RCPA Research Award 2010-2011 Final Progress Review

Name: Dr Craig Wallington-Beddoe

Degree/Institution/Year: PhD, The University of Sydney, Year 2

Research Project Title: New Therapeutic Strategies for Acute Lymphoblastic Leukaemia

A. Brief lay summary of findings to date:

This original project aimed to examine how effective the drug FTY720 was for treating acute lymphoblastic leukaemia (ALL). This research comprised the first year (2010) of the PhD. Although FTY720 worked well in an in vitro setting it failed to produce positive results when given to mice with human ALL. The in vitro data was published in the journal Autophagy (2011;7:7-15) and the negative in vivo data is in the final revision process for the journal PLoS ONE. Please refer to the 2010 report for further information. Year 2 (2011) focused on the roles of enzymes called sphingosine kinases in ALL. Specifically, it was found that inhibition of these enzymes results in the death of leukaemia cells in vitro and in vivo and prolonged the survival of mice with human ALL. Subsequent work will further define the roles of these enzymes in ALL.

B. Summary of research against plan to date:

As mentioned, FTY720 will no longer be investigated as a potential therapeutic for ALL patients. The remainder of the PhD will examine the roles of sphingosine kinases in supporting leukaemia cell growth and survival. In short, the sphingosine kinases, of which there are two isoforms (SK1, SK2), catalyse the formation of sphingosine 1-phosphate (S1P), a pro-survival lipid, from the precursor sphingosine. SK1 has been shown to be over-expressed in several cancers including leukaemia and inhibition of SK1 is a potential anti-cancer strategy. Inhibition of SK2 also appears promising and new SK2 inhibitors are slowly emerging.

The PhD year 2 (2011) research findings against the project aims are outlined below:

<u>Aim 1:</u> To determine the dependency of ALL cells on SK1/SK2-generated S1P in both in vitro and in vivo settings.

<u>In vitro:</u>

Application of the combined SK1/SK2 inhibitor SKI II and the selective SK2 inhibitor ABC294640 to ALL cells produced a reduction in cellular proliferation as measured by ^{3}H-thymidine incorporation in all cell lines (REH, NALM6, LK63, ALL1, 2070 and TOM1) tested with IC_{50} values of 1μM - 7μM for SKI II and <40μM for ABC294640. Viability, measured by flow cytometry using annexin V and propidium iodide (PI) staining, was also reduced in all cell lines except the Ph^{+} ALL1 and 2070 cells treated with SKI II with IC_{50} values ranging from 2μM to >10μM for SKI II and 50-60μM for ABC294640. Both agents significantly

reduced intracellular S1P concentrations by 24 hours as determined by ELISA, thereby confirming the ability of these compounds to inhibit SK1 or SK2 activity.

A search for agents that synergized with the SK inhibitors revealed that when Ph^+ ALL cells were treated with the combination of imatinib and either ABC294640 or SKI II, a further reduction in cell death occurred than with either agent alone, thereby enhancing the therapeutic effect of ABC294640 and overcoming resistance seen with SKI II alone. Furthermore, the combination of mildly cytotoxic concentrations of ABC294640 and the novel pan histone deacetylase inhibitor AR-42 or the proteosome inhibitor bortezomib were found to significantly increase leukemic cell death at 24 and 48 hours in Ph^+ and negative ALL cells.

These results suggest that inhibition of sphingosine kinases has relevance to the treatment of ALL, particularly in combination with novel non-chemotherapeutic agents.

<u>In vivo:</u>

(i) Efficacy of the SK2 inhibitor ABC294640

In vivo assessment of the SK inhibitors was determined by injecting NOD/SCID $IL2\gamma_c^{-/-}$ mice with 2-5 million human B-ALL cells and treating with 100 mg/kg/day ABC294640 or vehicle by intraperitoneal injection for 21 days after which all animals were sacrificed. Assessment of leukaemia in blood, bone marrow and spleen was determined by flow cytometry using antibodies to human CD19 and murine CD45. Significant reductions in the levels of leukaemia in all examined tissues were found in ABC294640-treated animals using three different human ALL xenografts, including the Ph^+ positive xenograft 2070. Average absolute levels of leukaemia in the bone marrow of ABC294640-treated mice for xenografts 2070, 1345 and 0398 were reduced by 40% ($p = 0.00007$), 55% ($p = 0.004$) and 72% ($p = 0.000001$) respectively. No overt toxicity was noted. Furthermore, ABC294640 also significantly extended the survival of mice with xenograft 0398 ($p = 0.012$). When imatinib was combined with ABC294640 using the Ph^+ xenograft 2070, significantly reduced leukaemia levels were observed in mice receiving combination treatment, resulting in significantly prolonged survival ($p = 0.001$).

