

Investigating the Toxicity and Mechanisms of Non- Protein Amino Acids

Kate Samardzic

BMedSc (Hons)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (Science) from:

School of Life Sciences,

University of Technology Sydney

June 2020

DECLARATION

I, Kate Samardzic, certify that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by an Australian Government Research Training Program.

Signature of candidate

Production Note:

Signature removed prior to publication.

Date

16/6/2020

ACKNOWLEDGEMENTS

The journey toward a successful PhD is not an easy one and, thankfully, one that I didn't walk alone. I will be forever grateful to those who have supported me through the highs and lows of this journey, and it's only fitting that they are recognised in this section.

First and foremost, I'd like to thank my family. Your support transcends my PhD; you have been there all my life, but, you have been especially wonderful over the past decade. Mum and Dad's initial confusion when I graduated from my undergraduate degree and told them that no, I wouldn't be getting a job yet turned to mild suspicion when after Honours graduation I admitted it would still be a few more years before I had a real job. Well, finally the time has come, and it wouldn't have been possible without you. You've provided a roof over my head and kept me fed while I was working late in the lab or deep in writing mode. You provided the essentials so I could focus on doing what I love. You printed out each paper I wrote and read them enthusiastically, even though you admitted later to not entirely understanding all of it. Most importantly, though, you taught me not to stress and worry as much as I otherwise might have. Your voices saying "don't stress", "take it easy," and "take your time" gave me the strength to finish this.

Michelle, my sister. You are also my confidant, proofreader, cheerleader, motivational speaker, stylist and inspiration. Even though you have lived overseas for the past four years, I never felt your absence. You were with me every step of this journey, and I think you know more about my research than anyone else.

My friends, both in and out of UTS, made the whole process bearable. Some of my favourite times over the past four years were spent with you. You listened to me complain about my experiments, took me out dancing to take my mind off things and very sincerely enquired about the health of my cells. Special mentions to my long-distance best friend Maddy, who helped me practice every single presentation I had to do during this PhD, and Vince, who, as own personal guru, provided me with endless

emotional support, advice and love. To my lab members, Brendan, Joel, Jake, Carly (and honorary member Nat) thank you so much for the support and friendship over the years.

I'd also like to thank the markers for taking the time to review this thesis and the Australian Government and UTS Graduate Research School for the Postgraduate Award Scholarship and Thesis Completion Equity Grant I received throughout my PhD candidature.

Finally, no one knows the naïve and inexperienced scientist I started as like my two supervisors; Ken Rodgers and Matt Padula. Ken, thank you for the opportunity to join your lab and work on this project. It has continued to excite me over the years and my time in your lab has nurtured my love of research. Matt, thank you for your omnipresence in the lab. It was always so helpful to be able to find you at any time and talk to you about anything (even though you often wished I didn't).

Thank you to all those I've listed here and the many others who know who they are. This has been one of the most challenging yet rewarding tasks I've ever undertaken.

LIST OF PUBLICATIONS

Rodgers, K.J., **Samardzic, K.**, and Main, B.J. (2015) Toxic Nonprotein Amino Acids, in *Plant Toxins*. Springer. p. 1-20.

Samardzic, K., and Rodgers, K.J. (2017). Oxidised protein metabolism: recent insights. *Biol Chem*, 398: p. 1165-1175.

Rodgers, K.J., Main, B.J., and **Samardzic, K.** (2017). Cyanobacterial Neurotoxins: Their Occurrence and Mechanisms of Toxicity. *Neurotox Res*, 33: p. 168-177.

Samardzic, K. and K.J. Rodgers. (2019). Cytotoxicity and mitochondrial dysfunction caused by the dietary supplement L-norvaline. *Toxicol in Vitro*, 56: p. 163-171.

Samardzic, K. and K. J. Rodgers (2019), Cell death and mitochondrial dysfunction induced by the dietary non-proteinogenic amino acid L-azetidine-2-carboxylic acid (Aze). *Amino Acids*, 8: p. 1221-1232.

Giannopoulos, S., **Samardzic, K.**, Raymond, B.A., Djordjevic, S.P., and Rodgers, K.J. (2019) L-DOPA causes mitochondrial dysfunction in vitro: a novel mechanism of L-DOPA toxicity uncovered. *Int J Biochem Cell Biol*, 117.

