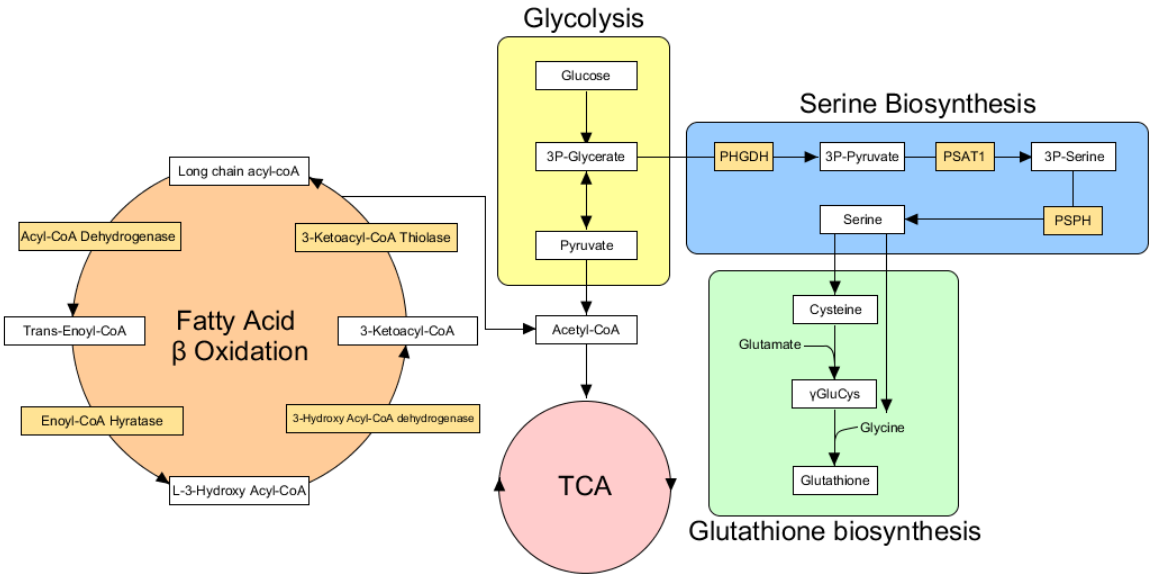

Figure 3 Energy metabolism in the cell: L-serine biosynthesis, glycolysis and glutathione biosynthesis interlinking pathways.

1.6 Energy-related metabolic disturbances in MND

Patients with MND are typically lean with a normal or low body mass index (BMI) (Ngo et al., 2014) with 50% of patients reported having increased resting energy expenditure and hypermetabolism (Bouteloup et al., 2009). Interestingly, MND patients with high BMI, dyslipidemia or type 2 diabetes mellitus may have slower clinical progression or delayed onset of symptoms (Jawaid et al., 2014). On the other hand, having low BMI, an active lifestyle and low cholesterol levels may have the opposite effect and increase risk or worsen prognosis (Jawaid et al., 2014). Indeed, clinical trials where patients were on a high-fat diet were shown to increase body fat and stabilise MND patients (Dorst et al.,

2013). Another trial found a high calorie/high carbohydrate diet significantly reduced mortality and slowed MND progression (Wills et al., 2014). The protective effects of these metabolic diseases on the progression of MND may be due to their pathogenic role in altering energy metabolism in cells.

Motor neurons are incredibly sensitive to energetic stress due to their low capacity to store energy and high energetic demand (Le Masson et al., 2014) and as a result, neurons are constantly producing ATP to meet their cellular demands. 90% of ATP in the CNS is generated in the mitochondria through the tricarboxylic acid cycle (TCA cycle, also known as the Krebs cycle or citric acid cycle) and uses glucose as the main energy source (Hyder et al., 2013). However, neurons must find a balance between providing sufficient ATP for energy demand and maintaining adequate levels of antioxidants, as glucose is used to produce glutathione through the pentose phosphate pathway (PPP) (Figure 3).

In MND patients, this delicate energy balance is disrupted; the energy expenditure of the cell exceeds that of energy uptake. Fibroblasts taken from sMND patients displayed a decreased ability to catabolise carbohydrates and down-regulation of two key glycolytic enzymes, phosphoglucomutase 2 like 1 and phosphoglycerate kinase (Raman et al., 2015). In times of energetic stress, neurons can upregulate glycolysis by redirecting glucose from the PPP and sacrificing antioxidant production (Rodriguez-Rodriguez et al., 2012). This may lead to the development of oxidative stress seen in post-mortem brain samples of fMND and sMND patients where motor neurons eventually die due to excessive oxidative stress (Ferrante et al., 1997). A mass spectrometry based metabolomics study found that 23 metabolites related to hypermetabolism, oxidative damage and mitochondrial dysfunction were increased in the plasma of MND patients (Lawton et al., 2012). The 23 metabolites include pyruvate and L-carnitine which have been explored as potential MND treatments.

Targeting metabolism has become a key strategy in treating MND. Examples of such include FDA-approved drugs Riluzole (Chowdhury et al., 2008), which improves glucose uptake in motor neurons, and Edavarone, which alleviates oxidative stress (Abe et al., 2017). Several prospective drugs tested clinically or on SOD1 mice have aimed to improve energy production through several mechanisms; providing alternate fuel

(pyruvate, ketone bodies or triglycerides), improving mitochondrial function (dichloroacetate or L-carnitine), or assisting with energy buffering and transport (creatine) (Vandoorne et al., 2018). Most of these metabolic treatments have multiple mechanisms of action which makes them ideal candidates.

1.7 Aims of the thesis and overview

Neurodegenerative diseases impose substantial medical and public health burdens on populations throughout the world (Larkindale et al., 2014). Their prevalence and incidence rise dramatically with age, and numbers will likely increase with lengthening life spans (Al-Chalabi et al., 2014). In the case of MND, diagnosis to death takes on average 4 years (Hu et al., 2009). This devastating disease is completely unexpected in 9 out of 10 patients as there is no family link (Byrne et al., 2011). Identifying factors in our environment or lifestyle that might precipitate this disease remains a great medical challenge. Exposure to algal blooms are increasing in line with the incidence of MND (Koreivienė et al., 2014). The epidemiological studies linking hot spots of MND to exposure to cyanotoxins are quite compelling and further investigation into BMAA and its isomers, currently known as 'emerging toxins,' are therefore important (Caller et al., 2009; Field et al., 2013; Masseret et al., 2013).

Only a handful of studies have investigated the combined toxicity effects of BMAA, 2,4-DAB and AEG, including a brief investigation of cell viability and ER stress marker expression in SH-SY5Y cells treated with different combinations of the three toxins (mentioned previously in section 1.4.1) (Main & Rodgers, 2018) and two studies conducted by Rubia Martin which used simplex axial mixture design on NSC-34 cells and Zebrafish (Martin et al., 2022; Martin et al., 2019). Interestingly in the NSC-34 cells, a combination of all three toxins, BMAA, 2,4-DAB and AEG resulted in the most significant drop in cell viability via MTS assay and a similar pattern is seen in caspase 3/7 activity (Martin et al., 2019). However, in a zebrafish model, 2,4-DAB was found to be the most potent toxin (only 50% viability) while BMAA and AEG decreased viability by 16% and 8% respectively (Martin et al., 2022). Shotgun proteomics of the 2,4-DAB-exposed zebrafish revealed several canonical pathways involved with cellular processes including gluconeogenesis and glycolysis pathways were found to be inhibited while the NOS signalling pathway was activated, suggesting 2,4-DAB-induced protein damage. MND-associated proteins SOD1 and ubiquilin 4 were significantly downregulated (Martin et al., 2022).

One of the keys to a better understanding of MND and its complications lies within the intricate metabolic balances that become disrupted under pathological stress. Thus, the goal of this thesis is to look at BMAA and its isomers specifically and how they may be involved in the development of MND from a metabolic perspective. In the literature, most proteomic and metabolic studies have only examined BMAA alone to identify its impact on cell metabolism and rarely in combination with its isomers (Beri et al., 2017; Frøyset et al., 2016).

Since the lowest observable adverse effect level (LOAEL) varies depending on the model used for toxicity assessment, in Chapter 2 of this thesis we identify the most toxic combination of BMAA and 2,4-DAB in the SH-SY5Y cell model using cell viability assays. We then use this combination to carry out proteomic analysis to investigate which pathways and proteins are impacted. AEG was initially considered for toxicity and proteomic assessments however, studies performed by our laboratory indicated that there was no toxicity in this particular model even in the highest concentration used (2 mM) or when in combination with the other two isomers. Thus, it was not used in subsequent studies and the focus was on BMAA and 2,4-DAB exclusively. Treatment conditions used were 48 hours as the standard time point as it has been shown protein aggregation occurs at this point (Dunlop et al., 2013) and it might allow us to identify changes that occur with chronic rather than acute exposure to these cyanotoxins. Chapter 2 further aims to investigate pathways that have been perturbed by BMAA and 2,4-DAB, focusing on the L-serine biosynthesis pathway as it was found to be a significantly impacted in the Ingenuity pathway analysis (IPA). The pathway is of particular interest as L-serine has been in the spotlight along with BMAA due to its protective effects against the NPAA. Chapter 2 has been submitted as a manuscript for publication and is presented in the formatted form.

In Chapter 3, the aims are to investigate and quantify key metabolites which may be perturbed by BMAA and 2,4-DAB including amino acid levels and levels of the antioxidant glutathione. There has been no research conducted on the impact of 2,4-DAB on the amino acid and antioxidant levels nor any findings on the combined effects of BMAA and 2,4-DAB. Amino acids are crucial building blocks required for protein synthesis as well as purine and pyrimidine formation for nucleotide synthesis, thus any

changes in amino acid levels may reflect on cell metabolism on a global level. Chapter 3 has been submitted as a manuscript for publication.

Cyanobacterial toxins BMAA and 2,4-DAB perturb the L-serine biosynthesis pathway in SH-SY5Y neuroblastoma cells: a proteomic study

Chapter Overview

The isomers of BMAA are often neglected and studies often investigate the mechanisms of toxicity individually. In this study, toxicity assays assessed the overall toxicity of BMAA and 2,4-DAB individually and in combination, with the most toxic combination then being used in proteomic investigations. IPA analysis revealed significant perturbations in pathways associated with energy metabolism and L-serine biosynthesis which were further investigated on the gene expression level. The first enzyme involved in L-serine biosynthesis was significantly decreased in expression in 2,4-DAB-treated and the combined-treated cells and was rescued when cells were exposed to the glycolytic metabolite, pyruvate.

Certificate of Authorship and Originality

This paper has been submitted for review. I certify that the work presented in this chapter has not been previously submitted as part of the requirements for a degree. I certify that I have carried out most of the experimental work and data analysis presented in this paper.

The other authors listed in the manuscript have contributed in the following way:

- Alessandro Castorina: Provided guidance on experiments.
- Mehdi Mirzaei: Carried out proteomic analysis on samples prepared at UTS.
- Kenneth J. Rodgers: Proofread and edited the manuscript, assisted in manuscript direction and provided concepts and guidance on the experiments.

Primary author: Lisa Pu

Signed: Production Note:
Signature removed prior to publication.

Dated: 29/06/2022

Cyanobacterial toxins BMAA and 2,4-DAB perturb the L-serine biosynthesis pathway in SH-SY5Y neuroblastoma cells: a proteomic study

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Highlights

- Cell toxicity was observed in SH-SY5Ys cells treated with a combination of 500 μM BMAA and 500 μM 2,4-DAB
- Following proteomic analysis, Ingenuity pathway analysis of BMAA plus 2,4-DAB-treated cells revealed significant enrichment in the L-serine biosynthesis pathway and pathways associated with energy production
- RT qPCR showed that the expression of L-serine biosynthesis enzyme PHGDH was decreased in 500 μM 2,4-DAB and further decreased in the combined treatment
- PHGDH expression levels were restored to normal levels with pre-treatment of cells with pyruvate

Abstract

The non-protein amino acid β-N-methylamino-L-alanine (BMAA) was linked to the high incidence of a complex neurodegenerative disorder reported on Guam in the 1940s which was proposed to have resulted from exposure to cyanobacterial toxins. More recently, exposure to cyanobacterial toxins has been linked to clusters of sporadic motor neurone disease worldwide. BMAA however, is only one of several isomers that are produced concurrently by cyanobacteria. The BMAA isomer L-2,4-diaminobutyric acid (2,4-DAB) has previously been demonstrated to cause excitotoxicity, lathyrism and secondary ammonia toxicity. In this study, we evaluated the combined toxicity of BMAA and 2,4-DAB in human neuroblastoma cells. Using an AlamarBlue assay, 2,4-DAB was found to be more cytotoxic than BMAA but BMAA increased the toxicity of 2,4-DAB. The most toxic combination examined was equimolar concentrations of BMAA (500μM) and 2,4-DAB (500μM) which decreased cell viability by 32.8%. We performed a proteomic investigation using Ingenuity Pathway Analysis (IPA) on cells treated with equimolar concentrations of BMAA and 2,4-DAB and found significant enrichment in the L-serine biosynthesis pathway and pathways associated with energy production. We then evaluated the effects of BMAA, 2,4-DAB individually and in combination on the enzymes of the L-serine biosynthesis pathway. We found a significant decrease in the expression and protein levels of 3-phosphoglycerate dehydrogenase (PHGDH), the first

enzyme in the pathway. PHGDH uses 3-phospho-D-glycerate (3PG) an intermediate in the glycolytic pathway as a substrate. Co-incubation of cells with L-serine restored expression levels of PHGDH and pre-treatment of cells with the glycolytic product, pyruvate prevented the decrease in expression of PHGDH. This is the first study to link BMAA and 2,4-DAB to the L-serine biosynthesis pathway and supports the view that L-serine is protective against these cyanobacterial toxins.

Keywords: β-methylamino-L-alanine; L-2,4-diaminobutyric acid; neurotoxins; motor neuron disease; amyotrophic lateral sclerosis; L-serine biosynthesis; pyruvate

1. Introduction

Cyanobacteria are a ubiquitous, ancient, photosynthetic bacteria found on every continent (Büdel et al., 2016), and capable of surviving in extreme environments (Cox et al., 2009; Sompong et al., 2005; Taton et al., 2006). Almost all cyanobacterial strains have been found to be capable of producing a wide range of compounds that are toxic to humans (Dittmann et al., 2013) including the non-protein amino acid β-methylamino-L-alanine (BMAA) and its isomers L-2,4-diaminobutyric acid (2,4-DAB) and N-(2-aminoethyl) glycine (AEG) (Cox et al., 2005). BMAA is often the least abundant isomer (Violi et al., 2019) and has been reported to cause oxidative stress (Liu et al., 2009), acute excitotoxicity (Weiss et al., 1989), and be misincorporated into proteins in place of L-serine (Dunlop et al., 2013) and cause ER stress (Main & Rodgers, 2018; Okle et al., 2013). 2,4-DAB is up to 10-fold more abundant than BMAA (Violi et al., 2019) with toxicity that includes excitotoxicity similar to BMAA (Weiss et al., 1989), laythrism (Ressler et al., 1961) and secondary ammonia toxicity in rats (O'Neal et al., 1968). AEG is the most abundant isomer and has been reported in some studies to be the most potent toxin, more so than BMAA as toxicity was reported at concentrations as low as 30 μM (Schneider et al., 2020). This appears to be very dependent on the assay or organism as other studies have reported that AEG is the least toxic (Main & Rodgers, 2018).

The connection between BMAA and neurodegenerative diseases was first made on the island of Guam in the 1940s (Garruto et al., 1981; Reed et al., 1966) where the incidence of a neurodegenerative disease with features of amyotrophic lateral sclerosis (ALS), Parkinson's disease and Alzheimer's was reported to be 50 to 100 times that of the global population (Kurland & Mulder, 1954). BMAA was hypothesised to be present in the brain of the native population following the consumption of cycad flour and flying foxes that fed on cycad seeds and were a local delicacy (Banack et al., 2006; Cox et al., 2003; Whiting, 1963). An early study in macaques showed that orally administered BMAA resulted in motor neuron dysfunction, supporting the proposed link between BMAA and neurological disease (Spencer et al., 1987). A more recent study involving vervets fed with BMAA (210 mg kg^{-1} d^{-1}) for 140 days identified an increase in neurofibrillary tangles (NFTs) and β-amyloid deposits in BMAA-dosed vervets which were reduced in abundance when the animals were co-fed with L-serine (Cox et al., 2016; Davis et al., 2020). L-serine was initially shown to reduce levels of radiolabelled BMAA in proteins

in vitro and to decrease the levels of autofluorescent bodies in BMAA-treated cells (Dunlop et al., 2013). L-serine was subsequently shown to protect against proteotoxic stress *in vitro* (Main et al., 2016) and to ameliorate BMAA-induced electrophysiological impairment in rats (Cai et al., 2018). In the vervet study, levels of BMAA in proteins did not change in the presence of L-serine (Cox et al., 2016) so while these studies have identified an interaction between BMAA and L-serine the mechanisms involved are not clear.

