HUNTER MEDICAL RESEARCH INSTITUTE

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COMBATT DMG: COMBINED ANTI-TUMOR TARGETING OF DIFFUSE MIDLINE GLIOMAS

Informational
DIPG, Childhood (Brain Cancer)
Affiliation: The Cure Starts Now
Requested Funding: $145,640

EXECUTIVE SUMMARY

1. **Scientific Merit**

Strategic advantage: **There is a clear imperative to develop better treatment options for children with DMG;** a goal that constitutes a major focus of PI Dun's and PI Cain's laboratories. Our unique approach to this problem centers on the application of high-resolution quantitative proteomic and gene knockout technologies, to dissect the signaling pathways dysregulated in DMG, as modeled in patient-derived cell lines obtained locally and from our international collaborators (Monje, Nazarian, Mueller, Hulleman, and Carcaboso). Together we have 26 different DMG cell lines under active investigation. This collaborative research paradigm centers around global identification of key pathways driving epigenetic dysregulation and the subsequent impact on signalling axes that sustain mitogenesis and cell survival. This work now must *be the focus of DMG research* if we are to indeed improve patient outcomes.[1]

Hypothesis: Based on our preliminary results, we hypothesize that simultaneous targeting of cell migration/dissemination (PKC-MARCKS) in combination with proliferation/metabolism (PI3K-Akt) will inhibit the diffuse and intrinsic neoplastic sequela of diffuse midline glioma.

Clinical Significance: Pediatric high-grade gliomas of the brainstem – diffuse midline gliomas (DMG), represent the most aggressive primary tumor of the central nervous system (CNS); responsible for half of all brain cancer deaths in children.[2] The brainstem controls breathing and heart rate, as well as the nerves and muscles required for movement, speech and swallowing. Tumor location precludes resection, with pharmacological and immunological treatment approaches currently failing to increase survival. Radiotherapy provides temporary benefit and is considered palliative. DMG primarily affects children and young adolescents with a median age at diagnosis of 7 years - median overall survival (OS) is just 10 months post diagnosis.[2] In Australia and the United States, approximately 100 and 1,200 children under the age of 15 die from cancer every year, respectively – **20-25% of these children are patients with DMG.**[3, 4] A significant barrier facing clinicians (and those diagnosed with DMG) is the limited biological understanding of the disease; stereotactic biopsy at diagnosis has only recently been deemed safe (reviewed in [5]), but is beginning to provide insight into the drivers of disease at a posttranscriptional level.

Clinical opportunity: Mutations and gene amplification that activate the PI3K/Akt/mTOR signalling axis are frequent, and are thought to drive growth and proliferation of DMG.[1] Unfortunately, PI3K inhibitors have previously failed brain cancer patients due to their inability to cross the blood-brain barrier (BBB) and because of our limited understanding of mechanisms driving resistance.[6] The PI3K inhibitor '**paxalisib**' (previously GDC-0084) was developed to cross the BBB[7] with efficacy reported in clinical trials for glioblastoma.[8] Our laboratory was the first to test its efficacy specifically in DMG, in both *in vitro* and *in vivo* models (**Fig. 1a,b**), and to formally assess BBB penetration *in vitro* (**Fig. 2**). The combined clonality[9] and number of driver mutations seen at DMG diagnosis,[10] ensures that no monotherapeutic approach will provide long term durable response, or be able to resolve DMG, at disease progression. In recognition of this, and **for the first time**, we have **subjected DMG cells to quantitative phosphoproteomic profiling +/- paxalisib** to map the signalling pathways controlling the growth, survival and development of treatment resistance (**Fig. 1c**). Our study aimed to identify which of the spectrum of pathways (that could be simultaneously targeted at diagnosis and/or as the disease progresses and/or as resistance develops) become overactivated in patients following treatment. We have identified that inhibition of the PI3K/Akt/mTOR signalling axis **using paxalisib** and the commonly used mTOR inhibitor, rapamycin, drove **potent activation of Protein kinase C (PKC)** signaling to **promote tumor invasion and migration regulated by Myristoylated alanine-rich C-kinase substrate (MARCKS)** (**Figs. 1c, 2a-c**)**.** This provides sound rationale as to why targeting PI3K/Akt/mTOR has failed patients across various cancer settings. Biochemical investigation using 13 DMG cell lines revealed the MARCKS proteins to be highly phosphorylated in untreated cells, with levels significantly higher in tumors harbouring the more aggressive H3.3K27M mutation, compared to wt-H3 and H3.1K27M, providing clues as to why overall survival of these patients is 40% shorter.[2] Furthermore, we see a significant correlation between MARCKS activity and the expression of N-Cadherin (**Fig. 3**). *Thus, we hypothesize that determining the mechanistic bases for the increased PKC activity in DMG cells will provide important information for future simultaneous targeting to improve outcomes.*