CONFERENCE PROCEEDINGS

Algal Research Symposium, Sydney, NSW, Australia	2015
Oral Presentation	
“Phytotoxic Activity of Non-Protein Amino Acids Synthesised by Cyanobacteria”	
Society of Environmental Toxicology and Chemistry, Hobart, TAS, Australia	2016
Oral Presentation	
“Phytotoxic Activity of Non-Protein Amino Acids Synthesised by Cyanobacteria”	
FameLab, Sydney, NSW, Australia	2018
Oral presentation	
“Mitochondria: Cellular Superheroes and their Kryptonite”	
Multifaceted Mitochondria, San Diego, CA, USA	2018
Poster Presentation	
“Mitochondrial Dysfunction caused by Non-Protein Amino Acid Dietary Supplements”	
AMP Amplify PhD Pitch Competition, Sydney, NSW, Australia	2018
Oral Presentation	
“Supplement Dos and Don’ts”	
AussieMit, Melbourne, VIC, Australia	2018
Poster Presentation	
“Mitochondrial Dysfunction caused by Non-Protein Amino Acid Dietary Supplements”	

ABSTRACT

Non-protein amino acids (NPAAs) are amino acids not normally used in protein synthesis. However, a small subset of this type of amino acid, known as amino acid analogues, can be mistakenly utilised in protein synthesis due to their structural similarity to a canonical protein amino acid. This process has been implicated in the development of neurodegenerative disease. This research set out to investigate the toxicity and mechanisms of two lesser studied NPAAs which enter the human food chain and have the potential to contribute to the development of disease. These were L-norvaline (Nva), an analogue of the branched chain amino acids that is usually found in dietary supplements consumed by bodybuilders, and L-azetidine-2-carboxylic acid (Aze), an analogue of L-proline that is produced by sugar beets and enters the human food as a dietary supplement and as fodder for livestock.

Initial experiments sought to identify whether either Nva or Aze induced cell death using metabolic and imaging assays. Both NPAAs were cytotoxic to human neuroblastoma cells, confirming the rationale behind the present investigation and providing the impetus for further research. Cell death pathways were also investigated, and death was determined to be necrotic following Nva exposure, and both necrotic and apoptotic following treatment with Aze. The toxicity of these NPAAs to mitochondria was then characterised using immunofluorescence microscopy and bioenergetic flux assays. These revealed, for the first time, that Nva and Aze cause mitochondrial dysfunction. Distinct morphological changes and bioenergetic failure were common to both conditions, however, for Nva bioenergetic failure was only observed in the presence of a nitric oxide synthase inhibitor due to Nva's secondary action as an arginase inhibitor.

Finally, a proteomic characterisation of cells exposed to both NPAAs was performed to further elucidate the molecular mechanisms involved in their toxicity. This was the first study to perform this analysis in human cells treated with either NPAA and revealed disruptions to processes that precede protein translation. Changes to proteins involved in the processing of DNA and RNA during cell cycle progression indicate, for the first

time, that NPAA exposure exerts toxic effects upstream of protein translation. These results identify Nva and Aze as NPAAs with significant potential for toxicity that should, therefore, be consumed with caution. Furthermore, these results add to the existing knowledge of the mechanisms of both these individual NPAAs, and amino acid analogues in general.

CONTENTS

Declaration	ii
Acknowledgements	iii
List of Publications	v
Conference Proceedings	vi
Abstract.....	vii
List of Tables and Figures.....	xi
List of Abbreviations	xiii
Chapter one: Overview of the Thesis.....	17
1.1 Introduction	17
1.2 Aims of the Thesis	18
1.3 Dissertation Organisation.....	19
Chapter two: Critical Review	21
2.1 Amino Acids.....	21
2.2 Non Protein Amino Acids	23
2.3 Toxicity of NPAAs	25
2.3.1 NPAAs and Bacteria	26
2.3.2 Plant Defence	27
2.3.3 NPAA Exposure in Mammals	28
2.4 Neurodegenerative Disease	31
2.4.1 Mitochondria and Neurodegenerative Disease.....	33
2.5 Routes of Exposure to NPAAs.....	35
2.5.1 Biomagnification.....	35
2.5.2 Dietary Supplements	36
2.6 Mechanisms of NPAA Toxicity.....	38
2.6.1 Fidelity of Protein Synthesis	38
2.6.2 NPAA Misincorporation and Aggregation.....	40
2.7 Cellular Responses to Stress.....	44
2.8 Methods of Assessing Cellular Stress	46
Chapter three: Investigation into L-Norvaline Toxicity	49
Chapter four: Investigation into L-Azetidine-2-carboxylic acid Toxicity	60

