Chapter 2

Materials and methods

CONTENTS.

2.1 – MATERIALS.

The materials utilised for this study are listed alphabetically in **Table 2.1**.

Table 2.1 – Materials and manufacturer.

Material	Manufacturer	Location
1, 1 cyclohexanediacetic acid monoamide	Sigma-Aldrich	Poole, UK
1kb DNA ladder	New England Biolabs	Hitchin, UK
2-vinylpyridine	Sigma-Aldrich	Poole, UK
3-(N-morpholino) propanesulfonic acid (MOPS)	Fisher Scientific	Loughborough, UK
3-aminophenol	Sigma-Aldrich	Poole, UK
3-bromopropene	Sigma-Aldrich	Poole, UK
3-chloro-4-hydroxyphenylacetic acid	Sigma-Aldrich	Poole, UK
3-hydroxybenzoic acid	Apollo Scientific	Stockport, UK
4-2-hydroxyethyl-1-piperazineethanesulphonic acid (HEPES)	Fisher Scientific	Loughborough, UK
4-dimethylaminopyridine (DMAP)	Sigma-Aldrich	Poole, UK
4-hydroxy-3-methylacetophenone	Apollo Scientific	Stockport, UK
4-hydroxy-3-methyloxyphenylacetic acid	Apollo Scientific	Stockport, UK
4-hydroxyphenylacetic acid	Sigma-Aldrich	Poole, UK
4-methylbenzyl bromide	Sigma-Aldrich	Poole, UK
5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)	Sigma-Aldrich	Poole, UK
5-Sulphosalicylic acid	Sigma-Aldrich	Poole, UK
6X orange DNA electrophoresis running dye	Fisher Scientific	Loughborough, UK
15 kDa dialysis tubing	Sigma-Aldrich	Poole, UK
α-carbon labelled [^{14}C] α-ketoisovalerate	American Radiolabelled Chemicals (ARC)	London, UK
α-ketoisocaproate (KIC)	Sigma-Aldrich	Poole, UK
α-ketoisovalerate (KIV)	Sigma-Aldrich	Poole, UK
β-mercaptoethanol	Sigma-Aldrich	Poole, UK
γ-aminobutyric acid (GABA)	Sigma-Aldrich	Poole, UK

Acetic acid	Fisher Scientific	Loughborough, UK
Acetone (technical grade)	Fisher Scientific	Loughborough, UK
Acrylamide/Bis-acrylamide 37.5:1 solution (BioReagent)	Sigma-Aldrich	Poole, UK
Adogen® 464	Sigma-Aldrich	Poole, UK
Agar	Sigma-Aldrich	Poole, UK
Agarose (low EEO)	Sigma-Aldrich	Poole, UK
AgeI-HF restriction enzyme	New England Biolabs	Hitchin, UK
Alanine transaminase from porcine heart	Sigma-Aldrich	Poole, UK
All-stars scrambled siRNA	Sigma-Aldrich	Poole, UK
Ambion Silencer siRNA construction kit	Fisher Scientific	Loughborough, UK
Amido black 10B sodium salt	Sigma-Aldrich	Poole, UK
Ammonium persulphate (APS)	Sigma-Aldrich	Poole, UK
Ammonium sulphate	Fisher Scientific	Loughborough, UK
Ampicillin	Sigma-Aldrich	Poole, UK
Benzyl bromide	Sigma-Aldrich	Poole, UK
Bovine serum albumin (BSA) (standard grade, fraction V)	Seralabs	Hull, UK
Bromine	Fisher Scientific	Loughborough, UK
Bromophenol blue dye	Sigma-Aldrich	Poole, UK
CellTiter 96® Aqueous One Solution	Promega	Southampton, UK
Chloroacetic acid	Sigma-Aldrich	Poole, UK
Chloroform (molecular biology grade)	Sigma-Aldrich	Poole, UK
cOmplete protease inhibitor cocktail	Sigma-Aldrich	Poole, UK
Coomassie blue G dye	Sigma-Aldrich	Poole, UK
Custom DNA oligonucleotides	Eurofins Operon EWG	Germany
CutSmart buffer	New England Biolabs	Hitchin, UK
D-glucose	Sigma-Aldrich	Poole, UK
Dichloromethane (DCM; technical grade)	Fisher Scientific	Loughborough, UK
Diethyl ether (ether; technical grade)	Fisher Scientific	Loughborough, UK
Dimethylformamide (DMF; technical grade)	Fisher Scientific	Loughborough, UK
Direct-zol spin column	Cambridge Bioscience	Cambridge, UK
Disodium hydrogen phosphate	Fisher Scientific	Loughborough, UK
Dithiothreitol (DTT)	Sigma-Aldrich	Poole, UK

DNase I enzyme	Sigma-Aldrich	Poole, UK
Dulbecco's Modified Eagle's medium (DMEM)	Fisher Scientific	Loughborough, UK
EcoRI-HF restriction enzyme	New England Biolabs	Hitchin, UK
EndoGro-MV complete culture media kit	Fisher Scientific	Loughborough, UK
Ethanol (molecular biology grade)	Sigma-Aldrich	Poole, UK
Ethanol (technical grade)	Fisher Scientific	Loughborough, UK
Ethidium bromide solution	Fisher Scientific	Loughborough, UK
Ethyl acetate (technical grade)	Fisher Scientific	Loughborough, UK
Ethylenediaminetetraacetic acid (EDTA)	Fisher Scientific	Loughborough, UK
Ethylene glycol	Sigma-Aldrich	Poole, UK
Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA)	Fisher Scientific	Loughborough, UK
Fatal bovine serum (FBS)	Fisher Scientific	Loughborough, UK
Fibroblast Growth Factor β	Fisher Scientific	Loughborough, UK
Gateway recombination kit (LR Clonase II enzyme mix and Proteinase K)	Fisher Scientific	Loughborough, UK
Glutathione (oxidised), (GSSG)	Sigma-Aldrich	Poole, UK
Glutathione (reduced) (GSH)	Sigma-Aldrich	Poole, UK
Glutathione reductase	Sigma-Aldrich	Poole, UK
Glycerol	Fisher Scientific	Loughborough, UK
Glycine	Fisher Scientific	Loughborough, UK
Hank's buffered salt solution (HBSS)	Fisher Scientific	Loughborough, UK
Hydrogen peroxide	Sigma-Aldrich	Poole, UK
Hydrochloric acid	Fisher Scientific	Loughborough, UK
Hydroxymethylaminomethane (tris)	Sigma-Aldrich	Poole, UK
Imidazole	Sigma-Aldrich	Poole, UK
Isopropanol (molecular biology grade)	Sigma-Aldrich	Poole, UK
Isopropanol (technical grade)	Fisher Scientific	Loughborough, UK
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Carbosynth	Compton, UK
jetPRIME	VWR	Lutterworth, UK
Kanamycin sulphate	Fisher Scientific	Loughborough, UK
L-alanine	Fisher Scientific	Loughborough, UK
L-glutamine solution	Fisher Scientific	Loughborough, UK

L-isoleucine	Sigma-Aldrich	Poole, UK
L-Lactic Dehydrogenase from rabbit muscle	Sigma-Aldrich	Poole, UK
L-leucine	Sigma-Aldrich	Poole, UK
L-leucine dehydrogenase	Sigma-Aldrich	Poole, UK
Lithium carbonate	Sigma-Aldrich	Poole, UK
Lithium chloride	Sigma-Aldrich	Poole, UK
Luminata Forte ECL solution	Fisher Scientific	Loughborough, UK
Magnesium chloride	Fisher Scientific	Loughborough, UK
Magnesium sulphate	Fisher Scientific	Loughborough, UK
Mark12™ unstained ladder	Fisher Scientific	Loughborough, UK
Marvel milk powder	Tesco	Welwyn Garden City, UK
Methanol (technical grade)	Fisher Scientific	Loughborough, UK
Monosodium glutamate	Sigma-Aldrich	Poole, UK
Morpholine	Sigma-Aldrich	Poole, UK
N,N-dicyclohexylcarbodiimide (DCC)	Sigma-Aldrich	Poole, UK
NanoScript cDNA synthesis kit	PrimerDesign	Southampton, UK
Nickel sepharose 6 fast flow beads	Fisher Scientific	Loughborough, UK
Nicotinamide adenine dinucleotide (NADH)	Melford	Ipswich, UK
Nicotinamide adenine dinucleotide phosphate (NADPH)	Melford	Ipswich, UK
Nitrocellulose membrane (marked)	Sigma-Aldrich	Poole, UK
Nuclease-free water	Sigma-Aldrich	Poole, UK
Opti-mem	Fisher Scientific	Loughborough, UK
Oxone	Sigma-Aldrich	Poole, UK
p-toluoyl chloride	Sigma-Aldrich	Poole, UK
PCR primers	Eurofins Operon EWG	Germany
PDVF membrane	Fisher Scientific	Loughborough, UK
Petroleum ether (100-120°C; extra pure)	Fisher Scientific	Loughborough, UK
Phosphoric acid	Fisher Scientific	Loughborough, UK
Potassium carbonate	Sigma-Aldrich	Poole, UK
Potassium chloride	Fisher Scientific	Loughborough, UK
Potassium dihydrogen phosphate	Fisher Scientific	Loughborough, UK

