Specific Aims

Neuroblastoma is the most common extracranial solid tumor of childhood, accounting for 15% of childhood cancer-related deaths [1]. Survival for patients with high-risk neuroblastoma (HR NB) is 50%, and patients who relapse present with chemotherapy resistant disease with little chance for cure, underscoring the need for innovative therapies that target resistance mechanisms [2].

Chemotherapy and radiation kill tumor cells by activating mitochondrial apoptosis [3]. Evasion of mitochondrial apoptosis is a mechanism of therapy resistance in HR NB [4]. The Goldsmith lab has shown that human derived NB cell lines depend on mitochondrial resident anti-apoptotic proteins Bcl-2 or Mcl-1 for survival [5]. They found that Mcl1 or Bcl2 dependence is defined by which pro-survival protein is binding to and sequestering the pro-death BH3 protein, Bim. They also showed that the select Bcl-2 inhibitor Venetoclax (ABT-199) can disrupt Bim:Bcl2 interactions, cause HR NB cell line apoptosis *in vitro,* and induce complete tumor regression of aggressive NB xenografts when combined with chemotherapy, leading to a first in pediatric phase 1 trial (clinicaltrials.gov, NCT03236857) [6].

Primary HR NB tumors are very heterogeneous and therefore homogeneous cell lines grown in culture may not accurately represent the entirety of the bulk tumor from whence it came. We therefore sought to utilize a more sophisticated model, patient derived xenografts (PDXs), to evaluate the true susceptibility of NBs to Bcl-2 family targeting therapies. I have so far characterized the anti-apoptotic dependencies of 16 CHOA HR NB PDXs, using co-immunoprecipitation of Bcl-2 pro-survival members, to determine Bim:Bcl2 family interactions. Resultant data suggest that PDXs derived from newly diagnosed post-induction therapy tumors or relapsed (post-chemotherapy) NBs had different Bim protein-protein interactions than homogeneous derived cell line results. We find that in PDXs, Bim interacts with multiple pro-survival members, such that Bim was found bound to Bcl-2, Bcl-xL, and Mcl-1 in the same tumor. This is significant as prior studies of NB cell lines grown in culture have not identified Bcl-xL dependency and multi-protein dependencies rarely occurred. Our overall goal is to seek a mechanistic understanding of neuroblastoma survival dependencies in a model that more faithfully mimics the human condition in order to leverage Bcl2-family inhibitors for clinical use. *We hypothesize that HR NBs harboring multiple Bim and Bcl-2 pro-survival protein interactions contribute to chemotherapy and Bcl-2 inhibitor resistance in HRNB and that disrupting the multiple Bim interactions through targeted inhibition will lead to cancer cell apoptosis and increased sensitivity to therapy.* To that end, we propose the following aims:

Aim 1: Dissect the essential BCL-2 family protein interactions in the heterotypic tumor environment of patient derived xenografts compared to cell lines. We will perform co-IP to determine Bim PPI patterns, and compare Bcl-2 dependency results to genomic sequencing results to define patterns of apoptosis resistance based on/driven by genomic characteristics.

Aim 2: Determine the "functional anti-apoptotic" dependence of tumors bearing multiple Bim-protein-protein interactions (Bim PPI). We will utilize targeted inhibitors of Bcl2, Bcl2/Bcl-xL, and Mcl1 in different combinations using a novel tumor slice culture apoptosis assay to determine the true antiapoptotic members driving survival in HR NBs with multiple Bim PPI.

Understanding the mechanisms of chemotherapy resistance in HRNB is critical for developing effective new therapy combinations that can improve survival rates and reduce the negative side effects of current therapies.

