Developing the most potent direct blockers of MYC/MAX interactions available using Phylomer Peptides

The Proposed Approach

It is widely recognised that targeting MYC with small-molecule antagonists is unlikely to succeed. However, there is evidence that peptide-based molecules (such as "Omomyc" developed by team member L. Soucek) can specifically interfere with critical interactions required for Myc activity and induce tumour cell death. Although peptides against intracellular targets are generally unsuitable for clinical application, we have developed technologies that can be applied to overcoming their limitations such as inability to efficiently cross cell membranes and instability in physiological fluids. Our team has diverse skills and access to unique technology platforms, reagents and animal models to develop and validate peptide-based antagonists of Myc.

We propose the development of new protein based therapeutics directly targeting Myc protein interactions using Omomyc inspired biologics molecules. Moreover, addressing a key challenge with intracellular biologics, these agents will be delivered into cells using a new technology shown to have superior cell penetration and endosomal escape. Potency of such direct Myc-targeted therapies will be further enhanced by simultaneous targeting of BCL-2 family prosurvival proteins with small molecules which are in development for treatment of blood cancers. Proof of concept of such a therapeutic synergy has already been demonstrated (see below).

TEAM PERSONNEL

Laura Soucek (Vall d'Hebron Institute of Oncology (VHIO - http://www.vhio.net/research-team/en_laura-soucek.php)

Specific Contributions

- Access expertise around Omomyc.
- Access to validated animal models for testing Myc inhibitors (skin, pancreatic and lung cancer, as well as glioblastoma)
- Access to inhaled delivery platform for lung cancer

Dr Soucek's work was instrumental in demonstrating the feasibility and efficacy of Myc inhibition *in vivo*. She has developed and validated the potential use of Omomyc in multiple genetic cancer models, including skin, pancreatic and lung cancer, as well as glioblastoma[1-5]. Targeting Myc with Omomyc has proven to be a dramatically effective strategy in different types of cancer and genetic make-up, and to cause very mild, well tolerated and completely reversible side effects in normal tissues.

Andreas Strasser (Walter and Elisa Hall Institute (WEHI), Melbourne - http://www.wehi.edu.au/people/andreas-strasser)

Doug Fairlie (Olivia Newton-John Cancer Research Institute (ONJCRI), Melbourne - http://www.onjcri.org.au/doug-fairlie/)

Specific Contributions

- Decades of expertise in genetic models to determine role of Myc in tumour survival from apoptosis and rescue from senescence

- Expertise in identifying cooperative pathways for Myc-driven tumour survival (BCL-2, MCL-1 etc.)
- Sophisticated animal models of lymphoma and leukaemia
- Expertise in developing small molecules and peptides (linear and stapled) targeting BCL-2 family prosurvival proteins, which are known to synergise with Myc inhibition
- Strong relationships with pharmaceutical companies developing small molecules targeting pro-survival proteins which may improve potency of Myc inhibitors.

Andreas Strasser, WEHI

The transcription factor c-MYC (and its close relatives) drive cellular metabolic activity and cell proliferation under optimal growth conditions [6]. However, when growth conditions are not optimal, such as when growth factors or nutrients are limiting, c-MYC will enhance the predisposition of cells to undergo apoptosis [7-9]. Accordingly, activation of the 'intrinsic' (also called 'mitochondrial' or 'BCL-2-regulated') apoptotic pathway serves as a tumour suppressive process in MYC-driven lymphoma development (and probably also in the development of other cancers). For example, Professor Strasser has shown that over-expression of pro-survival BCL-2 [10] or loss or pro-apoptotic BIM [11] or PUMA [12] greatly accelerate MYC-driven lymphoma development.

Professor Strasser has also examined which prosurvival BCL-2 family members, expressed under endogenous control (i.e, not over-expressed) are required for the development of Myc-driven lymphomas. Such studies showed that loss of BCL-2 has no impact on Myc-driven lymphoma development [13] but loss of both alleles of BCL-XL [14] or pharmacological inhibition of BCL-XL [15] substantially inhibited tumorigenesis. Even more remarkably, loss of only one allele of Mcl-1 almost completely abrogates MYC-driven lymphomagenesis (S Grabow, submitted). Interestingly, concomitant loss of BIM can restore MYC driven lymphoma development when BCL-XL is absent [16] or one allele of *Mcl-1* is missing (S Grabow, submitted). This demonstrates that MCL-1 and to a lesser extent BCL-XL are critical for survival of MYC-over-expressing cells undergoing neoplastic transformation, by inhibiting pro-apoptotic BIM.

