End of Grant Report

Report at the end of the grant by Christopher's Smile for the 3 year position of Post Doctorate Researcher in the Paediatric Drug Development Team at the Institute of Cancer Research

Christopher's Smile

coz kids get cancer too

Registered Charity 1129906

Contents

1. Position

Title	Post Doctorate Researcher in the Paediatric Drug Development Team
Role Description	
Start of Grant	May 2011
Period of Grant	3 Years
Name(s) of person in position	Evon Poon
Grant funding over period of grant	£194,898
Matched funding over period of grant	£0

Overview of role during grant period

Dr Evon Poon was funded by Christopher's Smile to identify novel therapeutic strategies to treat neuroblastoma and medulloblastoma in children. During her three-year funding period, Evon's role was to identify molecularly-targeted drugs that specifically target tumour cells with elevated levels of the MYCN oncoprotein. Cancer cells that aberrantly express high levels of MYCN have many aspects of cellular function fundamentally altered. This makes them extremely aggressive but also presents therapeutic opportunities – cancer cells become "oncogene addicted" and dependent on the expression of MYCN for survival. Evon's focus has been on drugs that target MYCN stability through inhibition Aurora-A kinase and *MYCN* expression through inhibition of cell cycle kinases, with the highest specificity for CDK2 and CDK9.

Evon identified these drugs as therapeutic targets in neuroblastoma and medulloblastoma by gaining insights into the way the levels of MYCN are controlled at the molecular level. Results arising from this work on inhibition of Aurora-A kinase using a novel drug, MLN8237, in neuroblastoma have recently been published in *Cancer Cell*, one of the world's leading medical research journals. This work has been extended to studies in relapsed medulloblastoma in children that is defined by loss of the p53 tumour suppressor and aberrant expression of *MYCN*. This study is currently under review in *Cancer Cell*. Evon has recently concluded her work using cell cycle kinase inhibitors and a manuscript is under preparation. Evon has also tested the combination these novel drugs with existing chemotherapeutics or other novel molecularly-targeted drugs that target MYCN via different mechanisms. This work has shown that combinations offer improved survival in pre-clinical models of paediatric cancer.

2. Objectives

The attrition rate for novel therapeutics is exceedingly high, in part relating to inefficient pre-clinical drug development approaches and also to a lack of suitable pre-clinical cancer models in which clinically relevant trials can be conducted. We have developed excellent models of neuroblastoma and medulloblastoma to test novel therapeutic strategies and the

objectives in this project were to establish a practical approach for the introduction of novel clinical-candidate agents into existing and amenable clinical trials regimens. The Royal Marsden is a partner in the BEACON-neuroblastoma trial (**BE**v**AC**izumab added to temozolomide +/- irin**O**tecan for children with refractory/relapsed **N**euroblastoma), which aims to find the best chemotherapy regimen for children with recurring or resistant neuroblastoma. For those children who do not respond to treatment with temozolomide/irinotecan in relapse (which is presently the standard strategy used to treat children with relapsed neuroblastoma) BEACON permits the incorporation of new molecularly-targeted drugs in combination with either temozolomide/irinotecan or whichever single agent is found to be most effective in a presently ongoing randomized clinical trial. The study is a joint trial between Cancer Research UK, the Innovative Therapy for Children with Cancer (ITCC) and the International Society of Paediatric Oncology European Neuroblastoma Group (SIOPEN). Seven countries across Europe will take part in the trial once they have regulatory approval.

Direct targeting of MYC proteins (MYC and MYCN) is difficult and has thus far not been achieved. As our understanding of the biology of MYC proteins has increased, however, a number of strategies to target MYCN indirectly and mutated ALK kinase (another oncoprotein that is associated with *MYCN*-amplified high-risk neuroblastoma) have been developed as summarized in **Figure 1** below. These novel therapeutic strategies, which are underpinned by drugs that are either already in or will soon enter clinical trials target distinct aspects of MYCN biology and their combinatorial use, either with other targeted drugs or chemotherapeutic agents represent a *bone fide* approach to improve survival in poor-outcome neuroblastoma and medulloblastoma. We are very happy that the first clinical trials of MYCN-targeted drugs have now been driven in-part by our work with Aurora A kinase inhibitors such as MLN8237, which Evon identified as the first effective agent that targets MYCN. MLN8237 is in trials alone and combined with chemotherapy in the USA Children's Oncology Group.