L-serine is classified as a conditionally-essential amino acid and can be derived from four sources: dietary intake, biosynthesis, recycling and from L-glycine (de Koning et al., 2003). However, *de novo* synthesis of L-serine accounts for 73% of all L-serine sources in humans (Kalhan & Hanson, 2012). It is the precursor to other amino acids such as L-glycine and L-cysteine which are both used in the formation of the antioxidant glutathione. Furthermore, L-serine is the dominant source of one-carbon groups for purine synthesis where the purines generated are further used in various methylation reactions (de Koning et al., 2003). L-serine can be taken up from the extracellular space by amino acid transporters or synthesised *de novo* via the phosphoserine pathway (Reid et al., 2018). 3-phosphoglycerate dehydrogenase (PHGDH) is the first enzyme in the phosphoserine pathway. PHGDH knock-out mice display severe brain malformation and die after embryonic day thirteen (Metcalf et al., 2018) highlighting the absolute necessity of L-serine, synthesized *de novo* for nervous system development and health. The neuroactive substance D-serine is produced from the isomerisation of L-serine in neurons (de Koning et al., 2003) and L-serine serves as a building block for phosphatidylserine and sphingolipids, both of which are important components of the plasma membrane (de Koning et al., 2003). Patients suffering from PHGDH deficiencies displayed several neuropathologies which were only modestly improved by L-serine supplementation, suggesting *de novo* synthesis of L-serine is required for proper neuro-function and development (Jaeken et al., 1996). Neurons, in particular, are largely dependent on the L-serine biosynthesis pathway as L-serine is not readily transported across the blood brain barrier, thus defects or downregulation of enzymes involved in the biosynthetic pathway would have pronounced detrimental effects on the brain (Smith et al., 1987).

Despite the fact that BMAA and its isomers 2,4-DAB and AEG are almost always found together in nature making human exposure to a single isomer unlikely, only a few studies

have investigated the toxicity of cyanotoxin mixtures (Main & Rodgers, 2018; Martin et al., 2022; Martin et al., 2019). While Main did not find a synergistic increase in toxicity in the combined treatments when compared to the single treatments, caspase-3 activity, lysosomal protease activity and ER stress markers were significantly increased in the BMAA and 2,4-DAB combination when compared to the single treatments (Main & Rodgers, 2018). A simplex axial mixture study performed by Martin using NSC-34 cells found that equimolar concentrations of BMAA, 2,4-DAB and AEG resulted in the highest decrease in cell viability and highest caspase-3/7 activity (Martin et al., 2019). A follow-up study using the same experimental design in zebrafish showed that 2,4-DAB resulted in the lowest viability, lower than any of the combinations with toxic load taken into account (Martin et al., 2022). In the present study, we aimed to look at the effects of BMAA, 2,4-DAB and combined on the proteomic level and further examined changes identified in the enzymes involved in the L-serine biosynthesis pathway using western blotting and RT qPCR. SH-SY5Y neuroblastoma cells were used as the model as it lacks active NMDA receptors and can be used to investigate the non-excitotoxic effects of BMAA and 2,4-DAB (Okle et al., 2013).

2. Materials & Methods

2.1 Reagents and chemicals

Dulbecco's Modified Eagle's Medium (DMEM) high glucose (D5796), DMEM high glucose with sodium pyruvate (D6546), Minimum Essential Medium Eagle (EMEM) (M2279) and BMAA were purchased from Sigma Chemical Co., St. Louis, MO. 2,4-DAB was purchased from Toronto Research Chemicals, Toronto, ON.

2.2 Cell culture

SH-SY5Y human neuroblastoma cells (American Tissue Culture Collection, catalogue number CRL-2266) were cultured as follows: cells were maintained in DMEM supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Australia origin, Sigma Chemical Co.) and 2 mM GlutaMAX (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C with 5% CO_2. Cells were maintained in 75 cm^2 flasks between passages

19 to 28 and then transferred to 96-well plates, 6-well plates, 25 cm^2 flasks or 175 cm^2 flasks for treatment. When being treated with BMAA and 2,4-DAB, EMEM media was used, which does not contain L-serine.

2.3 Cell viability of BMAA and 2,4-DAB treated SH-SY5Y cells

Cells were seeded at 30,000 cells/well in 96-well plates in DMEM and allowed to adhere overnight. Treatment was performed with four replicates of the following concentrations of BMAA and 2,4-DAB found in Table 1. Cells were treated for 48 hours before incubating with 10% AlamarBlue v/v (Thermo Fisher) for 2 hours at 37 °C, with 5% CO_2. Fluorescence was read at 570 nm for excitation and 585 nm for emission on a Tecan Infinite M1000 pro. Fluorescence was normalised to the amount of protein in each well as determined by the bicinchoninic acid (BCA) assay.

Table 1. Concentrations of BMAA and 2,4-DAB used in the cell viability assay.

	BMAA (µM)	**2,4-DAB (µM)**
Single treatments	125 250 500 1000	 125 250 500 1000
Combined treatments	50 100 500 500 1000	500 1000 500 50 100

2.4 Proteomic investigation of SH-SY5Y neuroblastoma cells exposed to equimolar concentrations of BMAA and 2,4-DAB

2.4.1 Sample preparation

SH-SY5Y cells were treated at 80% confluency in 175 cm^2 flasks for 48 hours with 500 µM BMAA plus 500 µM 2,4-DAB (n=3). Cells were harvested with 7 mL of TrypLE Express Enzyme (1x), no phenol red (Thermo Fisher Scientific) and then washed three times with warm PBS for 5 min per wash. The PBS wash was removed after centrifuging for 5 min at 1000 × *g* at room temperature. Cell pellets were snap frozen in liquid nitrogen and stored in the -80 °C freezer.

Cells were lysed in lysis buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 1 mM EDTA) with a protease and phosphatase inhibitor cocktail (10 µg/mL aprotinin, 10 µM leupeptin, 1 mM PMSF, 1 mM $NaVO_3$, 100 mM NaF, 1 mM Na_2MoO_4 and 10 mM $Na_4P_2O_7$). Samples were probe sonicated thrice at 50 Hz for 15 seconds with 20 seconds between each pulse. Insoluble matter was removed with centrifugation at 15,000 × *g*, 4 °C for 10 min. The supernatant was transferred into new tubes and proteins were reduced with 5 mM DTT for 15 min at room temperature and further alkylated with 10 mM iodoacetamide for 30 min at 4 °C shielded from light. The alkylation reaction was then quenched with an additional 5 mM DTT for 15 min, shielded from light.

Detergents and contaminants were removed using a chloroform-methanol precipitation protocol (Wessel & Flügge, 1984). The protein pellet was resuspended in 200 µL of 8 M Urea in 50 mM Tris (pH 8.8). A BCA protein assay (Pierce, Rockford, USA) was performed to determine the amount of protein needed for digestion. 150 µg of protein was used in a dual digestion process with Lys-C at a 1:100 enzyme to protein ratio overnight at room temperature and then with Trypsin at a 1:100 enzyme to protein ratio for at least 4 hours at 37 °C. Peptides were acidified with a final concentration of 1% trifluoroacetic acid (TFA) (pH 2 to 3) and purified using SDB-RPS 3M-Empore stage tips (Humphrey et al., 2018).

A 10plex TMT experiment was performed with some samples duplicated for balancing. The dried peptides were resuspended in 200 mM HEPES (pH 8.2) and peptide concentrations were measured with a MicroBCA protein assay kit (Thermo Scientific, Rockford, IL). TMT labelling was performed with 0.2 mg of 20 µL reagent with 70 µg of sample peptides. Labelling was performed at room temperature for an hour with occasional vortexing. The TMT reaction and reverse tyrosine labelling were stopped with 8 µL of 5% hydroxylamine per sample, followed by vortexing and incubation at room

temperature for 15 min. Samples were combined, dried down with vacuum centrifugation, fractionated with basic reverse phase (RP) isocratic step elution with RP spin columns (Pierce) and loaded onto RP cartridges. Elution was performed with 10 mM ammonium bicarbonate using 12 fractionation steps with the following acetonitrile (ACN) concentrations: 5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, 30, 40, 80% ACN. These fractions were then pooled into six subsets (fractions 1-7, 2-8, 3-9, 4-10, 5-11, 6-12) and were dried and desalted using SDB-RPS 3M-Empore stage tips.

2.4.2 Proteomic analysis on the mass spectrometry

Fractionated peptides were reconstituted in 30 µL of 0.1% formic acid (FA) and 10 µL of the samples were analysed on an Orbitrap Fusion Tribrid-MS (Thermo Scientific) equipped with a UHPLC Proxeon chromatography system. The peptides were separated with a gradient from 6-30% ACN with 0.125% FA at a flow rate of 400 nl/min for 3 hours. Each survey full scan (400-1400 m/z) was acquired in the Orbitrap (with 120,000 resolution, at 400 m/z, AGC of 2 $x10^5$). MS3 fragmentation was performed using higher-energy collisional dissociation (HCD) with 55% collision energy and reporter ion detection of 150,000 ions with AGC, 60,000 resolution and maximum ion accumulation time of 150 ms in the Orbitrap. Peptide fragmentation and reporter ion spectra collection were performed using the synchronous precursor selection method (McAlister et al., 2014). In this method, the 10 most intense ions were isolated and MS2 analysis was performed using CID fragmentation with the following settings: normalised collision energy of 35%, 4 $x10^3$ AGC, 0.5 Da isolation window and maximum ion accumulation time of 150 ms with 40 seconds of dynamic exclusion. Post MS2 scan, precursor isolation was performed for MS3 analysis using a 2.5 Da window and fragmented in the ion trap with the same CID settings as above, except with an AGC setting of 8,000. Multiple fragment ions (or sequential precursor selection (SPS) ions) were isolated and then fragmented by HCD with a normalised collision energy of 37.5%. Fragmented ions were selected based on the previous MS2 scan. MS2-MS3 was conducted using the SPS methodology (McAlister et al., 2014).

The raw data files were converted to mzxml format and erroneous charge states and monoisotopic m/z values were corrected using a published method (Huttlin et al., 2015). MS/MS spectra were assigned sequences using a Sequest algorithm (Eng et al., 1994) with searches performed against the human Uniprot database with reverse sequences

filtered out. Data searches were performed using cysteine carbamidomethylation and TMT on the N-terminal of peptides, lysine residues as static modifications and oxidation of methionine as dynamic modifications (20 ppm precursor ion tolerance, 0.8 Da fragment ion tolerance for CID). Sequest matches were filtered using linear discriminant analysis applied to a false discovery rate (FDR) of 1% at the peptide level based on matches to reversed sequences, as reported above (Eng et al., 1994). Quantification of peptides using TMT reporter ions was conducted with an established method (McAlister et al., 2012). Proteins were regarded as changing in abundance based on a two-sample t-test $p<0.05$ and fold change threshold of >1.3 for increased abundance and <0.77 for decreased abundance. Pathway enrichment analysis was carried out on proteins significantly changing in abundance using Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com). Identified proteins were matched to corresponding genes using the Ingenuity Pathway Knowledge base (IPKB) with interaction networks ($p>0.05$) and molecular and cellular ontology based on known protein-protein interactions in published literature (curated knowledge base). Networks were identified on the most common functional groups present. Canonical pathway analysis was used to identify function-specific genes that are significantly present within the networks.

2.5 RT qPCR with L-serine biosynthesis enzyme expression levels

SH-SY5Y cells were maintained in DMEM, or DMEM with 0.11 g/L sodium pyruvate supplementation before treatment. Cells were treated at 70% confluency in triplicate 25 cm^2 flasks for 48 hours with 500 µM BMAA, 500 µM 2,4-DAB and 500 µM BMAA plus 500 µM 2,4-DAB in EMEM. After treatment, cells were harvested with 2 mL per flask of TrypLE. Cell pellets were washed three times with PBS. During the final wash, each sample was aliquoted in half and stored at -80 °C for western blotting while the other half proceeded to RT qPCR. The PBS was removed after centrifuging for 5 min at 900 × *g*, room temperature.

RNA was isolated using TRI reagent from Sigma as per the manufacturer's guidelines. RNA quality was checked using the Nanodrop 1000 (Thermo Fisher) and with a bleach gel (Aranda et al., 2012) before proceeding to cDNA synthesis. 1 µg of RNA was reversed transcribed using the Tetro cDNA synthesis kit from Bioline. RT qPCR was performed

using the Bio-Rad CFX96 Real-Time System coupled with a C1000 Thermal Cycler. 15 ng of cDNA was used with the SensiFAST™ SYBR® No-ROX kit (Bioline) and duplicate technical replicates were averaged during statistical analysis. The experiment was repeated three times for a total of n=9.

Custom primers were designed using NCBI Primer-BLAST, MFEprimer 3.0 and Oligo analyser and synthesised by Sigma (Table 2). Post-run analysis was performed using Bio-Rad CFX Manager and fold change was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) and normalised to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). A melt curve analysis was performed to ensure there was no RNA degradation and to check for primer specificity.

Table 2. Primers PHGDH, phosphoserine aminotransferase 1 (PSAT1), phosphoserine phosphatase (PSPH) and GAPDH were designed using NCBI Primer-BLAST with melting temperatures between 58 to 63 °C, primer size between 18 to 30 base pairs and primers spanning across two exons.

NCBI accession number	Gene name	Direction	Sequence	Melting temperature (Tm)
NM_006623.3	PHGDH	Forward	5'GGGATGAAGACTATAGGGTATGAC'3	61
		Reverse	5'CAAAGGTGTTGTCATTCAGCAAG'3	
NM_058179.4	PSAT1	Forward	5'AGGATTCTACGTTTGTCCAGT'3	60
		Reverse	5'TGTGACAGCATTATACAGAGAGG'3	
NM_004577.3	PSPH	Forward	5'AAATCTGTGGCGTTGAGGAC'3	61
		Reverse	5'ACTTACCAGCTCCCTTATGC'3	
NM_001289745.3	GAPDH	Forward	5'CAGCCTCAAGATCATCAGCA'3	61
		Reverse	5'TGTGGTCATGAGTCCTTCCA'3	

2.6 RT qPCR with L-serine treated SH-SY5Y cells

SH-SY5Y cells were seeded into 6-well plates at 280,000 cells/well in DMEM and left to adhere overnight. Treatment was performed with nine replicates of the following concentrations: 500 µM BMAA plus 50 µM L-serine, 500 µM 2,4-DAB plus 50 µM L-serine and 500 µM BMAA plus 500 µM 2,4-DAB plus 100 µM L-serine. After 48 hours of treatment, cells were washed thrice with PBS. 1 mL of TRI reagent was then added

and aspirated several times to ensure cell lysis before being transferred into tubes. RNA was isolated using TRI reagent from Sigma as per the manufacturer's guidelines. cDNA synthesis and RT qPCR were performed as previously described.

2.7 Western Blots for PHGDH protein

Samples were taken out of the -80 °C freezer to defrost. 25 mL of RIPA lysis buffer (Thermo Fisher Scientific) was mixed with half a tablet of protease inhibitor cocktail (Roche, Basel, Switzerland). 110 µL of the lysis buffer was added to each tube. Samples were probe sonicated twice on ice at 40% power for 30 sec using the Qsonica Q125 sonicator. The lysates were then subjugated to centrifugation at 10,000 × *g* for 10 min. 10 µL of the lysate was used for protein quantification (BCA assay). The remaining supernatants were stored at -20 °C until they were ready for western blotting.