(ii) Leukaemic development and progression using SK1 or SK2 gene deleted ALL cells:

Wild-type (WT), SK1 knockout (SK1 KO) and SK2 knockout (SK2 KO) C57/BL6 mice were used to accurately assess the in vivo involvement of sphingosine kinases in ALL development and progression. Whole bone marrow was harvested from each of the above murine strains and the primitive B-cell fraction purified using antibodies to murine B220/CD45R, CD19 and CD11b by flow cytometry. These cells then underwent ex-vivo transduction using a generated murine retrovirus containing the proto-oncogene BCR/ABL in the presence of mouse bone marrow stroma and salutatory cytokines. The transduced cells of each genotype were harvested and injected into cohorts of WT C57/BL6 mice. WT recipients of WT-transduced cells developed aggressive leukaemia and inferior survival compared to WT recipients of SK1 or SK2 KO-transduced cells, which developed less aggressive disease with increased survival. Recipients of SK1 KO-transduced cells appeared to have the most favourable survival amongst all groups.

These results confirm that inhibition of sphingosine kinases in an in vivo setting has significant anti-leukaemic effects and that inhibition of SK1 may be a more important target than SK2 in reducing leukaemic burden.

<u>Aim 2:</u> To investigate the functional roles of SK1 and SK2 on cell growth, survival and death. Specifically, to determine mechanisms of cell death upon SK1 and SK2 inhibition.

SKI II resulted in caspase-dependent cell death, as determined by flow cytometric assessment of intracellular caspase-3 cleavage and apoptotic morphology on light microscopy, with cell death prevented by pre-incubation with 100μM of the pan-caspase inhibitor Z-VAD-FMK. However, ABC294640 induced caspase-3 cleavage at lower than expected levels and cell death was not prevented by Z-VAD-FMK. Preliminary work with the selective SK1 inhibitor (SK1-I) also resulted in little caspase-3 cleavage.

These results are difficult to interpret accurately, however, it appears that inhibition of SK1 or SK2 alone in ALL cells results in caspase-independent cell death whereas inhibition of both SK isoforms is caspase-dependent.

<u>Aim 3:</u> To investigate the interaction of the S1P signalling pathway with the development of autophagy in ALL cells.

This aim has been partially addressed during year 1 (2010) where it was found that the phosphorylated form of the drug FTY720, a S1P receptor type 1 super agonist, resulted in autophagy, a cell survival process, which partially protected ALL cells from cytotoxic agents such as vincristine (Wallington-Beddoe et al. Autophagy 2011;7:707-15). Inhibition of autophagy using the chemical inhibitor 3MA resulted in abrogation of this protective effect and subsequent cell death when ALL cells were challenged with non-phosphorylated FTY720.

This suggests that S1P signalling through the S1P1 receptor has pro-survival effects through autophagic induction and by extension that the natural agonist S1P also induces such effects. These results are in agreement with the established fact that S1P is a pro-survival lipid mediator.

C. Future Directions:

<u>Aim 1, In vitro:</u>

Testing of the selective SK1 inhibitor (SK1-I) is being finalised for inclusion in a third manuscript detailing the aforementioned work on SK inhibitors, to be submitted within the next few months.

<u>Aim 1, In vivo (ii):</u>

This experiment is currently being repeated to ensure the results of the initial experiment are reproducible and statistical significance between groups is reached.

<u>Other work to be undertaken:</u>

1. *Preparation of a third manuscript containing the work on sphingosine kinases is almost complete with expected submission by March 2012.*
2. *The role of ERK signalling in activating sphingosine kinases and the potential for dual inhibition as a potential therapeutic strategy.*
3. *The role of the transcription factor CTGF in sphingolipid signalling.*

D. Timeline summary for remaining duration of research:

2012 (Year 3):

Aims 1 to 3 will be continuously refined throughout 2012.