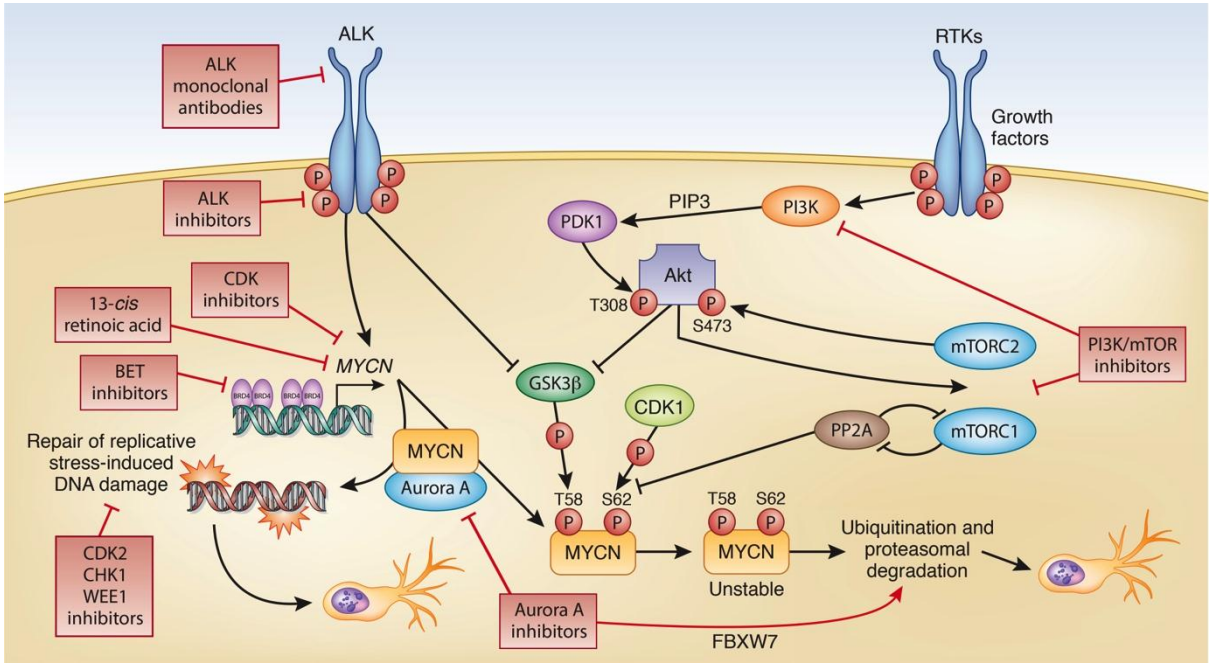

Figure 1. Targeted therapy of MYCN. Current approaches to targeted therapy for NB include several modalities including the following: (i) small-molecules that inhibit stabilization MYCN via activated

signaling pathways (ALK, PI3K/mTOR, and mTORC1/2 inhibitors) or protection from proteasomal degradation (Aurora-A). (ii) Molecules that inhibit MYCN expression such as 13-cis retinoic acid, BET bromodomain inhibitors or CDK inhibitors. (iii) Drugs that target factors that are synthetic lethal in MYCN amplified NB such as CHK1 and WEE1. (iv) Immunotherapeutic strategies that target ALK.

3. Work Undertaken

Year 1

Evon tested a number of novel molecularly-targeted therapies in neuroblastoma and medulloblastoma cell lines using *in vitro* assays. She identified a several agents including the Aurora-A kinase inhibitor (MLN8237) and the CDK inhibitor (CYC065) that were selective and potent for neuroblastoma and medulloblastoma cells that amplification of the *MYCN* gene and/or elevated expression of MYCN protein. She spent the remainder of Year 1 elucidating their mechanisms of action at the molecular level.