Research strategy

Patients with HR NB undergo extensive therapy with chemotherapy and radiation which causes significant physical and emotional challenges for children and their families [2]. Patients with relapsed disease have poor survival due to chemoresistance [7]. Chemotherapy and radiation activate a pro-apoptotic subset of Bcl-2 proteins, called BH3 proteins (Bid, Bim), that translocate to the mitochondria to bind to pro-apoptotic Bax and Bak to induce their oligomerization, leading to mitochondrial release of cytochrome c and irreversibly committing the tumor cell to die. Pro-survival Bcl-2 members (Bcl-2, Bcl-xL, BcL-W, Mcl-1) can sequester pro-death BH3 proteins to prevent them binding to Bax/Bak binding to inhibit apoptosis. This study aims to understand this mechanism of chemoresistance to improve therapy and lessen the suffering of children.

Specific Aim 1: Dissect the essential BCL-2 family protein interactions in the heterotypic tumor environment of PDXs compared to cell lines. *We hypothesize that Bcl-2 family protein interactions in PDXs will more closely recapitulate that of the heterotypic primary tumor compared to homogenous tumor derived cell lines.* The Goldsmith lab has shown that NB cell lines depend on Bcl-2 or Mcl-1 sequestering and de-activating Bim for survival [5, 6]. Preliminary data generated during my first rotation suggest that different PDX tumors display Bim binding patterns unique from cell lines with Bim often bound to multiple Bcl-2 family members within a single PDX (Figure 1). Furthermore, Bim protein interactions in PDX are identical to that seen in the primary tumor tissue compared to a cell line derived from the PDX that failed to show the Bim protein protein interactions (Bim PPI). We therefore seek to answer the question of what contributes to this diverse Bim-PPI phenotype. We have so far found a potential correlation between chemotherapy treatment and PDX's bearing multiple Bim-PPI.

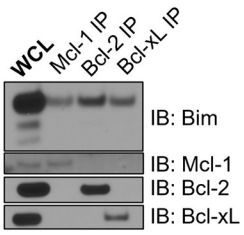

Figure 1. Co-IP of Bcl-2 family survival proteins from a relapsed neuroblastoma PDX. IB;immunoblot, WCL;whole cell lysate

One possibility is that selective pressure from chemotherapy could promote survival of a clonal population that sequesters Bim with multiple Bcl-2 family members on a single cell. Alternatively, there may be subpopulations of cells within the same tumor that have Bim sequestered by different family members. In either case, this implicates likely tumor resistance to monotherapy with a Bcl-2 anti-apoptotic inhibitor. To address this question, we will utilize a cell line derived from one of the multiple Bim-PPI PDX that maintained the same multiple Bim-PPI profile at early passage. We will isolate, grow up and characterize single cell clones from that cell line and determine their Bim dependency through co-immunoprecipitation (Co-IP). To begin to understand the mechanism of this multi-dependent phenotype, we have submitted samples of all Aflac NB PDXs for whole exome sequencing. Genetic profiles from single and multi-dependent PDX will be compared to explore the potential that a genetic mutation may influence Bim binding patterns to multiple partners. For example, dysregulation of the MEK/ERK pathway via EGFR inhibition switches Bim PPI from Mcl-1 to Bcl-2 [8], and mutations or loss of function in genes in this pathway may mediate Bim's preferred binding partner through post-translational modification of Bim.

Expected results, Potential Limitations and Alternative Strategies. Results will determine whether there are multiple Bim interactions occurring within the same NB cell or if there is sub-clonal variation of Bim PPI's within a tumor and whether common genomic alterations in PDX's correlate to unique Bim PPI phenotypes. This information will help determine potential targetable

drivers of chemoresistance in NB. Clonal cell lines will be verified with flow cytometry for NB cell surface markers CD56, CD81, and GD2. Quality control and statistical analysis for whole exome sequencing of PDXs will be performed by Novogene.