Goals: Gliomas originating in the pontine region of the brainstem disseminate along the neuraxis into other midline structures such as the thalamus, through white matter tracts into the cerebellum and leptomeningeal, and are found in

supratentorial extensions.[11] This extreme level of cell motility is a hallmark of the disease, and plays a major role in the failure of standard of care radiation therapy; however may provide us with a novel treatment target. This project aims to build on our pilot data that shows an important role for the MARCKS protein, one of the primary downstream phospho-targets of PKC.[12] Herein, we report that PKC activated in response to potent PI3K inhibition, drives MARCKS phosphorylation and N-cadherin activity and may influence dynamic remodeling of the actin cytoskeleton. As MARCKS is expressed at its highest levels in the brain during embryonic development,[13] we aim to determine its role in the gliomagenesis of DMG using molecular and pharmacological inhibition, cell biology and sophisticated proteomics techniques. Additionally, PI3K activity is linked to insulin signaling; reduced therapeutic benefitis seen *in vivo* using PI3K mutant xenografts when stimulated with insulin.[14] PKC signaling is activated in response to hyperglycemia-induced accumulation of diacylglycerol (DAG) or calcium ions (Ca^{2+})[15] with both of these second messengers produced by components of the PI3K/Akt signaling axis. Therefore, we aim to better characterize the emerging link between PI3K and PKC signaling, to increase the likelihood of treatment success using strategies targeting multiple components of these oncopathways.

2. **Feasibly**

Design and methods of proposed study: The preliminary data that underpins this application, shows potent activation of Protein kinase C – driving MARCKS phosphorylation in response to inhibition of PI3K/Akt signalling using paxalisib (in trials for DMG NCT03696355) and mTOR inhibition using rapamycin (sirolimus). This was determined by quantitative high-resolution phosphoproteomic profiling in PI Dun's laboratory.[16-20] Unexpectedly, 11/13 untreated DMG cell lines harbored high-level MARCKS activity; which corresponded to levels of N-Cadherin expression (**Fig. 3**). These data highlight the necessity to perform rigorous mechanistic studies to determine whether targeting PKC/MARCKS offers clinical relevance. In the first year of the project we will (***Aim 1)*** elucidate the molecular mechanisms underpinning PKC/MARCKS activity in response to PI3K inhibition. To determine the role PKC/MARCKS plays in DMG survival, we will subject 3 x DMG cell lines to molecular studies where we will knockdown and knockout expression (established in PI Dun's[20-22] and PI Cain's laboratory[23]). Further, we will assess growth, proliferation and migration following therapeutic activation of

PKC/MARCKS using known activating compounds (Phorbol 12-myristate 13-acetate (PMA), insulin, PI3K/Akt/mTOR inhibitors), alongside pharmacological inhibition of PI3K/Akt (paxalisib) and PKC (enzastaurin, midostaurin). Cultures showing altered growth, proliferation, migration will then be investigated by quantitative proteomics, to map the communication networks and structural elements that underpin the diffuse and intrinsic growth pattern of DMG. As insulin drives resistance to PI3K inhibitors, and second messengers that activate PKC are produced during insulin signaling, we will investigate the potential of anti-glycemic strategies alone, and in combination, with PI3K and PKC inhibition (**0-12 months**). In the second year of the project (***Aim 2***) we will investigate whether molecular or pharmacologic inhibition of PKC/MARCKS increases therapeutic response to: PI3K inhibition (paxalisib) and anti-glycemic approaches. Hyperglycemia drives both PI3K[14] and PKC[15] activity in a range of tumors, we will investigate whether limiting blood sugars synergizes with PI3K and PKC inhibitors *in vivo* (**12-24 months**).