Year 2

In Year 2, the novel molecular-targeted therapeutic agents identified from Year 1 *in vitro* studies (with a focus on MLN8237) were tested in models of neuroblastoma and medulloblastoma. Evaluation was also carried out to assess the toxicity of the drugs and whether the mechanisms of actions that were identified using *in vitro* assays in Year 1 were present in *in vivo* models.

Year 3

In Year 2 and 3, Evon concluded her study with CYC065. Evon's work with MLN8237 was extended to *in vivo* models of medulloblastoma. MLN8237 and CYC065 were combined either with conventional chemotherapeutic agents or other molecular-targeted therapies to achieve maximum survival benefit. Studies have focused on combinations that incorporate the DNA alkylating agent temozolomide and the DNA topoisomerase inhibitor irinotecan, which for the backbone for treatment of relapsed high-risk neuroblastoma in the BEACON trial.

4. Key Achievements

Year 1

Identification of selective and potent novel molecular-targeted therapeutics for neuroblastoma and medulloblastoma cell lines were identified using *in vitro* assays. MLN8237 and CYC065 were selected for further study.

Year 2

Evon found that MYCN forms a complex with the Aurora-A kinase, which protects MYCN from proteasomal degradation. Although stabilization of MYCN does not require the catalytic activity of Aurora-A, MLN8237 (but not a structurally distinct inhibitor MK5108) disrupts the Aurora-A/MYCN complex and promotes degradation of MYCN via the Fbxw7

ubiquitin ligase. Disruption of the Aurora-A/MYCN complex via MLN8237, or its analogue MLN8054, inhibits MYCN-dependent transcription, correlating with tumour regression and prolonged survival in a cancer model of *MYCN*-driven neuroblastoma (**Figure 2**).