Potassium hydroxide	Sigma-Aldrich	Poole, UK
Potassium osmate	Sigma-Aldrich	Poole, UK
Potassium phosphate dibasic	Fisher Scientific	Loughborough, UK
Precision diamond DNA stain	Promega	Southampton, UK
PrecisionFAST mastermix (SYBRgreen dye, high ROX)	PrimerDesign	Southampton, UK
ProSafe+ scintillation fluid	Meridian biotechnologies	Epsom, UK
PureLink HiPure Plasmid Miniprep Kit	Fisher Scientific	Loughborough, UK
Pyridine (technical grade)	Fisher Scientific	Loughborough, UK
Pyridinium p-toluenesulphonate	Sigma-Aldrich	Poole, UK
Pyridoxal L-phosphate (PLP)	Sigma-Aldrich	Poole, UK
Rat tail collagen type 1	Sigma-Aldrich	Poole, UK
Resveratrol	Sigma-Aldrich	Poole, UK
RNAiMAX	Fisher Scientific	Loughborough, UK
siGLO Green NTsiRNA	Fisher Scientific	Loughborough, UK
siRNA sequences	Eurofins Operon EWG	Germany
Sodium bisulphite	Fisher Scientific	Loughborough, UK
Sodium chloride	Fisher Scientific	Loughborough, UK
Sodium dodecyl sulphate (SDS)	Fisher Scientific	Loughborough, UK
Sodium fluorescein	Sigma-Aldrich	Poole, UK
Sodium hydrogen carbonate	Fisher Scientific	Loughborough, UK
Sodium pyruvate solution	Fisher Scientific	Loughborough, UK
Sodium hydroxide	Fisher Scientific	Loughborough, UK
Sodium phosphate dibasic	Fisher Scientific	Loughborough, UK
sodium valproate	Sigma-Aldrich	Poole, UK
Spectra ladder	Sigma-Aldrich	Poole, UK
Sulphur	Fisher Scientific	Loughborough, UK
Sulphuric acid	Fisher Scientific	Loughborough, UK
Stellar competent cells	Clontech	France
T4 ligase kit	Promega	Southampton, UK
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich	Poole, UK
Thrombin enzyme (restriction grade)	Sigma-Aldrich	Poole, UK
Transwell 0.4 µm pore size polyester bucket inserts	Sigma-Aldrich	Poole, UK

Tri-reagent	Sigma-Aldrich	Poole, UK
Trichloroacetic acid (TCA)	Fisher Scientific	Loughborough, UK
Triethanolamine	Sigma-Aldrich	Poole, UK
Trimethylamine	Sigma-Aldrich	Poole, UK
Tripsin-EDTA	Fisher Scientific	Loughborough, UK
Tryptone (pancreatic digest of casein)	Sigma-Aldrich	Poole, UK
Triton X-100	Sigma-Aldrich	Poole, UK
Trypan blue dye solution	Fisher Scientific	Loughborough, UK
Tween-20	Sigma-Aldrich	Poole, UK
Ultra low DNA ladder	New England Biolabs	Hitchin, UK
Urea	Fisher Scientific	Loughborough, UK
XhoI restriction enzyme	New England Biolabs	Hitchin, UK
Yeast extract	Fisher Scientific	Loughborough, UK
Zymoclean DNA purification kit	Cambridge Bioscience	Cambridge, UK

2.1.1 – Equipment.

Agilent 6890N gas chromatograph and 5973N mass selective detector were obtained from Agilent (Stockport, UK). Econo-columns, the midi-gel SDS-PAGE and transfer systems were obtained from Bio-rad (Berkeley, USA). StepOnePlus RT-PCR system, nanodrop 1000 spectrophotometer, and the Jenway 6300 spectrophotometer was purchased from Fisher Scientific (Loughborough, UK). Äkta pure purification system, HiTrap Q HP anion exchange column, PD10 column, and Sephadex G-25 column was obtained from GE healthcare (Little Chalfont, UK). Wallac 1414 WinSpectral scintillation counter was purchased from Gemini BV (The Netherlands). Spectrum BX with Golden Gate attenuated total reflection (ATR) attachment and Lambda XLA spectrophotometer were purchased from Perkin-Elmer (London, UK). FluorChem Q system was purchased from ProteinSimple (Oxford, UK). EndOhm-6, EndOhm-12, and EVOM were obtained from World Precision Instruments (Hitchin, UK). Fluostar OPTIMA spectrophotometer was obtained from BMG LABTECH (Aylesbury, UK). Eclipse 50i microscope was acquired from Nikon (Kingston, UK).

2.1.2 – Software.

Schrödinger suite 2012 demonstration version was obtained from Schrödinger LLC (USA). Graphpad Prism 6.07 was purchased from GraphPad Software, Inc. (USA). Dock blaster 1.6.0 was operated using the online software (http://blaster.docking.org) and ZINC12 database (Irwin & Stoichet, 2005).

2.1.3 – Plasmids.

pCW57.1 was a gift from David Root (Addgene plasmid # 41393). Tet-pLKO-puro was a gift from Dmitri Wiederschain (Addgene plasmid # 21915). pLKO-BCAT1 was a gift from Bernhard Radlwimmer (DKFZ German Cancer Consortium, Germany). BCAT2 pENTR/D-TOPO was synthesised to order by Fisher Scientific (Loughborough, UK). eGFP and BCAT1 expression plasmids were a gift from Matthew Harris (UWE, UK).

2.1.4 – Primers.

The primer sequences used in this study are summarised in **Table 2.2**.

Table 2.2 – Primer sequences.

Primer	Sequence
H1	TCGCTATGTGTTCTGGGAAA
pCEP_Forward	AGAGCTCGTTTAGTGAACCG
BCAT1 Forward	TCCGCAGAGTGTACCGGAGA
BCAT1 Reverse	TGAGGACCACTCCACCGTCA
BCAT2 Forward	ATGGGCCCACCGTGTTAGTG
BCAT2 Reverse	CTCCAGCACCCCATCTTCGTG
GAPDH Forward	TGCACCACCAACTGCTTAGC
GAPDH Reverse	GGCATGGACTGTGGTCATGAG

2.1.5 – Antibodies.

The antibodies used in this study are summarised in **Table 2.3**.

Table 2.3 – Antibody list.

Antibody	Manufacturer code	Manufacturer	Concentration used
Occludin Mouse polyclonal	*ab168957*	Abcam (Cambridge, UK)	*1:3000*
GRx Mouse monoclonal	*ab55059*	Abcam (Cambridge, UK)	*1:2000*
TRx Mouse monoclonal	*ab57675*	Abcam (Cambridge, UK)	*1:2000*
AUH Rabbit monoclonal	*ab155980*	Abcam (Cambridge, UK)	*1:2000*
BCKDHA Rabbit polyclonal	*ab90691*	Abcam (Cambridge, UK)	*1:1000*
GDH-1 Rabbit monoclonal	*ab166618*	Abcam (Cambridge, UK)	*1:2000*
VE-Cadherin Rabbit polyclonal	*ab57675*	Abcam (Cambridge, UK)	*1:5000*
PDI Rabbit polyclonal	*5601-100*	Cambridge Bioscience (Cambridge, UK)	*1:2000*
ZO-1 Rabbit polyclonal	*21773-1-AP*	Cambridge Bioscience (Cambridge, UK)	*1:1000*
hBCATc Rabbit polyclonal	*Custom synthesis*	Insight Biotechnology (Wembley, UK)	*1:3000*
hBCATm Rabbit polyclonal	*Custom synthesis*	Insight Biotechnology (Wembley, UK)	*1:5000*
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Mouse monoclonal	*SC-47724*	Santa Cruz Biotechnology (California, USA)	*1:10,000*
Goat anti-rabbit IgG – HRP linked	*PI-1000*	*Vector Labs (Peterborough, UK)*	*1:3000 – 1:10,000*
House anti-Mouse IgG – HRP linked	*PI-2000*	Vector Labs (Peterborough, UK)	1:3000 – 1:10,000

2.1.6 – Cell lines.

The SH-SY5Y neuroblastoma cell line and the C6 rat glioma cell lines were obtained from ATCC (Teddington, UK). The hCMEC/D3 cell line was a gift from Pierre-Olivier Couraud (Institut Cochin, Université René Descartes, Paris, France).

2.2 – METHODS.

2.2.1 – Purification of hBCAT proteins.

The hBCAT genes were previously cloned into the pPET28a plasmid with a C-terminal his-tag (Davoodi *et al.*, 1998). These plasmids were transformed in BL21 DE3 *E. coli* and supplied as glycerol stocks, which were used in this study. Several strains were utilised: Wild type (WT) hBCATc and hBCATm isoforms, CXXC motif mutants for each isoform (C335S, C338S and C335S/C338S for hBCATc; C315A, C318A, C315A/C318A and C315S/C318S for hBCATm) as well as a C219S mutant of hBCATc.

For culture, sterile 2X YT media (1.6% Tryptone, 1 g yeast extract, 0.5% NaCl; 12 mg/L kanamycin sulphate; acidified to pH 7.0 with HCl) was prepared. A loop of *E. coli* glycerol stock was used to inoculate 100 mL of media and cultured at 37°C at 155 RPM for 18 hours. The suspension was then added to 1 L of sterile 2X YT and incubated at 30°C with 155 RPM agitation for an additional 18 hours. Transcription was induced by addition of 1 mL of 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) to the media and subsequent incubation at 30°C at 180 RPM for 4 hours. The suspension was centrifuged at 12000 xg for 15 minutes at 4°C, the supernatant discarded, and the bacteria pellets snap frozen at -80°C until required.

Bacterial pellets were resuspended in 20 mL of either extraction buffer A (10 mM Tris-HCl pH 8.0, 100 mM Na_2HPO_4, 1 mM β-mercaptoethanol) for WT hBCATc and mutants, or buffer AU (10 mM Tris-HCl pH 8.0, 100 mM Na_2HPO_4, 5 M urea and 1 mM β-mercaptoethanol) for WT hBCATm and mutants. The suspension was then sonicated

for 10 minutes with a 15 second pause for every 15 seconds of sonication and was then centrifuged at 12000 xg for 10 minutes at 4°C and the supernatant transferred to a fresh flask. To ensure complete lysis and protein collection, the pellet was resuspended, sonicated, and centrifuged again as previously described, and the supernatant added to the first flask of supernatant. Ni-NTA agarose (7 mL) was equilibrated in either buffer A or AU, and added to the supernatant before the solution stirred gently for 1 hour at 4°C.