Three parts of the sample were mixed with one part of Laemmli buffer 4X, heated at 85 °C for 5 min and centrifuged at 10,000 × *g* for 5 min to remove any insoluble proteins. A total of 45 µg protein was then loaded into a 10-well Novex™ WedgeWell 10-20% Tris-Glycine gel with 10 µL of SeeBlue® Plus2 Pre-Stained Protein Standard ladder loaded in the first lane. The gel was left to run at 225 V for 40 min before being wet transferred onto an Amersham™ Hybond ECL nitrocellulose 0.45 µm membrane (Amersham Biosciences, Buckinghamshire, UK) at 20 V for 1 hour. Membranes were immersed in Ponceau S (Sigma-Aldrich) for 5 min to check for transfer quality and blocked overnight with 5% non-fat milk in PBS-T at 4 °C with constant agitation. Primary monoclonal antibodies were diluted at 1:15,000 and 1:1,500 in PBS-T for beta-actin (Cat. No. BLR057F Abcam, Melbourne, Victoria) and PHGDH (Cat. No. 66350 Cell Signaling Technology, Danvers, MA) respective and membranes incubated for 1 hour. Membranes were then incubated in the secondary antibody goat anti-rabbit IgG peroxidase (Cat. No. A6154 Sigma-Aldrich) diluted 1:6000 in PBS-T for 1 hour and imaged with Clarity™ Western ECL Substrate (Bio-Rad, Hercules, CA) on an Amersham Imager 600. Band intensity was analysed with the software on the Amersham Imager 600 and the target protein was normalised to the housekeeping protein, beta-actin.

2.8 Bicinchoninic acid assay for protein normalisation

Following the AlamarBlue assay endpoint, the reagent was removed from the wells and the cells were washed three times with phosphate buffered saline (PBS). 50 µL of 0.02% Triton™ X-100 (Sigma-Aldrich, Castle Hill, NSW, Australia) was added to each well and the plate was freeze-thawed at -80 °C. A solution consisting of 4% (w/v) copper (II) sulphate ($CuSO_4$) and BCA solution purchased from Sigma-Aldrich was mixed in a 1:50 ratio and 100 µL was added to each well. The plate was covered and left to incubate for 30 min at room temperature before being read on the Tecan Infinite M1000 pro at an absorbance wavelength of 562 nm. Cell viability values were then normalised to the protein absorbance values.

Following on from the western blot protocol, 10 µL of sample lysate was added per well in a 96-well plate with triplicates. A 7-point calibration curve was made with bovine serum albumin (Cat. No. A9418 Sigma-Aldrich) at 25, 37.5, 50, 125, 250, 375, 500 and 1000 µg/mL. 10 µL of each standard was loaded per well. A solution consisting of 4% (w/v) $CuSO_4$ and BCA solution was mixed in a 1:50 ratio and 100 µL was added to each well. The plate was shielded from light and left to develop for 45 min at 37 °C and read on the Tecan Infinite M1000 pro at an absorbance wavelength of 562 nm.

2.9 Statistical analysis

Statistical analysis was performed using Prism version 8 (GraphPad software, CA, USA) using one-way ANOVA with Dunnett's multiple comparison post-test and RT qPCR fold change was calculated in Microsoft Excel 2016.

3. Results

3.1 Viability of SH-SY5Y cells was significantly reduced when cells were exposed to 2,4-DAB but exposure to BMAA resulted in no changes in viability

Cell viability was measured by the reduction of resazurin to resorufin in metabolically active cells (AlamarBlue cell viability reagent). Fluorescence changes were measured to provide optimal sensitivity. There was no significant change in viability with increasing concentrations of BMAA (Figure 1A). A significant decrease in cell viability was

observed with 2,4-DAB at the higher concentrations examined (Figure 1B): 500 µM ($P<0.001$) and 1000 µM ($P<0.0001$).

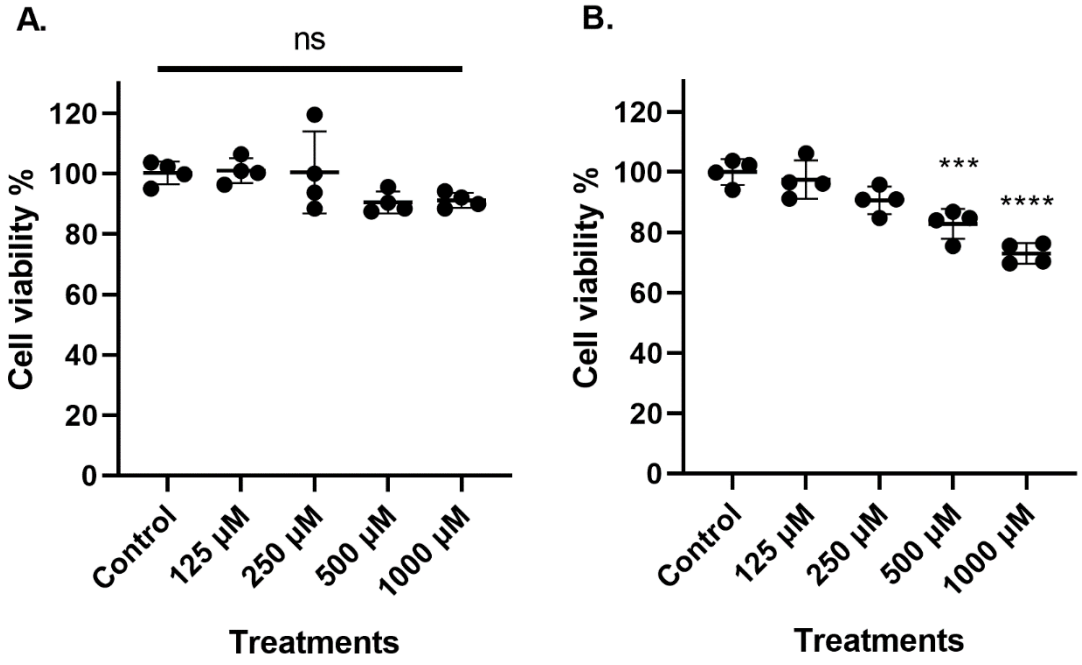

Figure 1. Cell viability of SH-SY5Y neuroblastoma cells treated with increasing concentrations of (A) BMAA and (B) 2,4-DAB for 48 hours in EMEM. Cell viability was measured against control cells (untreated) using the AlamarBlue assay and normalised to cellular protein. Statistical analysis using one-way ANOVA with Dunnett's multiple comparison test and plotted as mean ± SD. **P<0.01; ***P<0.001; ****P<0.0001; ns = non-significant (n=4).

3.2 Combined treatment of SH-SY5Y cells with BMAA and 2,4-DAB resulted in a significant decrease in cell viability

Cell viability was measured by monitoring fluorescence changes from the reduction of resazurin to resorufin in metabolically active cells using the AlamarBlue reagent. All the combinations of BMAA and 2,4-DAB examined significantly reduced cell viability relative to untreated cells (Figure 2). Equimolar concentrations of 500 µM BMAA and 500 µM 2,4-DAB resulted in the lowest cell viability ($P<0.0001$). Based on these data, equimolar concentrations of BMAA and 2,4-DAB were used in subsequent studies.

Despite not being toxic individually at concentrations of 500 µM BMAA and 50 µM 2,4-DAB when combined they resulted in a significant reduction in viability.

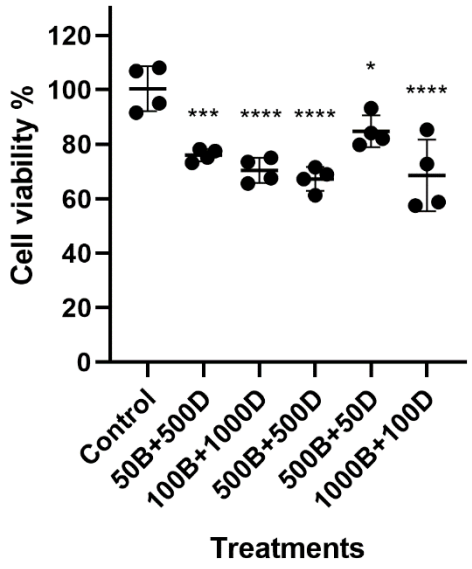

Figure 2. Cell viability of SH-SY5Y neuroblastoma cells co-treated with a range of concentrations of BMAA (B) and 2,4-DAB (D) for 48 hours in EMEM. Cell viability was measured against the untreated control cells using the AlamarBlue cell viability assay. Statistical analysis was carried out using one-way ANOVA with Dunnett's multiple comparison test and plotted as mean ± SD. *P<0.05; ***P<0.001; ****P<0.0001 (n=4).

3.3 Proteomic pathway analysis of SH-SY5Y neuroblastoma cells exposed to BMAA and 2,4-DAB

Approximately 3,500 proteins were identified in cells exposed to equimolar concentrations of BMAA (500 µM) and 2,4-DAB (500 µM) for 48 hours. Differentially abundant proteins were identified if they were statistically significant (student t-test $p<0.05$) and differed by at least ± 30% (FC > 1.3 or FC < 0.77). Out of 3,500 proteins, 276 proteins were significantly changing in abundance (Figure 3A). Heat map analysis of the differentially abundant proteins illustrates a consistent increase and decrease in abundance of the proteins within the control and treatment groups (Figure 3B). IPA canonical pathway analysis (Figure 3C) has found a significant enrichment of pathways involved with L-serine biosynthesis ($p=0.000071$) and energy production (fatty acid β-oxidation, $p=0.000086$, glycolysis, $p=0.002$). The L-serine biosynthesis enzymes were

further examined with RT qPCR and western blotting to validate the changes seen in the proteomic data. IPA analysis of biological functions (Figure 3D) identified an over-representation of proteins associated with neurological disorders, protein synthesis and degradation, mitochondrial dysfunction and cellular apoptosis.

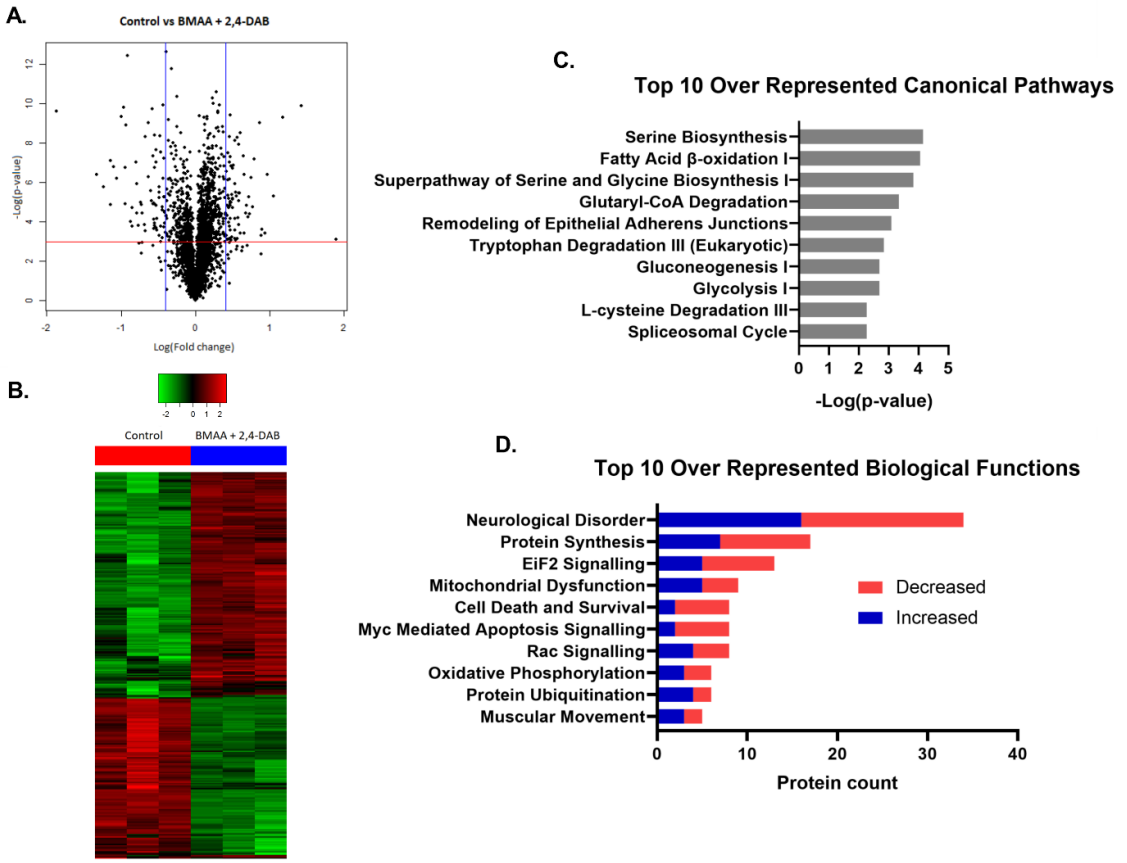

Figure 3. (A) Volcano plot representation of protein abundance and significance in 500 µM BMAA plus 500 µM 2,4-DAB exposed groups. Each dot represents a single quantified protein. An FC cut-off is applied at the 1.3 and 0.77 ratios (BMAA plus 2,4-DAB/control) and a significance cut-off is applied at $P<0.05$. (B) Heat map of the log-transformed ratios of differentially abundant proteins. Green to red colour scale indicates relative decreases or increases in protein abundance respectively. (C) Top 10 most enriched canonical pathways identified by IPA functional analysis of differentially abundant proteins. Full table available under Supplementary Table 1. (D) Top 10 most enriched disease and biological functions as identified by IPA.

3.4 Changes in expression of the enzymes involved in the L-serine biosynthesis pathway in cells incubated with BMAA and 2,4-DAB

Quantitative PCR was used to measure the expression of the three enzymes involved in the L-serine biosynthesis pathway. SH-SY5Y cells treated with 500 µM 2,4-DAB showed a significant decrease in PHGDH expression ($P<0.01$) but BMAA had no effect at this concentration (Figure 4A). Combined treatment of 500 µM BMAA with 500 µM 2,4-DAB resulted in a greater decrease in PHGDH expression ($P<0.0001$) (Figure 4A). There was no significant change in the expression of the other two enzymes in the pathway, PSAT1 and PSPH, under the conditions tested.

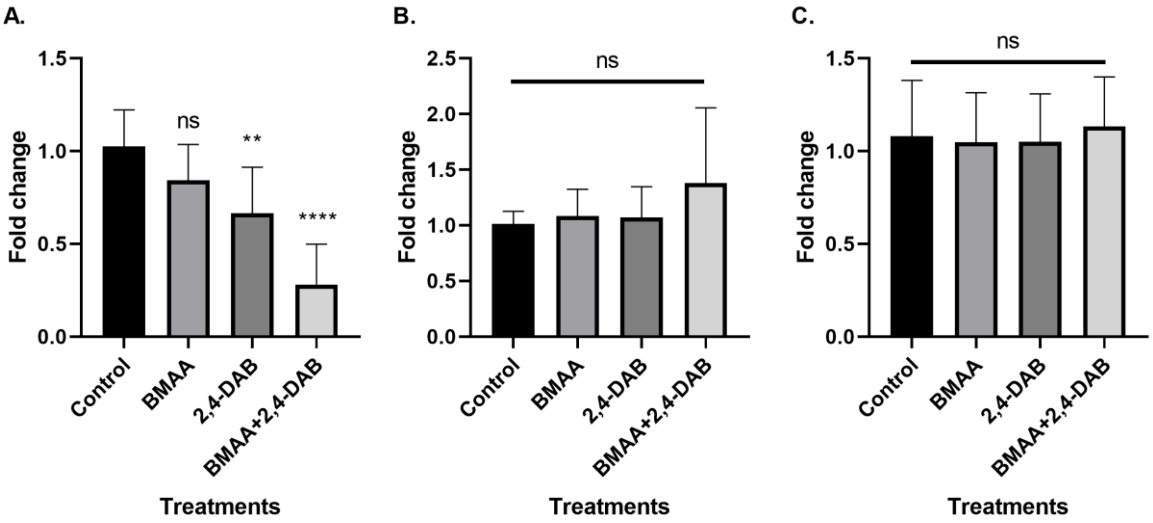

Figure 4. PHGDH (A), PSAT1 (B) and PSPH (C) expression in SH-SY5Y neuroblastoma cells treated in EMEM with 500 µM BMAA, 500 µM 2,4-DAB and 500 µM BMAA plus 500 µM 2,4-DAB for 48 hours. GAPDH was used as the housekeeping gene. The experiment was repeated three times for a total of n=9. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison test and plotted as mean ± SD. **P<0.01; ****P<0.0001; ns = non-significant.

3.5 Expression of PHGDH in SH-SY5Y cells treated with BMAA and 2,4-DAB is increased when the medium is supplemented with L-serine.