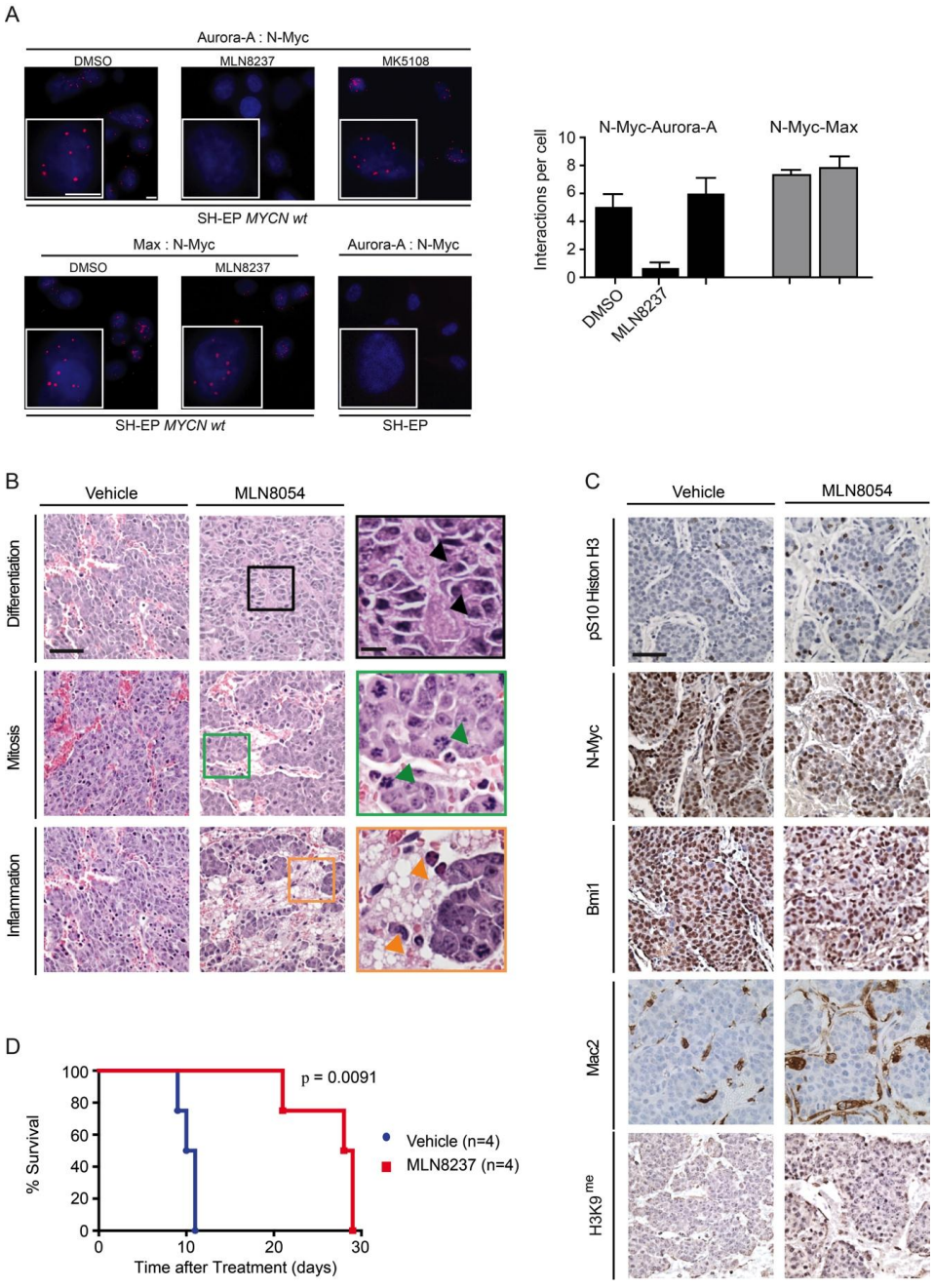

Figure 3. Therapeutic targeting of the MYCN/Aurora-A interaction inhibits tumour growth and prolongs survival in neuroblastoma. (A) Proximity ligation assays analyzing N-Myc/Aurora-A and N-Myc/Max complexes in SH-EP cells stably expressing N-Myc. Blue, DAPI staining of nuclei; red dots, PCR amplification products indicating complex formation of N-Myc with Aurora-A or with Max as indicated. Scale bars indicate 10 mm. Where indicated, MLN8237 (500 nM) or MK5108 (1,000 nM) was added for 4 hr (n = 3). SH-EP cells not expressing N-Myc serve as negative control. Lower panel shows mean +SD of triplicate biological replicates. (B) Hematoxylin and eosin staining of sections of tumours treated with vehicle or 40 mg/kg MLN8054 at day 3 (high-magnification views are shown on the right). Black arrows (top row) indicate neuronal differentiation with the presence of variably maturing ganglion-like cells. Green arrows (middle row) indicate mitotic cells. Orange arrows

(bottom row) show a multivacuolated histiocytic component around tumours. Scale bars represent 50 mm (left and middle) or 10 mm (right). (C) Immunohistochemistry of treated tumours using indicated antibodies. Scale bar represents 50 mm. (D) Kaplan-Meier plot documenting effectiveness of cancer models treated with 30 mg/kg MLN8237 versus vehicle.

Year 3

Evon has found that neuroblastoma cell lines with *MYCN* amplification and/or high levels of MYCN are highly sensitive to the CDK inhibitor CYC065. CYC065 blocks neuroblastoma cell proliferation, induces apoptosis and downregulates MYCN protein. CYC065 inhibits transcriptional activity of neuroblastoma cells, importantly MYCN transcriptional activity. This work will now extended to include combinatorial studies in relapsed medulloblastoma.