The gel was transferred to an 18 mL econo-column column and washed sequentially with 3 column volumes of buffer A or AU, buffer B (10 mM Tris-HCl pH 7.4, 100 mM Na_2HPO_4, 500 mM NaCl, 20% glycerol, 5 mM β-mercaptoethanol) and buffer C (10 mM Tris-HCl pH 6.0, 100 mM Na_2HPO_4, 1.5 M NaCl, 20% glycerol, 5 mM β-mercaptoethanol). A final wash with 10 mL of buffer C50 (10 mM Tris-HCl pH 6.0, 100 mM Na_2HPO_4, 750 mM NaCl, 10% glycerol, 77 mM imidazole, 5 mM β-mercaptoethanol) was followed by protein elution with 10-20 mL of buffer D (10 mM Tris-HCl pH 6.0, 100 mM Na_2HPO_4, 750 mM NaCl, 10% glycerol, 770 mM imidazole, 5 mM β-mercaptoethanol).

This was exchanged into thrombin buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl) in a G25 column and incubated with 2 units of thrombin at 200 RPM rotation for 1 hour at 37°C. The solution was centrifuged at 4000 xg for 5 minutes at 4°C and the supernatant exchanged into Mono Q buffer (10 mM K_2HPO_4, 500 mM NaCl; adjusted to pH 8.0 with 10 mM K_2HPO_4) in a G25 column. The hBCAT protein was collected, KIC added to a final concentration of 2 mM, and the protein loaded onto a Hi-trap Q HP anion exchange column at a flow rate of 1 mL min^{-1} using the Äkta protein purification system. The bound protein was eluted over a gradient of 0 M to 0.5 M NaCl in Mono Q buffer at a flow rate of 1 mL min^{-1}, and collected in 0.5 mL fractions. These were then transferred to 12-14 kDa molecular weight cut-off dialysis tubing pre-equalibrated in stable buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM KIC, 5 mM glucose, 1 mM EDTA, 5 mM DTT) and dialysed against stable buffer with constant gentle stirring for 18 hours at 4°C. After this

period the stable buffer was discarded and replenished with fresh stable buffer, and the protein solution dialysed for a further 1 hour at 4°C. The protein solutions were collected and stored in 500 µL aliquots at -20°C in a 30% glycerol solution, and analysed for protein concentration using the Bradford assay (**Chapter 2.2.2**). Quadrupole time-of-flight mass spectrometry (QTOF-MS) of WT hBCAT proteins was conducted by Insight Biotechnology (Wembley, UK) as part of a standard procedure for preparing antigens before custom antibody synthesis.

2.2.2 – Protein estimation using the Bradford assay.

Fresh working solution of dye was prepared from 1.5 mL stock solution (330 mg Coomassie blue G, 66.6 mL phosphoric acid, 33.3 mL ethanol), 4 mL phosphoric acid, 1.9 mL ethanol and 92.6 mL water. Standards of 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 µg/µL BSA in deionised water were prepared, while unknown concentration protein samples were diluted to between 1 in 5 and 1 in 50 in deionised water.

To a 96-well plate was transferred, 200 µL of working dye solution and 5 µL of sample (unknown protein concentration sample, standard, or water blank; N=3), and absorbance at λ 620 nm quantified using a spectrophotometer (Fluostar OPTIMA). The absorbance of the blank sample containing no protein was subtracted from each standard or sample. Unknown protein concentrations were calculated from a calibration plot of the standards using Microsoft Excel software.

2.2.3 – SDS-PAGE gel electrophoresis.

Reduced samples were prepared by combining 10 µg protein, 6.25 µL 4X LDS running buffer (150 mM Tris-HCl pH 8.5, 2% LDS, 10% glycerol, 0.51 mM EDTA, 0.22 mM blue G250, 0.175 mM phenol red), 5% β-mercaptoethanol, and adjusting to volume to 25 µL with 200 mM Tris solution (pH 7.8). Each sample was then incubated at 80°C in a water bath for 10 minutes and 20 µL loaded into a 4-12% polyacrylamide gel (30% acrylamide, 1.5 M Tris-HCl pH 8.8, 0.5 M Tris-HCl pH 6.8, 10% SDS, 10% APS, TEMED), protein

ladder (Spectra ladder) was loaded into a separate well. The gel tank was filled with running buffer (25 mM Tris-HCl pH 6.8, 192 mM glycine, 0.1% SDS) and the gel electrophoresed at 100 V for 20 minutes, and 175 V for 60 minutes.

2.2.4 – Estimation of reaction thiols using the 5,5'-dithiobis-2-nitrobenzoic acid assay.

A fresh solution of DTNB was prepared by dissolving 160 mg DTNB in 20 mL DTNB buffer (50 mM HEPES, 1 mM EDTA, adjusted to pH 7.5 with HCl). The exact concentration of DTNB was calculated by the addition of 100 mM DTT to 1 mL of DTNB solution and monitoring of absorbance at 412 nm in a spectrophotometer (Jenway 6300); when the absorbance no longer continued to increase, the value was recorded. The concentration of the solution was calculated using a molar coefficient of 13700 M^{-1} cm^{-1} and the Beer-Lambert law equation.

To calculate the number of free thiol groups in hBCAT the protein was first exchanged into a non-reducing buffer of 20 mM HEPES and 1 mM EDTA using a PD10 column. The protein concentration was calculated by measuring the absorbance at 280 nm in a spectrophotometer (Jenway 6300) and using the molar coefficient of 86300 M^{-1} cm^{-1} for hBCATc and 67300 M^{-1} cm^{-1} for hBCATm. The hBCAT sample (2 µM) was titrated against a 100-fold molar excess of DTNB and the absorbance change at 412 nm in a spectrophotometer (Jenway 6300) monitored over 10 minutes, or until the absorbance was stable. The Beer-Lambert law was used to calculate the number of free thiols at each time point, again using a molar coefficient of 13700 M^{-1} cm^{-1}.

2.2.5 – Validation of synthesised chemical structure using spectroscopy.

The Agilent 6890N gas chromatograph was used with an Agilent 5973N mass selective detector (MSD) for GC-MS analysis. A HP-5MS 30 m x 250 µm x 0.25 µm column was used. Flow-rate was 1 mL/min helium a splitless inlet was used with a temperature of

310°C. For each analysis, the initial temperature was 100°C and this increased linearly to 200°C over a period of 30 - 40 minutes depending on individual compound optimised conditions. The MSD was in negative ion mode with a mass scan range of 50 - 400 m/z.

All compounds analysed by FT-IR were either solid or semi-solid and were analysed without further preparation using attenuated total reflection sampling.

The LC-MS analysis was completed by the University of Bath LC-MS service. An Agilent Eclipse XDB-C18 5 µm, 4.6 x 150 mm column was used for LC. This was coupled to an ESI-TOF MS in positive ion mode, with a mass scan range of 50 - 750 m/z. The sample was analysed in duplicate, with a calibrant injected at the start of each LC-MS run to ensure accurate mass data.

The Varian 400-MR nuclear magnetic resonance (NMR) spectrometer was used at Bristol University for NMR spectroscopy. Specific usage parameters are detailed in **Table 2.4**. Usage of the spectrometer was kindly provided by Dr Craig Butts.

2.2.6 – Gabapentin synthesis.

The method for gabapentin synthesis was adapted from Kumar *et al.* (2008). A 250 mL round-bottom flask was charged with 45 mL of a 7 M solution of NaOH and the solution cooled to -8°C. A total of 3.5 mL of bromine was added drop-wise over a period of 40 minutes. A solution of 10 g 1,1-cyclohexanediacetic monoamide in 10 mL of 7 M NaOH was slowly added with stirring for 1 hour, with the temperature maintained between -8°C and -5°C. The solution was then heated to 35°C for one hour before cooling to 15°C. By monitoring pH using wide spectrum pH indicator paper, the solution was acidified to pH 5 with 10 M HCl and washed with 50 mL dichloromethane (DCM). The aqueous layer was collected, acidified further to pH 2 with 10 M HCl and stirred gently for 4 hours. The gabapentin hydrochloride precipitate was collected by filtration, and the solid dried by heating in an oven at 60°C for 18 hours. The crude solid was purified by dissolving in absolute ethanol, stirring for 2 hours and then filtering the solution to remove impurities.

***Table 2.4* – Parameters for usage of NMR spectroscopy.**

Parameter	Value
Spectrometer frequency	399.773 MHz
Pulse width	9.8 µs
Acquisition time	4.089 s
Number of points	65536 points
Sweep width	8012.8 Hz
Recycle delay	2.0 s
Temperature	25.0 °C
Solvent	CD_3OD

Ethanol was removed using a rotary evaporator, generating gabapentin hydrochloride crystals as needles. To obtain the free base, the hydrochloride salt was dissolved in 200 mL of ethyl acetate and the pH adjusted to pH 7.2 using trimethylamine, again monitoring the approximate pH using wide spectrum pH indicator paper. The precipitate that formed was filtered from solution, and dried in an oven for 18 hours at 60°C before the product was analysis by GC-MS. The final yield was recorded as 9%.

2.2.7 – Computer simulation of structure based ligand discovery.

The crystal structures of hBCATm in the PMP form (PDB ID: 1KTA. Yennawar *et al.*, 2002) and hBCATc bound to a specific inhibitor (PDB ID: 2ADJ. Hu *et al.*, 2006) were imported into the molecular visualisation package UCSF Chimera (Petterson et al., 2004) and optimised by removing water molecules and heteroatoms not essential for catalysis. The structure of hBCATm was then imported into Dock Blaster (Irwin *et al.*, 2009), the active site identified, and a variety of compounds from the Zinc database (Irwin & Stoichet, 2005) analysed using high throughput screening (HTS) for favourable binding in the active site. The molecule which was the most energetically favourable and thus a potential ligand was identified. Using Schrödinger suite 2012, as described in Wu *et al.* (2011), the crystal structures of hBCAT were optimised using the protein preparation wizard (Epik version 2.3; Impact version 5.8; Prime version 3.1) (Sastry *et al.*, 2013). In addition, the structure of the potential ligand was drawn and optimised using Maestro (version 9.3) and Ligprep (version 2.5). A simulation of the potential ligand binding to both isozymes of hBCAT was then conducted using the XP setting of Glide version 5.8 (Friesner *et al.*, 2006) and an evaluation of the binding of the compound to hBCAT obtained. The chemical structure of the compound was gradually changed within the software and the binding to hBCAT simulated again using the same settings as

previously described until a compound was found for which it was energetically favourable to bind to hBCATm but unfavourable to bind to hBCATc.

