CHARACTERISATION OF PROTEASE-RESISTANT, NON-MATRIX-BINDING VARIANT OF IGFBP-2 AS A POTENTIAL TUMOUR GROWTH INHIBITOR

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ABSTRACT

The insulin-like growth factor (IGF) system includes two ligands (IGF-I and IGF-II), which bind and activate the type 1 IGF receptor (IGF-1R). IGF-II also binds with high affinity to the structurally similar insulin receptor isoform A (IR-A). Activation of these receptors elicits mitogenic responses for normal growth and development.

IGF bioavailability is modulated by high-affinity binding proteins (IGFBPs). When in complex with IGFBPs the IGF are unable to interact with their receptors. IGF is released from the IGF:IGFBP complex via IGFBP interaction with extracellular matrix (ECM) or through proteolysis of IGFBPs, increasing bioavailability of IGF. This effectively increases IGF-1R and IR-A activation (in the case of IGF-II).

In cancer, expression of IGF-I, IGF-II, and IGF-1R is upregulated, while compensatory signalling via IGF-II/IR-A (in the event of perturbed IGF/IGF-1R signalling) was documented. Additionally, tumours overexpress IGFBP specific proteases to accelerate IGF release. Collectively, mitogenic signalling via this system is upregulated, thereby promoting tumour growth, survival and metastasis.

The main objective was to investigate the use of IGFBPs as potential anti-cancer agents. Here, IGFBP-2 was used to sequester IGF as it has similar affinities for both ligands. Two novel modified IGFBP-2 analogues were designed to block mitogenic signalling of IGF-1R and IR-A by impairing their IGF releasing abilities. The protease resistant (PR) mutant was produced by deleting residues 114-170 of the linker domain (which contains cleavage sites), while the protease resistant/non-matrix binding (PR/NMB) mutant was generated by further mutation of the PR mutant by Lys→Ala substitutions at positions 180, 181, 227, 234, and 237 (disrupting the ECM binding sites).

The initial work was dedicated to *in vitro* characterisation of modified IGFBP-2. Truncation of the linker domain in PR and PR/NMB IGFBP-2 significantly improved resistance to tumour proteases: plasmin, MMP-1 and MMP-7. However, there is evidence for the presence of MMP cleavage sites outside the truncated linker domain. Furthermore, IGF

binding did not alter rate of IGFBP-2 cleavage by the aforementioned proteases. Additionally, IGFBP-2 was cleaved by plasmin C-terminally to Arg56, Lys150, Arg156, Lys158, Arg188, and Arg287. Together these data provided a detailed understanding of the mechanisms of proteolytical degradation of IGFBP-2, which in a cancer setting may increase IGF bioavailability.

Additionally, both IGFBP-2 mutants exhibited low affinity to ECM components including vitronectin, fibronectin and heparin, which are known to be involved in tumour progression. Lack of ECM binding discourages dissociation of the IGF:IGFBP-2 complex hence preventing IGF-mediated signalling.

An *in vitro* proliferation assay demonstrated that these mutants significantly inhibited proliferation of HT29 cells despite the presence of exogenous IGF-I. Additionally, growth of breast cancer xenograft tumours treated with PR/NMB IGFBP-2 was significantly inhibited. Interestingly, sequestration of IGF by IGFBP-2 did not trigger a compensatory increase of IGF-I in the circulation, an event commonly accompanying treatment using anti-IGF-1R monoclonal antibodies to perturb IGF signalling. Furthermore, analyses of the tumours indicated that PR/NMB IGFBP-2 treatment disrupted vascularisation with a corresponding decrease in the expression of *VEGFA,* a major angiogenic factor.

Through development of these IGFBP-2 mutants, the importance of IGFBP-2 proteolysis and matrix binding in the control of IGF action was confirmed. This research also provided an understanding of the mechanism by which mutant IGFBP-2 inhibits tumour growth. Ultimately, this data can be used to develop mutant IGFBP-2 as a therapeutic agent.