Evon's work with MLN8237 paved the way for this drug to be used in pre-clinical testing for a study recently concluded and in review for *Cancer Cell*. A comprehensive clinical and biological investigation of serial medulloblastoma biopsies obtained at diagnosis and relapse revealed that combined *MYC* gene family amplifications and P53 pathway defects commonly emerged, and all patients in this group died of rapidly progressive disease post-relapse. To study this genetic interaction, we investigated a model of MYCN-driven medulloblastoma and found spontaneous development of *Trp53* inactivating mutations. Abrogation of Trp53 function in this model produced aggressive tumours that mimicked the characteristics of relapsed human tumours with combined P53-MYC dysfunction. Restoration of p53 activity, genetic and therapeutic suppression of MYCN with MLN8237 all reduced tumour growth and prolonged survival (**Figure 3**).

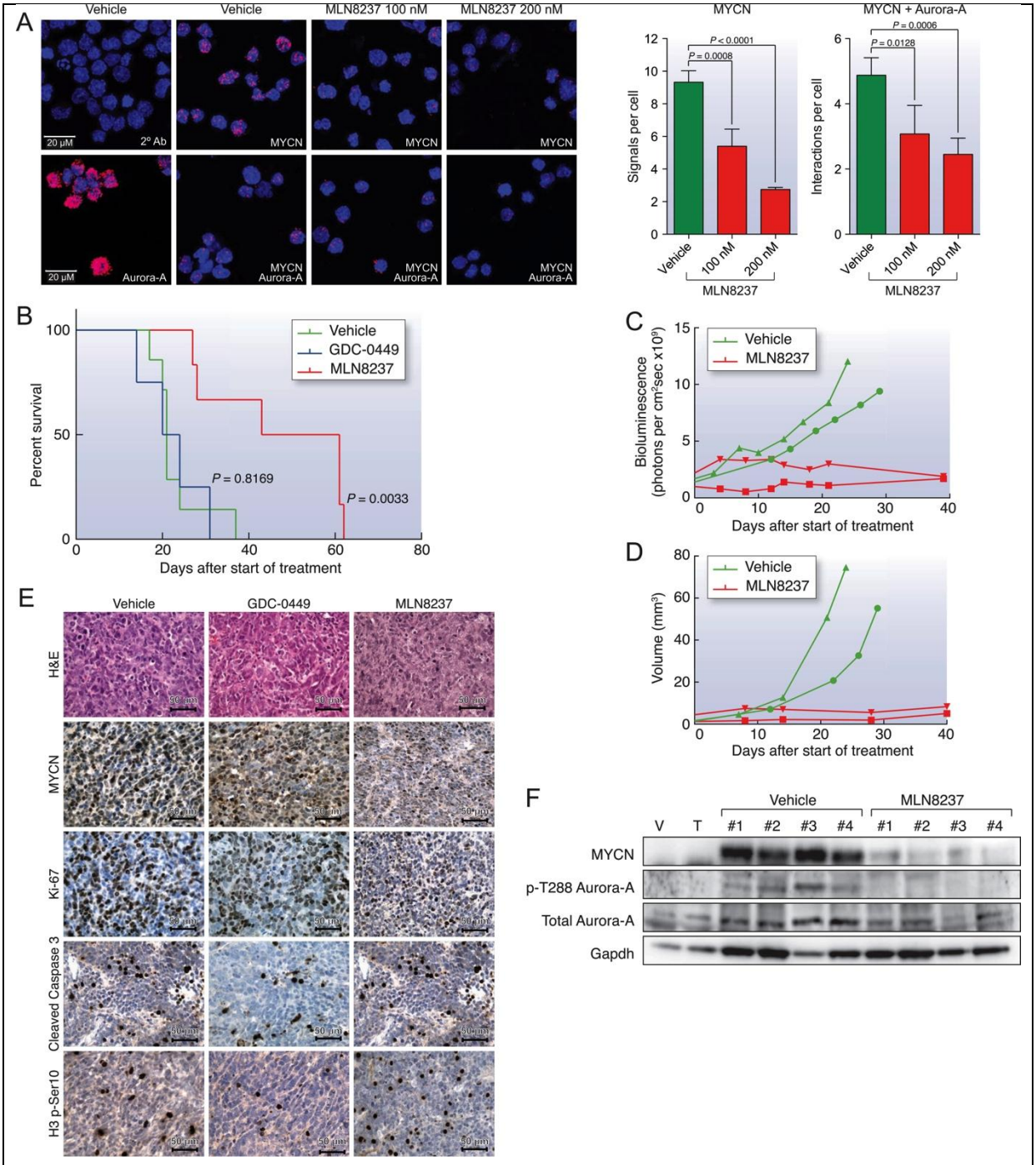

Figure 3. Therapeutic targeting of the MYCN/Aurora-A interaction inhibits tumour growth and prolongs survival in relapsed medulloblastoma. (**A**) Proximity ligation assay (PLA) analyzing MYCN/Aurora-A complexes in GTML/*Trp53*$^{KI/KI}$ neurospheres. Left panel shows close proximity (< 40 nm) of antibody conjugated PLA probes that have been ligated, amplified and detected with complementary fluorescent probes. Red dots represent the presence of MYCN or Aurora-A protein, or MYCN/Aurora-A interactions as indicated. Scale bars indicate 20 µm. Where indicated, MLN8237 was added for 48 hr. Right panel shows mean values of signals (red dots) per cell representing MYCN expression or MYCN/Aurora-A interactions. Values are derived from triplicate biological replicates and error bars represent standard deviations. *P*, unpaired t-test. (**B**) Kaplan-Meier survival for treatment with MLN8237 (30 mg kg^{-1}, n = 6), GDC-0449 (50 mg kg^{-1}, n = 4) or vehicle (n = 7) as indicated. (*P*, Log rank test.) (**C**) Longitudinal bioluminescent (firefly luciferase) imaging shows stable firefly luciferase expression after treatment with MLN8237 (50 mg kg^{-1}) compared to vehicle. (**D**)

Longitudinal MRI analysis of tumour volume on the axial plane. (**E**) H&E and immunohistochemical staining indicating levels of MYCN protein, cell proliferation (Ki-67), apoptosis (Cleaved caspase 3), or mitotic activity as measured by phosphorylated Ser10 on histone H3 (H3 p-S10) after treatment with GDC-0449 or MLN8237. Scale bars represent 50 µm. (**F**) Immunoblotting of MYCN protein levels, and total and phosphorylated Thr288 on Aurora-A (p-T288 Aurora-A) in MLN8237-treated tumour tissues. For (**E**) and (**F**) animals were treated with vehicle, GDC-0449 or MLN8237 for 48 hrs and samples taken 2 hrs after final administration of agent.

