Assessment of Critical Survival Mechanisms Exploited by *BCR-ABL1*+ Cells to Evade Tyrosine Kinase Inhibitor-Induced Death;

Determination of Novel Therapeutic Targets in Chronic Myeloid Leukaemia

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Declaration

I, Lisa Schafranek, certify that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Schafranek L, Leclercq TM, White DL and Hughes TP. Clarithromycin enhances dasatinib-induced cell death in chronic myeloid leukemia cells, by inhibition of late stage autophagy. *Leuk Lymphoma* 2013; 54: 198–201.

Schafranek L, Nievergall E, Powell JA, Hiwase DK, White DL and Hughes TP. Sustained inhibition of STAT5, but not JAK2, is essential for TKI-induced cell death in chronic myeloid leukemia. Leukemia advance online publication, June 27, 2014; doi:10.1038/leu.2014.156; accepted article preview online May 12, 2014.

Conference Presentations

Oral Presentations

Schafranek L, Nievergall E, Powell JA, Hiwase DK, Leclercq T, Hughes TP and White DL. New evidence that transient Bcr-Abl inhibition commits cells to death in a time- and STAT5-dependent manner despite reactivation of Bcr-Abl. NDLR, March 2014, Noosa, QLD (oral presentation)

Schafranek L. Cannibalistic Cancer. 3 Minute Thesis Competition, July 2013, University of Adelaide, Australia (oral presentation). **Faculty Finalist**

Schafranek L, Leclercq TM, White DL and Hughes TP. Macrolide Antibiotic Clarithromycin targets TKI-induced autophagy in CML cells. HAA Oct, 2012 Melbourne, Australia (oral presentation).

Schafranek L, Leclercq TM, White DL and Hughes TP. Overcoming resistance to tyrosine kinase inhibitors in chronic myeloid leukaemia by blocking autophagy with clarithromycin. Medical Staff Society Research Prize, May, 2013, Adelaide, Australia (oral presentation).

Schafranek L, Hiwase H, Powell J, Melo J, White D, Hughes T. Constant Exposure to Low Dose Dasatinib Is Sufficient for Induction of Apoptosis in CML Cells. HAA Oct, 2011 Sydney, Australia (oral presentation) **Awarded HAA 2011 non-member travel grant.**

Poster Presentations

Schafranek L, Nievergall E, Hiwase H, Powell J, White D, Hughes T. Direct inhibition of STAT5 in combination with transient Bcr-Abl inhibition commits cells to apoptosis despite reactivation of Bcr-Abl. ASH Dec 2013, New Orleans, USA (poster presentation). **ASH Abstract Achievement Award**

Schafranek L, Nievergall E, Hiwase H, Powell J, White D, Hughes T. STAT5 inhibition is critical to the commitment of chronic myeloid leukemia cells to apoptosis regardless of Bcr-Abl reactivation. CPCM Symposium, August 2013, National Wine Centre, Adelaide Australia (poster presentation)

Schafranek L, Nievergall E, Hiwase H, Powell J, White D, Hughes T. Inhibition of activated STAT5 sensitizes chronic myeloid leukemia cells to TKI treatment and commits cells to apoptosis despite reactivation of Bcr-Abl, independent of JAK1/2. FHS

Conference, July 2013, University of Adelaide, Australia (poster presentation). **Awarded Florey Medical Research Foundation Prize**

Schafranek L, Nievergall E, Hiwase H, Powell J, White D, Hughes T. Commitment of CML Cells to Apoptotic Cell Death Depends On the Length of Exposure to Das and the Level of STAT5 Activity. ASH Dec 2012, Atlanta, USA (poster presentation)

Schafranek L, Leclercq TM, White DL and Hughes TP. Clarithromycin increases the sensitivity of chronic myeloid leukaemia cells to dasatinib. FHS conference, August 2012, University of Adelaide, Australia (poster presentation).

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Schafranek L, Leclercq TM, White DL and Hughes TP. Clarithromycin increases the sensitivity of chronic myeloid leukaemia cells to dasatinib. CPCM Symposium, July 2012, National Wine Centre, Adelaide, Australia (poster presentation).

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Scholarships and Awards

American Society of Hematology Abstract Achievement Award

For the abstract entitled "Direct inhibition of STAT5 in combination with transient Bcr-Abl inhibition commits cells to apoptosis despite reactivation of Bcr-Abl. ASH Annual Conference, New Orleans, USA.