2.2.8 – Trial synthesis of 3-[(chloroacetyl)oxy]benzoic acid.

A 150 mL 2-necked round-bottom flask was charged with 30 mL anhydrous DCM, 7.05 g (75 mmol) chloroacetic acid, and 0.6 g (5 mmol) 4-Dimethylaminopyridine (DMAP), and the solution cooled to 0°C with an ice bath with gentle stirring. To this 11 g (54 mmol) dicyclohexylcarbodiimide (DCC) in 10 mL anhydrous DCM was first added, followed by the slow addition of 6.9 g (50 mmol) hydroxybenzoic acid in 20 mL anhydrous DCM. The solution was stirred at 0°C for 5 minutes, and then 3 hours at room temperature. This was filtered and the filtrate retained, dried over $MgSO_4$ and the solvent evaporated under pressure with a rotary evaporator, generating a crystalline yellow oil. This was recrystallised with hot acetone to generate yellow crystals suspended in oil, which were analysed by GC-MS.

2.2.9 – Trial synthesis of amide based hBCATm inhibitor.

A 250 mL 2-necked round-bottom flask was charged with 150 mL ethyl acetate, 3 g (27 mmol) aminophenol, and 3.3 mL (40 mmol) pyridine. This was cooled to 0°C and over 10 minutes, a total of 3.3 mL (25 mmol) p-toluoyl chloride diluted in an equal volume of ethyl acetate was added. This was stirred for 15 minutes before the solution was washed twice with 40 mL of 1 M HCl, twice with 25 mL deionised water, and dried over $MgSO_4$. The organic solvent was then evaporated under vacuum using a rotary evaporator to yield a white amorphous solid, which was dried to constant weight in an oven at 60°C for 6 hours. The compound did not require any further purification and was identified as N-(3-hydroxyphenyl)-4-methylbenzamide by FTIR and GC-MS.

In a 250 mL round-bottom flask, 100 mL of anhydrous acetone, 5 g (22 mmol) N-(3-hydroxyphenyl)-4-methylbenzamide, 1.9 mL (33 mmol) 3-bromopropene and 6.08 g (44 mmol) K_2CO_3, was combined and the suspension refluxed for 6 hours. The volume of

acetone was then reduced to a third by evaporating under vacuum and 50 mL of ether was added. This was washed once with 50 mL of a 1 M NaOH solution, and twice 50 mL deionised water before the organic solution was dried over $MgSO_4$ and evaporated under vacuum to yield a pale yellow crystalline powder, which was dried to constant weight in an oven at 60°C. The compound was identified as 4-methyl-*N*-[3-(prop-2-en-1-yloxy)phenyl]benzamide by FT-IR and LC-MS.

A 150 mL 3-neck round-bottom flask was charged with 100 mL ethylene glycol and 5 g (19 mmol) 4-methyl-*N*-[3-(prop-2-en-1-yloxy)phenyl]benzamide. The solution was heated to 175°C for 6 hours or until the reaction was deemed complete, both by a change of colour and spectrographically using UV-Vis spectroscopy. Here, during the reaction the colour of the solution would change from a strong yellow, through orange and then to a deep red/burgundy colour. Serial samples were taken and diluted in ethanol for UV/Vis spectroscopy analysis, and heating would continue for one hour after the total disappearance of the peak at λ 270 nm. The solution was cooled to 0°C in an ice-water bath and 150 mL deionised water added, which caused the product to immediately precipitate from solution. The product was extracted three times with 50 mL ether, and the combined extracts washed three times with 50 mL deionised water, once with 50 mL saturated NaCl solution and then dried over $MgSO_4$. The solvent was evaporated under vacuum to yield a red/brown amorphous powder with a slightly waxy texture which was dried to constant weight in an oven at 60°C. The mixture was not purified further and was identified as the two isomers of the aromatic Claisen rearrangement product of 4-methyl-*N*-[3-(prop-2-en-1-yloxy)phenyl]benzamide by FTIR spectroscopy and LC-MS.

A 100 mL round-bottom flask was charged with 25 mL DMF, 14 mg (37.4 µmoles) K_2OsO_2 and 4.6 g (14.96 mmoles) Oxone®, and the suspension stirred for 30 minutes. The mixture was then cooled in an ice-water bath to 10°C and 1 g (3.74 mmol) of the aromatic alkene from the previous step added over 10 minutes. The ice-water bath was then removed and the mixture allowed to return to room temperature with stirring for 6

hours. The resulting mixture was transferred to a separating funnel and 25 mL of a saturated aqueous $NaHSO_3$ solution added. The resulting slurry was extracted three times with 50 mL ethyl acetate, the organic extracts combined, and then washed twice with 50 mL of 1 M HCl, twice with 50 mL deionised water, and once with 50 mL of a saturated NaCl solution, before the organic was dried over $MgSO_4$ and evaporated under vacuum. The resulting oil was analysed by GC-MS and identified as starting material.

2.2.10 – Ester intermediate used for synthesis of phenylacetic acid dervived hBCATm inhibitors.

The reaction was completed with each of three different carboxylic acid starting materials: 4-hydroxyphenylacetic acid, 3-chloro-4-phenylacetic acid, and 4-hydroxy-3-methoxyphenylacetic acid. In total, four different compounds were synthesised from these carboxylic acid starting materials by coupling either benzyl bromide or 4-methylbenzyl bromide to the starting material. The final products were: 4-(benzyloxy)phenylacetic acid (BOPAA), 4-(benzyloxy)-3-chloro-phenylacetic acid (Benzofenac), 3-chloro-4-[(4-methylbenzyl)oxy]-phenylacetic acid (MeBenzofenac), and 4-(benzyloxy)-3-hydroxy-phenylacetic acid (3OMeBOPAA). The synthesis took place in three steps, protection of the carboxylic acid group by synthesis of a methyl ester, esterification with benzyl bromide or 4-methylbenzyl bromide, and finally deprotection of the methyl ester by saponification.

First, a 250 mL 2-necked round-bottom flask was charged with 5 g of carboxylic acid starting material, 62.5 mL methanol, and 0.5 mL concentrated H_2SO_4 and refluxed for 1 hour. The solution was allowed to cool and neutralised with 0.1 M sodium hydrogen carbonate. The product was extracted twice with 30 mL ethyl acetate, the extracts combined and washed three times with 20 mL of deionised water and 20 mL of saturated NaCl solution. The organic extract was dried over $MgSO_4$ and the solvent evaporated under vacuum.

For the second step a 100 mL round-bottom flask was charged with 30 mmol of the methyl acetate, 35 mmol benzyl bromide or 4-Methylbenzyl bromide, 50 mL DMF, and 8.3 g (60 mmol) K_2CO_3 and the suspension stirred for 4 hours at room temperature. This was then diluted with 100 mL of ethyl acetate and washed five times with 50 mL of deionised water, once with 50 mL of a saturated NaCl solution, dried over $MgSO_4$ and the solvent evaporated with a rotary evaporator.

For the final step, 20 mmoles of product from the previous step, was refluxed in 20 mL of 6 M NaOH for 15 minutes. The solution was cooled to room temperature and then acidified slowly with 1 M HCl until material no longer precipitated. The product was filtered from the solution and washed with ice cold deionised water before being dried to constant weight at 60°C in an oven to yield the free acid product. Synthesis was confirmed by GC-MS and FT-IR.

2.2.11 – Morpholide intermediate used for synthesis of benzyloxyphenylacetic acid dervived hBCATm inhibitors

Synthesis of 3-methyl-4-[(4-methylbenzyl)oxy]-phenylacetic acid (3MeBOPAA) was achieved by a separate method which utilised morpholine. A 250 mL round-bottom flask was charged with 10 g (66 mmol) 4-Hydroxy-3-methylacetophenone, 13 g (70 mmol) 4-methylbenzyl bromide, 75 mL dimethylformamide, and 18.25 g (132 mmol) K_2CO_3, and stirred for 4 hours at room temperature. The product was extracted twice with 50 mL of diethyl ether and the extracts combined. This was then washed twice with 50 mL deionised water and once with 50 mL of a saturated NaCl solution, before the organic solved was dried over $MgSO_4$ and evaporated under vacuum to yield 1-3-methyl-4-(4-methylbenzyl)oxy-phenylethanone.

A 100 mL round-bottom flask was charged with 10 g of the phenylethanone product, 4.5 mL morpholine, and 1.6 g elemental sulphur, and refluxed for 6 hours. Once cooled to room temperature 20 mL deionised water was added and the product extracted twice

with 25 mL diethyl ether. The solvent was dried over $MgSO_4$ and evaporated with a rotary evaporator to yield 2-3-methyl-4-[(4-methylbenzyl)oxy]phenyl-1-(morpholin-4-yl)ethanethione.

Finally, 9 g (25 mmol) 2-{3-methyl-4-[(4-methylbenzyl)oxy]phenyl}-1-(morpholin-4-yl)ethanethione and 100 mL of a 2 M KOH solution was added to a 250 mL round-bottom flask and the suspension refluxed twice for 6 hours. This was acidified to pH 4 with 50 mL of 5 M HCl, extracted twice with 50 mL diethyl ether, washed twice with water, once with saturated NaCl solution, and then dried over $MgSO_4$. The solvent was evaporated to yield 3-methyl-4-[(4-methylbenzyl)oxy]phenylacetic acid. This was recrystallised using hot petroleum ether and the purified product extracted by filtration before drying to constant weight at 50°C in an oven. Synthesis was confirmed by GC-MS analysis.

2.2.12 – Radioactive substrate BCAT activity assay.