Specific Aim 2: Determine the "functional anti-apoptotic" dependence of tumors bearing multiple Bim-protein-protein interactions. *Hypothesis: Mcl-1 and Bcl-xL mediate resistance to apoptosis in chemotherapy treated NBs. Combination inhibition of Bcl-2, Mcl-1, and Bcl-xL will displace Bim and promote apoptosis in multi-dependent relapsed PDX.* We plan to utilize a novel slice culture system to test relapsed tumors with multiple Bim-PPI. This system preserves the intratumor heterogeneity and allows for scalable testing of a range of drugs and concentrations per tumor. My pilot data demonstrated that PDX tumors that are primarily dependent on Bim:Bcl-2 are susceptible to ABT-199 (Bcl-2 inhibitor) by a dose-dependent increase in apoptosis using our novel tumor slice culture system (figure 2). Slices of multi-dependent PDX tumors will be exposed to combinations of Bcl-2 specific inhibitor ABT-199 and Mcl-1 inhibitor S63845 (Diagram 1). We will also test a Bcl-xL proteolysis targeting chimera (protac) DT2216. Inhibition of Bcl-xL causes destruction of platelets in the clinic [9]. This protac specifically targets Bcl-xL to the Von Hippel-Lindau (VHL) E3 ubiquitin ligase for degradation. DT2216 does not have the same platelet destructive activity of Bcl-xL small molecule inhibitors because platelets have low VHL expression [10]. I verified that our PDX express VHL suggesting this protac may be effective in neuroblastoma. PDX with low VHL expression will be used as a negative control. Tumor slices will be assessed for apoptosis as evidenced by PARP cleavage by western blot and Cleaved Caspase 3 activation through immunofluorescence. The contribution of Bcl-xL to Bim protein-protein interactions will be assessed by Co-IP of Bim:Bcl-xL. **Statistical analysis.** Drug vs vehicle response will be determined by standard error of multiple technical replicates using an unpaired Student *t* test, with $P < 0.05$ considered statistically significant.

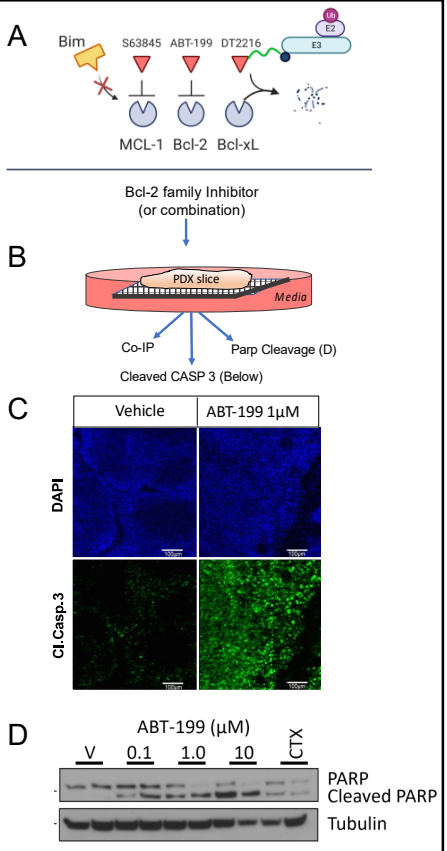

Figure 2. A. Bcl-2 family inhibitors and mechanisms. **B.** Diagram of PDX slice culture plan. **C.** Fluorescence detection of cleaved caspase 3 as indicator of NB PDX apoptosis following 24 hr culture with ABT-199. **D.**PARP cleavage as second quantifiable mode of apoptosis detection post ABT-199 in slice culture.

Expected results, Potential Limitations and Alternative Strategies. Results will show that multi-dependent neuroblastoma PDX can be forced to undergo apoptosis with combination targeted therapy and show whether secondary Bim-PPI are involved in chemoresistance to single agent therapy. PDX tumors grown in immunocompromised mice require 2-3 weeks to reach sufficient size. To eliminate downtime, Goldsmith lab members will assist by injecting mice ahead of time and in a timeframe such that full tumors are available for immediate experimentation during my research rotation.