Florey Medical Research Foundation Prize

For the presentation of the abstract entitled "Inhibition of activated STAT5 sensitizes chronic myeloid leukemia cells to TKI treatment and commits cells to apoptosis despite reactivation of Bcr-Abl, independent of JAK1/2", FHS conference, Adelaide, Australia

Faculty of Health Science, 3 Minute Thesis Finalist

For the presentation entitled "Cannibalistic Cancer", University of Adelaide, 2013

Medical Staff Society Research Prize Finalist

For the presentation entitled "Overcoming resistance to tyrosine kinase inhibitors in chronic myeloid leukaemia by blocking autophagy with clarithromycin", Royal Adelaide Hospital Medical Staff Society, Adelaide, Australia 2013.

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PhD Scholarship, Leukaemia Foundation of Australia 2010-2013

Support for the educational and professional development of researchers and other professionals undertaking a PhD. The award is to support research in Australia into the causes, treatment and care of people with leukaemia, lymphoma, myeloma and related blood disorders and is awarded on the merits of the applicant and project proposal.

Abbreviations

µg – Microgram/s

µL – Microlitre/s

µM – Micromolar

14-C – Carbon-14 radioactive isotope

3-MA –3-Methyladenine

7-AAD – 7-Aminoactinomycin D

Ab – Antibody

ABL1 – Abelson murine leukaemia virus human homologue 1 gene

ACD – Acid Citrate Dextrose Acid

Akt – a serine threonine kinase also known as protein kinase B

ALL – Acute Lymphoblastic Leukaemia

AMPK – AMP-activated protein kinase

-AP – Alkaline Phosphatase Conjugated Antibody

AP – Accelerated Phase

APS – Ammonium Persulfate

Ara-C – Arabinofuranosyl Cytidine

ATCC – American Type Tissue Culture Collection

ASK1 – Apoptosis signal-regulating kinase 1

ATP – Adenosine Triphosphate

BAD – Bcl-Xl/Bcl-2 associated death promoter

BAX – Bcl-2 associated X protein

Bcl-X_L – B-cell lymphoma extra large

Bcl-2 – B-cell lymphoma 2

BC – Blast Crisis

BCR – Breakpoint Cluster region

BCR-ABL1 – BCR-ABL1 oncogene

Bcr-Abl – Bcr-Abl oncoprotein

Bim – Bcl-2 interacting mediator of cell death

BM – Bone Marrow

BSA – Bovine Serum Albumin

C – Degrees Celsius

CAM – clatithromycin

CCyR – Complete Cytogenetic Remission

CFU – Colony forming unit

CFU-GM – Colony forming unit granulocytes and macrophage

CFC – Colony forming cells

Chk2 – checkpoint kinase 2

CML – Chronic Myeloid Leukaemia

CP –Chronic Phase

CO_2 – carbon dioxide

CrkL – C1T10 regulator of kinase like

Ctrl – control

CQ – chloroquine

Das – Dasatinib

DMSO – Dimethyl Sulphoxide

DNA – Deoxyribonucleic Acid

EDTA – Ethylenediaminetetraacetic Acid

Erk – Extracellular signal related kinase

e.g. – exempli gratia

et al. – et alia

FACS – Fluorescence Activated Cell Sorting

FBS – Foetal Bovine Serum

FDA – Food and Drug Administration

FSC – Forward scatter

FLT-3 ligand – FMS-like tyrosine kinase 3 ligand

Gab2 – GRB2-associated-binding protein 2

G-CSF – granulocyte-colony stimulating factor

GF – growth factor

5GF – five haematopoietic growth factors (G-CSF, GM-CSF, IL-3, IL-6, SCF)

6GF – six haematopoietic growth factors (G-CSF, IL-3, IL-6, SCF, TPO and Flt3-ligand)

GM-CSF – Granulocyte Macrophage Colony-Stimulating Factor

GMP – Granulocyte macrophage progenitors

Grb2 – Growth factor receptor-bound protein 2

Glut3 – glucose transporter 3

h – Hour/s

HBSS – Hanks Balanced Salt Solution

HSC – Haemopoietic stem cells

HSCT – Haemopoietic stem cell transplantation

IC50 – Inhibitory Concentration 50

i.e. – id est

IFN – Interferon IM – Imatinib IUR –

IL-3 – Interleukin-3

IL-6 – Interleukin-6

IM – Imatinib mesylate

IMDM I– scove's modification of Dulbecco's medium.