A working dilution of substrate was prepared by mixing 150 dpm/nmole α-carbon [U-^{14}C]KIV, 100 µL of 100 mM non-radioactive KIV, 600 µL of 200 mM L-isoleucine and the volume brought to 1 mL with deionised water, depending on the quantity of radiolabelled KIV required. Inhibitor compounds were dissolved in deionised water with the exception of the BOPAA family compounds which were dissolved ion dimethyl sulfoxide (DMSO).

Glass vials were prepared containing 100 µL of 100 mM potassium phosphate, 20 µL of 100 mM DTT, 20 µL of 5 mM PLP and 50 µL of the working substrate dilution (**Figure 2.1**). The reaction solution was complete with either 300 µL deionised water for kinetic analysis without inhibitor to assess normal hBCAT activity, 300 µL of inhibitor solution (**Table 2.5**) to assess the impact of inhibitors on hBCAT activity, or 310 µL of water as a negative control sample, which would not contain hBCAT enzyme. Each vial was produced in duplicate. Filters were prepared from filter paper soaked in 5 M KOH and placed in wells suspended by the lid of the glass vials to capture released [^{14}C]CO_2.

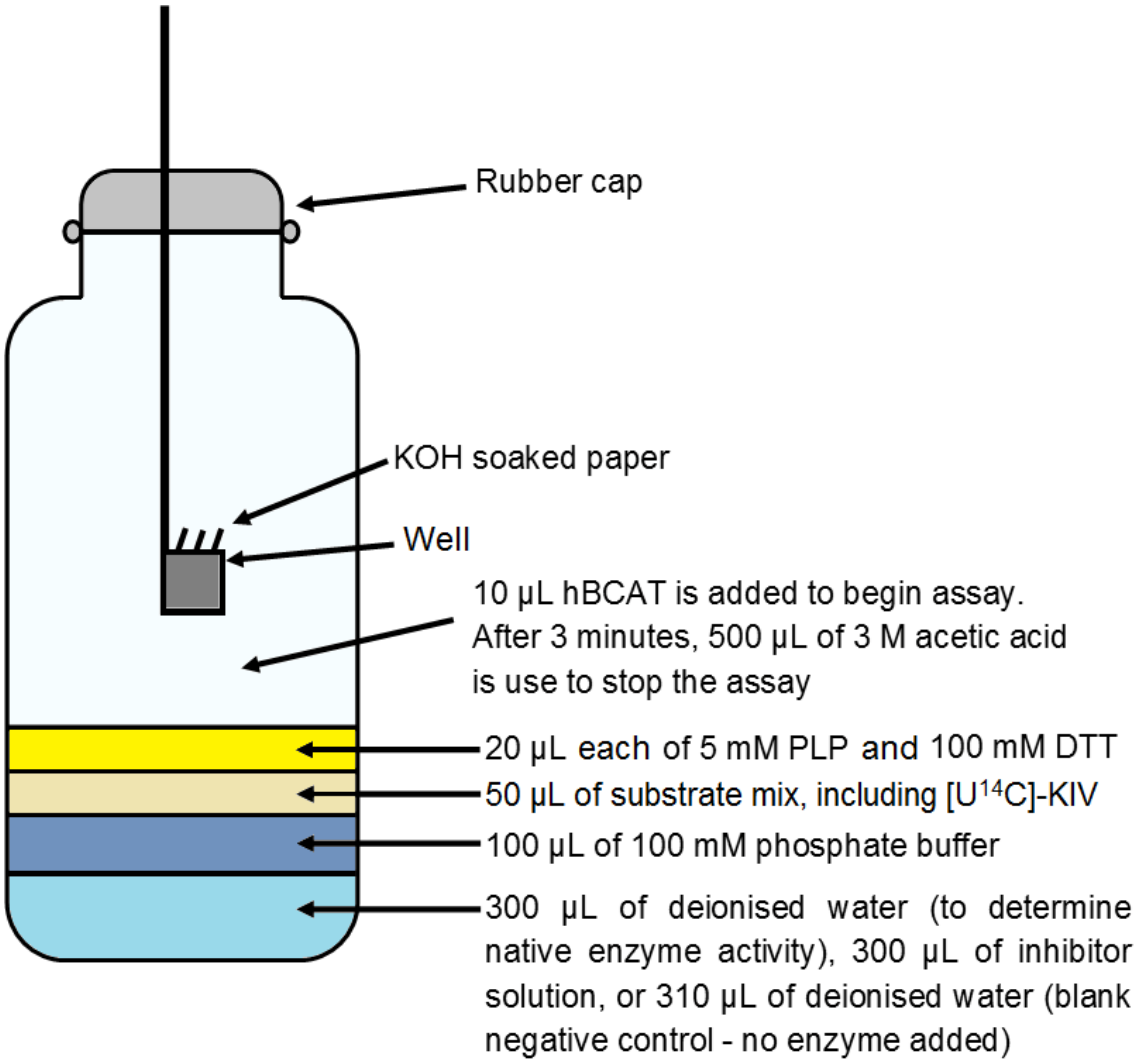

***Figure 2.1* – The components of the hBCAT kinetic assay.** Glass vials were prepared containing radiolabelled substrate, phosphate buffer, a reducing agent and a co-factor. The assay was initiated by the additional of hBCAT enzyme, conducted at 37°C for 3 minutes with gentle shaking, and stopped by addition of acetic acid. Aqueous solutions of compounds of interest were prepared and their effect on transamination evaluated by comparison to the native enzyme activity. Finally, a negative control reaction was conducted alongside all assays to determine the radioactive background, which would be subtracted from all results.

Table 2.5 – Concentrations of inhibitors analysed using the radioactive method.

Inhibitor compound	Concentration (mM)
Gabapentin	20
Valproic acid	2, 5, 10, 15, 20
Dipropylglycine	4, 10, 20, 30, 40
Memantine	2, 10
γ-aminobutyric acid (GABA)	60
$LiCO_3$	40
BOPAA	10
3OMeBOPAA	10
3MeBOPAA	10
Benzofenac	10
MeBenzofenac	10
Hydroxyphenylacetic acid (HPAA)	0.5, 1, 2

The glass vials were incubated in water at 37°C with 100 RPM shaking for 3 minutes, after which 10 µL of enzyme in buffer (50 mM HEPES, 1 mM EDTA) was added to all vials except the negative control. The vials were incubated for 3 minutes before the reaction was stopped by adding 500 µL of 3 M acetic acid. The rubber lids were placed on the vials, and 500 µL of 30% hydrogen peroxide added before the vials were sealed, and incubated for 1 hour at 37°C with 100 RPM shaking. The rubber lids were removed from the glass vials and the filter papers discarded. Tubes containing 3 mL of scintillation fluid (ProSafe+) were prepared and 150 µL of each sample transferred into each respective tube before mixing. The DPM for each sample was monitored as an average over 3 minutes using a β-radiation scintillation counter (Wallac 1414 Winspectral).

2.2.13 – BCAT coupled enzyme assay.

The experiment was adapted from a method by Cooper et al. (2002). A series of reaction buffers were prepared of L-leucine (0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 10.0, 13.33 mM) in BCAT reaction buffer (10 mM K_2HPO_4, 50 mM ammonium sulphate, 5 µM PLP, 5 mM KG, 50 µM NADH, 5 mM DTT, 1 U L-leucine dehydrogenase; adjusted to pH 7.4 with 10 mM KH_2PO_4). To investigate the effect of benzofenac on BCAT transamination some reaction buffers also contained 0.25, 0.5 or 1.0 mM of the compound. Wells of a clear flat-bottom 96-well plate were first filled with 195 µL BCAT reaction buffer, before the rapid addition of 5 µL of 50 ng/µL hBCATc or 80 ng/µL hBCATm enzyme. The plate was quickly transferred to a spectrophotometer (Fluostar OPTIMA) and the absorbance at λ 340 ± 12 nm was assayed at least every 30 seconds (depending on the number of wells on the plate) for at least 2 minutes.

2.2.14 – ALT activity assay.

A series of reaction buffers were prepared by serial dilution of L-alanine (11.72, 23.44, 48.88, 93.75, 187.5, 375, 750 mM) in ALT reaction buffer (200 mM MOPS, 0.001% Triton-X100, 25 µM PLP, 10 mM KG, 50 µM NADH, 100 mU L-lactic dehydrogenase;

adjusted to pH 7.5 with NaOH). To evaluate the impact of inhibition by benzofenac, a range of concentrations (0.25, 0.5, or 1.0 mM) were added to the ALT assay. Next, 195 µL reaction buffer was transferred to wells of a clear 96-well plate, before the rapid addition of 5 µL of 10 mU/µL ALT enzyme to initiate the reaction. The absorbance was monitored immediately at λ 340 ± 12 in a spectrophotometer (Fluostar OPTIMA) at least every 30 seconds (depending on the number of wells on the plate) for at least 2 minutes. The velocity of a blank reaction containing no L-alanine was subtracted from the velocity of each of the samples. Kinetic analysis was conducted using Microsoft Excel software to create a Lineweaver-Burk Plot, or Graphpad Prism for non-linear regression modelling and calculation of K_m and V_{max} values.

2.2.15 – Synthesis of siRNA.

The Ambion Silencer siRNA construction kit was used to produce siRNAs and synthesis closely followed the procedure outlined in the instruction manual. All consumables used in methods containing nucleic acids are certified DNase and RNase-free by the manufacturer. The mRNA transcripts of BCAT1, BCAT2 and BCKDHA were identified using the NCBI human gene database. Each gene had several splice variants but did not exhibit any other polymorphism and so the shortest variant was used for each template. The gene was scanned for 'AA' dinucleotide sequences and these highlighted along with the next 19 bases towards the 3' end. These potential siRNA targets were checked for similarity to other human genes using NCBI BLAST; any similarity greater than 16 bases discounted the sequence. Three sequences were chosen for each gene, one near the beginning of the gene, one in the middle, and one near the end of the gene. Sense and antisense single stranded DNA (ssDNA) oligonucleotide sequences were designed using the RNA sequences to act as templates for RNA synthesis. The sense sequence was identical to the 12 base RNA sequence except that all uridine bases were exchanged for thymidine bases and the T7 promoter sequence 5'-CCTGTCTC-3' was added to the 3' end of the sequence (**Figure 2.2**). For the antisense template, the 19

bases after the dinucleotide AA start were reversed and the bases inverted, and then the T7 promoter sequence was added to the 3' end of the sequence. The sequences were then chemically synthesised and purified by HPLC by an external laboratory (Eurofins Genomics, Germany).