We then examined if the BMAA-induced decrease in expression of the enzyme PHGDH could be modulated by the addition of L-serine to the treatment media. When SH-SY5Y

cells were treated with 500 µM DAB or 500 µM BMAA plus 500 µM 2,4-DAB, the expression of PHGDH was significantly decreased by 35.2% and 72.6% respectively. The expression of PHGDH however, was restored to control levels in the presence of L-serine (50 µM and 100 µM respectively) (Figure 5A).

3.6 Effect of L-serine on the expression of PSPH in SH-SY5Y cells treated with BMAA and 2,4-DAB.

The final enzyme in the L-serine biosynthesis pathway (PSPH) is responsible for negative feedback in the presence of excess L-serine. In the control cells, the addition of L-serine resulted in a significant decrease in the level of expression of PSPH (Figure 5B). The presence of 500 µM BMAA, or 500 µM 2,4-DAB, or 500 µM BMAA and 500 µM 2,4-DAB had no impact on the decrease in expression of PSPH in the presence of L-serine.

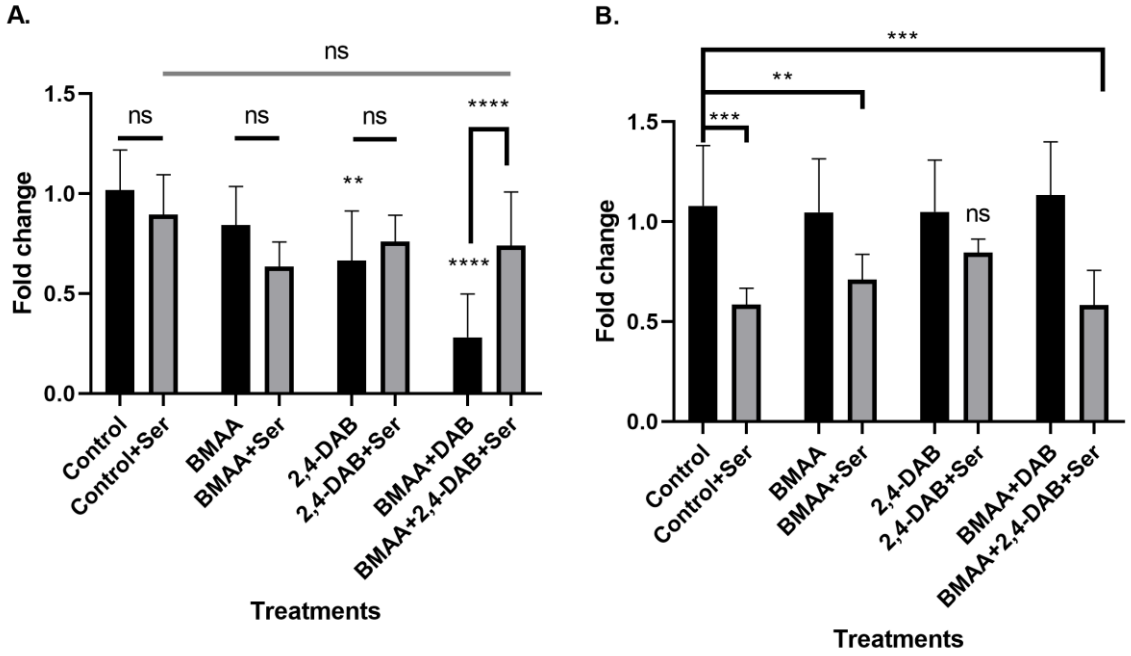

Figure 5. PHGDH (A) and PSPH (B) expression in SH-SY5Y neuroblastoma cells treated in EMEM for 48 hours with 500 µM BMAA plus 50 µM L-serine; 500 µM 2,4-DAB plus 50 µM L-serine; 500 µM BMAA plus 500 µM 2,4-DAB plus 100 µM L-serine compared to the same conditions without the presence of L-serine. GAPDH was used as the housekeeping gene. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Statistical analysis was performed using one-way ANOVA with Sidak's multiple comparison test and plotted as mean ± SD. **P<0.01; ***P<0.001;

****P<0.0001; ns = non-significant (n=9). Black significance represents significance relative to the control, grey significance represents significance relative to the control plus L-serine.

3.7 Pre-treatment of SH-SY5Y cells with pyruvate prevented the decrease in expression of PHGDH enzyme in the presence of 2,4-DAB and 2,4-DAB and BMAA

SH-SY5Y cells were cultured in DMEM supplemented with pyruvate (0.11 g/L) before treatment in EMEM media with BMAA and 2,4-DAB as described previously (Figure 4A). When cells were pre-treated with medium containing pyruvate they were protected against the decrease in expression of PHGDH in response to 2,4-DAB as well as BMAA plus 2,4-DAB (Figure 6).

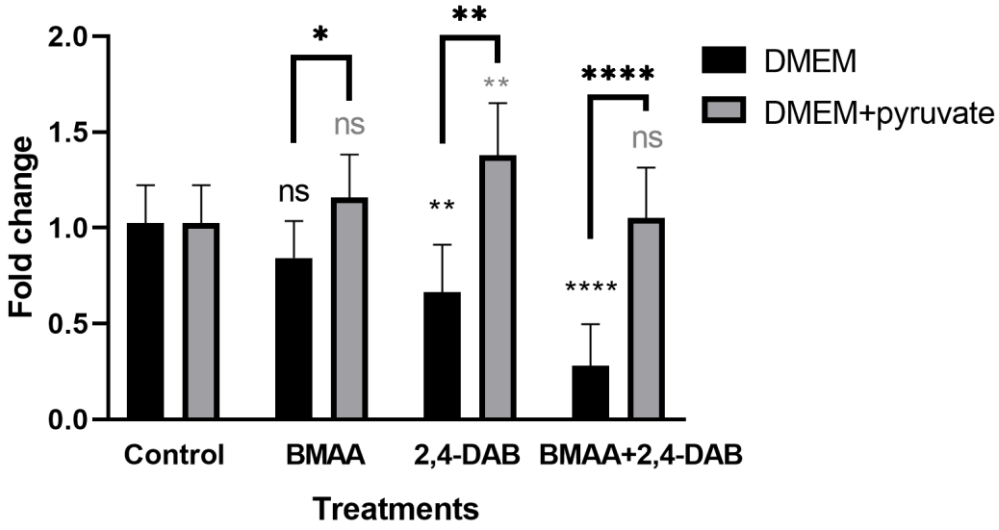

Figure 6. PHGDH expression in SH-SY5Y neuroblastoma cells cultured in DMEM and DMEM with sodium pyruvate prior to treatment in EMEM containing 500 µM BMAA, 500 µM 2,4-DAB and 500 µM BMAA plus 500 µM 2,4-DAB for 48 hours. GAPDH was used as the housekeeping gene. The experiment was repeated three times for a total of n=9. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Statistical analysis was performed using one-way ANOVA with Sidak's multiple comparison test and plotted as mean ± SD. *P<0.05; **P<0.01; ****P<0.0001; ns = non-significant. Black significance represents significance relative to the DMEM control, grey significance represents significance relative to the DMEM plus pyruvate control.

3.8 PHGDH protein levels by western blotting

PHGDH protein levels were significantly decreased by 63% in 500 µM BMAA plus 500 µM 2,4-DAB when compared to the untreated control (Figure 7B).

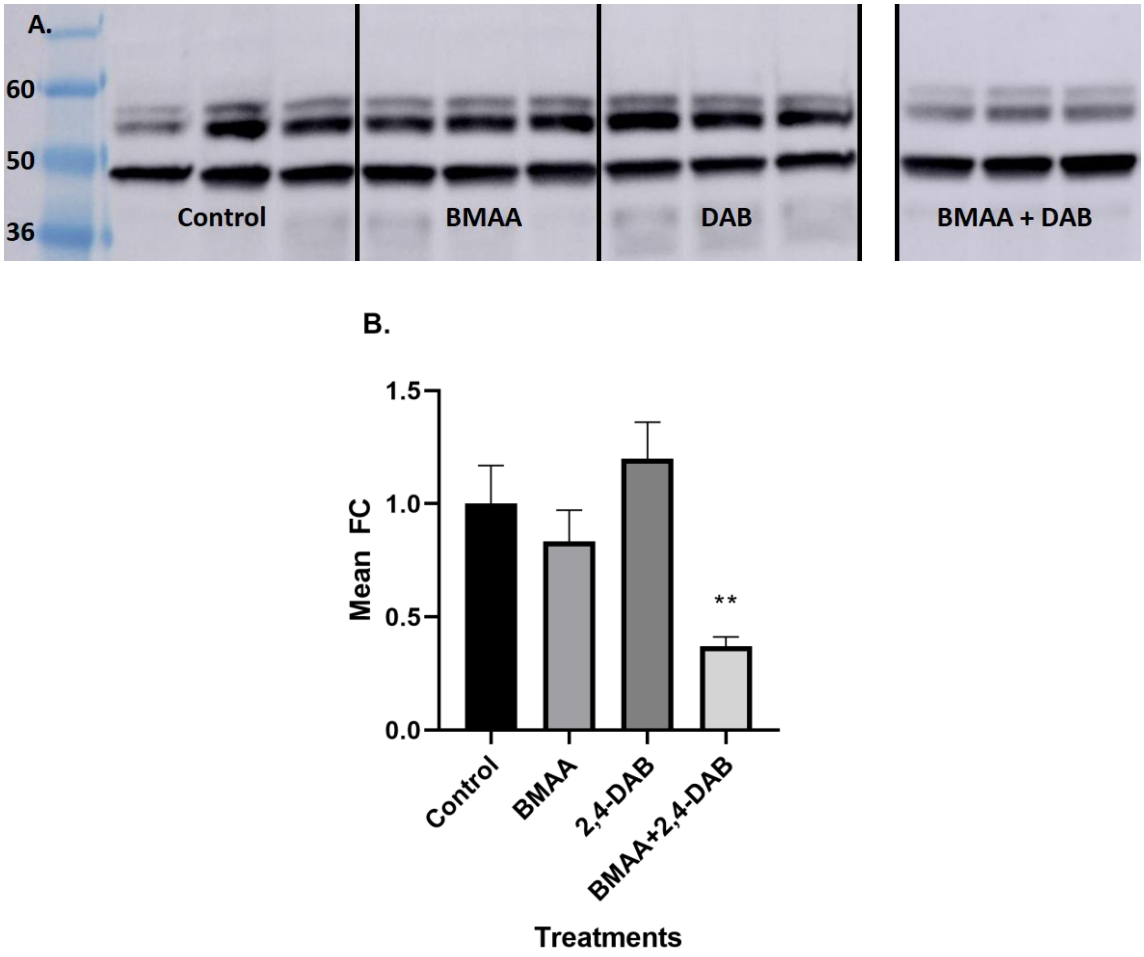

Figure 7. PHGDH protein levels in SH-SY5Y samples used for RT qPCR. Cells were treated in EMEM with 500 µM BMAA, 500 µM 2,4-DAB and 500 µM BMAA plus 500 µM 2,4-DAB for 48 hours. 45 µg of protein was probed with a PHGDH antibody. Figure 6A: A representative western blot of PHGDH (MW 57 kDA) and beta-actin (MW 41 kDA) on two separate 10-well blots. Figure 6B: Band density was calculated using Amersham Imager 600 software and normalised to beta-actin. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison test and plotted as mean ± SD. **P<0.01 (n=3).

4. Discussion

In this *in vitro* study, we found BMAA alone was not toxic to SH-SY5Y cells at any concentration up to 1000 μM (Figure 1A), 2,4-DAB however, caused a significant decrease in toxicity in a dose dependant manner above 500 μM (Figure 1B). Treatment with equimolar concentrations of BMAA and 2,4-DAB (500 μM) resulted in the lowest cell viability of 67.3% at 48 hours. Despite not being significantly toxic to the cells alone, BMAA increased the toxicity of 2,4-DAB, for example, 50 μM 2,4-DAB was not toxic but in combination with BMAA (500μM), there was a significant decrease in viability. In the proteomics analysis, caspase-3 (CASP3), which is used as an apoptotic marker, was significantly elevated in cells co-treated with BMAA and 2,4-DAB (FC=1.38, $p<0.001$). Since SH-SY5Y neuroblastoma cells lack NMDA receptors, the toxicity we observe here is not excitoxicity related (Okle et al., 2013). It is likely the mechanisms of toxicity differ between the two isomers based on *in vivo* data since 2,4-DAB has been shown to cause lathyrism and secondary ammonia toxicity in rats (O'Neal et al., 1968; Ressler et al., 1961) while BMAA is capable of causing protein misfolding, proteotoxic stress (Dunlop et al., 2013) and oxidative stress (Liu et al., 2009). However, they are both able to induce excitotoxicity by excessive stimulation of GABA receptors (Weiss et al., 1989). Our toxicity data is in agreement with the simplex axial mixture study on zebrafish larval viability where 2,4-DAB toxicity was greater than that of BMAA (Martin et al., 2022). Our data showed that BMAA was not toxic at concentrations up to 1000 μM, contrary to a previous report using the same cell line and viability assay which reported significant toxicity at 250 μM and above, however in the previous study AlamarBlue fluorescence was not normalised to cellular protein (Main & Rodgers, 2018). Normalising to cellular protein is carried out to control for differences in cell number in different wells in the cell culture plate but would exclude cells that had lysed during the culture period. Normalising to cellular protein would therefore give a more accurate estimate of the toxicity of the treatments at the 48 hours' time-point but would not account for cell death at earlier time points. Thus, the results portray a snapshot of overall cellular metabolic activity at this specific time-point in cells that are still viable. Another study using an MTT assay for toxicity reported no significant viability changes in SH-SY5Y cells treated up to 2000 μM BMAA for 48 hours (Okle et al., 2013), the assay used in the present

studies however (AlamarBlue) is a redox indicator that estimates viability from broad changes in enzyme activity in the cell (Rampersad, 2012).

Treatment with equimolar concentrations BMAA plus 2,4-DAB (500 μM) which resulted in the highest toxicity was used in subsequent proteomic studies. IPA analysis of the proteomic data revealed a disturbance in protein homeostasis in BMAA and 2,4-DAB-treated cells with an increase in abundance of proteins involved in ubiquitination and a decrease in proteins associated with protein synthesis (Figure 3D). We have previously shown BMAA has the ability to cause damage to proteins and increase ubiquitination in differentiated SH-SY5Y cells (Quinn et al., 2021). It was suggested that BMAA produces non-native proteins by being incorporated into the protein in place of L-serine resulting in aggregate-prone proteins potentially promoting neurofibrillary tangles or amyloid plaques as reported in primates exposed to BMAA (Cox et al., 2016). The proteomic data would support the view that BMAA and 2,4-DAB induce proteotoxic stress in cells and cells respond by decreasing protein synthesis rates.

Of particular interest in the proteomic study, given the link already established between BMAA and L-serine (Bradley et al., 2018; Cox et al., 2016; Dunlop et al., 2013), were changes in the L-serine biosynthesis pathway resulting from BMAA and 2,4-DAB treatment, so this was investigated further using western blotting and RT qPCR. Proteomic analysis revealed that the L-serine biosynthesis pathway was significantly enriched in cells exposed to BMAA and 2,4-DAB (Figure 3C) where the levels of PHGDH, the first enzyme in the biosynthesis pathway was significantly decreased to 91%. RT qPCR mRNA expression levels of the L-serine biosynthesis enzymes were consistent with the proteomic data on PHGDH levels; 2,4-DAB alone significantly reduced PHGDH expression and when combined with BMAA resulted in an even more profound decrease (Figure 4A). There were no significant changes in PSAT1 or PSPH at the level of gene expression (Figures 4B and 4C). BMAA and 2,4-DAB did not appear to allosterically inhibit the L-serine biosynthesis pathway since no changes were identified in the expression of the PSPH enzyme which is responsible for negative feedback inhibition in response to L-serine (de Koning et al., 2003). It was clear, however, that L-serine decreased the expression of PSPH and it was not impacted by the presence of BMAA or 2,4-DAB (Figure 5B). It would therefore seem unlikely that BMAA and 3,4-DAB would impact the L-serine biosynthesis pathway through a negative feedback effect.