References

1. Park, J.R., A. Eggert, and H. Caron, *Neuroblastoma: biology, prognosis, and treatment.* Hematol Oncol Clin North Am, 2010. **24**(1): p. 65-86.
2. Pinto, N.R., et al., *Advances in Risk Classification and Treatment Strategies for Neuroblastoma.* J Clin Oncol, 2015. **33**(27): p. 3008-17.
3. Sarosiek, K.A., T. Ni Chonghaile, and A. Letai, *Mitochondria: gatekeepers of response to chemotherapy.* Trends Cell Biol, 2013. **23**(12): p. 612-9.
4. Wong, R.S., *Apoptosis in cancer: from pathogenesis to treatment.* J Exp Clin Cancer Res, 2011. **30**(1): p. 87.
5. Goldsmith, K.C., et al., *Mitochondrial Bcl-2 family dynamics define therapy response and resistance in neuroblastoma.* Cancer Res, 2012. **72**(10): p. 2565-77.
6. Tanos, R., et al., *Select Bcl-2 antagonism restores chemotherapy sensitivity in high-risk neuroblastoma.* BMC Cancer, 2016. **16**: p. 97.
7. Goldsmith, K.C. and M.D. Hogarty, *Targeting programmed cell death pathways with experimental therapeutics: opportunities in high-risk neuroblastoma.* Cancer Lett, 2005. **228**(1-2): p. 133-41.
8. Nalluri, S., et al., *EGFR signaling defines Mcl(-)1 survival dependency in neuroblastoma.* Cancer Biol Ther, 2015. **16**(2): p. 276-86.
9. Schoenwaelder, S.M., et al., *Bcl-xL-inhibitory BH3 mimetics can induce a transient thrombocytopathy that undermines the hemostatic function of platelets.* Blood, 2011. **118**(6): p. 1663-74.
10. Khan, S., et al., *A selective BCL-X(L) PROTAC degrader achieves safe and potent antitumor activity.* Nat Med, 2019. **25**(12): p. 1938-1947.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Douglas J Saforo

eRA COMMONS USER NAME (credential, e.g., agency login): DJSAFO

POSITION TITLE: Pediatric Resident Physician MD, PhD

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Louisville, Louisville, KY	BS	05/2013	Biology
University of Louisville School of Medicine, Louisville, KY	MD, PhD	05/2021	Medicine/Pharmacology & Toxicology
Emory University School of Medicine, Atlanta, GA		06/2024	Pediatric Residency

A. Personal Statement

My goal is to pursue a career in academic medicine and pediatric oncology with the aim of improving the lives of children and their families affected by cancer. I am deeply committed to advancing our understanding of cancer and developing novel therapies to combat this devastating disease. My research interests lie in solid tumor biology and the development of new therapeutics, and I am fortunate to have had the opportunity to work in the lab of Dr. Kelly Goldsmith, who has a successful track record of translating new therapeutics into early phase clinical trials for pediatric solid tumors like neuroblastoma.

I began pursuing this career path during my undergraduate studies at the University of Louisville, where I enrolled in science-heavy coursework and participated in the R25 Cancer Education Program. During this time, I learned basic laboratory techniques and I conducted research on a novel series of small molecule inhibitors for ovarian cancer inhibition, which resulted in my selection as one of five undergraduates nationally by Pfizer to present at the Society of Toxicology meeting.

During my medical school and PhD research, I became interested in focusing on physiologically relevant in-vitro and -in-vivo models. My graduate research focused on incorporating elements of the tumor microenvironment, specifically the stromal extracellular matrix and physiological hypoxia into a 3D cell culture system. Using this system, I was able to isolate and characterize a stem-like cell from primary lung cancer biopsies and found that this cell promoted a metastatic phenotype in lung cancer.

My current research at Emory University builds upon these experiences and focuses on identifying mechanisms of chemoresistance in high-risk neuroblastoma. Specifically, I am studying survival protein interactions in patient-derived xenografts to explore how these interactions can be disrupted to kill cancer cells. With the guidance of Dr. Goldsmith, I submitted an abstract of my preliminary work, which has been accepted for presentation at the Advances in Neuroblastoma Research conference.

