Title: Robotic screening for novel therapies for diffuse pontine gliomas

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Lay Summary
Diffuse pontine gliomas are the most devastating of all brain tumours. They mostly affect young children and, as there are no effective treatments, almost all will die of their tumour within 12 months. In this project we will collect tumours from patients, grow the cells in the laboratory, and then use robotic technology to rapidly screen thousands of molecules to determine which ones are able to kill these tumour cells. Ultimately we aim to discover innovative new treatments for this destructive and intractable cancer.

Background
New treatment strategies for malignant gliomas are urgently needed, given the marginal increases in patient survival seen over the past two decades. Malignant gliomas rank among the most deadly of all cancers, and whilst initial treatment with surgery, radiotherapy and chemotherapy often produces some palliation of symptoms, the highest grade tumours almost universally recur with unrelenting progression to death.[1] One of the most aggressive types of these tumours is the diffuse intrinsic pontine glioma (DIPG). Due to their location within the brainstem they are not amenable to surgical resection, chemotherapy offers no benefit, and radiotherapy only has palliative value. They strike the youngest of patients, including children and young adults and almost all die within 1 year of diagnosis.[2] Even novel agents such as temozolomide, VEGF inhibitors and EGFR inhibitors have shown no therapeutic benefit. Clearly new and innovative strategies are urgently needed to counter these devastating gliomas.

A principle problem with improving treatment outcomes for DIPG has been the scarcity of biological material through which the underlying oncogenic pathways can be identified. Due to their sensitive location within the brainstem, and due to their inherently poor prognosis these malignant gliomas are rarely biopsied. Attention has therefore recently been placed on obtaining tumour tissue from autopsy specimens in order to improve our understanding of the underlying biology, and ultimately to facilitate the development of novel therapeutics. Recently published studies have shown that the collection of autopsy samples is feasible, acceptable to parents, and yields high quality specimens that can be adequately processed for genome wide analysis at both the RNA and DNA levels.[3] Monje and Wong *et al* (Stanford) have also shown that viable stem cells can be grown from these autopsy specimens and cell lines subsequently cultured.[4] While these studies, currently being pursued internationally, will enhance our understanding of the biology of these tumours, the reality is that given the infancy of this field of research it may be decades before this improved biological knowledge will ultimately be translated to improved therapeutic strategies. Also of concern is that no such studies are currently being performed within Australia.

Objectives
We therefore seek to develop a novel and innovative approach that capitalizes on the benchmark provided by the recent autopsy studies, as well as on our own research strengths, to rapidly develop novel therapeutic strategies for these devastating tumours. We propose to leverage off our expertise in glioma research and high-throughput molecular screening to develop a high-throughput screening program for DIPG. The program will utilize our national and international collaborations with leading researchers in the field, as detailed below, to enhance its chance of success. Our overall objective is to develop new treatment strategies for DIPG through the identification novel small molecules that specifically inhibit DIPG propagation. Our primary hypotheses are that an effective screening readout can be developed for DIPG, and that this readout can be used in a high-throughput screen to discover innovative new therapeutic agents. Our primary aims are to collect autopsy DIPG specimens within

Australia, to culture DIPG cells *in vivo*, to develop an effective readout of cellular viability, and to perform an initial screen of 10,000 small molecules to establish the feasibility of high-throughput screening, and to ultimately identify new therapies for this intractable disease.

In our first specific aim we will propagate DIPG cells and assess their viability for use in a high throughput screen. We will initially collect autopsy DIPG specimens in collaboration with leading paediatric oncology centres within Australia who have agreed in principle to participate in the study. Consenting patients who die within close proximity to an affiliated pathology service will undergo brain-only autopsy as close as possible to time of death. Harvests will be performed up to 24 hours after the time of death, given the success in culturing DIPG cells demonstrated by other investigators up to this time point (personal communication, M. Monje). Samples of both tumour and normal brain will be collected. We will aim to collect tumour specimens from at least 5 individual patients. Tumour samples will be used fresh when collected locally or stored at room temperature and transported in Hybernate (-A) media (Invitrogen) with Antibiotic / antimycotic agent added. Propagation of cells will be undertaken using the expertise aquired in our laboratory with other glioma specimens, [5] modified according to the protocol of our international collaborators who have successfully demonstrated the feasibility of this approach in DIPG specimens.[4] In brief, cells will be washed, acutely dissociated in oxygenated artificial cerebrospinal fluid (CSF) and subject to enzymatic dissociation.[6] Thawed or fresh tumour cells will then briefly be placed in tumour sphere media (TSM) to allow for recovery following enzymatic dissociation. To maximise the likelihood of achieving viable cells, two differing methods will be employed simultaneously following dissociation. Cells will therefore be split, with half plated to assess viability for direct neurosphere formation, and half preserved for in vivo propagation. Plated cells will be suspended in tumour stem cell media with growth factors (EGF, FGF, and PDGF) with media replaced every 2-4 days as reported.[4] In vivo propagation will be simultaneously be achieved by serially passaging cells via stereotactically placed orthotopic xenografts in NOD-SCID mice, as we have previously described.[5]