The oligonucleotides were supplied lyophilised and were dissolved in nuclease-free water to 100 µM calculated using the synthesis quantity information sheet supplied. To synthesise each siRNA ssDNA oligonucleotide template, the sense and antisense ssDNA strands were first extended to double stranded DNA (dsDNA) with an exo-klenow enzyme fragment. They were then transcribed in separate reactions before the transcripts combined to prevent an excess of one particular strand being constructed over the other. First, 2 µL T7 promoter primer, 6 µL DNA hybridisation buffer and 2 µL of either the sense or antisense template DNA oligonucleotide solution were combined in a nuclease-free PCR tube. This was heated to 70°C in hot block for 5 minutes, and then incubated at room temperature for 5 minutes. Next, 2 µL 10X Klenow reaction buffer, 2 µL 10X dNTP mix, 4 µL nuclease-free water, and 2 µL Exo-Klenow enzyme fragment were mixed into the solution, all of which were supplied by the kit. This was then incubated in a dry cabinet heater at 37°C for 30 minutes.

The single stranded RNA (ssRNA) transcripts of the DNA templates were then synthesised. To achieve this, 2 µL of sense or antisense DNA template, 4 µL of nuclease-free water, 10 µL of 2X NTP mix, 2 µL of 10X T7 reaction buffer, and 2 µL of T7 enzyme mix were mixed gentle in a fresh nuclease-free PCR tube and incubated in a cabinet heater for 2 hours at 37°C to hybridise the ssRNAs to dsRNA. The promoter sequence and DNA templates were digested by adding 6 µL of digestion buffer, 48.5 µL of nuclease-free water, 3 µL of RNase, and 2.5 µL of DNase to the PCR tube, and mixing gently before incubation in a cabinet heater for 2 hours at 37°C. The desired dsRNA was purified using the kit supplied spin columns and eluted in 100 µL of nuclease-free water heated to 75°C.

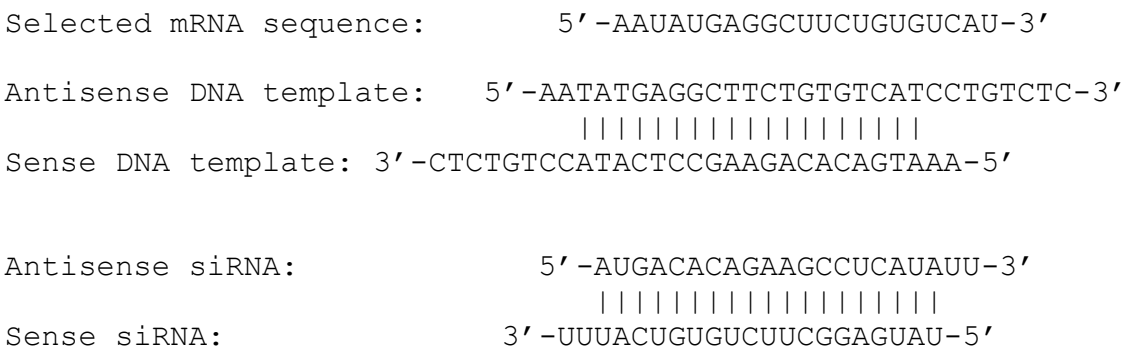

***Figure 2.2* – Design of DNA templates for siRNAs to silence a target gene.** A targeted section of the BCAT2 gene is shown. Sense and antisense DNA oligonucleotide templates were constructed, as described, which would produce a 21 nucleotide double stranded RNA (dsRNA) containing a 'UU' overhang. The T7 promoter region (5'-CCTGTCTC-3') was removed by digestion before siRNAs were used.

2.2.16 – Mammalian cell culture.

The human SH-SY5Y neuroblastoma cell line was cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS) and 2.5 mM L-glutamine (complete DMEM) in Corning T75 flasks until required for specific experiments. Passage numbers 3-25 were used for experiments in this thesis. The growth media of cells was changed every three days and cells were grown until 80% confluent, before subculture. Cells were grown in an incubator at 37°C with 5% CO_2 and 80% humidity. For subculture, growth media was first aspirated and the cells washed briefly with phosphate buffed saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH adjusted to 7.4 with HCl), before the addition of enough trypsin-EDTA solution (0.25% trypsin, 0.1 mM EDTA) to coat the surface of the growth vessel, and incubated for 5 minutes at 37°C. Complete media was then added to neutralise the trypsin-EDTA solution, and the combined suspension collected and centrifuged at 1000 xg for 5 minutes. The supernatant was discarded and the pellet resuspended in 50 µL complete media per cm^2 of original growth surface area. A 50 µL aliquot of this suspension was then transferred into a 500 µL centrifuge tube and combined with 50 µL of 0.4% trypan blue dye solution, the cell density was counted and calculated using a glass haemocytometer and microscope. Cells were seeded onto the surface of either a Corning 96-well plate at 5000 cells per well, a Corning 6-well plate at 1 x 10^5 cells per well, a Corning T25 flask with 2 x 10^5 cells, or a Corning T75 flask with 2 x 10^6 cells.

The human hCMEC/D3 endothelial cell line was cultured in EndoGro-MV media with the addition of the supplement kit (0.2% low serum supplement, 5 ng/mL human RGF, 10 mM L-glutamine, 1 µg/mL hydrocortisone hemisuccinate, 0.75 U/mL heparin sulphate, 50 µg/mL ascorbic acid, and 5% FBS) and 1 ng/mL Fibroblast Growth Factor β (complete EndoGro) in Corning T75 flasks unless being used for specific experiments. The endothelial cells were cultured on a surface coated with 10 µg/cm^2 rat tail collagen type 1. Passage numbers 30-35 were used for experiments. The growth media of cells was

changed every three days and cells were allowed to grow to 100% confluence. When cultured for evaluation of permeability or for tight junction protein expression, the endothelial cells were seeded in complete EndoGro media supplemented with 10 mM LiCl and 10 µM resveratrol. After three days this media was removed and changed for fresh media containing LiCl and resveratrol, while on the sixth day post-seeding this media would then be removed and changed for fresh complete EndoGro without LiCl and resveratrol. When cells were grown on Corning transwell 0.4 µm pore size polyester bucket inserts, defined volumes of cell growth media or saline solution were used both inside the insert and outside within the well of the plate. For 6-well plate inserts, the well contained 2.6 mL volume, while the insert contained 1.5 mL, conversely for 12-well plate inserts, the well contained 1.5 mL volume and the insert 500 µL. The inserts were seeded with 1 x 10^5 cells per cm^2 of growth surface area.

Rat C6 glioma cells were cultured in complete DMEM on Corning T75 flasks until required for specific experiments. Passage numbers 15-20 were used for experiments. The growth media of cells was changed every three days and cells were grown to 80% confluence before subculture. When grown on transwell inserts the C6 cells were grown on the underside, this was achieved by transferring 500 µL of complete media containing 10 x10^3 cells to the underside of the standing 6-well plate insert and incubating in a sterile box within an incubator for 4 hours. The inserts could then be restored to the wells of the 6-well plate and grown as normal in cell growth media.

2.2.17 – Transient cell transfections of expression plasmids and siRNA.

JetPrime and RNAiMAX transfection reagents were trialled for siRNA transfection in the SH-SY5Y neuroblastoma and hCMEC/D3 endothelial cell lines. All expression plasmid transfections were conducted using the JetPrime transfection reagent. The hCMEC/D3 cells were transfected on subculture, while the SH-SY5Y cells were subcultured and then transfected 48 hours after seeding, at between 50 and 70% confluence. The SH-SY5Y cell media was replenished with fresh media 1 hour before transfection.

To prepare the transfection of siRNA or DNA, the nucleic acid was first diluted in the appropriate dilution buffer; JetPrime buffer for JetPrime, or opt-mem for RNAiMAX (**Table 2.6**). The transfection reagent was then added, the solution vortexed mixed and then incubated at room temperature for 15 minutes. The entire solution was then distributed evenly over the well of cells and the plate gently swirled to mix the solution with cell media. Two controls were used for every transfection; one well of a plate would be treated with a 'mock transfection' solution containing only transfection buffer and reagent, without siRNA, while a second well would be transfected with either 100 nM of a non-targeting siRNA sequence or 50 ng eGFP expression plasmid.

2.2.18 – Spin-column purification of RNA from cultured cells.

Adherent cells were first washed with PBS, and then treated with 100 µL tri-reagent per cm^2 of cell growth area. Cells were homogenised by repeated pipette aspiration and the suspension centrifuged at 12000 xg for 5 minutes. The supernatant was then transferred into a Direct-zol spin column within a collection tube and centrifuged at 12000 xg for 30 seconds. The flow-through was discarded and the RNA washed once by addition of 400 µL of pre-wash buffer before centrifugation at 1000 xg for 1 minute. The genomic DNA digested by applying 80 µL of DNAse solution (30 U DNAse I, 75 µL of supplied DNA digestion buffer) directly to the column matrix and incubating the reaction for 15 minute at room temperature. The column was then centrifuged at 1000 xg for 1 minute and the RNA purified by sequential washing; twice with 400 µL of direct-zol pre-wash buffer, and once with 700 µL of wash buffer, with the solution drawn through the column each time by centrifugation at 1000 xg for 1 minute. For RNA elution, the column was transferred to a fresh tube and 30 µL of nuclease-free water applied to the column matrix before centrifugation at 1000 xg for 3 minutes to elute RNA. The sample tube was transferred to an ice water bath and either used immediately for cDNA synthesis or stored at -20ºC.