5. Objectives not met during grant period

Evon has been very successful in meeting her primary objectives, however with further funding we would have been more successful. In the future, we have further work to do in the area of MYCN targeting. With respect to aurora kinase, we have identified and published the mechanism by which aurora kinases can theoretically destabilise and eliminate MYCN protein. A preliminary compound (MLN8237) which addresses this mechanism is in two clinical trials which are ongoing in the USA Children's Oncology Group. Ideally we would have liked this trial to occur within the UK And EU (ITCC), but for logistical reasons this was not possible. Importantly, our work also predicts that MLN8237 is not a structurally perfect compound to activate the mechanism of MYCN degradation and ongoing work within the next 3 years is focused on identification and medicinal synthesis of more potent MYCN inhibitors based on our initial success in this area. Additionally, Evon's work, in collaboration with Martin Eilers (Wurzburg) identified a set of initial proteins that interact directly with MYCN, each of which represent known or novel pharmaceutical targets of great interest. Evon's work will now focus on some of the initial hits in this screen, to develop the preliminary data that will establish which proteins present druggable interactions that we can potentially take forward to clinical use. Finally, all of this work would have benefitted from greater access to medicinal chemistry compounds and promising new cancer drugs, which are in general still difficult to obtain in the academic setting. We ideally need a "drugs fund" to permit continuous access to these compounds, which represent a considerable and unsustainable expense for research laboratories. Most importantly, in order to most effectively perform research with drugs that could reach clinical trials, academic research laboratories need unrestricted access to these tools/drugs without any conflicts-of-interest that could arise if the drugs are obtained through material transfer agreements from for-profit entities that own them. This limits the types of research that can be performed and potentially also the unbiased selection of the most effective therapeutics in a purely academic setting.