Following the development of signs of tumour growth, mice will be sacrificed and tumour harvested and dissociated as above. The viability of passaged cells will be assessed utilising a colorimetric MTS assay.[5] MTS assays will be performed with CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corp, Madison, WI). Briefly, 1×10^3 cells will be plated in 100 microliters of medium in 96 well microtiter plates and incubated for 24 hours. Cellular proliferation will be measured at 0,1,3,5 and 7 days by adding 20 microliters of labeling reagent to each well and incubated at 37° for 2 hours. The absorbance will be read at 490 nm with a 96-well plate reader, with absorbance established as directly correlated with final cell number. Based on prior experience with serially passaged glioma cells, it is estimated that approximately 1 in 5 tumours will yield viable cells following serial passaging.

In the second specific aim, an automatic read-out will be established and a 10,000 high-throughput small molecule screen will be performed to identify novel anti-tumour agents. The initial screen will be performed utilizing a DIPG neurosphere culture already successfully propagated by our collaborators at Stanford (Monje et al), which they have generously agreed to share with our institute. First, the optimal conditions for developing an automated cell-based automatic readout will be established. Viable cells will be grown as described above, and then seeded at differing cellular densities in both 96-well and 384-well plate formats and proliferation assessed with varying concentrations of the CellTiter MTS labeling reagent with absorbance read at 490 nm on 96- and 284-well plate readers. Measurements will be taken to determine optimum read-out conditions at 24, 48, 72 and 96 hours of incubation. The same conditions will be used to test a culture of human neural stem cells, derived from embryonic cell lines, supplied by our collaborator at the Telethon Institute (WA, Australia), Dr N Gottardo. These cells will be used as a control for the molecular screen in order to facilitate the identification of active agents that are non-specific and therefore potentially toxic. High concentrations of the cytotoxic agent doxorubicin will be used as the positive control, as this drug has been previously shown to inhibit glioma proliferation and induce apoptosis (although has no clinical role due to poor penetration of the blood brain barrier).[7] The MTS assay will be employed and absorbance read on a plate reader will be used as a surrogate for final cell number and as an indicator of successful inhibition of cellular proliferation and / or induction of cell death.

Once this read-out system has been optimized we will proceed to confirm the read-out in other cell lines harvested from autopsy specimens and perform the initial high-throughput screen. Viable cells harvested after propagation in xenograft will be grown in the same multi-well format and read-out

conditions assessed with CellTiter MTS labeling reagent to determine the ability of the read-out to apply across multiple cell lines. We will then proceed to the high-throughput screen. To facilitate this we will utilize the newly established Australian Cancer Research Foundation (ACRF) Drug Discovery Centre for Childhood Cancer at our institute. The Centre has state-of-the-art robotic equipment (including Hamilton MicroLab STAR and Nimbus automation liquid handling systems, Molecular Devices SpectraMax plate readers, Biotek ELx405 plate washers, and Thermo Multidrop 384 and Multidrop Combi liquid dispensers), advanced data management systems (ActivityBase), a 160,000 chemical compound library and local and international collaborators with expertise in high-throughput screening to ensure optimal outcomes. For this proposed pilot study an initial robotic screen of 10,000 small molecules will be performed using final cell number assessed by MTS as the measure of drug activity. Screens will be performed on the cell line provided by Monje, on primary cells propagated from one additional autopsy specimen, and on a control neurosphere culture. Those small molecules identified as "hits" in the primary screen will be independently re-tested in triplicate at a single concentration to eliminate false positives. Molecules that inhibit both DIPG and normal neural stem cells will be discounted due to non-specific toxicity. Additional controls will be provided by other cell lines run through the same 10,000 small molecule screen in parallel for other experimental programs. Specifically, we are currently running a high-throughput screen for MLL rearranged leukaemia, and for MRP inhibitors in neuroblastoma. Any hits that are positive in all the differing cancer cell lines will be discounted due to non-specific toxicity and the likelihood of a low therapeutic ratio. The "hits" displaying reproducible inhibitory activity in DIPG cells only will be tested in a full dose response titration using the MTS assay in 96-well plate format with varying concentrations of the putative inhibitory agent. Based on prior screens we anticipate that approximately 10 - 20 small molecules may be identified for further testing.