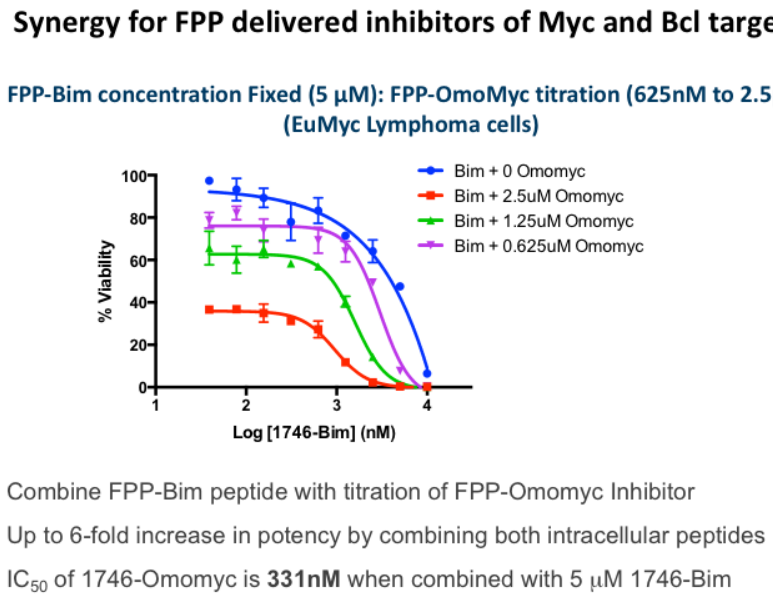
Using inducible gene knockout technology and treatment with BH3 mimetic drugs, Professor Strasser has examined which prosurvival BCL-2 family member(s) is/are essential for the sustained survival and expansion of different hematopoietic cancers, including MYC-driven lymphomas. Surprisingly, although BCL-XL is critical for the development of MYC-driven lymphoma, it is not needed for the sustained survival of malignant lymphomas [17]. In contrast, MCL-1 is essential for the sustained survival and growth of MYC-driven lymphomas (both mouse and human) [17], acute myeloid leukaemia (AML) driven by diverse oncogenic lesions (including MYC over-expression) [18] and T cell lymphoma triggered by loss of p53 [19]. These results suggest that MYC-driven cancers have an Achilles heel that can be exploited therapeutically – an exquisite dependence on pro-survival MCL-1. Small molecule inhibitors of MCL-1 are being developed and it will be exciting to test their impact on a broad range of cancers with deregulated MYC expression.

Doug Fairlie, ONJCRI

Dr Fairlie, together with Professor Strasser and colleagues at WEHI, have been involved in several projects involving Genentech and Abbvie (formerly Abbott Laboratories) to develop

small molecule inhibitors of BCL-2 proteins (i.e. "BH3-mimetics") leading to identification of several novel and potent compounds targeting various family members[20-22] (some undergoing formal pre-clinical development) and the first structural data on how this class of compounds engage their targets [20] [23]. Due to the absence of small-molecule MCL-1 inhibitors, the Fairlie lab has also developed highly specific and potent peptide-based tool reagents to target MCL-1 in manner similar to that shown for BH3 mimetic compounds [24,25]. Using the Bim_S2A MCL-1 targeting sequence they idenified, human Myc-driven lymphoma cell lines were shown to be sensitive to MCL-1 antagonism, even in mutant P53 backgrounds [17] confirming the genetic evidence for MCL-1 dependency of these tumours [17] and providing proof-of-principle for drugs targeting this axis. Similar results using this ligand were also obtained in AML cell lines driven by diverse oncogenic lesions (including MYC over-expression) [18]. More recently they have shown that Phylogica's FPP technology can be applied to effectively deliver BH3 peptides into MYC-driven lymphoma cell lines (derived from Eμ-*myc*-transgenic mice) (E. Lee, W.D. Fairlie unpublished) to induce apoptosis. Critically, they also showed that combining MYC inhibition (using FPP-Omomyc) with targeting the BCL-2-regulated intrinsic apoptosis pathway (using FPP BIM peptides) resulted in significant synergy, with up to 6-fold enhanced cell death (E. Lee, W.D. Fairlie unpublished – see figure 5 below).

