**ESA Postdoctoral fellowship award 2018 – Final Report Jan 31st 2019**

**Title:** A new paradigm for endocrine therapy to treat breast cancer

**Background:** The estrogen receptor (ER) is the key driver of 70-75% of breast cancers (BC). Endocrine therapy (ET) has been used to treat BC for >70 years. Newer ET strategies involving hormone deprivation (aromatase inhibitors) or ER targeting (Selective ER Disruptors and Modulators (SERDs/SERMs)) have improved survival outcomes, but acquired resistance remains a major contributor to BC deaths. Recent studies demonstrate that ER typically remains active in endocrine-resistant BC.

Our groups published data indicate that ER can be “reprogrammed” in endocrine-sensitive cancers to drive tumour suppressive transcriptional programs, rather than activating genes that promote tumour growth and survival. This novel reprogramming strategy has strong potential to improve BC outcomes. A major unanswered question is whether ER can be reprogrammed to inhibit endocrine-resistant BC. Additionally, recent research demonstrates that methylation of ER DNA binding sequences contributes to endocrine resistance. In light of these findings, I postulate that epigenetic remodelling is essential for optimal therapeutic ER reprogramming in BC.

**Hypotheses**: (a) Redirecting ER away from oncogenic DNA-binding sites toward tumour suppressive sites will inhibit growth of endocrine-sensitive and -resistant BC cells, and (b) Epigenetic remodelling will enhance ER reprograming strategies to improve their efficacy.

**Aim 1: (a) Evaluate repurposed and novel candidate reprogramming compounds for their capacity to inhibit proliferation and promote differentiation in endocrine-sensitive and -resistant BC cell lines, and (b) determine their efficacy in the presence or absence of epigenetic modifying drugs.**

**Aim 2: (a) Evaluate lead compounds for their capacity to reprogram ER in endocrine-sensitive and -resistant BC cell lines, and (b) determine the efficacy of ER reprogramming in the presence or absence of epigenetic modifying drugs.**

**Methods and Results:**

This project first proposed to test 20 nuclear receptor ligands (compounds) for their capacity to supress growth and promote a more differentiated phenotype of 4 human ER positive breast cancer cells lines, including endocrine-sensitive and –resistant sub-types. It also aimed to determine if supressing DNA hypermethlyation could enhance this effect and allow for ER to “reprogram” from oncogenic DNA binding sites, to those associated with a more normal DNA binding profile. After receiving this award in early 2018 I was able to establish a successful collaboration with Cell Screen SA at Flinders University to carry out a high content image based high throughput screen. This project was expanded to cover additional novel and repurposed compounds (n=38) that were selected from preliminary studies by our lab and other collaborators. This project was also expanded to incorporate a much broader range of cell lines (n=8), in order to identify compounds that are efficaous across a larger range of ER positive endocrine-sensitive (T-47D, ZR-75-1, MCF7) and –resistant (MCF7-LTED, MCF7-TAMR, BT474) breast cancer models. Additionally, ER-negative breast cancer cell lines (MDA-MB-453, MFM-223) were incorporated to act as negative cell line controls, enabling identification and exclusion of compounds that do not supress growth via the ER.

Before the high throughput screen could begin at Cell Screen SA, a rigorous optimisation process was carried out at The University of Adelaide, in order to identify the optimal conditions for the high throughput screen. For each of the 8 cell lines, the optimal dose and duration of the DNA demethylation agent Decitabine, which could reduce DNA methylation without significant cell toxicity was determined. DNA methylation was quantified by 5-methyl-cytosine staining on a DNA dot blot and DNMT1 protein expression by Western blot. Once optimal conditions were established for the DNA demethylation treatment, an extensive cell seeding density experiment was performed to establish the ideal starting cell quantity in 384-well plates for a 7 day high throughput screen growth experiment. Ten different seeding densities were tested across 8 cell lines, and predicated cell numbers were based on each cells unique doubling time. Treatment groups included media only, 0.3% DMSO (the final DMSO percentage in the high throughput screen), 1nM Cabazitaxel and 10nM Fulvestrant (control compounds). Cells were seeded Day 0, and drugs added on days 1 and day 3. On day 6 cells were fixed and stained with DAPI and imaged using a fluorescent microscope.

Once a complete optimisation of the experimental conditions had been established and validated, n=38 compounds, 2 controls, and several DMSO only (vehicle) treatment groups were prepared in single 6x 10-fold doses (from 100pm🡪10uM) within 384-well plates by Compounds Australia, Sydney. The screen was performed in 2 cohorts. Cohort 1: T47D, ZR-75-1, BT474, MDA-MB-453. Cohort 2: MCF7, MCF7-LTED, MCF7-TAMR, MFM-223. Cell were cultured with either Vehicle (DMSO) or optimal doses of DNA demethylation agent and delivered to Cell Screen SA. Using robotic operators cells were seeded on day 0 into 384 well plates and drugs were added to either Vehicle or DNA demethylation treated cells on days 1 and 3. On day 6, cells were fixed and DAPI stained for nuclear count and cellular morphology (cell shape and size) quantification using high content image based screening. Cell counts normalised to DMSO were generated for growth inhibition, and mean cell roundness and cell area were determined versus DMSO control for changes in morphology (indicative of differentiation).