STATEMENT OF ORIGINALITY

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief it contains no material that has previously been published by any other person except where due reference is made. The research that was carried out as detailed in this thesis was under the supervision of Associate Professor Dr. Briony Forbes and Associate Professor Dr. Grant Booker. The author consents to this thesis being made available for photocopy or loan, subject to the provisions of the Copyright Act 1968. The author consents for the digital version of this thesis to be made available on the web, via the University's digital research repository, the Library catalogue, and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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LIST OF PUBLICATIONS, CONFERENCE ATTENDENCE AND AWARDS

Publication:

Soh, C. L., McNeil, K., Owczarek, C. M., Hardy, M. P., Fabri, L. J., Pearse, M., Delaine, C. A. & Forbes, B. E. 2014. Exogenous administration of protease-resistant, non-matrix-binding IGFBP-2 inhibits tumour growth in a murine model of breast cancer. Br J Cancer, 110**,** 2855-64. DOI: 10.1038/bjc.2014.232

Awards:

2014 ASBMB Best Student Publication Award 2014, Centre for Neuroscience, Flinders University of South Australia.

GRS-IGF Society Student Travel Grant 2014

2013 Combio 2013 Student Travel Award

Conference Presentations:

Oral

2014 Exogenous administration of protease-resistant, non-matrix-binding IGFBP-2 inhibits tumour growth in a murine model of breast cancer

- The 7th International Congress of the GRS and IGF Society, Singapore.

Poster

2014 Proteolysis of IGFBP-2 in a Cancer Context

- APG Student Awards 2014, Adelaide, SA, Australia.
- CNS Collaborators' Day, Adelaide, SA, Australia.

2013 Investigating protease resistant IGFBP-2 as a potential tumour growth inhibitor

- Combio, Perth, WA, Australia.
- ASMR SA Annual Scientific Meeting, Adelaide, SA, Australia.
- APG Student Awards, Adelaide, SA, Australia.

2012 Protease resistant IGFBP-2 as a potential tumour growth inhibitor

- Combio, Adelaide, SA, Australia.
- ASMR SA Annual Scientific Meeting, Adelaide, SA, Australia.
- APG Student Awards, Adelaide, SA, Australia.

2011 Developing a protease resistant IGFBP-2 as a tumour inhibitor

- IGF-Oz 2011 Conference, Parkville, Vic, Australia.

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ABBREVIATIONS

ACN	Acetonitrile
Akt	Protein kinase B
ALS	Acid labile subunit
APMA	4-aminophenylmercuric acetate
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CD	Circular dichroism
CID	Collision induced dissociation
CM	Conditioned medium
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immuno sorbent assay
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
GH	Growth hormone
GLUT	Glucose transporter
GOI	Gene of interest
HBD	Heparin binding domain
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia inducible factor
HRE	HIF response element
HRP	Horseradish peroxidase
IAA	Iodoacetic acid
IGF	Insulin-like growth factor
IGF-1R	Type 1 IGF receptor
IGF-2R	Type 2 IGF receptor
IGFBP	IGF binding protein
IR-A	Insulin receptor isoform A
IR-B	Insulin receptor isoform B
JNK	c-Jun N-terminal kinase
LC-ESI	Liquid chromatography electrospray ionisation
MALDI	Matrix-assisted laser desoprtion ionisation
MAPK	Mitogen activated protein kinase
MGF	Mascot generic file
MMP	Matrix metalloproteinase
MOPS	N-morpholino)propanesulfonic acid
mTOR	Mammalian target of rapamycin
NLS	Nuclear localisation signal
NMR	Nuclear magnetic resonance
NTC	Non-template control
OD	Optical density
PAWS	Protein Analysis WorkSheet
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor

PI3K	Phosphoinositide-3 kinase
PMSF	phenylmethanesulfonylfluoride
PR	Protease resistant
PR/NMB	Protease resistant/non-matrix binding
PTEN	Phosphatase and tensin homolog
qPCR	Real-time polymerase chain reaction
rpHPLC	Reversed phase high performance liquid chromatography
RPLP0	Ribosomal protein P0 large
RT-PCR	Reverse transcription polymerase chain reaction
SA	Sinapinic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMI	Small molecule inhibitor
SPR	Surface plasmon resonance
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TK	Tyrosine kinase
TMB	Tetramethylbenzidine
VEGF	Vascular endothelial growth factor
WT	Wild-type

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