Chapter five: Proteomic Characterisation of SH-SY5Y Neuroblastoma Cells Following NPAA Exposure	78
5.1 Introduction	78
5.2 Methods	81
5.2.1 Reagents	81
5.2.2 Cell Culture	81
5.2.3 Treatment.....	82
5.2.4 Protein Precipitation and Mass Spectrometry	82
5.2.5 Generation of Protein Quantification Data	83
5.3 Results	84
5.3.1 L-Norvaline	84
5.3.2 L-Azetidine-2-carboxylic acid	91
5.4 Discussion.....	98
5.4.1 L-Norvaline	98
5.4.2 L-Azetidine-2-carboxylic acid	102
5.5 Conclusions	105
Chapter six: Concluding Remarks and Future Directions.....	108
Appendix.....	112
References	130

LIST OF TABLES AND FIGURES

Figure 1. Structural representation of an amino acid molecule.	21
Table 1. Classification of protein amino acids.	22
Table 2. Table listing amino acids, their analogues and origins in nature	24
Figure 2. Structural representation of the protein amino acid L-tyrosine and L-DOPA.	25
Figure 3. BMAA bioaccumulation through the Guamian food chain.	35
Table 3. Table listing NPAA containing dietary supplements and their purported benefits.	37
Table 4. Structural representations of the dietary supplement NPAAs Nva and Aze and their corresponding protein amino acids.	38
Figure 4. Fidelity of protein synthesis.	40
Table 5. Characteristics of proteopathies.	43
Table 6. Cellular morphologies and proteins associated with cell death pathways.	45
Figure 5. Proteomic characterisation of Nva treated cells.	85
Figure 6. Enriched ClueGo Gene Ontology (GO) Cellular Compartment (CC) terms.	87
Figure 7. Enriched ClueGO Gene Ontology (GO) Biological Process (BP) terms.	88
Figure 8. GO BP terms associated with proteins interacting within ClusterONE significant clusters for the unique proteins in the untreated group.	89
Figure 9. GO BP terms associated with proteins interacting within ClusterONE significant clusters for the unique proteins in the Nva treated group	90
Figure 10. Proteomic characterisation of Aze treated cells.	92
Figure 11. Scatter plot of LFQ intensity ratios following Aze treatment.	93
Table 7. Differentially expressed proteins in SH-SY5Y neuroblastoma cells following Aze 24 h treatment.	94

Figure 12. GO BP terms associated with proteins downregulated following Aze treatment.	96
Figure 13. GO BP terms associated with proteins upregulated following Aze treatment.	97

***Please note that the Tables and Figures listed above include those in chapters one, two and five only.**

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
ALS-PDC	Amyotrophic Lateral Sclerosis- Parkinsonism-Dementia Complex
aaRS	aminoacyl transfer ribonucleic acid synthetase
BCA	bicinchoninic acid
BP	Biological Process
CC	Cellular Compartment
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DMEM	Dulbecco's Modified Eagle's Medium
EMEM	Eagle's Minimum Essential Medium
EF-Tu	elongation factor Tu
ER	endoplasmic reticulum
<i>E. coli</i>	<i>Escherichia coli</i>
FDR	false discovery rate
fALS	familial Amyotrophic Lateral Sclerosis
FBS	foetal bovine serum
GO	Gene Ontology
HSP	heat shock protein
HD	Huntington's disease
IRES	internal ribosome entry site

DAB	L-2, 4-diaminobutanoic acid
L-DOPA	L-3,4- dihydroxyphenylalanine
Aze	L-azetidine-2-carboxylic acid
Nva	L-norvaline
BMAA	L- β - <i>N</i> -methylaminoalanine
BOAA	L- β - <i>N</i> -oxalylamino-L-alanine
LFQ	label free quantification
LeuRS	leucyl-transfer ribonucleic acid synthetase
LC-MS	liquid chromatography-mass spectrometry
mRNA	messenger ribonucleic acid
MS	Multiple Sclerosis
NO	nitric oxide
NPAA	non-protein amino acid
OXPHOS	oxidative phosphorylation
PD	Parkinson's disease
PBS	phosphate buffered saline
PI	Propidium Iodide
ROS	reactive oxygen species
RFC	replication factor C
RNA	ribonucleic acid
sALS	sporadic Amyotrophic Lateral Sclerosis
SOD1	superoxide dismutase 1
SLE	Systemic Lupus Erythematosus

THOC	THO complex
tRNA	transfer ribonucleic acid
TCEP	tris(2-carboxyethyl)phosphine
UPR	unfolded protein response
VCP	valosin containing protein
ZnF	zinc finger

***Please note that the abbreviations listed above include those in chapters one, two, five and six only**