The role of increase hBCATm in the endothelial cells of patients with Alzheimer's Disease.

Thomas Edward Forshaw

Department of Biological, Biomedical and Analytical Sciences,

University of the West of England, Bristol.

September 2016

A thesis submitted in partial fulfilment of the requirements of the University of the West of England, Bristol for the degree of Doctor of Philosophy.

This work was partly funded by BRACE

(Bristol Research into Alzheimer's and Care of the Elderly).

Copyright disclaimer

ACKNOWLEDGEMENTS.

This thesis, and the research contained within, could not have been achieved without my friends, family, colleagues, and supervisory team. I would like to take a moment to thank them all for everything they have done for me.

Firstly, I would like to thank my director of studies, Prof. Myra Conway, for her continued support, enthusiasm, and belief in me. I have learnt such an incredible amount over the past four years, and it was only possible through Myra's teachings and hard work. A childhood dream has literally come true.

I must also thank my second supervisors, Dr. Annabelle Hodson for her expert advice in chemistry, and Dr. Carolyn Paul for her expertise in the blood-brain barrier and contribution to the building of a cell culture model. Again, their hard work has made this possible.

Academically I would like to thank Dr. Craig Butts at the University of Bristol for use and training in NMR spectroscopy, Dr. James Costello for advice on drug design and chemical synthesis, Dr. Man-Kim Cheung for his guidance on molecular biology, and Dr. Anneke Lubben at the University of Bath for providing LC-MS analysis of compounds. Thank you to Team Conway, past and present - Chris, Matt, Jon, Maya, Mo, Marcela, Anna, Grace, Fred, and Mai. I had the pleasure of working with four undergraduate students in my third year and tutoring them on laboratory techniques. They also helped provide data for this thesis and I would like to thank Bethany Samways, Elsa Aguiar, Jovi Workman, and Matt Heyda. I would also like to thank the technical team at both UWE and UoB.

Finally (but very importantly), my friends and family. Karen, Alan, Michael, Siân Parry (incredible patience), James Billett, Morgan Lewis, Max Hughes, Jason Redican (stealer of socks), Abbey May, Jon Quirk, Niamh Ní Branghaile, Gareth Hughes, Liz Bowler, Simon Andrews, Mike Devey, Strong Coffee, Mars Skittles, Thatchers Cider, The Ratties, and all who have been there with me on my journey.

Dedicated to the memory of Thomas William Joynes.

Rest in peace, granddad.

ABSTRACT.

Introduction and aims: The branched-chain aminotransferase (BCAT) enzymes are important in the regulation of brain L-glutamate. A unique function of these aminotransferases is their regulation by the redox environment, where our group have shown their function as oxidoreductases, and their ability to refold misfolded proteins in particular when S-glutathionylated. Our group recently showed a significant increase in the level of these proteins in Alzheimer's disease brain. An increase in BCAT activity could generate excess L-glutamate, contributing to the excitotoxic environment observed under pathogenic conditions. Alternatively, we hypothesize that if this protein is modified in response to cellular stress, it may play a more prominent role in regulating redox status or protein folding. To address these questions this project focussed on the design of chemical inhibitors and knock-down models together with a co-culture model of the human cerebrovasculature and specifically targeted key metabolic and redox pathways.

Methods: Several chemical inhibitors based on 4-Benzyloxyphenylacetic acid were identified using the DockBlaster and Schrödinger software suites, synthesized and structurally verified using proton nuclear magnetic resonance (NMR) spectroscopy. The functional impact and specificity of these inhibitors was assessed using the BCAT radiolabelled assay and a coupled-enzyme assay. In tandem, siRNA was used to knock-down both isoforms in SH-SY5Y cells, and validated using western blot analysis and RT-PCR. The impact of BCATm knock-down on the expression of redox proteins, in addition to selected metabolic proteins, was assessed by western blot analysis. Functional redox assays, including glutathione concentration and metabolic activity, were also used to investigate the impact of human BCAT (hBCAT) expression in neuronal cells. Finally, a model of the blood-brain barrier (BBB) was developed and validated for studies into the role of mitochondrial hBCAT (hBCATm) in brain microvasculature.