Consistent with the RT qPCR data that showed a decrease in expression of PHGDH in cells exposed to BMAA and 2,4-DAB, western blotting revealed a significant decrease in PHGDH protein in the cells, which could result in a decrease in L-serine levels in cells and could impact a number of other pathways including the synthesis of purines via the folate-mediated one-carbon pathway as well as L-serine for the synthesis of sphingolipids and glutathione (de Koning et al., 2003). Interestingly, despite 2,4-DAB alone causing a decrease in the expression of PHGDH, there was no change in PHGDH protein in the cells at the same time point (48 hour) which could be due to cellular processes such as translational delays following an induced state change, availability of resources for protein biosynthesis or translation rate modulation (Liu et al., 2016). Interference with the L-serine biosynthesis pathway by BMAA and 2,4-DAB may have important downstream effects as it is known that PHGDH deficiencies have resulted in various neuropathologies in patients (Jaeken et al., 1996).

It has been proposed that BMAA competes with L-serine for protein incorporation and supplementation with L-serine having protective effects against BMAA toxicity (Davis et al., 2020; Dunlop et al., 2013). Supplementation of culture medium with L-serine fully restored PHGDH expression levels in BMAA and 2,4-DAB-treated cells when compared to the controls with the same L-serine supplementation but did not fully restore PHGDH expression levels to the original untreated control (Figure 5A) This may be similar to another study where patients with PHGDH deficiency mildly improved after L-serine supplementation and demonstrated that a defective L-serine biosynthesis pathway cannot be compensated for by exogenous L-serine when compared to control patients with no L-serine supplementation (Jaeken et al., 1996). L-serine supplementation did reduce the expression of PSPH in all treatments but no negative feedback from BMAA of 2,4-DAB was evident (Figure 5B).

Several energy production pathways were significantly enriched in the IPA analysis of BMAA and 2,4-DAB-treated cells; namely fatty acid β-oxidation, glycolysis pathways (Figure 3C) and oxidative phosphorylation (Figure 3D). Supplementation of the culture medium with pyruvate prior to treatment with BMAA and 2,4-DAB prevented the decrease in PHGDH expression observed when the basal medium was used (Figure 6). Pyruvate is the terminal metabolite of the glycolysis pathway which is linked to the L-serine biosynthesis pathway through the intermediate 3-phospho-D-glycerate (3PG)

(Tabatabaie et al., 2010). Pyruvate is used as an additive in tissue culture media for its antioxidant properties and as an additional energy supplement and might have contributed to the restoration of PHGDH expression in more than one way. It is possible the presence of additional pyruvate might have provided an additional substrate required for energy production via the TCA cycle potentially replacing a pyruvate deficiency caused by 3PG being re-directed into the L-serine biosynthesis pathway. Motor neurons have a low capacity to store energy, they are constantly producing ATP through the tricarboxylic acid (TCA) cycle to meet their high energetic demand (Le Masson et al., 2014). However, under energetic stress neurons sacrifice antioxidant production in favour of upregulating glycolysis by redirecting glucose from the pentose phosphate pathway (PPP) (Rodriguez-Rodriguez et al., 2012). The supplementation of pyruvate may have made additional substrates available for the TCA cycle to produce ATP whilst not sacrificing glucose required for the PPP or serine biosynthesis pathway. The glycolytic pathway has been previously shown to be inhibited in zebrafish embryos exposed to 500 μM of 2,4-DAB for 4 days (Martin et al., 2022). Pyruvate is one metabolite that has been considered for therapeutic use in ALS treatments but one on which very few studies have been conducted (Vandoorne et al., 2018). The metabolite, pyruvate, plays a multifaceted role in energy and antioxidant production and is capable of restoring PHGDH expression levels which were decreased by BMAA plus 2,4-DAB.

5. Conclusions

Our study provides evidence that 2,4-DAB induces more toxicity than BMAA and more so when they are combined. Proteomic analysis using IPA has shown enrichment in L-serine biosynthesis and energy production pathways of cells exposed to BMAA plus 2,4-DAB. PHGDH expression and protein levels were confirmed to be significantly decreased in both RT qPCR and western blots respectively. Lastly, we have shown supplementation with pyruvate prior to treatment was able to resolve the decrease in PHGDH expression. Our results highlight the importance of investigating BMAA and isomers and their effects on L-serine biosynthesis enzymes and energy metabolism in particular.

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6. Appendix A. Supplementary data

The following is Supplementary data to this article:

Proteomic data generated from BMAA plus 2,4-DAB-treated cells were analysed using IPA. The full table of significantly enriched canonical pathways can be found below.

Supplemental Table 1. All IPA significantly enriched canonical pathways.

Ingenuity Canonical Pathways	**-log (p-value)**	**zScore**	**Ratio**	**Molecules**
Serine Biosynthesis	4.15E00	NaN	4.00E-01	PSAT1,PHGDH
Fatty Acid β-oxidation I	4.06E00	NaN	9.38E-02	SCP2,HSD17B4,HADHA
Superpathway of Serine and Glycine Biosynthesis I	3.83E00	NaN	2.86E-01	PSAT1,PHGDH
Glutaryl-CoA Degradation	3.34E00	NaN	1.67E-01	HSD17B4,HADHA
Remodeling of Epithelial Adherens Junctions	3.09E00	NaN	4.41E-02	TUBG1,ZYX,ARPC3
Tryptophan Degradation III (Eukaryotic)	2.84E00	NaN	9.52E-02	HSD17B4,HADHA
Gluconeogenesis I	2.69E00	NaN	8.00E-02	ENO2,ALDOA
Glycolysis I	2.69E00	NaN	8.00E-02	ENO2,ALDOA
L-cysteine Degradation III	2.27E00	NaN	5.00E-01	GOT1,
Spliceosomal Cycle	2.27E00	NaN	5.00E-01	U2AF2,
Epithelial Adherens Junction Signaling	2.15E00	NaN	2.05E-02	TUBG1,ZYX,ARPC3
Aspartate Biosynthesis	2.09E00	NaN	3.33E-01	GOT1,
Glutamate Degradation II	2.09E00	NaN	3.33E-01	GOT1,
Tetrahydrobiopterin Biosynthesis I	2.09E00	NaN	3.33E-01	GCH1,
Tetrahydrobiopterin Biosynthesis II	2.09E00	NaN	3.33E-01	GCH1,
L-cysteine Degradation I	1.97E00	NaN	2.50E-01	GOT1,
Cell Cycle: G1/S Checkpoint Regulation	1.89E00	NaN	3.12E-02	RPL5,CDKN2C
Caveolar-mediated Endocytosis Signaling	1.81E00	NaN	2.82E-02	ARCN1,COPG2
Aspartate Degradation II	1.73E00	NaN	1.43E-01	GOT1,
Ketolysis	1.62E00	NaN	1.11E-01	HADHA,
Sucrose Degradation V (Mammalian)	1.62E00	NaN	1.11E-01	ALDOA,
Ketogenesis	1.58E00	NaN	1.00E-01	HADHA,

Bile Acid Biosynthesis, Neutral Pathway	1.46E00	NaN	7.69E-02	SCP2,
Mevalonate Pathway I	1.46E00	NaN	7.69E-02	HADHA,
Isoleucine Degradation I	1.43E00	NaN	7.14E-02	HADHA,
Phenylalanine Degradation IV (Mammalian, via Side Chain)	1.43E00	NaN	7.14E-02	GOT1,
γ-glutamyl Cycle	1.43E00	NaN	7.14E-02	GGCT,
Chondroitin Sulfate Degradation (Metazoa)	1.38E00	NaN	6.25E-02	GM2A,
phagosome maturation	1.38E00	NaN	1.67E-02	PRDX5,TUBG1
RhoA Signaling	1.37E00	NaN	1.64E-02	ARPC3,SEPT2
Dermatan Sulfate Degradation (Metazoa)	1.35E00	NaN	5.88E-02	GM2A,
Histamine Degradation	1.35E00	NaN	5.88E-02	ALDH1A3,
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	1.35E00	NaN	5.88E-02	HADHA,
Valine Degradation I	1.33E00	NaN	5.56E-02	HADHA,
Estrogen Receptor Signaling	1.33E00	NaN	1.56E-02	CTBP1,DDX5
Adipogenesis pathway	1.3E00	NaN	1.49E-02	CTBP1,RBBP7
Glutathione Redox Reactions I	1.3E00	NaN	5.26E-02	MGST3,
Oxidative Ethanol Degradation III	1.3E00	NaN	5.26E-02	ALDH1A3,
DNA Methylation and Transcriptional Repression Signaling	1.28E00	NaN	5.00E-02	RBBP7,
Fatty Acid α-oxidation	1.28E00	NaN	5.00E-02	ALDH1A3,
Putrescine Degradation III	1.26E00	NaN	4.76E-02	ALDH1A3,
Aryl Hydrocarbon Receptor Signaling	1.26E00	NaN	1.43E-02	ALDH1A3,MGST3
Ethanol Degradation IV	1.22E00	NaN	4.35E-02	ALDH1A3,
Tryptophan Degradation X (Mammalian, via Tryptamine)	1.22E00	NaN	4.35E-02	ALDH1A3,
Germ Cell-Sertoli Cell Junction Signaling	1.16E00	NaN	1.25E-02	TUBG1,ZYX
Dopamine Degradation	1.14E00	NaN	3.57E-02	ALDH1A3,
Superpathway of Cholesterol Biosynthesis	1.14E00	NaN	3.57E-02	HADHA,
Glutathione-mediated Detoxification	1.12E00	NaN	3.45E-02	MGST3,

Mitochondrial Dysfunction	1.11E00	NaN	1.17E-02	ATP5C1,PRDX5
Superpathway of Methionine Degradation	1.08E00	NaN	3.12E-02	GOT1,
Retinoate Biosynthesis I	1.07E00	NaN	3.03E-02	ALDH1A3,
EIF2 Signaling	1.06E00	NaN	1.09E-02	RPL4,RPL5
Ethanol Degradation II	1.05E00	NaN	2.86E-02	ALDH1A3,
Clathrin-mediated Endocytosis Signaling	1.05E00	NaN	1.08E-02	HSPA8,ARPC3
RAR Activation	1.03E00	NaN	1.05E-02	ALDH1A3,CRABP1
Estrogen Biosynthesis	1.01E00	NaN	2.63E-02	HSD17B4,
Noradrenaline and Adrenaline Degradation	1.01E00	NaN	2.63E-02	ALDH1A3,
tRNA Charging	1E00	NaN	2.56E-02	YARS,
Integrin Signaling	9.71E-01	NaN	9.66E-03	ZYX,ARPC3
Serotonin Receptor Signaling	9.52E-01	NaN	2.27E-02	GCH1,
LPS/IL-1 Mediated Inhibition of RXR Function	9.25E-01	NaN	9.05E-03	ALDH1A3,MGST3
Signaling by Rho Family GTPases	8.84E-01	NaN	8.55E-03	ARPC3,SEPT2
Unfolded protein response	8.68E-01	NaN	1.85E-02	HSPA8,
Actin Nucleation by ARP-WASP Complex	8.54E-01	NaN	1.79E-02	ARPC3,
Protein Ubiquitination Pathway	8.24E-01	NaN	7.84E-03	HSPA8,UBE2H
Retinoic acid Mediated Apoptosis Signaling	8.19E-01	NaN	1.64E-02	CRABP1,
Estrogen-Dependent Breast Cancer Signaling	8.06E-01	NaN	1.59E-02	HSD17B4,
Hypoxia Signaling in the Cardiovascular System	7.94E-01	NaN	1.54E-02	UBE2H,
Serotonin Degradation	7.82E-01	NaN	1.49E-02	ALDH1A3,
Xenobiotic Metabolism Signaling	7.78E-01	NaN	7.33E-03	ALDH1A3,MGST3
Ephrin B Signaling	7.48E-01	NaN	1.37E-02	CAP1,
Cyclins and Cell Cycle Regulation	7.22E-01	NaN	1.28E-02	CDKN2C,
Dopamine Receptor Signaling	7.22E-01	NaN	1.28E-02	GCH1,
Regulation of Actin-based Motility by Rho	6.62E-01	NaN	1.10E-02	ARPC3,
Chronic Myeloid Leukemia Signaling	6.54E-01	NaN	1.08E-02	CTBP1,

Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	6.54E-01	NaN	1.08E-02	ARPC3,
Glioma Signaling	6.34E-01	NaN	1.02E-02	CDKN2C,
Rac Signaling	6.12E-01	NaN	9.62E-03	ARPC3,
fMLP Signaling in Neutrophils	5.97E-01	NaN	9.26E-03	ARPC3,
Oxidative Phosphorylation	5.94E-01	NaN	9.17E-03	ATP5C1,
14-3-3-mediated Signaling	5.67E-01	NaN	8.55E-03	TUBG1,
CD28 Signaling in T Helper Cells	5.64E-01	NaN	8.47E-03	ARPC3,
HMGB1 Signaling	5.58E-01	NaN	8.33E-03	RBBP7,
Hereditary Breast Cancer Signaling	5.32E-01	NaN	7.75E-03	TUBG1,
eNOS Signaling	4.97E-01	NaN	7.04E-03	HSPA8,
Axonal Guidance Signaling	4.87E-01	NaN	4.61E-03	TUBG1,ARPC3
Aldosterone Signaling in Epithelial Cells	4.73E-01	NaN	6.58E-03	HSPA8,
Gap Junction Signaling	4.66E-01	NaN	6.45E-03	TUBG1,
Cdc42 Signaling	4.4E-01	NaN	5.99E-03	ARPC3,
Acute Phase Response Signaling	4.36E-01	NaN	5.92E-03	CRABP1,
RhoGDI Signaling	4.28E-01	NaN	5.78E-03	ARPC3,
Ephrin Receptor Signaling	4.26E-01	NaN	5.75E-03	ARPC3,
Sertoli Cell-Sertoli Cell Junction Signaling	4.18E-01	NaN	5.62E-03	TUBG1,
NRF2-mediated Oxidative Stress Response	4.14E-01	NaN	5.56E-03	MGST3,
Breast Cancer Regulation by Stathmin1	3.94E-01	NaN	5.24E-03	TUBG1,
Actin Cytoskeleton Signaling	3.54E-01	NaN	4.63E-03	ARPC3,
Huntington's Disease Signaling	3.36E-01	NaN	4.37E-03	HSPA8,
Glucocorticoid Receptor Signaling	2.8E-01	NaN	3.64E-03	HSPA8,
Molecular Mechanisms of Cancer	2.02E-01	NaN	2.74E-03	CDKN2C,

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Chapter 3: Changes to intracellular amino acid levels in SH-SY5Y cells exposed to the cyanotoxins BMAA and 2,4-DAB

Chapter Overview

The following chapter expands on the toxicity elucidated by the neurotoxins on a more metabolic level. Free amino acid and glutathione levels were quantified in cells exposed to BMAA, 2,4-DAB or the combination. There were significant increases in glutathione in the 2,4-DAB and combined treatment with greater markers for oxidation in the combined. One of the biggest changes was alanine levels being significantly decreased down to 40% of the control in the single treatments of BMAA and 2,4-DAB. This finding lends evidence to the neurotoxins' potential actions on alanine tRNA synthetases as suggested previously in the literature.

Certificate of Authorship and Originality

This paper has been submitted for review. I certify that the work presented in this chapter has not been previously submitted as part of the requirements for a degree. I certify that I have carried out all of the experimental work and data analysis presented in this paper.

The other authors listed in the manuscript have contributed in the following way:

- Jake P. Violi: Proofread the manuscript and provided guidance on experiments.
- Joel R. Steele and Matthew P. Padula: Proofread the manuscript and provided feedback on the manuscript.
- Kenneth J. Rodgers: Proofread and edited the manuscript, assisted in manuscript direction and provided concepts and guidance on the experiments.

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Changes to intracellular amino acid levels in SH-SY5Y cells exposed to the cyanotoxins BMAA and 2,4-DAB

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Abstract

The cyanotoxin β-methylamino-L-alanine (BMAA) is a non-protein amino acid (NPAA) that has been investigated for its potential role in the development of neurodegenerative diseases. Until recently, most studies have investigated the effects of BMAA in isolation, but it is almost always found in nature with its isomers. L-2,4-diaminobutyric acid (2,4-DAB) is a neurotoxic isomer of BMAA that is concurrently produced with BMAA by cyanobacteria. NPAAs are known to be capable of protein amino acid 'mimicry' either in protein synthesis or through other cellular metabolic pathways. The aim of this *in vitro* study was to investigate how BMAA and 2,4-DAB influenced intracellular amino acid levels. LC-MS/MS was used to quantify amino acid and glutathione levels. Cells were treated for 48 hours with BMAA (500µM), 2,4-DAB (500µM) and BMAA (500µM) plus 2,4-DAB (500µM). We identified two amino acids that changed in abundance in BMAA-treated cells, three in cells treated with 2,4-DAB alone and seven in cells treated with both BMAA and 2,4-DAB. There was evidence of oxidative stress in cells treated with BMAA and 2,4-DAB as indicated by elevated levels of glutathione and precursor amino acids (glutamic acid, glycine and serine) as well as changes in levels of amino acids sensitive to oxidative stress (proline). These data suggest that BMAA and 2,4-DAB impact significantly on amino acid homeostasis *in vitro*.