Table 2.6 – Quantity of material for siRNA and plasmid transfection.

Transfection type	Growth vessel	siRNA dilution buffer volume (µL)	Transfection reagent volume (µL)
siRNA	Corning 96-well plate	20	0.4
	Corning 6-well plate	200	4
DNA plasmid	Corning 96-well plate	20	0.5
	Corning 6-well plate	200	5

2.2.19 – cDNA synthesis from isolated RNA.

The nanoScript cDNA synthesis kit was used for cDNA synthesis from 2 µg of RNA as described by the manufacturer.

2.2.20 – Analysis of gene expression by RT-PCR.

The PrecisionFAST mastermix containing SYBRgreen dye was used for RT-PCR analysis of mRNA expression. A 96-well PCR plate was used with 20 µL PCR reactions. Standards were assayed in duplicate wells and samples performed in triplicate. To prepare the standards 1 µL of each of the experimental samples was pooled in a fresh reaction tube and serially diluted to 0.781, 1.563, 3.125, 6.25, 12.5, and 25 ng/µL. For experimental samples 125 ng of template was used for the PCR reaction.

A RT-PCR master mix was prepared in a fresh reaction tube containing 10 µL PrecisionFAST mastermix, 0.5 µL 10 µM forward primer, 0.5 µL 10 µM reverse primer, and 4 µL nuclease-free water, for each PCR reaction. Next, 5 µL of cDNA template and 15 µL of RT-PCR mastermix was transferred to wells in the PCR plate. The reaction was pulse centrifuged at 1000 xg and inserted into the StepOnePlus RT-PCR system. A 3 step protocol was used for the PCR reaction; HotStart enzyme activation for 1 minute at 95°C before 50 cycles of 95°C for 5 seconds, 55°C for 5 seconds, and 72°C for 10 seconds. A melt curve stage at the end of the 50 cycles ran from 60°C to 95°C. Fluorescence data was automatically analysed by the StepOnePlus software to output relevant C_T values and was processed further using Microsoft Excel. Results were normalised against GAPDH expression

2.2.21 – Agarose gel electrophoresis.

Agarose was prepared in 100 mL TAE (40 mM Tris-HCl pH 8.0, 20 mM acetic acid, and 1 mM EDTA) to a concentration between 0.8 and 2%. Analysis of large plasmids required a lower percentage of agarose in the gel, while analysis of short

oligonucleotides was conducted in a lower percentage gel. If required, ethidium bromide stain was added to the gel to a final concentration of 0.5 µg/mL.

The 6X orange running dye was diluted to 1X with DNA solution and 20 µL loaded into each well. An ultra low ladder or 1kb ladder was loaded into each gel. Nucleic acids were separated at 200 V in TAE buffer for 1 hour. If the precision diamond stain was used the gel was then incubated in 1X precision diamond solution in 1X TAE buffer for 20 minutes with gentle agitation. The bands were visualised using UV transillumination using the FluorChem Q Gel Dock.

2.2.22 – Extraction of mammalian cell lysate.

Media was aspirated from the culture vessel and the growth surface washed twice with PBS cooled to 4°C, and 10 µL per cm^2 of protein extraction buffer added to the cells (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1X cOmplete protease inhibitor cocktail). The growth surface was scraped, the whole of the suspension transferred to a reaction tube, and then incubated for 1 hour in an ice-water bath. The suspension was sonicated for 1 minute with a 10 second pause for every 10 seconds of sonication and was then centrifuged at 12000 xg for 10 minutes at 4°C. The supernatant was stored at -20°C until required.

2.2.23 – Protein estimate using amido black method.

Samples and standards were prepared in a reaction tube by combining 30 µL amido buffer (1 M Tris-HCl pH 7.5, 1% SDS) with the protein solution and bringing the total volume to 300 µL with deionised water. Standards contained 1, 2.5, 5, 7.5, and 10 µg of BSA, in addition to a blank with no protein. Proteins were precipitated by the addition of 60 µL of 60% trichloroacetic acid (TCA) before the suspension was briefly mixed, pulse centrifuged and incubated at room temperature for 5 minutes. A nitrocellulose membrane was placed on top of a glass apparatus and prepared by washing three times with 6% TCA solution. The total content of each protein solution was then passed

through a defined segment of the membrane and washed regularly with 6% TCA. The membrane was then stained with amido staining solution (0.1% amido black 10B sodium salt, 45% methanol, 10% acetic acid) for 2 minutes, and then destained to remove background with a repeating sequence of deionised water and destain buffer (90% methanol, 2% acetic acid) washes. Each sample segment was then cut from the membrane and the dye eluted in a reaction tube using 500 µL elution buffer (25 mM NaOH, 0.05 mM EDTA, 50% ethanol). The absorbance of each solution at λ 620 nm was quantified using a spectrophotometer (Fluostar OPTIMA).

2.2.24 – Wet protein transfer and western blot analysis of cell lysate.

A PDVF membrane was first activated by incubation with 100% methanol for 5 minutes before being soaked in transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% methanol). A sandwich was created with the SDS-PAGE gel, the PDVF membrane, and transfer buffer soaked sponges and blotting paper. Proteins were transferred at 30 V and 400 mA for 18 hours at 4°C.

Once the proteins had transferred to the membrane the membrane was removed and washed in Tris buffered saline (TBS) (50 mM Tris-HCl pH 7.5, 150 mM NaCl) for 5 minutes with gentle agitation before protein blocking with blocking solution (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20, 5% Marvel milk powder) for 1 hour at room temperature with gentle agitation. The blocking solution was aspirated and replaced with primary antibody diluted in fresh blocking solution (**Table 2.3**) before incubation for 18 hours at 4°C with gentle agitation. The solution was then aspirated and the membrane washed 4 times for 5 minutes with TBST (TBS, 0.1% Tween-20), before a secondary antibody diluted in blocking solution (**Table 2.3**) was added and the membrane incubated for between 1 hour at room temperature with gentle agitation. The membrane was then washed 4 times in TBST for 5 minutes. Imaging took place by treating the membrane with Luminata Forte ECL solution at a volume of 1 mL per 10 cm^2 for 1 minutes being

visualising the bands by using the Gel Doc system or by exposing to high sensitivity film and then developing and fixing the image.

2.2.25 – Estimate of cell viability and metabolic activity by MTS reduction assay.

A flat-bottom 96-well plate was first seeded with 5000 cells per well in 200 µL of complete media. Cell treatments (L-glutamate dissolved in deionised water or benzofenac dissolved in DMSO) were prepared by aspirating all of the cell growth media and then replenishing with the treatment compound solution in 100 µL of complete media. To measure the metabolic capacity in each of the wells, and thus estimate the relative cell number, 20 µL of CellTiter 96 Aqueous One Solution is added to each of the wells containing media and incubated for 1 hour at 37°C in the dark. The absorbance at λ 490 nm was quantified immediately using a spectrophotometer (Fluostar OPTIMA), the absorbance of two blank wells containing media, but no cells, was subtracted from each of the samples, and a relative cell number estimated by comparing each well.

2.2.26 – Cellular glutathione concentration assay using DTNB.

The experiment was adapted from a method by Rahman *et al.* (2007). Cell lysates were extracted as previously described (**Chapter 2.2.22**) from 5 x 10^6 SH-SY5Y cells except 500 µL of GSH extraction buffer (100 mM K_2HPO_4, 5 mM EDTA, 0.1% triton X-100, 0.6% 5-sulphosalicylic acid; adjusted to pH 7.5 with 100 mM KH_2PO_4) was used as opposed to the described protein extraction buffer.

To assay the total glutathione concentration of a sample, 20 µL of cell extraction was transferred, in duplicate, to wells in a clear flat-bottom 96-well plate. Serial dilutions of reduced glutathione in GSH extraction buffer were prepared (0.1016, 0.2031, 0.4063, 0.8125, 1.625, 3.25, 6.5 nM) and 20 µL transferred in duplicate to separate wells of the 96-well plate. Next, 120 µL DTNB/enzyme solution (1.68 mM DTNB, 10 U glutathione

reductase, 100 mM K_2HPO_4, 5 mM EDTA; adjusted to pH 7.5 with 100 mM KH_2PO_4) was transferred to each well and the plate incubated for 30 seconds at room temperature away from sources of light before the rapid addition of 60 µL β-NADPH solution (900 µM β-NADPH, 100 mM K_2HPO_4, 5 mM EDTA; adjusted to pH 7.5 with 100 mM KH_2PO_4). The absorbance at λ 410 ± 12 nm was assayed immediately using a spectrophotometer (Fluostar OPTIMA) heated to 25°C and measured again at least every 30 seconds (depending on the number of wells on the plate) for at least 2 minutes. The reactions of the standards were plotted on a graph of absorbance against time. The slope of a 0 nM blank reaction was subtracted from the slope of each of the standards, the steady-state rate of each reaction derived, and this plotted on a derivative graph against standard concentration; generating a standard curve. The steady-state reaction rate was calculated for each of the samples in the same way and the total glutathione concentration in the same calculated using the standard curve.

To assay the oxidised glutathione concentration of a sample the GSH was first blocked in a separate reaction. In a reaction tube, 100 µL sample extraction and 2 µL of 92 mM solution of 2-vinylpyridine in GSH extraction buffer was mixed and then incubated with 1000 RPM shaking for 1 hour. The reaction was then stopped by addition of 6 µL of 1.25 M triethanolamine in GSH extraction buffer, the reaction mixed thoroughly, pulse centrifuged, and then incubated at room temperature for 10 minutes. The assay then proceeded as for the total glutathione assay with the addition of 20 µL of sample to separate wells of a clear 96-well plate. For the standards, seven 1 in 2 serial dilutions of GSSG were used at a starting concentration of 17.15 nM. For GSSG concentration calculations, all values were halved as it is the GSH which is measured in the assay and during the assay the 1 mole of GSSG produced 2 moles of GSH. To calculate GSH present in a sample, the GSSG concentration was substracted from the concentration of total glutathione.