Results: For the first time we have identified a family of chemical inhibitors of hBCATm. In particular, benzofenac has a two-fold greater enzyme affinity for hBCATm (K_i=43 µM) than cytosolic hBCAT (hBCATc) (K_i=93 µM) and a four-fold greater inhibition relative to alanine transaminase (ALT; K_i=167 µM). These inhibitors will require further optimisation but have potential as tools to assess the cellular function of hBCAT. In separate studies, knock-down of hBCATm in SH-SY5Y neuronal cells demonstrated that hBCATm expression has an impact on the metabolic and redox status of the cell. In particular, knock-down caused a >70% decrease in glutaredoxin (GRx), thioredoxin (TRx), branched-chain α-keto acid dehydrogenase α-subunit (BCKDHA), and AU-rich binding homolog of enoyl-CoA hydratase (AUH) expression. Interestingly, this effect was attenuated when cells were treated with L-leucine, indicating that these mechanisms may be regulated by a metabolic signal. L-glutamate treatment was also found to significantly increase hBCATm expression, but decreased glutamate dehydrogenase (GDH) expression, except in cells overexpressing hBCATm, suggesting a metabolic synergy between the two enzymes. Finally, total glutathione concentration was significantly decreased on hBCATm knock-down, while sensitivity to L-glutamate toxicity was significantly increased.

Discussion: Results from this work have significantly contributed to the design of cellular models, which can be used to further investigate the role of BCAT in metabolic and redox metabolism. A model of the human blood-brain barrier, developed in this thesis, will also contribute to evaluating the endothelial role of hBCATm. The initial impact of limiting BCAT expression is both a decrease in the expression of key metabolic proteins and also the cellular reductants. Together this had an impact on cell survival. Knowledge of these pathways and their regulation will be important to our understanding, of not only the regulation of brain L-glutamate, but also the role of BCAT in protein folding and cellular redox status. These factors are fundamentally important to development of neurodegenerative conditions but also in tumour development such as gliomas.

ABBREVIATIONS.

3MeBOPAA – 3-methyl-4-[(4-methylbenzyl)oxy]-phenylacetic acid

3OMeBOPAA – 4-(benzyloxy)-3-hydroxy-phenylacetic acid

4EBP1 – Eukaryotic translation initiation factor 4E-binding protein 1

AAOA – Aminooxyacetic acid

Aβ – Amyloid β peptide

Aβ40 – Amyloid β peptide 1-40

Aβ42 – Amyloid β peptide 1-42

AD – Alzheimer's disease

AJ – Adherens junctions

Akt – RAC-alpha serine/threonine-protein kinase

ALT – Alanine transaminase

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPK – 5' adenosine monophosphate-activated protein kinase

APP – Amyloid precursor protein

APS – Ammonium persulphate

ARE – Antioxidant-response element

AST – Aspartate aminotransferase

ATF4 – Activating transcription factor 4

ATP – Adenosine triphosphate

AUH – AU RNA binding protein/enoyl-CoA hydratase

BBB – Blood brain barrier

BCAA – Branched-chain amino acids

BCAT – Branched-chain aminotransferase

BCAT1 – Branched-chain aminotransferase gene (cytosolic isoform)

BCAT2 – Branched-chain aminotransferase gene (mitochondrial isoform)