6. Contribution to published, peer reviewed scientific paper(s)

Small molecule inhibitors of aurora-a induce proteasomal degradation of N-myc in childhood neuroblastoma. Brockmann M,* **Poon E**,* Berry T, Carstensen A, Deubzer HE, Rycak L, Jamin Y, Thway K, Robinson SP, Roels F, Witt O, Fischer M, Chesler L, Eilers M. *Cancer Cell*. 2013 Jul 8;24(1):75-89. (*** Joint first authors**).

Evaluation of clinically translatable MR imaging biomarkers of therapeutic response in the TH-MYCN transgenic mouse model of neuroblastoma. Jamin Y, Tucker ER, **Poon E**, Popov S, Vaughan L, Boult JK, Webber H, Hallsworth A, Baker LC, Jones C, Koh DM, Pearson AD,

Chesler L, Robinson SP. *Radiology*. 2013 Jan;266(1):130-40.

The ALK(F1174L) mutation potentiates the oncogenic activity of MYCN in neuroblastoma. Berry T, Luther W, Bhatnagar N, Jamin Y, **Poon E**, Sanda T, Pei D, Sharma B, Vetharoy WR, Hallsworth A, Ahmad Z, Barker K, Moreau L, Webber H, Wang W, Liu Q, Perez-Atayde A, Rodig S, Cheung NK, Raynaud F, Hallberg B, Robinson SP, Gray NS, Pearson AD, Eccles SA, Chesler L, George RE. *Cancer Cell*. 2012 Jul 10;22(1):117-30.

Manuscripts submitted or in preparation:

Combined MYC and TP53 defects emerge at medulloblastoma relapse and define rapidly progressive, therapeutically targetable disease. Hill R, Kuijper K, Lindsey JC, Schwalbe E, Barker K, Boult JKR, Williamson D, Ahmad Z, Hallsworth A, Ryan SL, **Poon E**, Robinson SP, Ruddle R, Raynaud FI, Howell L, Kwok C, Joshi A, Nicholson SL, Crosier S, Wharton SB, Robson K, Michalski A, Hargrave D, Jacques TS, Pizer B, Bailey S, Swartling FJ, Petrie K, Weiss WA, Chesler L, Clifford SC. Submitted to *Cancer cell* (CANCER-CELL-D-14-00338).

AT7519 as a potential drug for MYCN-amplified neuroblastoma. Dolman MEM,* **Poon E**,* Marli E. Ebus ME, den Hartog IJM, van Noesel CJM, Sparidans RW, Kok RJ, Versteeg R, Caron HN, Chesler L, Molenaar JJ. Manuscript in preparation. (*** Joint first authors**).

Inhibition of the CDK pathway represses the transcriptional activity and function of MYCN in neuroblastoma. **Poon E**, *et al*. Manuscript in preparation.

7. Overview by Team Leader of achievements during grant period

Evon's contribution to the laboratory has been significant over the last three years. Not only has her work been published in high-impact journals but, more importantly, her findings are very likely to lead to advances using targeted, combinatorial approaches in the treatment of high-risk neuroblastoma and medulloblastoma. She has been a hard-working, conscientious and collegiate member of the team, always ready to give advice and training where required.

8. Overview by Department Head of achievements during grant period

Dr Poon's post-doctoral position funded by Christopher's Smile has been very successful. She has consistently performed at the highest level and this is reflected in her publication output. The kind of high-quality work she is conducting in the Paediatric Solid Tumours Biology and Therapeutics team is competitive at the international level. This work is an asset to the ICR, and is unique in its scale and novelty in the UK. Most importantly it is identifying novel therapeutics for children's cancer that are being deployed to clinical trials and may already be contributing to increased survival for children. This work is within the core mission of the ICR to conduct basic science research that contributes to improved outcomes for children with cancer. We very much appreciate the support that Christopher's Smile has given to this laboratory and to this area of research.