Current analysis of growth inhibition and cell morphology is still ongoing. Early results show that several compounds were significantly reducing growth: At least 50% growth inhibition versus the DMSO vehicle group (Table 1). Many of these compound “hits” overlapped across cell lines, and some were cell line specific. Notably, several of the “hit” compounds were only inhibiting growth at the highest dose tested (10uM), and therefore further analysis of their specificity will be followed up. Additionally, both ER negative cell lines MDA-MB-453 and MFM-223 demonstrated hits across n= 12 and 14 compounds, respectively, which were mostly supressing growth at the highest dose tested. This indicates these compounds may be inhibiting growth through non-specific (ie non-ER) mechanisms, or are simply toxic at the doses tested, and will likely be excluded for future exploration of ER DNA binding sites by ChIP-seq.

The interaction effect of adding a DNA demethylation agent to each of the compounds tested did not enhance growth inhibition in BT474, MCF7, MCF7-TAMR or either of the ER negative cell lines MDA-MB-453 and MFM-223. However, several compounds showed a significant interactive effective after treatment with a DNA demethylation agent in MCF7-LTED, T47-D and ZR-75-1 (Table 1). In MCF7-LTED cells, there were 3 compounds that demonstrated significant growth suppression after Decitabine treatment (Table 1). However, of particular interest is the unexpected result that treatment with Decitabine could *rescue* the growth of T47D and ZR-75-1 cells in the presence of n=2 and n= 4 compounds, respectively (Table 1). This result will be validated in these cell lines using a lower throughput “re-screen” in order to determine if these results are repeatable. Cell morphology changes will also be analysed and incorporated into a final selection pipeline to identify those compounds that can inhibit growth and promote a more differentiated phenotype. Due to the expansion of this project to incorporate additional compounds and a larger range of cell lines, ER reprogramming will be analysed by ChIP-seq in future studies this year.

Table 1: Number of compound hits from the high throughput screen in 8 cell lines tested, with or without DNA demethylation treatment.

|  |  |  |  |
| --- | --- | --- | --- |
| **Cell line** | **Number of hits** | **DNA demthylation effect?** | **Compound interaction with DNA demethylation agent Decitabine** |
| BT474 | 11 | 0 | NA |
| MCF7 LTED | 21 | 3 | 21827219 (Novel compound) supresses growth by ~40%  42190112 (Novel compound) supresses growth by ~40%  Enobosarm (AR ligand) supresses growth by ~40% |
| MCF7 | 21 | 0 | NA |
| MCF7 TAMR | 27 | 0 | NA |
| T47D | 12 | 2 | Progesterone (PR ligand) – ***enhances*** growth by 40-50%  Triamcinolone acetonide - ***enhances*** growth by 40% |
| ZR751 | 14 | 4 | 21827219 (Novel compound) - ***enhances*** growth by 40%  SARM SK33E (AR ligand) - ***enhances*** growth by 30-40%  SARM SK38C (AR ligand) - ***enhances*** growth by 20-40%  SARM SK51E (AR ligand) – ***enhances*** growth by 40%  Enobosarm (AR ligand) - ***enhances*** growth by 30-40% |
| 453 | 12 | 0 | NA |
| 223 | 14 | 0 | NA |

**Summary**

This ESA fellowship project identified several novel and repurposed nuclear receptor ligands that could significantly reduce growth of endocrine-sensitive and –resistant ER positive breast cancer cells, both selectively through the ER and via non-ER related mechanisms. Furthermore, it was identified that treatment of select ER positive cell lines with the DNA demethylation agent Decitabine could either enhance or repress growth inhibitory properties of nuclear receptor ligands, indicating a significant role of DNA methlyation on the growth and survival of ER positive breast cancer cells in response to endocrine therapies. These data will form the basis of a larger *ex vivo* and *in vivo* screens of leading treatment strategies using patient derived xenografts to begin later this year. This exciting new data will have the potential for rapid translation into the clinic to implement newer treatment treatment strategies for women with ER-positive breast cancer.

**Conferences attended/Abstracts submitted**

* Lorne Cancer Conference, Lorne, Victoria, February 2018
* South Australia Breast Cancer Study Group, Adelaide, The University of Adelaide AHMS building, October 2018
* American Association for Cancer Research annual meeting, Atlanta, Georgia, USA, March-April 2019