I am confident that my experiences have prepared me well to perform the meaningful work proposed in this study. I am deeply passionate about contributing to the advancement of pediatric oncology research and am excited to continue making a positive impact in the field.

B. Positions, Scientific Appointments, and Honors

Positions and Employment

2012-2013 R25 Undergraduate Cancer Research Assistant, University of Louisville
2013 Medical Student Research Assistant, University of Louisville School of Medicine

2014 T35 NIDDK Medical Student Research Trainee, University of Louisville School of Medicine
2015-2019 Graduate Research Assistant, University of Louisville School of Medicine

Other experience and Professional membership

Society of Toxicology, previous member
American Society for Pharmacology and Experimental Therapeutics, previous member
American Medical Association, current member
American Academy of Pediatrics, current member

Honors

2012 Research!Louisville Undergraduate First Prize Winner
2013 Pfizer Undergraduate Student Travel award for Society of Toxicology annual meeting
2013 Best Undergraduate Thesis award finalist for the Natural Sciences Division
2013 *Summa cum laude* with B.S in Biology with Biology Honors
2016 Keystone Symposia "Future of Science Fund" Scholarship to Keystone Symposia
2016 Outstanding Graduate/Professional Student Award
2018 Research!Louisville Doctoral Basic Science Graduate Student 2nd place award
2019 AAP/ASCI/APSA Joint Meeting Travel Award
2019 School of Medicine Student Diversity Award, one of two recipients
2021 Merck Manual Award for Scholarly Research, University of Louisville School of Medicine

C. Contributions to Science

1. Undergraduate research: At the end of my junior year, I was selected to participate in the R25 Cancer Education Program. My project was to test a novel series of compounds for efficacy in ovarian cancer inhibition. These compounds were identified in-silico to target the Anaphase Promoting Complex/Cyclosome (APC/C), which targets dozens of proteins for degradation and regulates many pathways and cell cycle processes. My role was to complete *in-vitro* testing of these candidate drugs in various human carcinoma and fibroblast cell lines. We demonstrated that 3 compounds targeting the APC/C could induce mitotic arrest and apoptosis in ovarian carcinoma cell lines, but not in transformed diploid fibroblasts. This work served as the foundation for multiple regional foundation grants from the Kentucky Lung Cancer Research Program and the Kentucky Science and Engineering Foundation, and these compounds are currently being investigated as a novel class of non-tubulin based mitotic inhibitors in lung cancer. From this work I produced a poster that was awarded first prize at the University of Louisville's annual research symposium in my competition category, and I was one of 5 undergraduates selected nationally by Pfizer to present at the Society of Toxicology meeting. I went on to complete an undergraduate Honors thesis based upon that work, which enabled me to graduate *summa cum laude* from the University of Louisville with Honors in Biology.

1. **Douglas J. Saforo**, Brian C. Sils, B. Frazier Taylor, John O. Trent, and J. Christopher States. Candidate Drugs Target the APC/C to Induce Mitotic Arrest in Ovarian Cancer. Abstract for poster presentation: 2012 Research Louisville, Louisville, KY.
2. States, JC, **Saforo, DJ**, Klimchak, NA, Trent, JO. Non-tubulin mitotic disruptors induce apoptosis in cancer cells. Abstract 304. The Toxicologist CD—An official journal of the Society of Toxicology, Volume 138, Issue 1, March 2014.
3. **Saforo, Douglas John**, "Targeting the anaphase promoting complex/cyclosome to induce mitotic arrest in ovarian cancer." (2013). *College of Arts & Sciences Senior Honors Theses.* Paper 22. http://doi.org/10.18297/honors/22