Figure 5: Validating synergistic inhibition with Phylomer FPP fusions targeting Myc and Bcl



Studies examining the effect of combined MCL-1 and MYC antagonism are about to commence. In parallel studies, Dr Fairlie's group has also been able to enhance (by >100-fold) the stability of BCL-2 targeting peptides though the incorporation of non-natural amino-acids (i.e., β-amino-acids) [26-30] and facilitate their cellular uptake by introducing hydrocarbon "staples" into the sequences [31]. Such peptides effectively entered and killed lymphoma cell lines [31]. However it is likely that the efficiency of stapled peptides could be improved if endosome escape can be enhanced. Phylogica has independently confirmed enhanced potency of stapled peptides using FPP sequences.

Paul Watt (Phylogica Ltd, Australia – www.phylogica.com)
Specific Contributions

- Access to Phylomer peptides for more efficient next generation intracellular delivery (FPPs), discovered using the 'endosome escape trap'
- Next generation inhibitors of protein protein interactions of N-Myc and c-Myc.
- Access to sophisticated genetic lead optimisation platforms for maturation of peptide leads combined with animal models for rapid lead validation (lymphoma and breast cancer)
- Translational focus targeting preclinical programs in blood cancers (specifically AML)
- Strong track record of discovery partnerships with Pharmaceutical industry

TECHNOLOGY

Phylomer Peptides – what they are and where do they come from?

Phylomer peptides are structure-rich peptides derived from biodiverse gene fragment libraries that have been constructed from the genomes of diverse bacteria [32]. These proprietary libraries represent the most structurally rich source of peptides available, as they contain hundreds of billions of peptides from thousands of distinct fold families [33 34]. Since December 2009, Phylogica Ltd, a small Australian biotech company developing this resource has entered into discovery alliances around access to Phylomer technology with Roche, MedImmune, Pfizer, Janssen and Genentech. Potent biologically active Phylomer peptides have already been identified against multiple transcription factors in the Myc pathway, including STAT5, N-Myc and c-Myc.

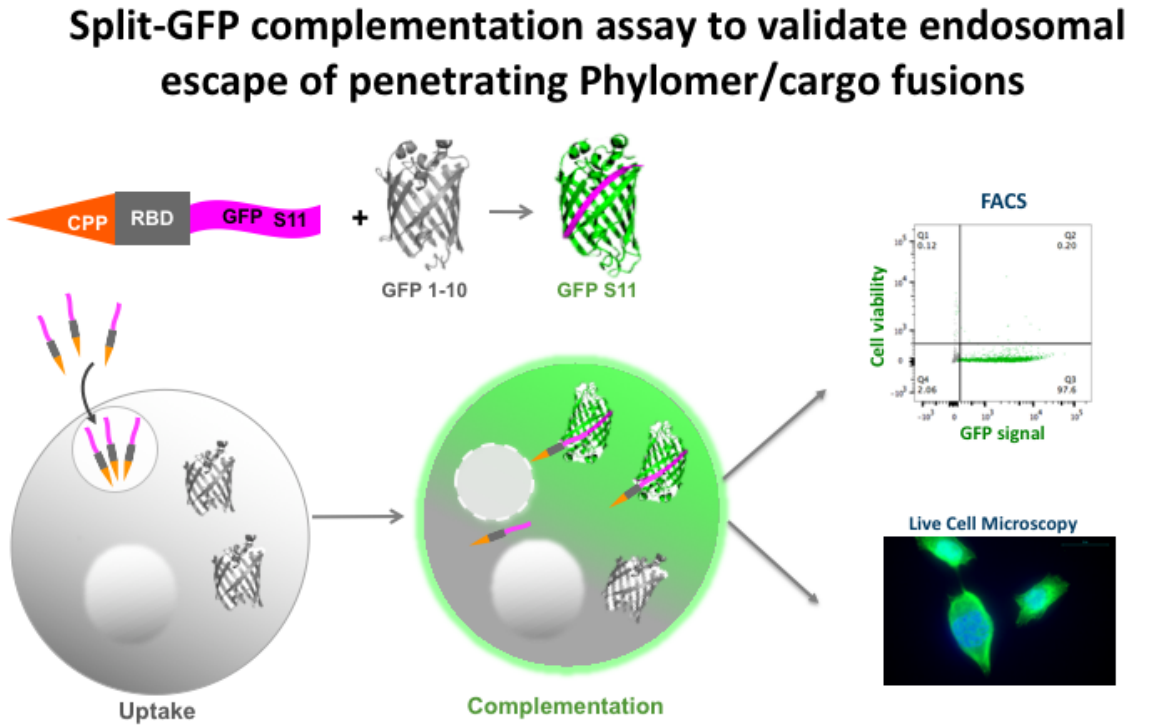
Phylomer FPP's as a source of Best-in-Class cell penetrating peptides to deliver Myc inhibitors