IUR – intracellular uptake and retention assay

kD – kilo Dalton

KD – Kinase Domain

JAK – Janus Kinase

JAKi – ruxolitinib, pan JAK inhibitor

JNK – c-Jun N-terminal kinase

L – Litre/s

LC3 – Microtubule-associated protein 1A/1B-light chain 3

LKB1 – liver kinase B1

M – Molar

mA – mili Amp (10-3 Amps)

MACS – Magnetic activated cell sorting

MAPK – Mitogen activated protein kinase

Mcl-1 – myeloid cell leukemia sequence 1

MFI – Mean Fluorescence Intensity

mg – milligram/s

min – Minutes/s

mL – Millilitre/s

mM – Millimolar

MMR – Major Molecular Response

MNC/s – Mononuclear Cell/s

mRNA – messenger RNA

mTOR – mammalian target of rapamycin

MW – Molecular Weight

ng – Nanogram/s

NIL – Nilotinib

nM – Nanomolar

O_2 – oxygen

OPT – optimal

p- – Phosphorylated Form of Protein

p62 – sequestosome 1 (SQSTM1)

PAGE – Polyacrylamide Gel Electrophoresis

PARP – Poly (ADP-ribose) polymerase

PB – Peripheral Blood

PBMNC/s – Peripheral Blood Mononuclear Cell/s

PBS – Phosphate Buffered Saline

PDGFR – Platelet-Derived Growth Factor Receptor

PE – Phycoerythrin

Ph – Philadelphia Chromosome

PI3-K – Phosphotidylinositol – 3-kinase

Pim – serine/threonine kinase

P-loop – Phosphate binding loop

p-value – Probability Value

PVDF – Polyvinylidene Difluoride

Pz – pimozide

rcf – Relative Centrifugal Force

RPMI – Roswell Park Memorial Institute (media)

RNA – Ribonucleic Acid

rpm – Revolutions Per Minute

STAT5 – Signal Transducer and Activator of Transcription 5

STAT5i – inhibitor of Signal Transducer and Activator of Transcription 5 r

SCF – Stem cell factor

SC – side scatter

SDS – Sodium Dodecyl Sulphate

sec – second/s

SEM – Standard Error of the Mean

SH – Src Homology Region

S/N – Supernatant

STD – standard

TBS – Tris Buffered Saline

TBST – Tris Buffered Saline +Tween®20

TKI/s – Tyrosine Kinase Inhibitor/s

Tyr – Tyrosine

U – Units

U/mL – Units Per Millilitre

Wash – drug washout

WCF – White Cell Fluid

Acknowledgments

One does not complete a PhD thesis without a great deal of encouragement, guidance and support, so there are a few people whom I would like to acknowledge and thank.

Thank you to my supervisors Tim Hughes, Deb White and Junia Melo. Early on in my PhD, I would often go into a meeting with my supervisors excited about my data and what it meant, only to come out overwhelmed with critiques and further experiments to perform and questions to answer. However, despite my initial misgivings to this process, I have fond memories of the challenging conversations and debates coming from those meetings. As mentioned by John Rasko in his special oration at NDLR 2012, Tim is one of the nicest guys in research: you have always challenged me to achieve my best and your clinical perspective has been invaluable. From the moment I came to this lab, Deb you have been an amazing mentor. You saw something in me, that at the time I couldn't see; the potential to be a great scientist and researcher. I finally feel like I can live up to that potential. Thank you for believing in me even when I didn't and pushing me to be a first-class researcher. You may still get that confused look when I reverse my sentence order after I get overly excited over a result or expressing a new theory, but hopefully I get it the right way around more often than not these days.

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Ever since I was little, my mum has instilled in me an infallible belief that I could do anything and always encouraged me to question everything, which may be why I ended up undertaking a PhD. This may have come back to bite her, as this now includes questioning her. Without her encouragement and support throughout my life I would not have been able to accomplish all that I have to date. Additionally, I give you huge "props" for reading my entire thesis for grammar and spelling. I'm glad that you now have at least a little bit of an understanding of what I've been up to the past few years. I also have an amazing big brother, who constantly provides me with food for thought on concepts relevant to my PhD and thankfully a range of other topics from philosophy to computer science to anime, but additionally provides me with delicious, nutritious food. I love you both so much and thank you for all that you have contributed to my life, above and beyond my PhD.