Keywords: β-methylamino-L-alanine; L-2,4-diaminobutyric acid; neurotoxins; motor neuron disease; amyotrophic lateral sclerosis; amino acids; glutathione

1. Introduction

Motor neurone disease (MND) or amyotrophic lateral sclerosis is a progressive degenerative disease characterised by the selective death of motor neurons in the brain and spinal cord (Ingre et al., 2015). In 90-95% of patients, the disease is sporadic, and the cause is unknown (Ingre et al., 2015). Genetic susceptibility, age, and exposure to environmental factors could each contribute to the risk of developing sporadic MND (Al-Chalabi et al., 2014; Al-Chalabi & Hardiman, 2013; Vucic et al., 2020). An understanding of the environmental contribution to sporadic MND is essential since it is the modifiable component of the overall risk. The discovery of a high incidence of a complex neurological disease on the island of Guam in the 1940s which had features of amyotrophic lateral sclerosis, Parkinson's disease and dementia (termed ALS/PDC) sparked interest in possible environmental causes (Kurland & Mulder, 1954). Guam had extensive cycad forests and cycad seeds were a traditional source of flour for tortillas (Banack & Cox, 2003). Analysis of the cycad seeds revealed that they contained a previously undiscovered amino acid known as 2-amino-3-(methylamino)-propanoic acid, now more commonly referred to as β-methylamino-L-alanine (BMAA) (Cox et al., 2003). BMAA was later found to bind to or associate with proteins and this might have contributed to its bioaccumulation through the food chain (Cox et al., 2003; Murch et al., 2004a) and its presence in the brain tissue of the deceased Guam residents (Murch et al., 2004b) . The actual source of BMAA was symbiotic cyanobacteria living in the roots of cycad plants (Vessey et al., 2005). Since then, BMAA has been detected in 97% of cyanobacteria worldwide (Cox et al., 2005), and in diatoms (Jiang et al., 2014) and dinoflagellates (Lage et al., 2014). Cyanobacteria are ubiquitous organisms that are capable of producing a wide array of toxins, indeed the residents of Guam would wash the cycad flour in flowing water for days to remove water-soluble acute toxins (Cox et al., 2003).

Since this discovery, BMAA has attracted attention as a possible neurotoxin capable of triggering sporadic MND in susceptible individuals. BMAA has a number of isomers including 2,4-diaminobutyric acid (2,4-DAB) and N-(2-aminoethyl) glycine (AEG) which are often neglected in MND research. These isomers are produced simultaneously by cyanobacteria and are often detected at a higher concentration than BMAA (Violi et al., 2019). It is within public health interests to study any adverse effects of these isomers

in conjunction with BMAA. Numerous studies have investigated the individual toxicity of the isomers with BMAA reported being potentially the most toxic. BMAA is found to cause oxidative stress at 3 mM (Liu et al., 2009), cause acute excitotoxicity by overstimulating the NMDA receptor (Weiss et al., 1989), misincorporate in place of serine in proteins (Dunlop et al., 2013) and result in elevated ER stress markers (Main & Rodgers, 2018). 2,4-DAB is not as thoroughly investigated as BMAA with its own toxicity profile that includes acute excitotoxicity similar to BMAA (Weiss et al., 1989), and is responsible for causing lathyrism and secondary ammonia toxicity in rats (O'Neal et al., 1968; Ressler et al., 1961). AEG is the most common naturally occurring isomer of BMAA and has been reported by some groups to be the most potent neurotoxin (Schneider et al., 2020). AEG was found to induce significant toxicity at only 30 μM and inhibit L-cystine uptake at 10 μM in primary mixed cortical cells (Schneider et al., 2020). However this would appear to be dependent on the toxicity model or assay, and some studies found AEG to be the least toxic even when in it was combined with other isomers (Main & Rodgers, 2018).

BMAA has been demonstrated to potentially misincorporate into proteins in place of the structurally similar amino acid L-serine resulting in protein misfolding and aggregation (Dunlop et al., 2013). This observation was based on *in vitro* studies using radiolabelled BMAA (Dunlop et al., 2013). BMAA is commonly found in both free and protein fractions in a range of samples. The 'protein fraction' however refers to molecules that are not soluble in 10% trichloroacetic acid (TCA) and so BMAA is often referred to as protein-associated, but it could also be associated with other macromolecules. Vervets fed with BMAA-dosed fruit for four months developed neurofibrillary tangles (NFT) and β-amyloid plaques which are hallmarks of neurodegenerative diseases (Cox et al., 2016). With L-serine supplementation, the formation of NFTs and plaques decreased significantly by 80-90%, levels of BMAA in the 'protein fraction' however were unchanged (Cox et al., 2016). L-serine is one of 20 canonical amino acids which are involved in many metabolic and biochemical processes in addition to protein synthesis (Boss & Erbe, 1982). The impact of BMAA's isomers on the metabolite level has not been performed as studies have only ever investigated BMAA itself (Engskog et al., 2017). The untargeted metabolomics study found differentiated SH-SY5Ys exposed to 250-1000 μM induced significant increases in alanine, aspartate and glutamate metabolism (Engskog et al., 2017). While differentiation of cells bares a closer

resemblance to neuron-like cells, it changes the phenotype of the cells, thus causing shifts in the metabolome including changes to oxidative stress response and mitochondrial metabolism (Schneider et al., 2011).

In this present study, we investigated the impact of BMAA, 2,4-DAB alone as well as their combined effect on intracellular amino acid levels in SH-SY5Y neuroblastoma cells after a 48 hour treatment period using a HILIC liquid chromatography triple quadrupole mass spectrometry (TQMS) method. In addition, we monitor levels of intracellular BMAA and the anti-oxidant glutathione.

2. Materials & Methods

2.1 Reagents and chemicals

Dulbecco's Modified Eagle's Medium (DMEM) high glucose (D5796), Minimum Essential Medium Eagle (EMEM) (M2279) and BMAA were purchased from Sigma Chemical Co., St. Louis, MO. 2,4-DAB was purchased from Toronto Research Chemicals, Toronto, ON.

2.2 Cell culture

SH-SY5Y human neuroblastoma cells (American Tissue Culture Collection, catalogue number CRL-2266) were cultured as follows: cells were maintained in DMEM supplemented media containing 10% heat-inactivated Foetal Bovine Serum (FBS) (Australia origin, Sigma Chemical Co.) and 2 mM GlutaMAX (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C with 5% CO_2. Cells were maintained in 75 cm^2 flasks and seeded at passage 24 into 12-well flasks for treatment. When being treated with BMAA and 2,4-DAB, DMEM media was replaced with L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, glycine, L-proline and L-serine deficient EMEM media.

2.3 Amino acids, glutathione and BMAA quantification

2.3.1 Amino acid standards

A standard mix was prepared from individual standards of 20 protein amino acids (L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine) all purchased from Sigma-Aldrich. Individual standards were made up in 20 mM HCl and 10 mM DTT at a concentration of 1000 µg/mL before being diluted and mixed to a final concentration of 10 µg/µL. The combined standard was aliquoted and stored at -20 °C until analysis. Glutathione and BMAA were made up in ultrapure water at a concentration of 1000 µg/mL, mixed to a final concentration of 10 µg/µL and stored at -80 °C and -20 °C respective until analysis. L-norvaline a non-protein amino acid, was made up to 50 µg/L (Sigma-Aldrich) and added to each amino acid standard mix to act as an internal standard (ISTD). The ISTD was further added to samples during preparation to allow us to account for sample loss during processing.

2.3.2 Sample preparation for LC-MS/MS analysis

SH-SY5Y cells were seeded at 150,000 cells/well in 12-well plates and allowed to adhere overnight. Treatment was performed in six replicates of the following concentrations: 500 µM BMAA, 500 µM DAB and 500 µM BMAA plus 500 µM DAB for 48 hours. Cells were then washed with 1 mL of PBS three times with the wash discarded. Norvaline ISTD at 50 µg/L was added per well and the wells were thoroughly scrapped with a cell scrapper before being transferred into Eppendorf tubes. An additional 400 µL of ultrapure water was added to each well and scrapped before being transferred to their respective tubes. Samples were then probe sonicated (Qsonica Q125 Sonicator) twice at 40% power for 30 seconds on ice. From each sample, 40 µL was taken to perform BCA protein quantification and analyte normalisation. TCA 100% w/v was added to each sample so that the final concentration was 10% w/v. After a brief vortex, the samples were centrifuged at 15,000 g, 4 °C for 15 min and the metabolite supernatant was transferred into new tubes. The pellet was discarded after being washed twice with ice-cold 10% TCA w/v with the subsequent washes added to their respective tubes with their supernatant. The metabolites were then freeze-dried for >48 hours at 0.1 mbar and -80 °C. The samples were reconstituted in 200 µL of 20 mM HCl and 10 mM DTT, briefly vortexed then centrifuged at 15,000 g at 4 °C for 15 min and stored at -80 °C until

analysis. Prior to LC-MS/MS analysis, the samples were diluted 1:10 with ACN to match the starting chromatographic conditions.

2.3.3 Protein quantification with bicinchoninic acid assay

Sample lysate was added to a 96-well plate in duplicates with 10 µL per well. A 7-point calibration curve was made with bovine serum albumin (Cat. No. A9418 Sigma-Aldrich) at 25, 37.5, 50, 125, 250, 375, 500 and 1000 µg/mL with 10 µL loaded into wells in triplicates. A solution consisting of 4% (w/v) $CuSO_4$ and BCA solution was mixed in a 1:50 ratio and 100 µL was added to each well. The plate was shielded from light and left to develop for 45 min at 37 °C and read on the Tecan Infinite M1000 pro at an absorbance wavelength of 562 nm.

2.3.4 Sample analysis by LC-MS/MS

Analyte quantification of the 20 protein amino acids, glutathione and BMAA were analysed on a Shimadzu Nexra X2 UHPLC coupled to a Shimadzu 8060 TQMS with a previously validated ACN adduct method of quantification (Violi et al., 2021). Liquid chromatography was performed using a Waters BEH Amide column (2.1 mm x 100 mm, 1.7 µm particle size). Solvent A consisted of 80 mM ammonium formate in ultrapure water plus 0.6% formic acid (FA) with solvent B consisting of ACN plus 0.6% FA. The analytes were chromatographically separated (Figure 1) and eluted via the following solvent B conditions: 0.00 min 90%, 3.50 min 90%, 5.50 min 80%, 9.25 min 80%, 9.30 min 70%, 11.20 min 70%, 11.20 min 90%, 14.00 min 90%. The flow rate was set to 0.8 mL/min with an injection volume of 5 µL and a column temperature of 30 °C.

The TQMS was operated in positive mode with electrospray ionisation (ESI). The operating settings for the TQMS were as follows: 0.1 kV interface voltage, 400°C interface temperature, 225°C desolation line (DL) temperature and 400°C heat block, 3 L/min nebulising gas flow, 17 L/min heating gas flow, and 3 L/min drying gas flow. Glutathione and BMAA multiple reactions monitoring (MRM) transitions were optimised to the most abundant transition while the MRMs for the 20 amino acids were acquired from a previous study (Violi et al., 2021) (Table 1). A dwell time of 20 ms was used for each transition.

Samples were run with a 9-point calibration curve (1 µg/L, 5 µg/L, 10 µg/L, 25 ug/L, 50 ug/L, 100 µg/L, 250 µg/L, 500 µg/L, and 1000 µg/L). The method and parameters have

previously been validated by Violi et al., 2021. Details on analyte linearity, LOD, LOQ, %RSD, MRM transitions and collision energy can be found in Supplementary data.

No carry-over was observed following injection of samples or standards. All samples and standards were run with triplicate injections and data analysis was performed using Shimadzu Labsolutions.

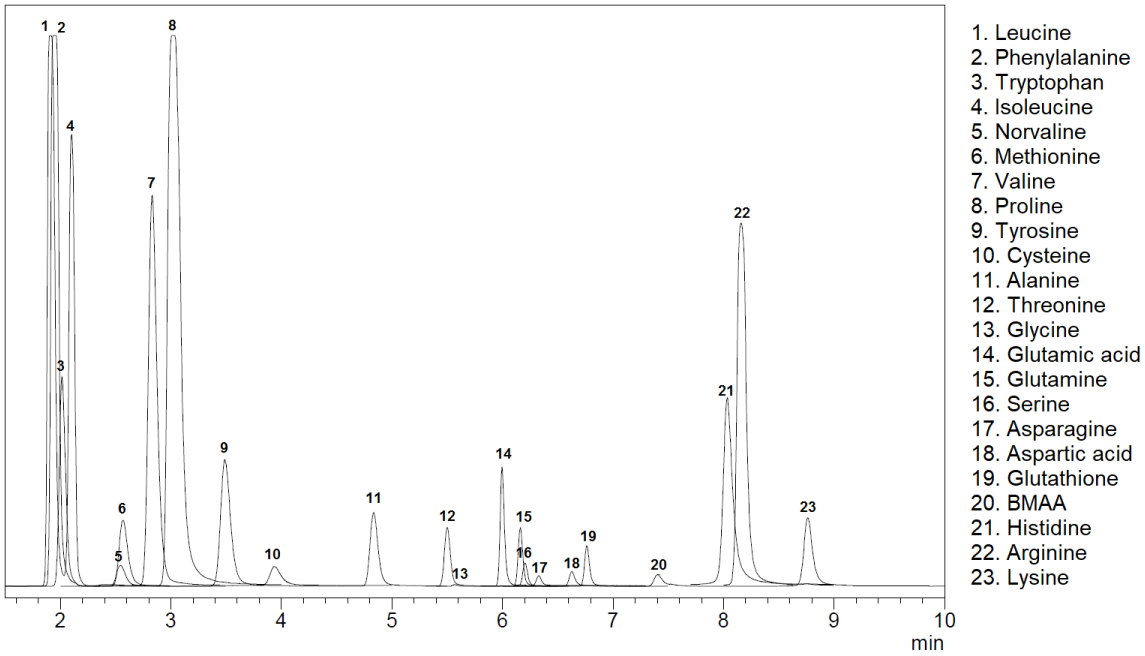

Figure 1. Total ion chromatogram (TIC) and retention times for the 22 analytes and the norvaline internal standard.

2.4 Statistical analysis

Statistical analysis was performed using Prism version 8 (GraphPad software, CA, USA) using one-way ANOVA with Dunnett's multiple comparison post-test with Grubb's test to remove outliers.

3. Results

3.1 Intracellular amino acid concentrations following BMAA and 2,4-DAB treatment

Amino acids were quantified in cell lysates using the validated method published by Violi (Violi et al., 2021). In this method, the adducts formed between the amino acids and

acetonitrile (ACN) can be used for quantification and can increase the detection sensitivity. BMAA and glutathione were added to the method, but for these analytes, there was no improvement in sensitivity by quantifying the adduct so their original protonated m/z transition values were used. Two normalisation points were utilised to ensure any variability was accounted for. L-norvaline was used as an internal standard to account for any sample loss during processing, and normalisation to the total protein concentration was performed to account for variation in cell numbers.

Following a 48 hour incubation with BMAA (500µM), 2,4-DAB (500µM) or the combined treatment, 12 of the 20 amino acids remained unchanged in all conditions relative to the control (Table 1).

In the BMAA-treated cells, only the intracellular concentrations of alanine and tyrosine differed from those in the untreated control cells. At 48 hours, levels of both amino acids had decreased significantly, with alanine levels at 38.7% of the control values and tyrosine levels at 57.1% of control values.

In 2,4-DAB-treated cells, there were significant changes in the abundance of three amino acids at 48 hours. Alanine levels were at 40.7% and tyrosine levels were at 72.3% of the control values. In contrast, aspartic acid levels had increased to 125.6% of control values.