While direct MYC inhibitors such as Omomyc have shown remarkable effects when expressed in Myc-driven tumours [2,4,5], their delivery in protein for as biotherapeutics has proved more challenging, even when fused to conventional cell penetrating peptides (CPPs) such as TAT, R9 or Penetratin. This is because the vast majority of biologics cargoes fused to such peptides remain trapped within endosomes [35], greatly limiting their potency, and hence their feasible future clinical applications, especially since CPP toxicity is often observed at the high concentrations typically required for biological activity [36]. Using a cytoplasmic split GFP complementation assay (Patent WO 2014/205518 A1) [37], Phylogica has recently shown that conventional cell penetrating peptides vary widely in their endosomal release efficiency. Moreover, these conventional CPP sequences are only efficient at higher, therapeutically irrelevant concentrations, probably due to an alternative pathway for cell penetration [38].

To address this issue, Phylogica has devised a new genetic screen for identifying very rare peptides capable of mediating more efficient endosomal release of entire T7 Phage nanoparticles. Phylomer peptides isolated using this 'endosome escape trap' are up 160 times more efficient at cytoplasmic delivery at lower concentrations, according to the split-GFP assay WO 2014/205518 A1, [37] and are referred to here as Functional Penentrating Peptides (FPP). These FPP sequences show no toxicity in mammalian cells, even at concentrations of 100µM (orders of magnitude above dosing concentrations) and are stable in 100% serum for more than 12 hours. In addition to entering cells by macropinocytosis,

Phylomer FPPs can also enhance endosomal escape of cargoes which are directed to particular receptors via receptor binding domains, providing an avenue for more selective targeting of cancer cells. Phylogica is currently developing a genetic 'CPP maturation trap', which allows for the screening of more than 10 million mutants of a CPP sequences using flow cytometry in order to select compounds with further enhancement of delivery efficiency.

Figure 1. A screen to measure the degree of endosomal escape



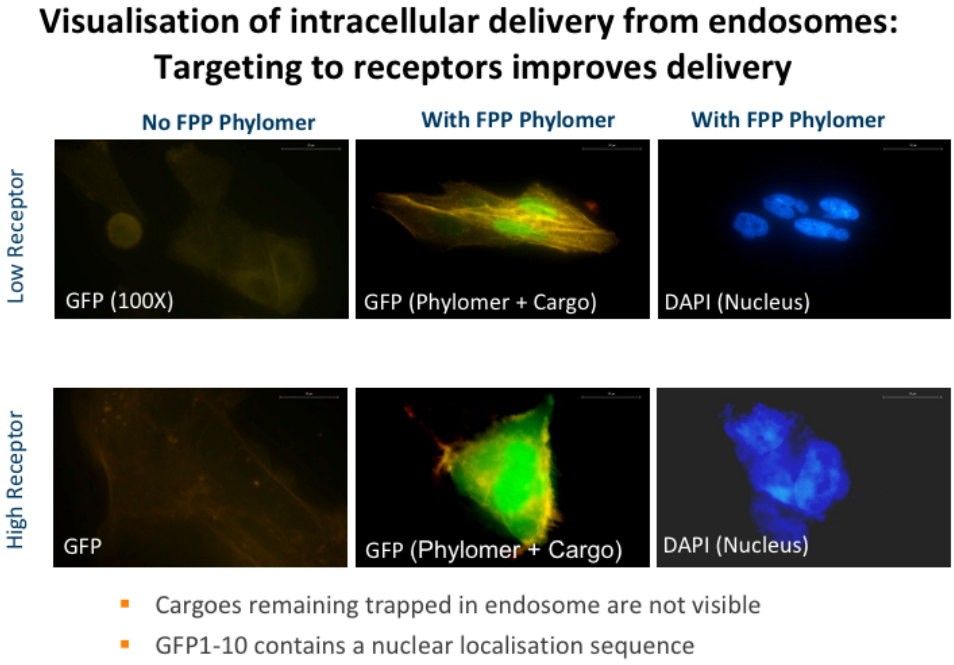
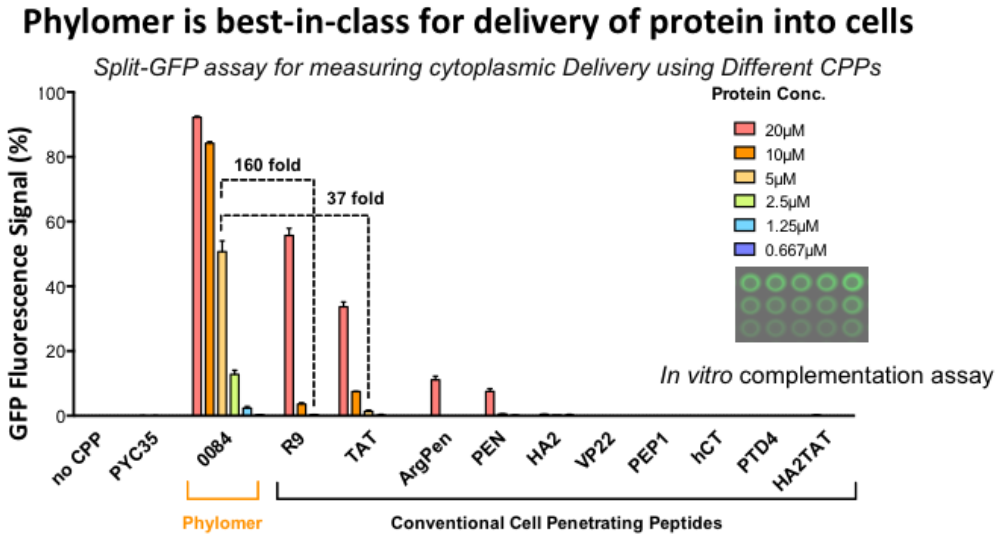


Figure 2. Phylomer FPP delivery to the cytoplasm is superior to conventional CPPs



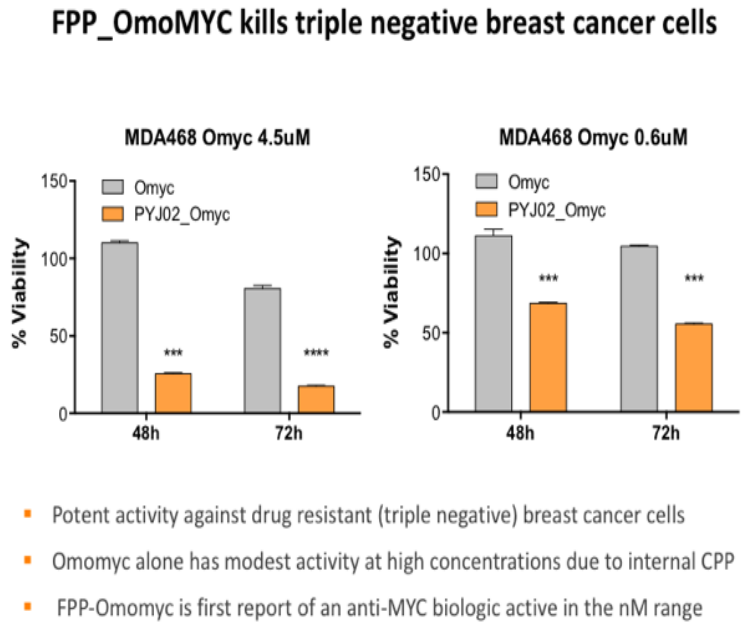
- New assay reveals more efficient Phylomer-derived CPPs defined as **'FPPs'**
- FPPs greatly superior to conventional CPPs– especially at lower concentrations
- Negatives still able to complement fluorescence when mixed with GFP1-10 *in vitro*

Milech *et al.*, (2015) *Scientific Reports* (in press)

Delivery of Omomyc into breast cancer cells using Phylomer derived FPP

Fusions of Omomyc to FPP Phylomers allowed for potent inhibition of breast cancer cell proliferation in the triple negative human breast cancer cell line MDA-MB-468. The figure below (Figure 3) shows that at submicromolar concentrations an FPP fusion to Omomyc (PY02_Omomyc) is active, while Omomyc alone is inactive at such concentrations.