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Abstract

Chronic myeloid leukaemia (CML) is a clonal myeloid proliferative disease that results from constitutive activation of the Bcr-Abl oncoprotein, which disrupts normal cellular signalling potentiating the survival and maintenance of *BCR-ABL1*+ cells. Tyrosine kinase inhibitors (TKIs), like imatinib, have revolutionised the treatment of CML and have become the model for therapy in other cancers. Imatinib treatment also founded the paradigm that potent and continuous dosing is required for optimal patient response in patients with CML. In contrast to imatinib, the second generation TKI dasatinib has a short half-life of only 3-5 h, nevertheless a once daily dosing regime is sufficient to achieve equivalent responses to twice daily dosing suggesting that continuous and complete inhibition of Bcr-Abl may not be required for optimal response to TKI therapy.

Despite initial studies indicating that a very brief exposure to a potent dose of TKI is sufficient to induce cell death in *BCR-ABL1*+ cells, recent studies have attributed this to sustained low-level inhibition of Bcr-Abl signalling due to inadequate drug washout. As reported in this thesis, experiments with low dose dasatinib treatment, which does not completely inhibit Bcr-Abl phosphorylation but is sufficient to induce cell death, demonstrated inactivation of STAT5 as a sensitive measure of Bcr-Abl activity. Here, it was also confirmed that <1 h exposure to potent TKI with adequate drug washout is insufficient to commit *BCR-ABL1*+ cells to death and it is established for the first time that at least 2 h of Bcr-Abl kinase inhibition are required. Furthermore, combinations of efficient TKI washout with specific inhibitors of STAT5, JAK and ERK ascertained sustained inhibition of pSTAT5, potentially independent of JAK2, as the determinant of commitment to cell death. Together, this research established that continuous,

complete inhibition of Bcr-Abl is not required to induce cell death, but that continuous blockade of STAT5, indicative of low-level threshold Bcr-Abl inhibition, is essential, thus challenging the imatinib paradigm.

Although most CML patients respond well to imatinib, only 40% of patients achieve a complete molecular response and some patients develop resistance. Blockade of Bcr-Abl signalling can drive cells to develop new survival mechanism, and amongst others, autophagy and the acquisition of extrinsic survival signalling have been implicated in resistance to therapy and/or persistent disease.

Studies presented in this thesis define a role for the activation of autophagy in response to tyrosine kinase inhibition of Bcr-Abl. Induction of autophagy by TKI was confirmed using established markers of autophagy, such as the conversion of LC3-I to LC3-II, degradation of p62 and cellular morphology. Blockade of anti-apoptotic proteins Bcl-2 and Bcl-xL along with activation of stress response pathways were revealed as potential mechanisms of autophagy induction, however, further investigation into these pathways is required. Importantly, the data presented here also established clarithromycin as a novel inhibitor of TKI-induced autophagy, advocating combination treatment with TKI therapy in resistant patients.

Recent observations that overexpression of cytokines and their receptors may contribute to *BCR-ABL1*+ cell persistence in CML patients undergoing TKI therapy. Here, the expression of IL-3 and GM-CSF cytokine receptors in *BCR-ABL1+* cell lines and chronic phase CML CD34+ progenitor cells was established and signalling through those

was confirmed to maintain STAT5 survival signalling, thereby protecting cells from TKI-induced death. Inhibition of JAK2 with ruxolitinib inhibited cytokine-dependent, but not Bcr-Abl-dependent, activation of STAT5 and neutralised cytokine-induced protection from cell death while having little effect in the absence of cytokines.

Together, the findings of this thesis established the critical mechanisms in Bcr-Abl-dependent and -independent signalling that may also be targeted in combination therapeutic approaches and provides an in-depth understanding of the potential clinical effectiveness of dose reductions during dasatinib therapy. These studies will have broad implications for the ongoing development of therapeutic strategies in CML, particularly in the setting of TKI-resistance, and will aid the goal of achieving a curative treatment for patients with CML.