BCKA – Branched-chain α-keto acid

BCKD – Branched-chain α-keto acid dehydrogenase

BCKDHA – Branched-chain α-keto acid dehydrogenase, E1α subunit

BiP – Binding immunoglobulin protein

Benzofenac – 4-(benzyloxy)-3-chloro-phenylacetic acid

BOPAA – 4-(benzyloxy)phenylacetic acid

BSA – Bovine serum albumin

CHOP – C/EBP [CCAAT/enhancer binding protein] homologous protein

cDNA – Complementary DNA

CSF – Cerebrospinal fluid

Cys – Cysteine

DCC – N,N-dicyclohexylcarbodiimide

DCM – Dichloromethane

ddDNA – Double stranded DNA

DMAP – 4-dimethylaminopyridine

DMEM – Dulbecco's Modified Eagle's medium

DMF – Dimethylformamide

DPM – Disintegrations per minute

DTNB – 5,5'-dithiobis-2-nitrobenzoic acid

DTT – Dithiothreitol

E-PLP – Enzyme-Pyridoxal L-phosphate complex

E-PMP – Enzyme-Pyridoxal monophosphate complex

FBS – Fetal bovine serum

EAAT1 – Excitatory amino acid transporter 1

EDTA – Ethylenediaminetetraacetic acid

eGFP – Enhanced green fluorescent protein

EGTA – Ethyleneglycoltetraacetic acid

eIF2α – Eukaryotic translation initiation factor 2 α

FT-IR – Fourier transform infrared

GABA – γ-aminobutyric acid

GAD – L-glutamate decarboxylase

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GC – Gas chromatography

GC-MS – Gas chromatography coupled mass spectrometry

GDH – Glutamate dehydrogenase

GLUT1 – Glucose transporter 1

GPx – Glutathione peroxidase

GRx – Glutaredoxin

GS – Glutamine synthase

GSH – Glutathione (reduced)

GSK3β – Glycogen synthase kinase 3 β

GSSG – Glutathione (oxidized)

HAPAA – 2-hydroxy-4-(4-methylbenzoyl)aminophenylacetic acid

hBCAT – Human branched-chain aminotransferase

hBCATc – Human branched chain aminotransferase (cytosolic isoform)

hBCATm – Human branched-chain aminotransferase (mitochondrial isoform)

HBSS – Hank's buffered salt solution

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HMG-CoA - 3-hydroxy-3-methylglutaryl-coenzyme A

HSPAA – 2-hydroxy-4-{[(4-methylphenyl)sulfanyl]carbonyl}phenylacetic acid

HTS – High-throughput screening

IC50 – Half maximal inhibitory concentration

IPTG – Isopropyl β-D-1-thiogalactopyranoside

IRS – Insulin receptor substrate

ISR – Integrated stress response

KA – Kainic acid

K_{cat} – Substrate molecules turned over per enzyme molecule, per second

KEAP1 – Kelch-like ECH [erythroid cell-derived protein with CNC homology]-associated protein 1

K_{eq} – Equilibrium constant

KG – α-Ketoglutarate

KIC – α-ketoisocaproate

KIV – α-ketoisovalerate

Klf15 – Kruppel-Like Factor 15

KMV – α-keto-β-methylvalerate

KMV - Keto-β-methylvalerate

LC-MS – Liquid chomatorgraphy coupled mass spectrometry

LDS – Lithium dodecyl sulphate

LNAAT – Large neutral amino acid transporter

MAP – Microtubule-associated protein

MAPT – Tau microtubule-associated protein gene

Mct – Monocarboxylic acid transporter

MeBenzofenac – 3-chloro-4-[(4-methylbenzyl)oxy]-phenylacetic acid

MG-CoA – 3-methylglutaconyl-CoA

MOPS – 3-(N-morpholino)propanesulfonic acid

MS – Mass spectrometry

MSUD – Maple syrup urine disease

mTOR – Mammalian target of Rapamycin

mTOR1 – Mammalian target of Rapamycin complex 1

MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NADH – Nicotinamide adenine dinucleotide

NADPH – Nicotinamide adenine dinucleotide phosphate

Na-Flu – Sodium fluorescein

NEM – N-ethylmalemide

NFT – Neurofibrillary tangles

NMDA – N-methyl-D-aspartic acid

NMDAR – N-methyl-D-aspartic acid receptor

NMR – Nuclear magnetic resonance (spectroscopy)

NO – Nitric oxide

Nrf2 – Nuclear factor (erythroid-derived 2)-like 2

P70S6K – Ribosomal protein S6 kinase beta-1

p-eIF2α – Phosphorylated eukaryotic translation initiation factor 2 α

P-gp – P-glycoprotein

PAG – Phosphate- activated glutaminase

PDI – Protein disulphide isomerase

Pe – Permeability coefficient

PERK – Protein kinase RNA-like endoplasmic reticulum kinase

PI3K – Phosphoinositide 3-kinase

pIC50 – negative logarithm of IC50 [Half maximal inhibitory concentration]

PLP – Pyridoxal L-phosphate

PMP – Pyridoxine monophosphate

PP18b – Placental protein 18b

PP2A – Protein phosphatase 2A

Rheb – Ras homolog enriched in brain

RNAi – RNA interference

ROS – Reactive oxygen species

RT – Retention time

SDS – Sodium dodecyl sulphate

shRNA – Short hairpin RNA

siRNA – Small interfering RNA

ssRNA – Single stranded RNA

SOD – Superoxide dismutase

TBS – Tris-buffered saline

TBST – Tris-buffered saline with Tween-20

TCA – Trichloroacetic acid

TEMED – Tetramethylethylenediamine

Tris – Hydroxymethylaminomethane

TJ – Tight junction

TOR – Target of Rapamycin

TR – Thyroid receptor

TRx – Thioredoxin

TSC1 – Tuberous sclerosis 1

U – Enzyme units

VDCC – Voltage dependent calcium channels

ZO – Zonula occludens

PRESENTATIONS AND PUBLISHED WORKS.

Oral presentation

"BCAT and the brain" Postgraduate research forum 2015, University of the West of England.

Poster presentations

"The importance of the AAA: Alzheimer's, autophagy and aminotransferases" Alzheimer's research UK 2015, University College London.

"Synthesis and testing of a selective hBCATm chemical inhibitor" CRIB forum 2015, University of the West of England.

"The design of enzyme inhibitors to aid investigation into the role of branched-chain aminotransferase in neurological disease" CRIB forum 2014. University of the West of England.

Publications

El Hindy, M., Hezwani, M., Corry, D., Hull, J., El Amraoui, F., Harris, M., Lee, C., **Forshaw, T.**, Wilson, A., Mansbridge, A., Hassler, M., Patel, V. B., Kehoe, P. G., Love, S., Conway, M. E. (2014) The branched-chain aminotransferase proteins: novel redox chaperones for protein disulfide isomerase--implications in Alzheimer's disease. Antioxid. Redox. Signal. 20(16): 2497-2513

Forshaw, T. E. & Conway M. E. Differential regulation of the hBCAT proteins by S-Nitrosation and S- Glutathionylation. (Submitted - awaiting publication)

CONTENTS.

CHAPTER ONE – INTRODUCTION AND MAIN AIMS.

CHAPTER TWO – MATERIALS AND METHODS.

FIGURE CONTENTS.

CHAPTER ONE – INTRODUCTION AND MAIN AIMS.

CHAPTER TWO – MATERIALS AND METHODS.

CHAPTER THREE – CHEMICAL SYNTHESIS AND ANALYSIS OF A SELECTIVE HBCATM INHIBITOR.

CHAPTER FOUR – THE ROLE OF HBCATM IN REDOX STATUS AND METABOLISM.

CHAPTER FIVE – *IN VITRO* MODELING OF THE HUMAN BLOOD-BRAIN BARRIER.

CHAPTER SIX – SYNOPSIS, CONCLUSION, AND FUTURE WORK.

TABLE CONTENTS.

CHAPTER ONE – INTRODUCTION AND MAIN AIMS.

CHAPTER TWO – MATERIALS AND METHODS.

CHAPTER THREE – CHEMICAL SYNTHESIS AND ANALYSIS OF A SELECTIVE HBCATM INHIBITOR.

CHAPTER FOUR – THE ROLE OF HBCATM IN REDOX STATUS AND METABOLISM.