The remaining experiments outlined in Section C will be undertaken during the year.

E. Progress report from primary supervisor:

Dr Wallington-Beddoe has continued to make excellent progress on his project during 2011. This has resulted in a publication in Autophagy and a second undergoing revision for resubmission to PLoS ONE. He has a third paper that is almost ready for submission to Blood. He has also given a number of poster and oral presentations at meetings based on his PhD studies. His laboratory work has been excellent. He reads and makes appropriate suggestions regarding the development of his project. He is highly motivated and more than suitably equipped to complete his PhD studies. He will have no difficulty completing his thesis and anticipate him completing within the 3-year period, possibly by publication. This is extremely impressive for a laboratory-based project.

F. Presentations, published articles and awards:

Awards:

1. *Leukaemia Foundation of Australia PhD (clinical) Scholarship (2010-2012)*
2. *Cancer Institute NSW Research Scholar Award (2010-2012)*
3. *The Royal College of Pathologists of Australasia Research Award (2010-2011)*
4. *Haematology Society of Australia and New Zealand Educational Grant (2011)*
5. *Haematology Society of Australia and New Zealand Travel Grants (2010/11)*

Peer-reviewed Journal Papers:

1. *Wallington-Beddoe CT, Hewson J, Bradstock KF, Bendall LJ. FTY720 produces caspase-independent cell death in acute lymphoblastic leukemia cells. Autophagy 2011;7:707-15.*

2. *Wallington-Beddoe CT, Don AS, Hewson J, Qiao E, Papa RA, Lock RB, Kenneth Bradstock KF, Linda J Bendall LJ. FTY720 Fails to demonstrate in vivo efficacy in a xenograft model of Ph^- B-lineage acute lymphoblastic leukemia. PLoS ONE. (under review)*

Conference Abstracts:

1. *Wallington-Beddoe CT, Bradstock K, Bendall L. FTY720 produces a marked reduction in cellular proliferation and viability in acute lymphoblastic leukaemia cell lines. New Directions in Leukaemia Research. Sunshine Coast, 2010.*

2. *Wallington-Beddoe CT, Bradstock K, Bendall L. Acute lymphoblastic leukaemia cell lines treated with FTY720 show a marked reduction in cellular proliferation and viability. Sydney Cancer Conference. Sydney, 2010.*

3. *Wallington-Beddoe CT, Bradstock K, Bendall L. FTY720 is an Effective Therapy for Acute Lymphoblastic Leukaemia Resulting in Caspase Independent Cell Death. International Society of Experimental Hematology. Melbourne, 2010.*

4. *Wallington-Beddoe CT, Hewson J, Bradstock K, Bendall L. FTY720 has Potent Anti-leukemic Effects on Acute Lymphoblastic Leukemia Cells and Results in Caspase Independent Cell Death. Haematology Society of Australia and New Zealand. Auckland New Zealand, 2010.*

5. *Wallington-Beddoe CT, Hewson J, Bradstock K, Bendall L. FTY720 has Potent Anti-leukemic Effects on Acute Lymphoblastic Leukemia Cells and Results in Caspase Independent Cell Death. American Society of Hematology. Orlando, Florida USA, 2010.*

6. *Wallington-Beddoe CT, Ho D, Bradstock K, Bendall L. Sphingosine Kinase Inhibition Represents a Promising Novel Strategy for the Treatment of Acute Lymphoblastic Leukaemia. Haematology Society of Australia and New Zealand. Auckland New Zealand, 2011.*

7. *Wallington-Beddoe CT, Ho D, Bradstock K, Bendall L. Sphingosine Kinase Inhibition has Pre-clinical Activity in Acute Lymphoblastic Leukemia. American Society of Hematology. San Diego CA, USA, 2011.*

8. *Wallington-Beddoe CT, Bradstock K, Bendall L. Sphingosine Kinase Inhibition has Pre-clinical Activity in Acute Lymphoblastic Leukaemia. New Directions in Leukaemia Research. Sunshine Coast, 2012.*

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