Figure 3. Developing more potent Myc inhibitors using an FPP fused to Omomyc



An FPP fused to Omomyc was also active in a syngeneic mouse model of triple negative breast cancer. The FPP-Omomyc fusion (1746-Omomyc) was able to shrink tumours when injected every second day into tumours induced by injection of T11 cells (Figure 4A). The tumours treated with FPP-Omomyc exhibited less proliferation and more apoptosis than those treated with either Omomyc alone or vehicle (Figure 4B).

Figure 4A. Validating FPP_Omomyc fusions in a syngeneic breast cancer model

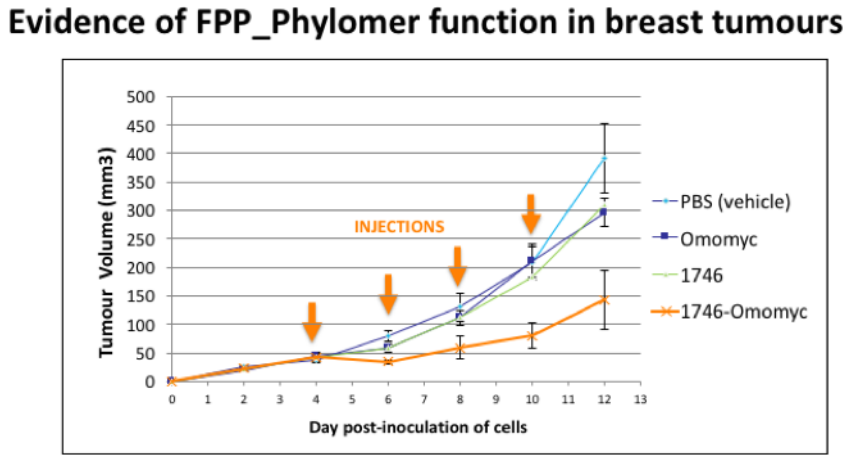
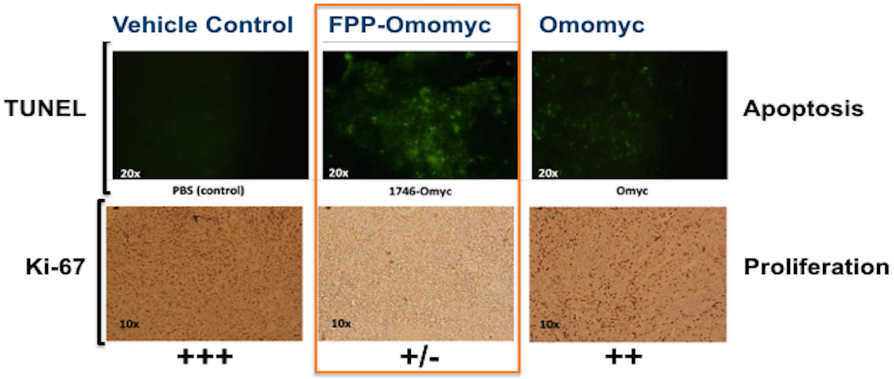


Figure 4B: Histology from T11 triple negative breast tumour specimens



Developing Next Generation Myc inhibitors

Several more potent FPP sequences have recently been identified from Phylomer libraries. Libraries of mutant variants of these FPPs will be subjected to genetic optimization using a flow cytometry assay. Multiple next generation Phylomer inhibitors of N-Myc and c-Myc have been tested in functional assays, where 52% show superior activity to the Omomyc Gold Standard. These hits are being validated using further *in vitro* and *in vivo* assays prior to further optimisation.

