The Swifty Foundation

Impact Report

Together Toward Hope

2023

Alex’s Lemonade Stand Foundation (ALSF) emerged from the front yard lemonade stand of 4-year-old Alexandra “Alex” Scott, who was fighting cancer and wanted to raise money to find cures for all children with cancer. Her spirit and determination inspired others to support her cause, and when she passed away at the age of 8, she had raised $1 million. Since then, the Foundation bearing her name has evolved into a national fundraising movement and is one of the leading funders of pediatric cancer research in the U.S. and Canada.

A letter from ALSF

Dear Patti, Al and Ginny,

All of us here at Alex's Lemonade Stand Foundation would like to sincerely thank you for your past support of Alex's mission to find new treatments and cures for childhood cancer.

Your partnership is helping researchers develop preliminary data, publish their findings, and push forward innovative treatment options. Because of your commitment to honoring Michael's legacy, we are closer to a day where no child will have to suffer from pediatric brain cancer.

We are truly honored to fight childhood cancer alongside the Swifty Foundation. Wishing you the best - and thank you for being the driving force behind life-saving cures! Please don't hesitate to reach out if you need anything from us here at ALSF.

Until there's a cure,

Liz and Jay Scott
Alex's Parents & Co-Executive Directors
Alex's Lemonade Stand Foundation

Crazy 8 Award: Small Molecule Degraders for Targeting Transcription Factor Drivers of Childhood Cancers

Team Leader: Charles Mullighan, MBBS (Hons), MSc, MD

Collaborators: Zoran Rankovic, PhD; M. Madan Babu, PhD, FRSC; Marcus Fischer, PhD; Jeffery M. Klco, MD/PhD; Paul A. Northcott, PhD; Martine F. Roussel, PhD

St. Jude's Children's Research Hospital

The overall goal for Dr. Mullighan's team is to expand and utilize a library of molecular glues to treat several of the deadliest forms of childhood cancer. The team at St. Jude's has been working towards this goal for the past two years. The multi-disciplinary team meets bi-monthly to share data, insights, reagents, and lessons. Their second progress report was provided March 31, 2023. Their aims and findings are summarized below.

Specific Aims

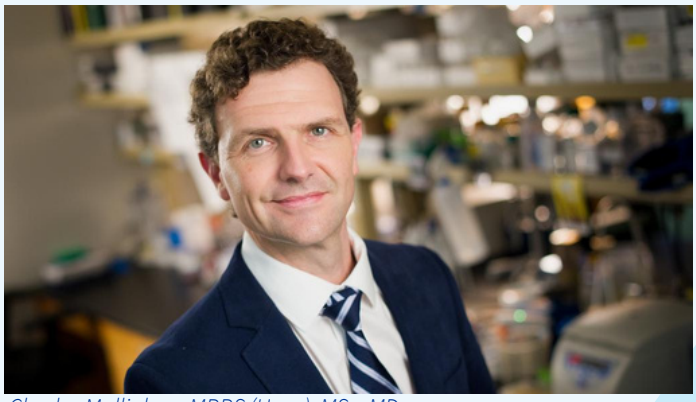
Aim 1: To identify novel oncogenic neosubstrates by phenotypic screening of the molecular glue library (MGL).

Aim 2: To develop and validate potent and selective MG degraders of medulloblastoma and acute leukemias oncogenic transcription factors (TFs) by targeted screening of the MGL.

Aim 3: To broaden the potential for the MG approach in pediatric cancer by further expanding the MGL chemical space coverage and size to 5,000 compounds.

Advancements for Leukemia

We have screened 3,626 compounds from the molecular glue library with chemical diversity based on Cereblon (CRBN, an E3 ubiquitin ligase) binding generated by the chemistry. Six ALL cell lines have been used in the phenotypic screening including two CRLF2-rearranged B-ALL, two hypodiploid B-ALL cell lines and two T-ALL cell lines. All of the cell lines have gone through a primary screen and the hits have been validated using dose-response plates generated from stock solutions and powder. Using MHH-CALL- 4-GSPT1-G575N cells resistant to GSPT1 degraders, we have identified two compounds that are cytotoxic independently of known targets of MGs. We are examining the therapeutic index (TI) of those compounds using hCD34+ cells from cord blood and PBMC from human donor and move forward to identify the potential protein targets if the therapeutic index is suitable.



Charles Mullighan, MBBS (Hons), MSc, MD

Advancements for Medulloblastoma

We generated several human MYC amplified Group 3 medulloblastoma lacking CRBN, and 293T cells expressing HiBiT-GFI1 and HiBiT-GFI1B constructs that permit cell based assays of the MG library to directly measure degradation of GFI1/GFI1B. We have also generated mouse Group 3 medulloblastoma lines over-expressing MYC+GFI1 or MYC+GFI1B neuronal progenitors in humanized cereblon I391V mutant mouse. We screened 7 additional sets of molecular glues containing 4,226 compounds in 3 human MYC amplified Group 3 medulloblastoma cell lines. Of the 4,226 compounds, 23 were further characterized. After treating human medulloblastoma lines HDMB03 and D425 with the compounds for 24 hours, cell lysates were immunoblotted with antibodies to GFPT1 and CK1α. We found that they were not degraders of the known CRBN neosubstrates GSPT1 and CK1α. The ability of the 23 compounds to act in a Cereblon-

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dependent manner was tested in two CRBN-KO human Group 3 medulloblastoma lines generated by CRISPR-Cas9 engineering. Two compounds showed efficacy in suppressing the proliferation of HDMB03 and D425 lines that were prioritized for mass spectrometry to identify their targets.

Chemistry Lead: Zoran Rankovic, PhD

Chemistry

The goal of aim 3 is to broaden the potential for temperature programmed desorption (TPD) in pediatric cancer by expanding the diversity coverage and size of the MGL to 5,000 compounds. The team has expanded the diversity and the size of the MGL from the previous 3,000 compounds to 4,200 new compounds and a total of 175 scaffolds, enriching with novel chemical scaffolds as well as novel building blocks. The design strategy for new compounds focused on building blocks with polar functionalities (basic amines, H-bond donors and acceptors) with the goal of generating interactions with amino acid residues in the beta-hairpin loop of C2H2 zinc fingers. Moreover, we introduced a covalent strategy, which includes adding covalent warheads to our CRBN binding scaffolds with the goal of identifying targets that can be degraded via this technology. We expect the library to contain over 5,000 compounds by end of June.

To identify structurally novel Cereblon binders, we used DNA-encoded library screening leading to the identification of SJ1042498, an allosteric cereblon binder. The allosteric binding has been confirmed by a nuclear magnetic resonance competition experiment with lenalidomide. This is an exciting advance, since the compound not only enables access to a novel chemical space quite distinct from thalidomide and its analogues, but also because it binds to a different binding pocket on the cereblon surface, which may widen the scope of cereblon neosubstrates to beyond what thalidomide analogues could offer. A fluorescent probe was synthesized and used to develop a fluorescent polarization (FP) assay, which is now used to establish the SAR and further optimize the cereblon affinity of this compound.

Structural Biology Lead: Marcus Fischer, PhD

Structural Biology

We used negative stain electron microscopy (ns-EM) to identify conditions amenable for imaging intact neosubstrate complexes for subsequent structure determination by cryo-EM. We are currently optimizing cryoEM conditions to image the CRBN+DDB1 complex bound to neosubstrate GSPT1 in the presence of the recruiting MG. Meanwhile, X-ray crystallography has proven successful. We pursued crystallographic studies of neosubstrates GSPT1 and CK1α bound to CRBN-DDB1 in the presence of certain molecular glues. While we were unsuccessful at obtaining crystals for those MGs thus far, we successfully crystalized two other complexes. We solved the quaternary crystal structure of the latter to 3.5 Å resolution. In addition to structural studies of the quaternary complex, we are pursuing crystallography

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studies of new MGs using the thalidomide binding domain (TBD) of CRBN. Readily crystallizable, yet structurally similar, the TBD alone allows insight into how candidate MGs bind to the thalidomide binding pocket. We have crystallized bacterial CRBN bound to thalidomide, exchanged the ligand for MG SJ41227, and recently collected data to 2.5 Å resolution. Data processing will reveal whether the ligand exchange was successful, as before, and how the MGs prime CRBN for interactions with the neosubstrate. Currently, we are screening conditions to generate TBD crystals of two MGs of interest for selectively degrading kinases CK1α and LCK.

Systems Biology Lead: Madan Babu, PhD, FRSC

Systems Biology

We previously constructed a comprehensive multiple sequence alignment of all the C2H2 domains found in the ~700 C2H2 zinc finger transcription factors in the human proteome. We analyzed the alignment for a conserved critical Gly151 and the conservation of thalidomide. This resulted in 19 C2H2 ZF with putative molecular glue degrons. Our previous analyses of the 665 ZF in the cancer DepMap dataset resulted in 5 top hits, 4 of which contain KRAB boxes and are likely transcriptional repressors. We screened ZNF658 against the St. Jude CBT library (600,000 drug-like molecules) and the enamine Molecular Glues library. We have generated a promising list of candidate structures, including both classical IMiD and novel scaffolds. We are currently performing careful secondary screening with ZNF658 and are continuing to screen additional models against the libraries previously mentioned and the St. Jude MG library. We have identified a shortlist of pediatric cell lines containing promising ZNF658 dependencies, including two, MOLM-13 (acute myeloid leukemia) and D341 (medulloblastoma), that our Chemistry team has recently screened against their MG collection. Once their analysis is complete, we will compare our predicted binding scores with their in situ activity assay results.

Impact of Findings

Finding new therapies for children with high-risk leukemia and medulloblastoma is of the utmost importance to improve outcomes for children with these diseases. As clonal alterations of ZnF transcription factors are common drivers of these diseases, therapeutic targeting of these is a logical, but challenging goal. MGs have attractive drug-like properties and achieving driver TF degradation would represent a valuable therapeutic advance. Accordingly, we have made substantial progress in all aims: screening and prioritizing MG hits in the cell line phenotypic screens; developing reagents for in vitro targeted TF degradation screening; developing novel mouse models for in vivo evaluation (syngeneic and xenograft); solving representative structures of MGs bound to the CRBN E3 ligase complex to provide mechanistic insight into mechanism of action; and exploiting our large genomic databases to prioritize additional targets for targeting. We have demonstrated potential anti-leukemic activity for GSPT1, CK1α and LCK directed MG in vivo that have been published, and IP filed. Clinical MG development is an extremely attractive but challenging and long term project, but we are gratified by the progress made and optimistic that we will identify additional MG hits for optimization and development.

Our consortium is unique in academia developing MG (in contrast to PROTACs). Our structural biology efforts have

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been integral in developing the MGL by providing key insights into how molecules from the MG library engage neosubstrates for degradation. Our recent quaternary structure illustrates how structural biology will impact the overall project in three ways: 1) a molecular understanding of complex formation can guide medicinal chemistry efforts to improve the binding affinity and selectivity of initial hits, e.g. by defining steric boundaries to avoid and exit vectors to exploit via medicinal chemistry; 2) it improves our understanding of how protein-protein interfaces are modulated by small molecules to form neo-interactions (for instance, our quaternary complex hints at important orthosteric and allosteric interactions beyond the structural degron that explains improved affinity, degradation efficiency and can be explored for ligand discovery); and 3) these efforts pinpoint ways to expand chemical space and improve the MG library that is the key driver of all aims of this project as well as provide new input for computational analysis and discovery.

Martine F. Roussel, PhD

Future Plans

For leukemia, we will continue the screening of the additional MGLs (total compounds will be ~ 4,000 compounds) generated by chemistry. We will extend the screening to identify compounds targeting IKZF1- N159Y. We will continue to generate the faithful cell line model for screening the compounds degrading BCL11B in T-ALL cells. The potential targets identified by the Babu bioinformatic group will be validated in ALL cells and moved forward for drug screening for potential degraders. We also plan to investigate novel neosubstrates through proteomics. For the hits showing promising effect in vitro, an in vivo study will be performed in the newly generated NSG-CRBN-V390E/I391V mice to access the efficacy and toxic effects at the same time.

For the medulloblastoma group, we will identify the protein targets from the two compounds identified by the unbiased screen of molecular glue libraries sets 1-10. This will include mass spectrometry and validation in vitro and in vivo. We will screen the sets 1-10 of molecular glue libraries on the mouse medulloblastoma line that expresses MYC and GFI1. In year 2 of the grant, we showed that tumor cells were completely dependent on GFI1 for proliferation. We expect that in this new set of targeted screens we will find specific GFI1 degraders.

For chemistry, we will complete the synthesis of 5,000 compounds in the MGL, by: 1) expanding around the allosteric binders identified from the DEL screening; and 2) rationally design libraries directed towards the program's high priority targets such as GFI-1. We will also continue expanding SAR around hits identified by phenotypic and targeted screening, as well as lead optimization efforts to support chemical probe identification for in-vitro and in-vivo validation studies. We will continue to solve individual and quaternary structures of proteins in the presence of relevant ligands. With the protein portfolio expanding as more targets emerge from our screens and subsequent proteomics, we will characterize these neosubstrates as we did for CK1α. Finally, confirmed allosteric hits will be further characterized structurally with the intention to identify novel allosteric site binding sites that then, in turn, can be targeted in addition to the canonical site to improve selectivity over off-targets.

ALSF's National Impact for 2022

Charity partners like the Swifty Foundation make it possible for us to continue Alex's dream of finding cures for all kids with cancer. With your support, we are funding the best, most promising research and giving hope to childhood cancer families across the country.

$26M+ total raised to help kids with cancer

83 grants funded

348 families assisted with travel costs through Travel for Care

1,300+ SuperSibs supported

Thank You

for all you do to help kids with cancer!