Progress Report & Funding Request

Project Title: Facilitates Chromatin Transcription Complex (FACT): A novel therapeutic target against Diffuse Intrinsic Pontine Glioma

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This project aims to test the hypothesis that targeting complex known as FACT (Facilitates Chromatin Transcription) represents a novel therapeutic approach for Diffuse Intrinsic Pontine Glioma (DIPG) and our objective is to develop effective combination strategies optimal for clinical translation. The specific research aims listed in the previously-submitted TCSN project proposal are:

(1) To examine the anticancer properties of CBL0137 in a panel of DIPG neurospheres.
(2) To evaluate the mechanism of action of CBL0137.
(3) To examine combination therapies which enhance the activity of CBL0137 against DIPG *in vitro.*
(4) To determine the therapeutic effect of CBL0137 as a single agent, with most effective combination (double agent combination) and with currently used treatment irradiation (triple combination) *in vivo*.

A larger grant was submitted to the NHMRC to comprehensively investigate the FACT complex as a therapeutic target in DIPG and Neuroblastoma, evaluate efficacy for a COG Phase I/II paediatric clinical trial and identify biomarkers. The specific aims of this project are:

(1) To investigate the potential of high FACT expression, and other specific biomarkers to predict and monitor CBL0137 response in DIPG and neuroblastoma.
(2) To evaluate combination therapies which enhance the ability of CBL0137 to inhibit DIPG and neuroblastoma proliferation *in vitro.*
(3) To evaluate the efficacy of CBL0137 in combination with chemotherapeutic drugs using *in vivo* models of DIPG and neuroblastoma.
(4) To assess biomarker efficacy by undertaking correlative biology studies on clinical specimens from patients treated in a Phase I/II clinical trial of CBL0137.

Progress on Aims:

We have investigated the cytotoxic effect of CBL0137 against a panel of 5 DIPG primary patient-derived neurosphere cultures, human healthy astrocytes (NHA), human healthy brainstem astrocytes (HBA) and healthy lung fibroblasts (MRC5) (**TCSN Aim 1/NHMRC Aim 2**). CBL0137 exhibited activity at low μM concentration (0.1-1.2μM) across all neurosphere cultures at concentrations that had no cytotoxic effect against adult-derived HBA cells and minimal toxicity against the foetal-derived healthy cells NHA and MRC5 (**Figure 1**).

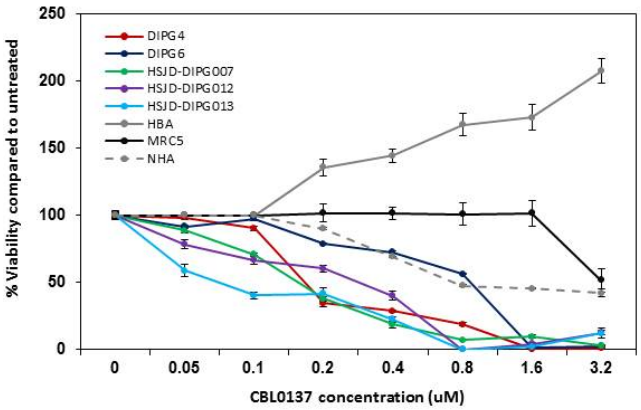

Figure 1: Cell viability assay of DIPG neurospheres and healthy cells treated with increasing concentrations of CBL0137 for 72h.

Furthermore flow cytometric experiments using Annexin/Propidium Iodide staining indicate that CBL0137 causes significant induction of apoptosis in a dose and a time dependent manner (**Figure 2**). In addition Caspase 3/7 induction is also observed upon CBL0137 treatment (Figure 3). These results further support our preliminary results

presented in TCSN project proposal and clearly support the potential of CBL0137 as a therapeutic strategy against DIPG **(TCSN Aims 1/2, NHMRC Aim 2)**.