3. **Expertise**

Research Team: Lead investigator PI Dun is a National Health and Medical Research Council (NHMRC) Investigator and Defeat DIPG Chadtough New Investigator. Trained by field-leading experts in phosphoproteomics and animal modeled cancer signaling (Prof Larsen SDU (2013-2014), Prof Cools KU Leuven (2014,2015)), and backed by continuous Cancer Institute NSW Early Career Fellowships (2014/16, 2017/19), Dr Dun established and leads the Cancer Signaling Research Group (CSRG), Hunter Medical Research Institute (HMRI) at the University of Newcastle (UON). Specializing in proteomic technologies and cancer signaling, PI Dun delved headlong into DMG research in 2018 when his daughter, Josephine, was diagnosed with DMG at two years of age. Realizing there was a distinct lack of knowledge of the post-translational landscape, and hence limited understanding into mitogenic processes, he commenced his own program of DMG research.

PI Cain is the Head of the Developmental and Cancer Biology research group in Hudson Institute's Centre for Cancer Research and a Chief Investigator for the Hudson Monash Paediatric Precision Medicine Program. PI Cain completed his PhD studies at Monash University in 2006, and moved to The Hospital for Sick Children in Toronto, Canada to complete postdoctoral training with Dr Norman Rosenblum in the Program of Developmental and Stem Cell Biology. Here, he

focused on the role of the Hedgehog signaling pathway in renal development and disease, developing specialized skills in developmental biology, mouse models of human disease, and congenital and paediatric disease. On his return to Australia in late 2010, he joined Hudson Institute of Medical Research has established a DMG CRISPR screening laboratory to assess the roles genes play in the neoplastic growth of DMG cells.

PI Duchatel is an early career postdoctoral researcher in the Cancer Signaling Research Group (led by PI Dun)[1, 20] holding a PhD in Neurobiology from the University of Newcastle. PI Duchatel has extensive expertise in brain pathologies and histological techniques, with established animal handling experience. Since joining PI Dun's laboratory PI Duchatel has received 3 platform presentation awards for his work in DMG, including the preliminary data that underpins this applications. PI Duchatel provides the hands-on capabilities of these studies and was trained by Prof Monje (Stanford University), Prof Ziegler (UNSW) and Dr Jason Cain (Hudson Institute). Dr Duchatel will lead *in vitro* and *in vivo* components of the study across all aims. PI Cain provided initial training to PI Duchatel when he and PI Dun established their DMG research program at UON, HMRI 2018.[1] In doing so, PI Dun was able guide daughter Josie's treatment when initial therapies targeting the genomic features (PRISM) of her tumor failed (relapse following PRISM therapies 37 weeks post diagnosis). This approach saw Josie survive 96 weeks – *more than double the median survival expectation*. PI Dun's lived experience provides unrivalled perspective to the importance of such research and will drive the project and team accordingly.

Capacity of Team: The powerful combination of specialist techniques and technologies, established cancer and emerging DMG expertise, and the unique access to novel chemicals assembled under the auspices of this proposal means that our goals are feasible, and their realization will provide mechanistic information that will impact on the design of future combined treatment strategies. This project will allow mentorship of the next generation of DMG researchers and thus generate a legacy for this program in terms of the human capital needed by the DMG field. Promotion of research findings to the media and general population will be actively undertaken via established and strong links with mainstream online and radio networks, as evidenced by frequent articles and interviews.