EXPECTED OUTCOMES

1) Using our Phylomer libraries and unique library screen approach, we will identify new MYC-targeting peptide sequences that are more potent than Omomyc.
2) Evaluate the efficacy of new MYC-targeting sequences coupled to our best cell-penetrating peptides in MYC-driven cancer cell lines *in vitro*
3) Evaluate the efficacy of new MYC-targeting sequences coupled to our best cell-penetrating peptides in MYC-driven cancer models *in vivo*, especially in blood cancer models (e.g. Eμ-myc model)
4) Expand on our preliminary studies demonstrating synergy between MYC and BCL-2 family inhibitors. MYC-driven cancer cell lines will be co-treated with our FPP-MYC Phylomers *plus* currently available BH3-mimetic drugs and novel BCL-2 family protein-targeting reagents developed in the Fairlie laboratory.

References

1. Soucek, L., Nasi, S. & Evan, G. I. Omomyc expression in skin prevents Myc-induced papillomatosis. *Cell Death and Differentiation* **11,** 1038–1045 (2004).
2. Soucek, L. *et al.* Modelling Myc inhibition as a cancer therapy. *Nature* **455,** 679–683 (2008).
3. Sodir, N. M. *et al.* Endogenous Myc maintains the tumor microenvironment. *Genes Dev* **25,** 907–916 (2011).
4. Soucek, L. *et al.* Inhibition of Myc family proteins eradicates KRas-driven lung cancer in mice. *Genes Dev* **27,** 504–513 (2013).
5. Annibali, D. *et al.* Myc inhibition is effective against glioma and reveals a role for Myc in proficient mitosis. *Nature Communications* **5,** 4632 (2014).
6. Soucek, L. & Evan, G. I. The ups and downs of Myc biology. *Current Opinion in*

Genetics & Development **20,** 91–95 (2010).
7. Askew, D. S., Ashmun, R. A., Simmons, B. C. & Cleveland, J. L. Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* **6,** 1915–1922 (1991).
8. Evan, G. I. *et al.* Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69,** 119–128 (1992).
9. Strasser, A., Elefanty, A. G., Harris, A. W. & Cory, S. Progenitor tumours from Emu-bcl-2-myc transgenic mice have lymphomyeloid differentiation potential and reveal developmental differences in cell survival. *The EMBO Journal* **15,** 3823–3834 (1996).
10. Strasser, A., Harris, A. W., Bath, M. L. & Cory, S. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. *Nature* **348,** 331–333 (1990).
11. Egle, A., Harris, A. W., Bouillet, P. & Cory, S. Bim is a suppressor of Myc-induced mouse B cell leukemia. *Proceedings of the National Academy of Sciences* **101,** 6164–6169 (2004).
12. Michalak, E. M. *et al.* Puma and to a lesser extent Noxa are suppressors of Myc-induced lymphomagenesis. *Cell Death and Differentiation* **16,** 684–696 (2009).
13. Kelly, P. N., Puthalakath, H., Adams, J. M. & Strasser, A. Endogenous bcl-2 is not required for the development of Emu-myc-induced B-cell lymphoma. *Blood* **109,** 4907–4913 (2007).
14. Kelly, P. N., Grabow, S., Delbridge, A. R. D., Strasser, A. & Adams, J. M. Endogenous Bcl-xL is essential for Myc-driven lymphomagenesis in mice. *Blood* **118,** 6380–6386 (2011).
15. Kelly, P. N., Grabow, S., Delbridge, A. R. D., Adams, J. M. & Strasser, A. Prophylactic treatment with the BH3 mimetic ABT-737 impedes Myc-driven lymphomagenesis in mice. *Cell Death and Differentiation* **20,** 57–63 (2013).
16. Delbridge, A. R. D., Grabow, S., Bouillet, P., Adams, J. M. & Strasser, A. Functional antagonism between pro-apoptotic BIM and anti-apoptotic BCL-XL in MYC-induced lymphomagenesis. *Nature Publishing Group* **34,** 1872–1876 (2015).
17. Kelly, G. L. *et al.* Targeting of MCL-1 kills MYC-driven mouse and human lymphomas even when they bear mutations in p53. *Genes Dev* **28,** 58–70 (2014).
18. Glaser, S. P. *et al.* Anti-apoptotic Mcl-1 is essential for the development and sustained growth of acute myeloid leukemia. *Genes Dev* **26,** 120–125 (2012).
19. Grabow, S., Delbridge, A. R. D., Valente, L. J. & Strasser, A. MCL-1 but not BCL-XL is critical for the development and sustained expansion of thymic lymphoma in p53-deficient mice. *Blood* **124,** 3939–3946 (2014).
20. Lee, E. F. *et al.* Conformational changes in Bcl-2 pro-survival proteins determine their capacity to bind ligands. *J Biol Chem* **284,** 30508–30517 (2009).
21. Sleebs, B. E. *et al.* Quinazoline sulfonamides as dual binders of the proteins B-cell lymphoma 2 and B-cell lymphoma extra long with potent proapoptotic cell-based activity. *J Med Chem* **54,** 1914–1926 (2011).
22. Sleebs, B. E. *et al.* Discovery of Potent and Selective Benzothiazole Hydrazone Inhibitors of Bcl-X L. *J Med Chem* **56,** 5514–5540 (2013).
23. Lee, E. F. *et al.* Crystal structure of ABT-737 complexed with Bcl-xL: implications for selectivity of antagonists of the Bcl-2 family. *Cell Death and Differentiation* **14,** 1711–1713 (2007).
24. Lee, E. F. *et al.* A novel BH3 ligand that selectively targets Mcl-1 reveals that apoptosis

can proceed without Mcl-1 degradation. *J. Cell Biol.* **180,** 341–355 (2008).
25. Lee, E. F. *et al.* Novel Bcl-2 homology-3 domain-like sequences identified from screening randomized peptide libraries for inhibitors of the pro-survival Bcl-2 proteins. *J Biol Chem* **284,** 31315–31326 (2009).
26. Boersma, M. D. *et al.* Evaluation of Diverse α/β-Backbone Patterns for Functional α-Helix Mimicry: Analogues of the Bim BH3 Domain. *J. Am. Chem. Soc.* **134,** 315–323 (2012).
27. Lee, E. F. *et al.* High-resolution structural characterization of a helical alpha/beta-peptide foldamer bound to the anti-apoptotic protein Bcl-xL. *Angew. Chem. Int. Ed. Engl.* **48,** 4318–4322 (2009).
28. Lee, E. F. *et al.* Structural basis of Bcl-xL recognition by a BH3-mimetic α/β-peptide generated by sequence-based design. *Chembiochem* **12,** 2025–2032 (2011).
29. Peterson-Kaufman, K. J. *et al.* Residue-Based Preorganization of BH3-Derived α/β-Peptides: Modulating Affinity, Selectivity and Proteolytic Susceptibility in α-Helix Mimics. *ACS Chem. Biol.* **10,** 1667–1675 (2015).
30. Smith, B. J. *et al.* Structure-Guided Rational Design of α/β-Peptide Foldamers with High Affinity for BCL-2 Family Prosurvival Proteins. *Chembiochem* **14,** 1564–1572 (2013).
31. Checco, J. W. *et al.* α/β-Peptide Foldamers Targeting Intracellular Protein-Protein Interactions with Activity in Living Cells. *J. Am. Chem. Soc.* **137,** 11365–11375 (2015).
32. Watt, P. M. Screening for peptide drugs from the natural repertoire of biodiverse protein folds. *Nat Biotechnol* **24,** 177–183 (2006).
33. Watt, P. M. Phenotypic screening of phylomer peptide libraries derived from genome fragments to identify and validate new targets and therapeutics. *Future Medicinal Chemistry* (2009). doi:10.4155/fmc.09.28
34. Milech, N. & Watt, P. The construction of 'phylomer' peptide libraries as a rich source of potent inhibitors of protein/protein interactions. *Methods Mol. Biol.* **899,** 43–60 (2012).
35. Al-Taei, S. *et al.* Intracellular Traffic and Fate of Protein Transduction Domains HIV-1 TAT Peptide and Octaarginine. Implications for Their Utilization as Drug Delivery Vectors. *Bioconjug Chem* **17,** 90–100 (2006).
36. Saar, K. *et al.* Cell-penetrating peptides: A comparative membrane toxicity study. *Anal Biochem* **345,** 55–65 (2005).
37. Milech, N. *et al.* GFP-complementation assay to detect functional CPP and protein delivery into living cells. *Sci. Rep.* **(In Press),** (2015).
38. Verdurmen, W. P. R., Thanos, M., Ruttekolk, I. R., Gulbins, E. & Brock, R. Cationic cell-penetrating peptides induce ceramide formation via acid sphingomyelinase: implications for uptake. *J Control Release* **147,** 171–179 (2010).