2. Medical School: I joined the MD/PhD program at the University of Louisville to continue to conduct quality cancer research with a translational emphasis. Shortly after entering the program, I enlisted in training under the NIDDK medical student training grant to study chemotherapy induced kidney injury. The major dose limiting toxicity for cisplatin, a common chemotherapy used in various cancers, is kidney injury. We investigated the potential of targeting the metabolism of sphingolipids as a mechanism to protect mouse kidney cells from chemotherapeutic-induced injury. This project employed several techniques concerning lipid metabolism and isolation, molecular biology techniques like overexpression and knockdown of genes using lentiviral delivery of shRNA, qPCR, and multiple proliferation and migration assays. We found that knockdown of neutral

ceramidase, an enzyme involved in metabolism of ceramide to sphingosine and upregulated in renal tubule cells, conferred enhanced proliferative and migratory properties to mouse embryonic fibroblasts, but did not enhance migration in baby mouse kidney cells. In addition to my *in-vitro work*, I assisted in the *in-vivo* investigation of potential for drugs to protect kidneys from cisplatin-induced acute and chronic kidney injury using physiologically relevant models. This work identified Suramin as a potential kidney protective agent in acute kidney injury and we refined a model of cisplatin-induced kidney injury that recapitulated the dosing regimen received by lung cancer patients in the clinic.

1. **Douglas Saforo**, Kumaran Sundaram, Cameron Conway, Gauri Patwardhan, and Leah J. Siskind. Sphingolipids as potential targets for the treatment of acute kidney injury. Abstract for poster presentation:2015 Southeastern Medical Scientist Symposium, Vanderbilt University, Nashville, TN.
2. Dupre TV, Doll MA, Shah PP, Sharp CN, Kiefer A, Scherzer MT, Saurabh K, **Saforo D**, Siow D, Casson L, Arteel GE, Jenson AB, Megyesi J, Schnellmann RG, Beverly LJ, Siskind LJ. Suramin protects from cisplatin-induced acute kidney injury. Am J Physiol Renal Physiol. (2016).
3. Sophia M Sears, Cierra N Sharp, Austin M Krueger, Gabrielle B Oropilla, **Douglas Saforo**, Mark A Doll, Judit Megyesi, Levi J Beverly, Leah J Siskind. C57BL/6 mice require a higher dose of cisplatin to induce renal fibrosis and CCL2 correlates with cisplatin-induced kidney injury. Am J Physiol Renal Physiol. (2020)

3. Graduate research: My PhD research focused on understanding the contributions of the tumor microenvironment of non-small cell lung carcinoma on metastasis. Lung cancer is the leading cause of cancer related death in the United States and worldwide. The tumor microenvironment is essential to tumor initiation and progression. However, the role and mechanism by which the extracellular matrix alters cancer development and metastasis is unknown. We used advanced in-vitro 3D cell culture techniques and in-vivo animal models to identify novel therapeutic targets. Using a human cell derived extracellular matrix and physiological hypoxia, we rapidly isolated and cultivated a stem-like cell from primary human lung cancer biopsies. We characterized these cells and found that the physiologically similar cell culture system was critical to maintaining their stem-like phenotype *in-vitro.* Further, these cells enhanced lung cancer cell proliferation and clonogenic potential and the extracellular matrix produced by these cells activated Epithelial to Mesenchymal Transition and 3D cell invasion through the Src mediated Focal Adhesion Kinase pathway. Direct interaction with these cells promoted lung cancer metastasis in *in-vivo*. This work highlighted an important mechanism of metastasis through cell-matrix interactions in lung cancer.

1. **Douglas J Saforo,** Levi J. Beverly, and Leah J. Siskind. *Tumor microenvironment mimetic culture aids isolation, expansion, and potency of tumor stromal progenitors from primary lung cancer resections*. Abstract for poster presentation: 2018 Research Louisville, Louisville, KY.
2. Shanice V. Hudson, Hunter Miller, Grace Mahlbacher, **Douglas Saforo**, Levi J. Beverly, Gavin E. Arteel, Hermann B. Frieboes. Computational/experimental evaluation of liver metastasis post hepatic injury: interactions with macrophages and transitional ECM. Scientific Reports. (2019)
3. **Douglas J Saforo,** Linda Omer, Andrei Smolenkov, Aditya Barve, Lavona Casson, Noland Boyd, Geoffery Clark, Leah J. Siskind, and Levi J. Beverly. Primary lung cancer samples cultured under microenvironment-mimetic conditions enrich for mesenchymal stem-like cells that promote metastasis. Scientific Reports. (2019).