In cells exposed to the combination of BMAA and 2,4-DAB, there was a significant increase in the intracellular concentrations of seven amino acids. Levels of alanine, aspartic acid, glutamic acid, glycine, proline, serine and threonine were all significantly increased with the greatest change being in glycine which was at 257.9% of control values. Despite both BMAA and 2,4 DAB causing a decrease in alanine concentrations, when cells were exposed to BMAA and 2,4-DAB together levels of alanine were 205.2% that of the untreated control cells. Similarly, despite treatment with both BMAA and 2,4 DAB alone resulting in significantly lower levels of tyrosine in cells, tyrosine levels were at control levels in cells exposed to BMAA and 2,4-DAB together.

Table 1. Intracellular amino acid levels as displayed as a percentage of change compared to the control. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 (n=6). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison post-test and Grubb's test and plotted as mean ± SD.

	BMAA	2,4-DAB	BMAA+2,4-DAB
Alanine	38.7 ± 3.0***	40.7 ± 3.4***	205.2 ± 9.5****
Arginine	96.6 ± 2.4	97.4 ± 2.3	116.2 ± 2.3
Asparagine	105.7 ± 2.6	116.9 ± 3.2	108.7 ± 1.9
Aspartic acid	118.9 ± 2.6	125.6 ± 3.8*	132.4 ± 2.9**
Cysteine	95.8 ± 2.4	90.6 ± 2.2	93.2 ± 1.3
Glutamic acid	102.8 ± 2.3	105.6 ± 3.0	130.6 ± 2.9**
Glutamine	102.6 ± 3.3	102.7 ± 3.4	107.0 ± 2.9
Glycine	58.6 ± 3.0	57.6 ± 2.8	257.9 ± 21.3**
Histidine	96.7 ± 2.4	89.5 ± 2.0	92.5 ± 1.2
Isoleucine	92.0 ± 3.0	95.1 ± 2.3	101.2 ± 2.5
Leucine	96.5 ± 2.6	98.6 ± 2.3	103.9 ± 2.0
Lysine	99.8 ± 2.4	98.4 ± 2.2	114.8 ± 1.8
Methionine	81.8 ± 3.4	100.8 ± 3.0	82.0 ± 4.2
Phenylalanine	97.3 ± 2.5	97.2 ± 2.6	96.3 ± 2.0
Proline	108.6 ± 2.3	112.7 ± 3.0	129.0 ± 2.4**
Serine	99.4 ± 3.7	121.0 ± 5.1	164.7 ± 5.5****
Threonine	95.5 ± 2.4	102.6 ± 2.7	130.3 ± 2.7**
Tryptophan	96.6 ± 3.6	97.0 ± 3.3	94.6 ± 1.8
Tyrosine	57.1 ± 2.4***	72.3 ± 4.4**	122.6 ± 5.2
Valine	88.3 ± 2.2	90.8 ± 2.2	99.5 ± 2.2

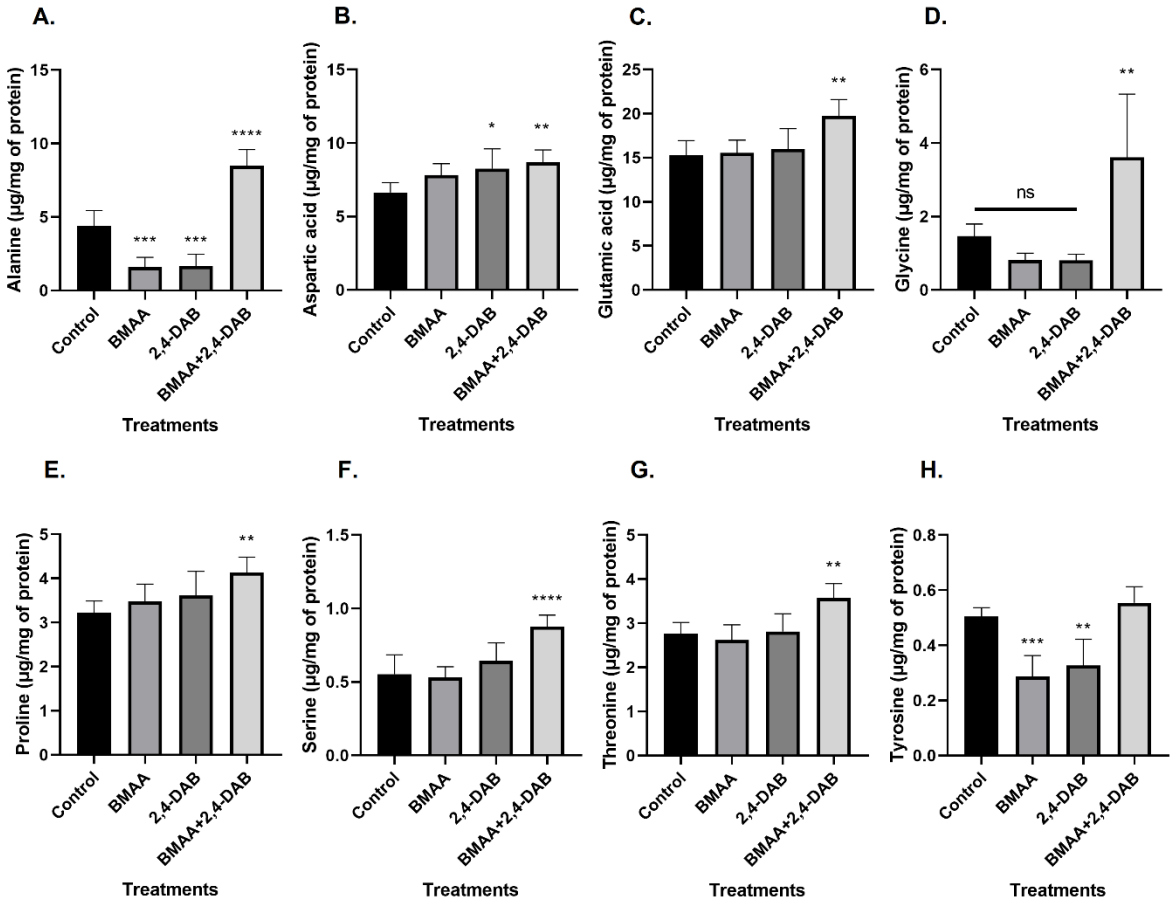

Figure 2. Intracellular (A) alanine, (B) aspartic acid, (C) glutamic acid, (D) glycine, (E) proline, (F) serine, (G) threonine and (H) tyrosine concentrations of all treatment conditions following 48 hour exposure to BMAA, 2,4-DAB and BMAA plus 2,4-DAB. Other amino acid concentrations can be found under supplementary. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison post-test and Grubb's test and plotted as mean ± SD. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 (n=6).

3.2 Glutathione concentrations following BMAA and 2,4-DAB treatment

Glycine and glutamic acid (along with cysteine) are substrates used in the formation of the anti-oxidant glutathione. Serine, however, can be used in the synthesis of cysteine, the third amino acid in the glutathione molecule. Since glycine, glutamic acid and serine were all significantly increased in cells exposed to BMAA and 2,4-DAB in combination we examined intracellular glutathione levels using the analysis method described previously. Only the reduced form of glutathione could be detected and not the oxidised

form since reducing agents (DTT) had been added into the samples for the analysis of amino acids, so the glutathione levels measured represent total glutathione in the cell. Glutathione levels in BMAA-treated cells were not significantly changed (112.3 ± 3.3%) but were significantly increased in 2,4-DAB-treated cells (133.8 ± 4.6%, $p<0.05$) and in cells treated with BMAA plus 2,4-DAB (168.8 ± 5.1%, $p<0.0001$) when compared the levels in the control cells.

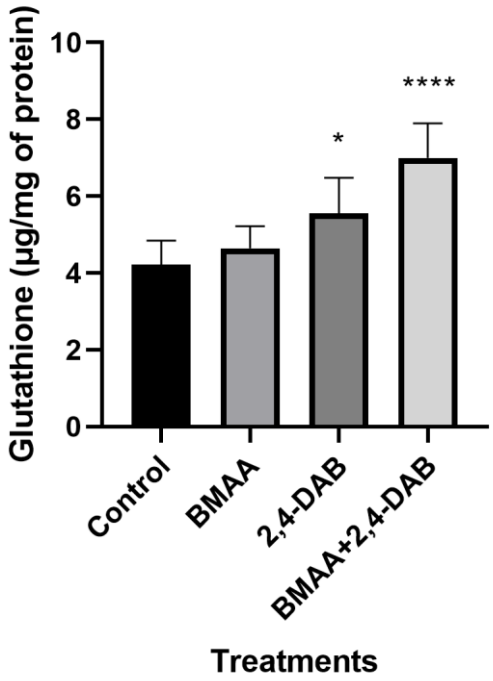

Figure 3. Quantification of total intracellular glutathione in SH-SY5Y cells after 48 hours. Cells were treated with medium only (control), BMAA (500µM), 2,4-DAB (500µM), and BMAA (500µM) plus 2,4-DAB (500µM) (n=6). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison post-test and Grubb's test and plotted as mean ± SD.

3.3 Intracellular BMAA concentrations in cells treated with BMAA and 2,4-DAB

No BMAA was detected in the control cells and cells treated with 2,4-DAB alone. Similar concentrations of BMAA were detected in BMAA-treated and BMAA plus 2,4-DAB-treated-cells (4.5 µg/mg and 4.3 µg/mg respectively) (Figure 4).

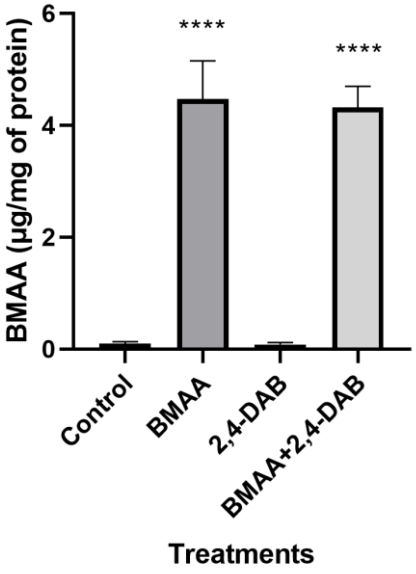

Figure 4. Quantification of intracellular BMAA in SH-SY5Y cells after 48 hours. Cells were treated with medium only (control), BMAA (500µM), 2,4-DAB (500µM), and BMAA (500µM) plus 2,4-DAB (500µM) (n=6). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison post-test with Grubb's test and plotted as mean ± SD.

4. Discussion

The amino acid L-type amino acid transporter 1 (LAT1) is functionally expressed in undifferentiated SH-SY5Y cells and has been shown to be at least partly responsible for BMAA uptake into this cell type (Okle et al., 2013). BMAA can also be transported into cells by the system Xc− in which glutamate is counter-transported into the extracellular space (Liu et al 2009, Albano and Lobner 2017). The intracellular concentration, and presumably uptake, of BMAA was not altered in the presence of its isomer 2,4-DAB in the culture medium (Figure 4). This observation is consistent with a study using primary rat cortical neurones that reported no difference in intracellular BMAA concentrations after 1 hour incubation with either BMAA or equimolar concentrations or BMAA and 2,4-DAB (Tan et al., 2018).

Total glutathione levels showed a small but significant increase in cells treated with 2,4-DAB-alone, but a much greater increase in cells treated with BMAA and 2,4-DAB (Figure 3) which coincided with a significant increase in the concentrations of glutamic

acid, glycine and serine levels in the cells (Figure 2C, 2D, 2F). Glutathione, a tripeptide consisting of cysteine, glycine and glutamic acid, is the major oxidant in the cell and can protect against singlet oxygen, superoxide and hydroxyl radicals (Pizzorno, 2014). It is present at around 5 mM and its synthesis is regulated by the cellular availability of cysteine (Pizzorno, 2014). Cysteine levels in the cell were unchanged (Supplementary Figure 1C) and this could reflect the abundance of extracellular cysteine present in the treatment medium, glutamate, serine and glycine on the other hand were not present in the culture medium and required *de novo* biosynthesis. *In vitro* studies have demonstrated a reduced dependence on *de novo* cysteine for the production of glutathione when levels in the culture medium are sufficient to support glutathione synthesis (Chen et al., 2020). The large increase in glycine levels could also be linked to the increase in both serine and threonine levels which can be converted to glycine through aldolase enzymes (Ogawa et al., 2000).

There was a significant increase in proline levels in the cells exposed to BMAA and 2,4-DAB (Table 1) and it has been suggested that the intracellular accumulation of proline is an adaptive response to oxidative stress (Krishnan et al., 2008). Proline is further oxidised to glutamate by mitochondrial enzymes (Kowaloff et al., 1977) which may be another contributor to the increase in glutamic acid (anionic form of glutamate) levels since both proline and glutamic acid were only significantly increased in the BMAA plus 2,4-DAB treated cells (Table 1).

Glycine, serine and threonine are amino acids required as one-carbon donors for purine synthesis through the coenzyme tetrahydrofolate while aspartate is used for pyrimidine synthesis (Zhang et al., 2008), thus changes in the abundance of these amino acids might reflect an impact of BMAA and 2,4-DAB on protein synthesis.

Tyrosine was the only amino acid that, despite being present in the culture medium and not requiring *de novo* synthesis, was lower in the treated cells than in the control cells. Both BMAA and 2,4-DAB individually caused a decrease in intracellular tyrosine concentrations (57.1% and 72.3% respectively), however, when cells were exposed to BMAA and 2,4-DAB together tyrosine levels remained at control levels (122.6%). Since tyrosine is derived from phenylalanine, a decrease in tyrosine levels would be expected to correspond to a decrease in phenylalanine levels upstream but phenylalanine levels were stable across all treatments.

Elevated glutamate levels may be toxic due to the over-excitation of glutamatergic NMDA receptors (Rush et al., 2012). BMAA has previously been suggested to drive glutamate release via the system Xc^- cysteine/glutamate antiporter (Liu et al., 2009). In the present study, elevated glutamic acid levels were only seen in the combined treatment with BMAA and 2,4-DAB. Glutamate neurotransmission can be further potentiated with the co-agonist glycine which was also elevated in the combined treatment (Johnson & Ascher, 1987). Furthermore, BMAA and 2,4-DAB are both capable of causing excitotoxicity through NMDA receptors (Weiss et al., 1989) due to the formation of the β-carbamate adduct which shares structural characteristics with glutamate, allowing it to interact with NMDA and other excitatory amino acid receptors (Myers & Nelson, 1990). While there has been no study that has investigated the formation of adducts by 2,4-DAB and bicarbonate, it is highly plausible as 2,4-DAB has similarly caused excitotoxicity in the presence of bicarbonate (Schneider et al., 2020; Weiss et al., 1989). It is noteworthy that SH-SY5Y neuroblastoma cells used in this study do not contain functional NMDA receptors and would therefore not be subjected to neuroexcitotoxicity (Okle et al., 2013) but the results here may contribute to the NMDA receptor activation seen in other studies (Schneider et al., 2020). BMAA and 2,4-DAB may contribute to acute excitotoxicity through the formation of β-carbamate adducts as well as elevating glutamate concentrations and elevated glycine potentiation of NMDA receptors

BMAA has been shown to charge alanyl-tRNA synthetase (Han et al., 2020) which could potentially reduce free alanine levels in the cell. Uncharged tRNA synthetases are known to regulate amino acids levels in cells (Yu et al., 2021) and NPAAs can interfere with this process. For example, the NPAA *meta*-tyrosine significantly decreased free phenylalanine levels in cells due to its ability to mischarge the phenylalanine tRNA synthetase (Zer et al., 2020). Interestingly when the toxins were combined, alanine concentrations were significantly increased. The conversion of excess aspartate to alanine through aspartate 4-decarboxylase could, however, be another source of alanine in the cells (Rathod & Fellman, 1985).

5. Conclusions

The culture medium used when treating cells with BMAA, 2,4-DAB and BMAA plus 2,4-DAB in combination, contained the essential amino acids, but was deficient in alanine, proline, asparagine, aspartic acid, glutamic acid, and serine. These 6 amino acids, therefore, required *de novo* synthesis, and of these amino acids, only the levels of asparagine did not change in at least one of the treatment groups suggesting that BMAA and 2,4-DAB have a profound impact on amino acid homeostasis. For the other amino acids, the culture medium would have provided a reservoir to allow intracellular levels to be maintained. This study examined amino acid levels at a single time-point and demonstrated that the NPAAs BMAA and 2,4-DAB modulated levels of some protein amino acids *in vitro*. Amino acid levels likely change over time as the cells attempts to maintain homeostasis and a time-course study should be performed to provide more information on the underlying mechanisms.

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6. Appendix A. Supplementary data

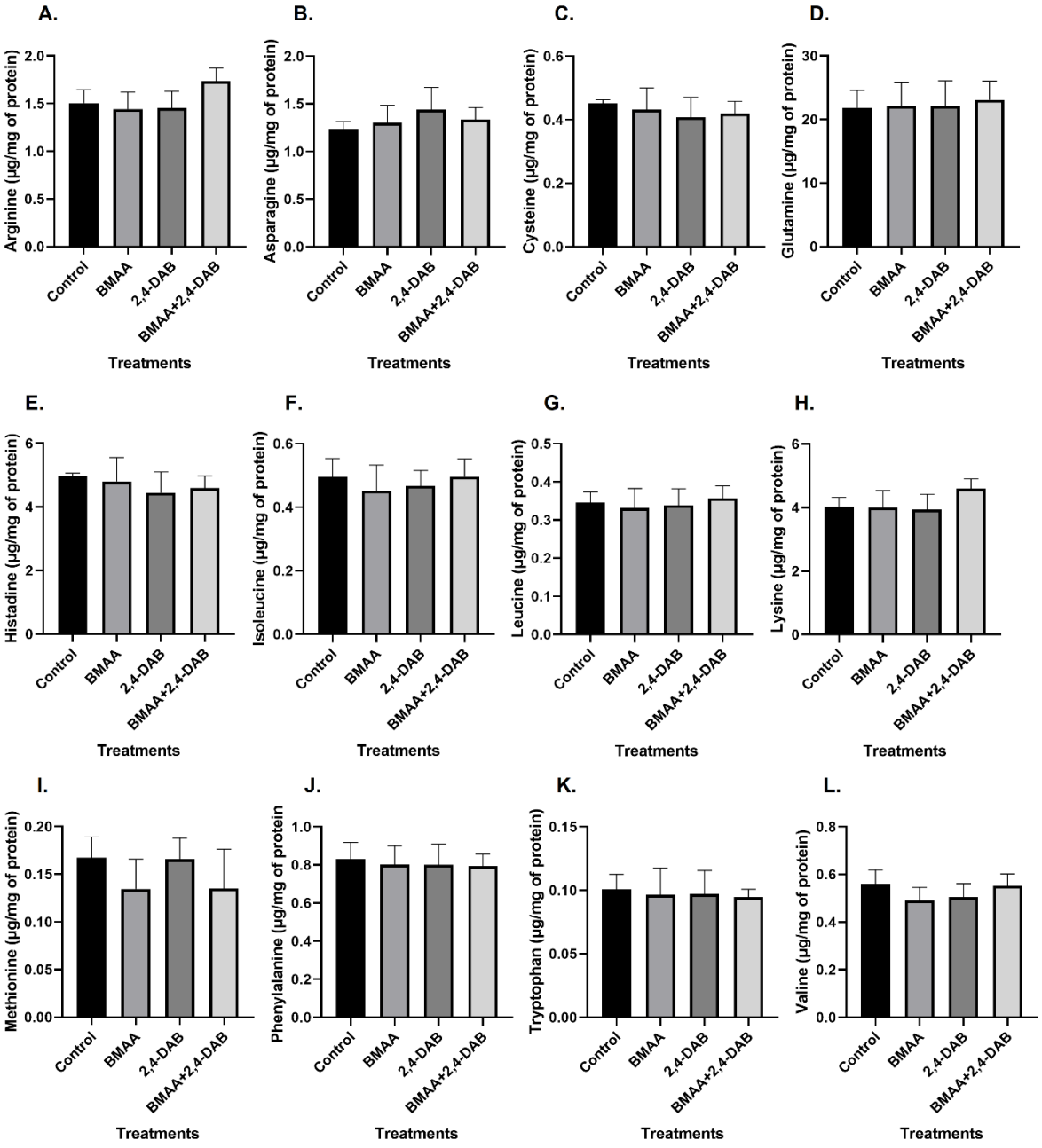
The following is Supplementary data to this article:

The amino acid adduct method has been previously validated by Violi et al., 2021 and expanded on with the addition of glutathione and BMAA to the method which uses the same conditions and parameters as the original method. Repeatability was determined via calculation of %RSD from 7 repeat injections of one point in the standard curve, with all metabolites having a % RSD values of <10% and regression values of R^2=0.99. LOD and LOQ were calculated with a signal to noise ratio of 3:1 and 10:1 respectively and can be found in Supplemental Table 1.

Supplemental Table 1. Linearity, LOD, LOQ, collision energy and MRM ion transitions for all metabolites, the internal standard and BMAA.

Analyte	Linearity (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Collision energy (eV)	MRM transition (m/z)
Alanine	0.10–1000	0.016	0.050	-29.4 -8.8	131.10 → 44.15 → 90.15
Arginine	10–750	1.9	5.7	-22 -12	175.20 → 70.15 → 60.15
Asparagine	5.0–750	0.73	2.2	-13.8 -9.8	174.10 → 87.15 → 133.10
Aspartic acid	5.0–1000	0.98	3.0	-22.1 -9.2	175.10 → 74.10 → 134.20
BMAA	5.0–1000	0.38	1.17	-17.00 -15.00	119.10 → 44.10 → 88.10
Cysteine	5.0–1000	0.58	1.8	-29.4 -8.8	163.10 → 59.25 → 122.15
Glutamic acid	5.0–750	1.5	4.5	-16 -21.9	148.10 → 84.10 189.10 → 84.10
Glutamine	5.0–750	0.47	1.4	-20 -14	147.10 → 84.10 → 130.10
Glutathione	25–1000	0.087	0.26	-13.7 -24.9	307.80 → 179.10 → 76.20

Glycine	10–750	3.1	9.5	-17.9 -7.2	117.20 → 76.20 → 30.15
Histidine	25–750	4.8	15	-15 -28	156.15 → 110.15 → 82.15
Isoleucine	0.10–250	0.030	0.091	-15.4 -8.8	173.20 → 86.25 → 132.20
Leucine	0.10–250	0.021	0.064	-15.4 -8.8	173.20 → 86.25 → 132.20
Lysine	10–750	2.9	8.7	-14 -30	147.20 → 67.10 → 84.15
Methionine	1.0–750	0.19	0.58	-30.3 -7.8	191.10 → 61.20 → 150.15
Norvaline	ISTD	0.02	0.06	-16.9 -8.2	159.10 → 72.05 → 118.20
Phenylalanine	0.10–250	0.033	0.098	-14.0 -8.7	207.10 → 120.15 → 166.20
Proline	0.10–500	0.016	0.050	-24.1 -9.8	157.00 → 70.10 → 116.20
Serine	1.0–750	0.11	0.32	-28.3 -17.6	147.15 → 60.15 → 42.15
Threonine	1.0–1000	0.19	0.58	-16.9 -9.2	161.10 → 74.20 → 120.20
Tryptophan	1.0–1000	0.18	0.56	-6.7 -22.9	246.10 → 72.05 → 118.20
Tyrosine	1.0–750	0.069	0.21	-35.1 -6.7	223.10 → 182.20 → 91.05
Valine	1.0–750	0.053	0.16	-16.9 -27.3	159.10 → 72.05 → 118.20



Supplemental Figure 1. Intracellular amino acid levels of all treatment conditions. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison post-test with Grubb's test and plotted as mean ± SD.

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Chapter 4: Concluding Remarks and Future Perspectives

It has been over 50 years since the identification of BMAA in cycad seeds (Vega & Bell, 1967). Since then, BMAA has been studied intermittently to evaluate its potential to cause neurodegenerative diseases. The hypothesis of how BMAA might impact neuronal function has been reshaped several times, with every growing piece of knowledge framing how the story flows. The origin of the story began on the island of Guam in the 1940s and was forgotten until almost 20 years later due to a lack of interest in the NPAA since it had failed to show neuropathology in neonatal rats (Polsky et al., 1972). Renewed interest in BMAA was sparked after a landmark study by Spencer in which macaques that had been exposed to BMAA for over 12 weeks developed several Parkinsonian features including gait disturbances; the authors however failed to find any neuropathological changes (Spencer et al., 1987). It was not until 15 years later that Cox hypothesised that the accumulation of BMAA in the food chain could have been responsible for the unusually high incidence of ALS/PDC cases in the Chamorros and was why high doses in Spencer's study were required to elicit a response (Banack & Cox, 2003; Cox et al., 2003). The hypothesis was scrutinised by critics who predicted that the amount of BMAA present in the food eaten by the Chamorros was insufficient to elicit neurotoxicity (Duncan et al., 1988). This led to a revised hypothesis that proposed that since BMAA was bound to proteins it could lead to more accumulation of BMAA than previously thought (Cheng & Banack, 2009). The past two decades of BMAA research have been even more eventful, with BMAA discovered to be produced ubiquitously by most strains of cyanobacteria (Cox et al., 2005), and that it bio-accumulated through aquatic food chains (Jonasson et al., 2010). Neuropathological hallmarks of MND were then identified in vervets exposed to BMAA (Cox et al., 2016) and a broadening focus of neurotoxicity research was extended to the isomers (2,4-DAB and AEG) (Main & Rodgers, 2018; Martin et al., 2022; Schneider et al., 2020).

One of the aims of this thesis was to use proteomics and pathway analysis to get a better understanding of the mechanisms behind how BMAA could induce the pathological

changes described in previous studies as well as any potential influence 2,4-DAB might have. Chapter 2 of this thesis identified several energy production pathways that were significantly impacted (fatty acid β-oxidation and glycolytic pathways) as well as the L-serine biosynthesis pathway in BMAA and 2,4-DAB-treated cells. These results suggest that these cyanotoxins could potentially cause dysregulation of energy metabolism, which is also a feature of MND. The protective effects of pyruvate as an antioxidant and energy source were shown to counter the toxicity of BMAA and 2,4-DAB. This could be explored further as there is evidence in the literature that highlights the importance of this metabolite in MND but little research has been conducted into its potential therapeutic value (Vandoorne et al., 2018). Pyruvate links the glycolytic pathway to the TCA cycle, and supplementation with pyruvate is capable of providing a substrate for the TCA cycle allowing the glucose metabolite 3PG to be used in L-serine biosynthesis (Figure 3). Thus L-serine production is not sacrificed for ATP production. Quantifying intracellular levels of pyruvate in cells exposed to BMAA and2,4-DAB using LC-MS/MS would be a good starting point to investigate this hypothesis. Quantitative pathway analysis of other energy metabolites relating to the fatty acid β-oxidation, glycolytic and TCA pathways may provide a clearer picture of the energy dynamics in cells exposed to BMAA and 2,4-DAB. LC-MS/MS can be used further to examine how fatty acid levels are modulated by exposure to BMAA and 2,4-DAB (Della Corte et al., 2015).

Studies from our laboratory provided evidence that BMAA could be mistakenly incorporated into proteins in place of the canonical amino acid L-serine (Dunlop et al., 2013). In the studies of Dunlop, radiolabelled BMAA was found in the protein fraction in a cell lysate but it is not known if it was peptide bonded into the polypeptide chain or associated with proteins in another way. Indeed, it is often overlooked that a trichloroacetic acid (TCA) precipitate contains other macromolecules that lose solubility in TCA and not just proteins. L-serine however has been shown to protect against BMAA in a range of *in vitro* and *in vivo* studies (Cox et al., 2016; Dunlop et al., 2013; Main & Rodgers, 2018) and a clinical trial on MND patients early data have been positive (Bradley et al., 2018). However, upon validation of the proteomic results relating to the enzymes involved in the L-serine biosynthesis pathway, the impact of 2,4-DAB on the first enzyme (PHGDH) was unexpected. 2,4-DAB, not BMAA, was found to significantly

lower the expression of PHGDH and when combined with BMAA, the decrease in expression was more profound. BMAA by itself was unable to elicit such a response on any of the enzymes, although we cannot discount that this could have occurred at an earlier time point. Interestingly, the expression of PHGDH was always decreased (but non-significantly) in BMAA-treated cells. Pre-treatment with pyruvate before BMAA exposure caused a significant increase in PHGDH expression relative to BMAA alone suggesting that despite not being significantly different to control samples PHGDH expression had been decreased. Chapter 2 has shown the importance of broadening the focus of toxicity to include BMAA isomers which has produced new evidence on potential interactions of BMAA and 2,4-DAB on L-serine biosynthesis which may suggest that BMAA and 2,4-DAB affect L-serine-related cellular metabolism outside of misincorporation. Further studies are required to investigate the impact of the neurotoxins on the activity of the PHGDH enzyme in cells. This can be carried out using commercially available kits. In addition, incubation of BMAA and 2,4-DAB with purified PHGDH would allow any direct effects on enzyme activity to be determined. These experiments were planned but were not possible due to the time lost from the lockdowns in Sydney due to COVID-19.

There has been very little research conducted on the effects of BMAA and its isomers at a metabolic level. One study performed untargeted metabolomics on BMAA-treated differentiated SH-SY5Y cells (Engskog et al., 2017) but no metabolomic studies have been performed on 2,4-DAB-exposed cells let alone on the combination with BMAA. There have been two proteomic studies by Martin which were performed on NSC-34 cells and a zebrafish model exploring the toxicity of the three isomers (BMAA, 2,4-DAB and AEG) (Martin et al., 2022; Martin et al., 2019). The second study found 2,4-DAB to be the most toxic out of all single and mixture designs and proteomic analysis of the 2,4-DAB-exposed zebrafish revealed several perturbed pathways including those involved with glycolysis, oxidative stress and the unfolded protein response (Martin et al., 2022).

In Chapter 3 of this thesis, an analysis of amino acid levels and antioxidant levels in cells exposed to BMAA, 2,4-DAB and combined found a disturbance in amino acid levels, particularly in the combined treatment. 2,4-DAB-exposure of cells caused a significant increase in levels of the antioxidant glutathione which corresponds with Martin's

proteomic findings of oxidative stress in 2,4-DAB-exposed zebrafish (Martin et al., 2022). A more robust indicator of oxidative stress was seen in the combined treatment with BMAA and 2,4-DAB as both glutathione and precursor amino acids used to synthesise the antioxidant were significantly elevated along with another amino acid marker of oxidation (L-proline).

Interestingly, BMAA and 2,4-DAB, alone and in combination, had a more profound effect on intracellular L-alanine levels than on L-serine. The neurotoxins both elicited significant decreases in L-alanine levels to 40% of the control levels. This observation supports the results of Han et al., 2020 who investigated the affinity of BMAA for tRNA aminoacylation. The study found BMAA has an affinity for the L-alanine tRNA synthetase and the resulting BMAA-alanyl-tRNA is capable of bypassing proofreading mechanisms. Protein synthesis in BMAA plus 2,4-DAB-treated cells were significantly decreased in the IPA performed in Chapter 2, which could be due to the interference of the toxins on the tRNA synthetases. Pulse-chase radiolabelling with amino acids to examine protein synthesis rates was an experiment that was planned but not possible due to COVID-19 lockdowns. We could determine whether BMAA and 2,4-DAB impact protein synthesis rates by measuring the rate of disintegrations per minute (DPM) present in proteins from radiolabelled amino acids that have been incorporated into protein following a short-term exposure to radiolabelled amino acids in the culture medium.

L-serine levels were found to be elevated in BMAA and 2,4-DAB-treated cells (Chapter 3) despite the PHGDH enzyme in the serine biosynthesis pathway having a decreased expression (Chapter 2). To fully explore this, we would initially carry out a time-course study examining L-serine levels in cells over a 48 hour period. The half-life of BMAA has been reported to be 48 hours, which would not impact the duration of the time course (Waidyanatha et al., 2018). Preliminary data we have shows that intracellular L-serine concentrations are decreased in cells treated with BMAA alone reaching the lowest level (37%) at 9 hours before increasing back towards basal levels (not shown). To explore this further we would perform a time-course study in cells treated with 500 µM BMAA, 500 µM 2,4-DAB and the combination. These studies were planned but due to the COVID-19 lockdowns in July to October 2021, were not possible. Chapter 3 provides new

insight into the fluctuations of amino acid metabolism and antioxidant capabilities in cells exposed to BMAA, 2,4-DAB and their combination.

In summary, the research presented in this thesis aimed to further understand the toxicity of BMAA and its isomers on the metabolic level and relate it to the metabolic disturbances seen in MND. New insight into the neurotoxins' roles in energy metabolism, L-serine biosynthesis and impacts on amino acids and antioxidants have opened up new possibilities for the venture in continuing to refine the story of the BMAA hypothesis.

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