Feasibility

The feasibility of autopsy specimen collection and processing has already been established in overseas institutes[3] and feasibility of collection within Australia has also been recently established with several patients having had autopsy specimens collected and sent overseas for laboratory research at parent request (personal communication N. Gottardo). The CI is a young investigator whose doctoral research was conducted as a Fulbright scholar at the Dana Farber Cancer Institute and which focused on the development of novel therapeutic strategies for malignant gliomas. His doctoral degree was awarded in 2009 and the work published in both the Journal of Clinical Investigation[5] (Impact Factor 17) and the Journal of Clinical Oncology[1] (Impact Factor 16), in both cases with the CI as first author. The feasibility of growing autopsy DIPG cells in culture has been established by others and the experience of the PI in the *in vivo* propagation of glioma neurospheres from primary patient samples will facilitate the translation of this model to DIPG cellular propagation. Our collaboration with the Stanford group will facilitate the rapid implementation of the project due to the ability to use cells already successfully propagated in culture. The CI's laboratory work is incorporated in the experimental therapeutics program of Prof Michelle Haber, director of the Children's Cancer Institute, Australia (CCIA). The CCIA represents the only research institute in Australia devoted solely to research into childhood cancer. Prof Haber is a leading international researcher in childhood malignancies with a track record of high-impact publications including recent papers in NEJM, JCO, Blood, JNCI and Nature Medicine. The housing of the new ACRF Drug Discovery Centre in the Institute, the expertise established with prior and current screening programs and the established collaborations with international experts in high-throughput screening (eg Andrei Gutkov, Roswell Park Cancer Institute) will provide the ideal environment for undertaking this exciting and innovative strategy. Prof Murray Norris at the CCIA has extensive experience in small molecule screening, and his expertise in the subsequent refining and authentication of lead compounds will ensure effective potential for translation. Our collaboration with Oren Becher at Duke (as described below) will facilitate the further extension of this research into the in vivo environment, and ultimately direct translation to the bedside.

Budget

A total budget of $100,000 is requested for the experiments described in this project. $15000 is requested towards the costs of collecting, shipping and storing DIPG post mortem specimens, including the cost of media, the cost of shipping companies and pathology costs for examining specimens. $35,000 is requested towards the costs of the *in vitro* and *in vivo* experiments required to propagate the DIPG cells, including the costs of consumables and mice as detailed below, and towards the costs of a 0.5 FTE research assistant for 6 months. A further $25,000 is requested to cover the cost of the experiments to optimise the assays to be used in, and the performance of, the

high-throughput screen (for consumables and towards the costs of a 0.4 FTE for a research assistant for 6 months) and a further $30,000 to cover the cost of the small molecules included in the high-throughput screen.

The consumables are those specifically required for this project, and funds requested are based on current experience in the applicant's laboratory. A major cost for the project maintaining is propagating primary patient tumour samples in culture, and developing the read-out assays for the throughput screen. The major components contributing to this expense include 96 well plates at $3.05 per plate, and Cell Titre 96 at $1000 per 100mL, plus the high cost of foetal calf serum, growth factors, tumor stem cell media, and Hibernate media. The *in vivo* experiments to propagate the cultures will require up to 12 mice, and based on prior studies, the cost for animals, housing and drug treatment is approximately $5000. Following the successful propagation of tumour cells, the cost of the high-throughput screen is $1 per small molecule screened, thus for a pilot screen of 10,000 molecules $10,000 is requested for the control and $10,000 for each of the two experimental arms.

Future Directions

Following the successful completion of this initial screen further funding will be sought to build on positive results. The success of this project will provide a platform for more comprehensive 160,000 drug screens to identify further potential therapeutics. Lead compounds will be able to be developed and characterized and tested extensively *in vitro* and *in vivo* for activity, toxicities and therapeutic ratios. Our collaboration with Oren Becher at Duke will provide us with access to his *in vivo* DIPG model to facilitate the further *in vivo* testing of target compounds, both for toxicity and for anti-tumour effect. Ultimately, this novel approach may lead to the rapid development of novel therapeutic strategies for a tumour that till now has proven devastating to patients and their families, with no known effective treatments.

Associate Investigators

Prof Michelle Haber is director of the CCIA, has an extensive track record in childhood cancer and runs the experimental therapeutics program at CCIA. She will mentor the CI in completing project from beginning to end.

Prof Murray Norris is deputy director of the CCIA and internationally renowned for his work on high-throughput screening and molecule diagnostics in childhood neuroblastoma and ALL. He will provide assistance in running the small molecule screen and in lead compound verification

Dr Michelle Monje (Stanford) has demonstrated the first successful *in vitro* neurosphere culture of DIPG cells. She will supply cells for culture and provide expert guidance in the propagation of autopsy specimens

Prof Andrei Gutcov (Roswell Park Cancer Institute) is a world expert in high-throughput screening and has collaborated extensively on previous projects with both screening and hit verification, toxicity testing and compound optimization.

Dr Oren Becher (Duke) has agreed to provide his DIPG *in vivo* model for further testing of identified hits. He will also be running a separate high throughput screen using cells obtained from his in vivo model. This will provide a unique opportunity to compare screens obtained from two different biological sources.

References

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