Spring 2021 Buchter Resident Research Award
Budget Template

Title: Targeting mechanisms of chemotherapy resistance in Neuroblastoma
PI: Douglas Saforo, MDPhD
Budget Prepared By: Douglas Saforo, MDPhD
Project Start Date: 7/1/2023
Project End Date: 6/30/2024
Length of Project (months): 12
Maximum budget $2,500

27.25%

Personnel	Role	% Effort	Cal Mo	Base Salary	Salary	Fringe	Total
Douglas Saforo	PI	0.00%	-		$ -	$ -	$ -
Kelly Goldsmith	Mentor	0.00%	-		$ -	$ -	$ -
			-		$ -	$ -	$ -
Subtotal							**$ -**

Non-Personnel			Quantity		
Laboratory Supplies					
Flow Antibodies					
CD81			1	$	176
CD56			1	$	269
GD2			1	$	133
Western Antibodies					
Mcl-1 (D35A5) Rabbit mAb			1	$	113
BIM			1	$	266
Bcl-2 (D55G8) Rabbit mAb (Human Specific)			1	$	228
IP Antibodies					
Mcl-1 IP antibody			1	$	205
BCL-xL (7B2.5) aMs			1	$	290
Inhibitors					
DT2216			1	$	198
Clinical Research Costs					
Other Expenses					
Pediatric Cores					
Flow Cytometry Core	Training	$88/hr (subsidized price)	2 hr	$	176
Flow Cytometry Core	Neuroblastoma cell surfac	$31/hr (subsidized price)	3 hr	$	93
Integrated Cellular Imagi	Confocal imaging	$31.8/hr (subsidized price)	4 hr	$	127
Integrated Cellular Imagi	Keyence Imaging	$43.5/hr (subsidized price)	5 hr	$	218
				$	**2,493**

Total Costs **$ 2,493**

Budget Justification: Budget Justification Personnel Douglas Saforo, MD, PhD, Principal Investigator Dr. Saforo is currently a Resident physician in the Department of Pediatrics at Emory University. He will be responsible for carrying out all aspects of the proposed work, including experimental design, acquisition of data, analysis and interpretation of data, writing and preparation of abstracts and manuscripts. He will also ensure this award is included in all publications, oral and poster presentations, and abstracts that result from this project. Kelly Goldsmith, MD, Mentor Kelly Goldsmith, MD, is an Associate Professor of Pediatrics at Emory University School of Medicine. Dr. Goldsmith serves as co-leader of the Discovery and Developmental Therapeutics Research Program at Winship Cancer Institute of Emory University, as director Neuroblastoma/MIBG Therapy Program and as clinical director, Aflac Precision Medicine Program at the Aflac Cancer and Blood Disorders Center of Children's Healthcare of Atlanta. Dr. Goldsmith will be responsible for providing mentorship and overseeing experimental design, data analysis, and assisting in writing abstracts and manuscripts. BUDGET JUSTIFICATION MATERIALS AND SUPPLIES Flow cytometry antibodies: $579 is requested to purchase neuroblastoma cell surface antibodies GD2, CD56, and CD81 for fluorescence activated cell sorting analysis. Western blot antibodies: $607 is requested for analysis of BCL-2 family survival protein expression in neuroblastoma PDX cell lines and tumor slices. Co-immunoprecipitation: $495 is requested for detection of protein-protein interactions between Bim and BCL-2 family survival proteins. Inhibitors: $198 is requested for the Bcl-xL proteolysis targeting agent DT2216 to be used to determine the contribution of Bcl-xL in neuroblastoma cell lines and PDX slices. PEDIATRIC CORE COSTS Flow Cytometry Core: $269 is requested for 2 hours of training and 3 hours of analysis of neuroblastoma cell surface markers using flow cytometry in 3 cell lines at the subsidized rate. Intergrated Cellular Imaging Core: The Integrated Cellular Imaging Core will be used for Keyence imaging of apoptosis in whole tumor slices (6 slices with drug exposures or controls) and confocal microscopy to confirm neuroblastoma cell specific apoptosis at a subsidized price of $345.