2.2.27 – Determining permeability of hCMEC/D3 monolayers.

Two methods were used to determine the permeability of hCMEC/D3 endothelial cells grown on transwell inserts. First, to measure trans-endothelial electrical resistance (TEER) and thus estimate membrane permeability the EVOM and EndOhm chamber system was used (World Precision Instruments, Hitchin). The EndOhm was sterilised with a 70% ethanol solution and rinsed thoroughly with sterile deionised water and then with complete EndoGro. The chamber was connected to the EVOM and left in the 'off' position for the electrode to equilibrate for 1 hour in complete EndoGro. The reading from the electrode was validated by measuring the TEER of a CaliCell insert which has a known resistance supplied by the manufacturer. Before the TEER of cell monolayers was assayed the cell media was aspirated and the cell surface washed carefully three times with HBSS (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 10 mM D-glucose, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$; supplemented with 1 mM sodium pyruvate). The inserts were filled with the appropriate volume of culture media pre-warmed to 25°C - 500 µL 12-well plate inserts, or 1.5 mL for 6-well plate inserts. They were then placed inside the corresponding EndOhm chamber filled with the appropriate volume of culture media pre-warmed to 20°C for that insert size - 1.5 mL 12-well plate inserts, or 2.6 mL for 6-well plate inserts. The lid of the EndOhm chamber as then carefully placed on the chamber, ensuring there were no bubbles between the electrode and the cell growth surface, and the resistance in Ω for the insert read from the EVOM meter. The resistance of a blank insert with no hCMEC/D3 cells growth, but a collagen coating (and if applicable, obverse C6 cell growth), was subtracted from the resistance of each insert with membrane growth and multiplied by the growth surface area of the insert to give a value in Ω x cm^2.

A second method to measure permeability was the use of a fluorescent dye to measure small compound migration from the insert (apical) side to the well (basal) side. The inserts were washed carefully three times with HBSS and then transferred to a fresh

plate. A precise volume of HBSS is required in the apical and basal side of the insert to maintain hydrostatic pressure. In 6-well plates, 2.6 mL of HBSS is required in the basal side, while 1.5 mL is required in the apical. While in 12-well plates, 1.5 mL of HBSS is required on the basal side, and 0.5 mL for the apical. The plate was transferred to an incubator under standard tissue culture conditions previously described (**Chapter 2.2.16**) and agitated at 100 RPM for 10 minutes. Concentrated sodium fluorescein solution in HBSS (NaFlu) was then added to the apical side to a 50 µM final concentration before the plate was returned to the incubator and agitated again at 100 RPM. Every 10 minutes thereafter for 40 minutes, 200 µL of solution was removed from the basal side of the insert and the sample transferred to a well of a 96-well Corning flat-bottomed black 96-well plate; each time 200 µL was removed from the basal side an equal volume of HBSS was used to replenish the volume. Once samples from four time points had been collected the inserts were washed with HBSS and restored to normal culture conditions with complete growth media.

The fluorescent emission of each well was detected in a spectrophotometer (Fluostar OPTIMA) using an excitation filter of λ 485 ± 12 nm and emission filter of λ 520 nm. The fluorescence of blank wells containing only HBSS was subtracted from each well. Using this the concentration (µM) of NaFlu over time (minutes) was plotted, and the slope of each graph used to determine the permeability coefficient (Pe) in $x10^{-3}$ M cm min^{-1} using the following equation:

$$\frac{\mathbf{1}}{\boldsymbol{sample}} - \frac{\mathbf{1}}{\boldsymbol{blank\ insert}} = \boldsymbol{PSe}$$

$$\left(\frac{\mathbf{1}}{\boldsymbol{PSe}}\right) \times \boldsymbol{insert\ growth\ area}\ \left(\boldsymbol{cm^2}\right) = \boldsymbol{Pe}$$

2.2.28 – Transforming of plasmids into Stellar competent cells.

Cells were heat shock transformed according to manufacturer's instructions.

2.2.29 – Plasmid preparation.

The PureLink HiPure Plasmid Miniprep Kit was used to purify plasmids replicated in *E. coli* and used according to manufacturer's instructions. A spectrophotometer (Nanodrop 1000) was used to assess concentration of DNA, as well as purity by the ratio of absorbance at λ 260 to 280 nm and 260 to 230 nm.

2.2.30 – Cloning of BCAT2 gene into pCW57.1 plasmid.

The gateway recombination system by Thermo-fisher was used to clone BCAT2 into the pCW57.1 expression plasmid. First, the entire BCAT2 gene was cloned into the pENTR/D-TOPO plasmid (entry clone) to order by an external laboratory (Fisher Scientific, Loughborough). In a reaction tube, 700 ng entry clone, 150 ng pCW57.1, and 2 µL of LR Clonase II enzyme mix was combined, and the volume brought to 10 µL with TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Following incubation at 25°C for 1 hour, 2 µg of Proteinase K solution was added and the tube further incubated at 37°C for 10 minutes. The expression plasmid created was then transformed into Stellar competent *E. coli* by adding 2 µL to the suspension of *E. coli* and proceeding as previously described (**Chapter 2.2.28**). The cell suspension was transferred to LB-agar plates containing 100 µg/mL ampicillin and incubated at 37°C for 24 hours. Isolated colonies were picked, transferred to 5 mL of LB (1% NaCl, 1% tryptone, 0.5% yeast extract) and incubated at 37°C with 200 RPM shaking for 18 hours. A glycerol stock was next prepared by combining 750 µL of the 5 mL cell suspension with 750 µL of 50% sterile glycerol in a 1.5 mL tube and snap frozen at -80°C. The remaining 4.25 mL of *E. coli* suspension was used in a plasmid preparation as previously described (**Chapter 2.2.29**) and the plasmid sequenced externally (Eurofins Genomics, Germany) using the pCEP-Forward primer.

2.2.31 – Synthesis of tet-pLKO-puro-BCAT2 shRNA plasmid.

Using validated siRNA sequences obtained in this thesis (**Chapter 4.3**), an shRNA sequence was designed which contained a XhoI hairpin loop and strong poly-A termination sequence. Complement DNA oligonucleotide sequences where designed with four bases on the 5' ends complement to the restriction sites AgeI and EcoRI, and were synthesised to order by an external laboratory (Eurofins Genomics, Germany). The lyophilised oligonucleotides where reconstituted in nuclease-free water to 0.1 nmol/µL, vortex mixed for 5 seconds, and pulse centrifuged. In a fresh tube, 11.25 µL of each of the complement nucleotides and 2.5 µL of 10X annealing buffer (100 mM Tris-HCl pH 7.4, 1 M NaCl) was combined and heated to 95°C in a hot block for 5 minutes. The reaction was allowed to cool slowly to room temperature, and 1 µL of this concentrated annealed oligonucleotide transferred to a fresh tube with 20 µL 10X annealing buffer and 379 µL nuclease-free water; this diluted oligonucleotide was stored at -20°C.

A fresh tube was charged with 1 µg tet-pLKO-puro plasmid, 5 µL 10X CutSmart buffer, 20 units EcoRI-HF restriction enzyme, 20 units AgeI-HF restriction enzyme, and prepared to a total volume of 50 µL with nuclease-free water. The reaction was incubated at 37°C for 1 hour and then the stopped by the addition of 10 µL 6X DNA loading dye. The entire 60 µL of solution was loaded onto a 0.8% TAE-agarose gel with 1 kb ladder in one lane, and the gel electrophoresed for 1 hour as previously described (**Chapter 2.3.8**).

The Zymoclean DNA purification kit was then used according to manufacturer's instructions, to gel purify the linearised plasmids, which resolve at 8500 bps. Concentration of DNA was assessed using a spectrophotometer (Nanodrop 1000), as well as purity by the ratio of absorbance at λ 260 to 280 nm and 260 to 230 nm.

The linearised plasmid and shRNA oligonucleotide insert were then ligated using T4 ligase. For this, 1 µL diluted shRNA insert, 20 ng linearised plasmid, 1 µL 10X ligase buffer, 1 µL T4 DNA ligase were combined in a reaction tube and the volume prepared

to a total 10 µL volume with nuclease-free water. This was incubated for 3 hours at room temperature and immediately transformed into Stellar competent cells as previously described (**Chapter 2.2.28**) using 4 µL of the ligation product. To select for ampicillin resistant clones, cells were spread across LB-amp plates containing 100 µg/mL ampicillin. Isolated colonies were picked, transferred to 5 mL of LB (1% NaCl, 1% tryptone, 0.5% yeast extract) and incubated at 37°C with 200 RPM shaking for 18 hours. A glycerol stock was prepared by combining 750 µL of the 5 mL cell suspension with 750 µL of 50% sterile glycerol in a 1.5 mL tube and snap frozen at -80°C. The remaining 4.25 mL was used in a plasmid preparation as previously described (**Chapter 2.2.29**).

Ligation and cloning of plasmid was confirmed by digestion. A fresh tube was charged with 1 µg tet-pLKO-puro-BCAT2 plasmid, 2 µL 10X buffer R, 1 µL XhoI restriction enzyme, and the total volume prepared to 20 µL with nuclease-free water. This was incubated at 37°C for 1 hour before the reaction stopped by addition of 4 µL 6X DNA loading dye and the entire 24 µL of solution loaded onto a 0.8% TAE-agarose gel with 1 kb ladder in one lane, and the gel electrophoresed for 1 hour as previously described (**Chapter 2.2.21**). The bands were observed and imaged using a UV transilluminator (FluorChem Q Gel Dock).

2.2.32 – Statistical analysis.

Densitometry data was calculated using Image J software and estimated relative to loading control (GAPDH or α-tubulin). Statistical analysis was conducted using the Graphpad Prism 6.07 software utility. Data was assumed normally distributed and an unpaired Student's t-test was used for statistical analysis of all results; with the exception of the MTS assay (**Chapter 4.3.5)** whereby a 1-way and 2-way statistical ANOVA test was used for analysis of two different groups of results.