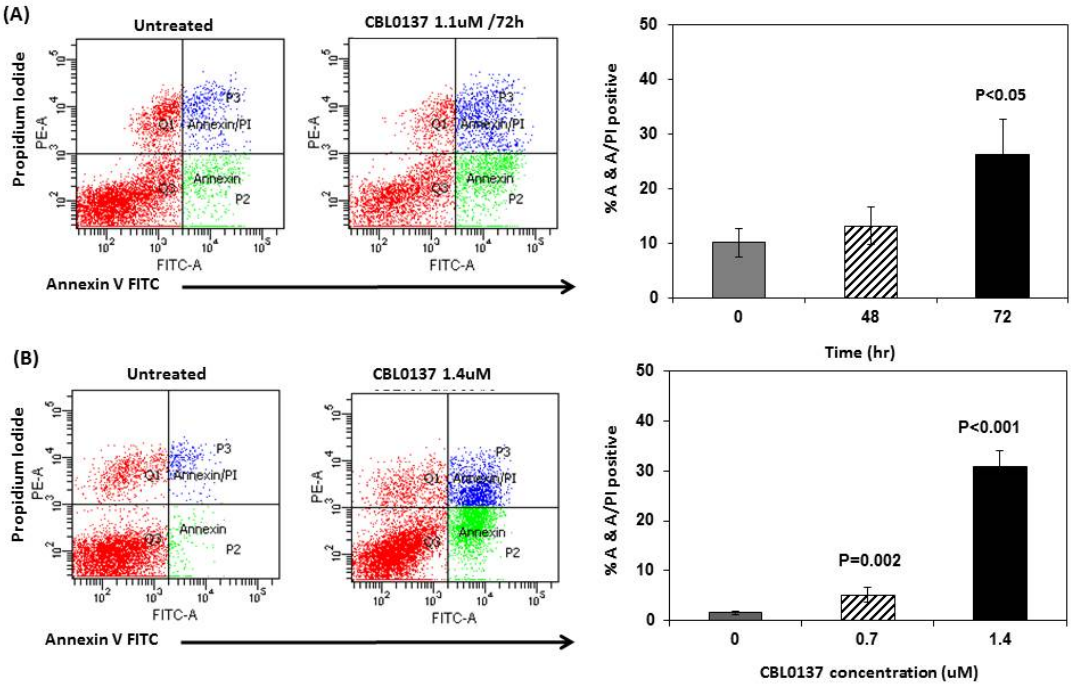

Figure 2: Induction of apoptosis in DIPG neurospheres treated with CBL0137; **(A)** DIPG6 cells treated with 1.1uM CBL0137 for 48 and 72h and stained with Annexin/PI; **(B)** DIPG4 cells treated with different concentrations of CBL0137 for 72h and stained with Annexin/PI.

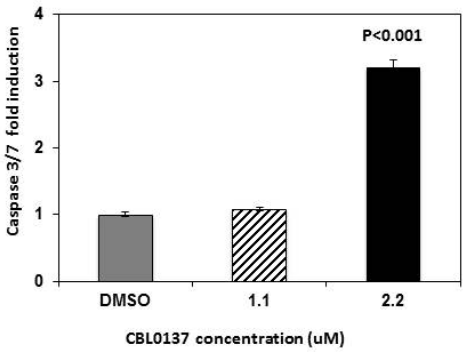

Figure 3: Caspase 3/7 induction in DIPG6 cells after 24h of CBL0137 treatment.

As previously discussed in the submitted TCSN grant we have assessed by western blot analysis the expression of the CBL0137 target, FACT, in both patient-derived neurospheres and in autopsy samples collected in Dr Ziegler's Australian DIPG autopsy study. Both FACT subunits, SPT16 and SSRP1, were highly expressed in 2 neurosphere forming DIPG cell lines compared to healthy brainstem astrocytes**.** Similar results were observed in 3 separate DIPG tumours collected at autopsy – with low levels of FACT expression in normal pons and cerebellum and high levels of protein seen in tumour tissue. We are currently investigating more DIPG cell lines and more tumour samples from our growing national bank of DIPG autopsy and from collaborators overseas (A/Prof Michelle Monje, Dr Angel Montero Carcaboso).

Additional western blot experiments have shown that CBL0137 enhances the expression of TP53 while they inhibit activation of NFkB (**Figure 4**). These results confirm previous published observations delineating the mechanism of action of this drug (**TCSN Aim 1/ NHMRC Aim 2**). We also intend to investigate whether targeting of the FACT complex by CBL0137 causes entrapment of the FACT complex in chromatin affecting thus NFkB function (**TCSN Aim 1/ NHMRC Aim 2**). Importantly we have also discovered that H3K27 methylation status is altered upon CBL0137 treatment indicating that this drug is potentially capable of causing epigenetic changes. Further experiments are planned to validate these findings, assess the effect of histone methylation across the genome and examine the differential effect of CBL0137 on DIPG cell lines with and without the H3.3K27M mutation (**NHMRC Aim 2**).