Proposed End Product: The anticipated timeline of data collection and analysis will be between 7/1/23 and 9/25/23. The goal of proposed project is to submit an abstract to the American Association for Cancer Research 2024 annual meeting by 11/23. The proposed research will provide additional data that will contribute to preparation of a manuscript to be submitted by February 2024 to allow time for addressing reviewer comments and resubmission with anticipated final acceptance by the end of the funding period.

Vertebrate Animals

Animal studies proposed in this project will be performed under the IACUC approved Goldsmith laboratory animal protocol (IACUC #PROTO201700089). All mice are housed in Emory HSRB animal facility.

Rationale for animal use: The process of tumor development is complicated and includes intricate events requiring the interaction of multiple cell types in an organized environment. Whenever possible, we use cultured cells to pilot and refine our experiments. However, there are well-defined differences between homogenous cell culture experiments and those that incorporate the heterogenic tumor microenvironment. Importantly, these experiments aim to limit the number of animals used for preclinical chemotherapeutic testing by intentionally using a ex-vivo slice culture system that will spare animals toxic effects from therapeutics.

1) Description of proposed use of animals:

Specific Aim 2: Determine the functional dependence of tumors bearing multiple Bim-protein-protein interactions. Our general approach will be to inject NSG mice with patient derived xenografts (PDX) of neuroblastoma single cell suspensions to allow development of tumors. All procedures are performed in a Class 2 biological safety cabinet using sterile technique. The mice are maintained in humidity- and temperature-controlled rooms appropriate for immunodeficient mice (aseptic) with 12-hour light and dark cycles. Briefly, male NOD-*scid* IL2Rg$^{-/-}$ (NSG) mice between 4 to 6 weeks of age will be injected subcutaneously to bilateral flanks with PDX cells suspensions of 5 million tumor cells mixed with a 1:1 ratio of RPMI:Matrigel. Mice receiving transplants will be monitored biweekly for tumor development. Once tumors are palpable, mice and tumor size will be monitored 3x/week by the investigator. Typical time for tumor growth of established low passage PDX is 14-21 days. Once tumors have reached sufficient size (~1000mm^3), mice will be euthanized. Excised tumors will then be used for ex-vivo slice culture drug exposure as described in Aim 2 of the research strategy or propagated into another mouse. We anticipate 6 animals will be used for PDX growth.

2) Justification of use of animals:

PDX models are essential for understanding the complex mechanisms of tumor progression and developing new approaches to treating cancer in humans. We have found key differences between PDX cell lines that are propagated in supraphysiologic conditions and those that are propagated only in mice. A focus of our study is to preserve the heterotypic cell-cell interactions found in the tumor microenvironment. Understanding survival mechanisms in the context of the tumor heterogeneity is paramount to the research study and future clinical application. Further, establishment of cell lines from PDX is timely and rarely successful. Mice are the lowest available species that can be used for this research. Overall, our use of mice in PDX models is essential for advancing our understanding of cancer and developing new treatments for patients.

3) Minimization of pain and distress: Animal behavior is monitored daily by our Laboratory Animal Technicians at Emory. This includes weekends and holidays when tumor bearing mice are under our care. All procedures are done with brief restraint, including tumor inoculation and tumor measurement. Our experiments do not proceed to a “survival end-point” defined by tumor symptoms, but are carried out to a tumor volume of 1,000 mm^3. In rare cases, a xenografted tumor may lead to overlying skin ulceration in which case the mouse will be euthanized. A veterinarian is available for emergencies, 24 hrs/day.

5) Method of euthanasia. Mice will be euthanized by administration of a lethal dose of CO_2, followed by a second mechanical method, such as cervical which are the recommendations of the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals.