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14. ABSTRACT

Deregulated activation of noncanonical (nc)NF-kappaB signaling has been identified to drive the proliferation of many hematologic cancers. The central activator of ncNF-kappaB signaling is NF-kappaB inducing kinase (NIK). We hypothesize that selective small molecule inhibitors of NIK may yield effective therapeutic strategies for the treatment of hematologic cancers. In this project, we will investigate two complementary approaches to develop selective NIK inhibitors and evaluate them in blood cancer cells with NIK accumulation. The first approach is to develop selective NIK chemical degraders through rational design of bifunctional proteolysis targeting chimeras (PROTACs) to restore the natural NIK degradation mechanism. The second approach is to develop first-in-class allosteric NIK modulators. Biochemical and crystallography studies are in progress to validate and fully characterize these inhibitors.

15. SUBJECT TERMS

NONE LISTED

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1. INTRODUCTION:

Aberrant noncanonical (nc)NF-κB signaling activation drives the proliferation of many hematologic malignancies. NF-κB-inducing kinase, or NIK, is the central activator of the ncNF-κB. Under normal conditions, NIK is a highly unstable protein and constantly degraded by ubiquitin proteasome system (**Fig. 1**). Aberrant stabilization of NIK protein has been identified to activate ncNF-κB in a variety of aggressive hematologic malignancies, such as multiple myeloma, classical Hodgkin lymphoma, and T-cell leukemia. Furthermore, stabilization of NIK has been demonstrated to play central roles in inducing drug resistance, such as ibrutinib-resistant mantle cell lymphoma and fludarabine-resistant chronic lymphocytic leukemia. ATP-competitive NIK inhibitors have been discovered with potent *in vitro* inhibitory activity. However, those compounds suffer from either suboptimal kinase selectivity or poor pharmacokinetic properties, making them unsuitable for further development. I propose two complementary approaches to develop novel NIK inhibitors and evaluate their anti-proliferative efficacy in hematologic cancers and toxicity in normal tissue cells. In Aim 1, selective NIK chemical degraders will be developed through rational design of bifunctional proteolysis targeting chimeras, or PROTACs, to restore the natural NIK degradation mechanism. In Aim 2, type III allosteric NIK inhibitors will be developed by targeting a less conserved back pocket behind the active site. Compounds from both aims will be rigorously evaluated for NIK degradation/inhibition, ncNF-κB inactivation, and anti-proliferative efficacy in cellular models of hematologic cancers.

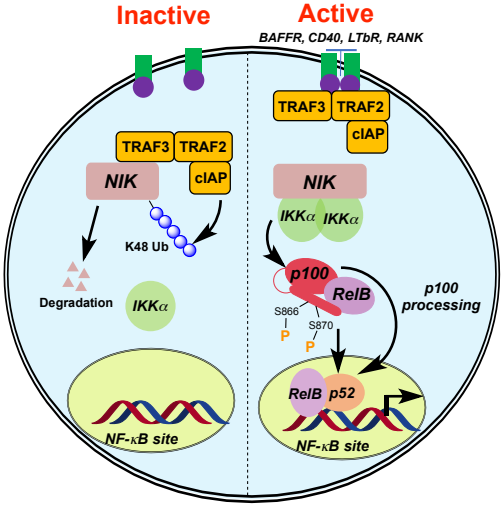


Figure 1. NIK stablization activates ncNF-κB.

2. KEYWORDS:

Noncanonical NF-κB; NF-κB-inducing kinase (NIK); Hematologic malignancies; Chemical degraders; Allosteric inhibitors.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Discover Selective NIK PROTACs
Major Task 1: Discovery and characterization of selective NIK PROTACs for NIK degradation
Subtask 1: Synthesis of designed NIK PROTACs with CRBN and VHL ligands.
Subtask 2: Characterize the enzyme inhibitory activities of synthesized PROTAC compounds against NIK
Subtask 3: Characterize the cytotoxicities of NIK-targeted PROTACS in multiple myeloma (MM) and mantle cell lymphoma (MCL) cells lines with/without activated NIK.
Subtask 4: Characterize the ability of synthesized PROTACs to induce NIK degradation in cells (DC_{50} and D_{max} values).
Milestone(s) Achieved: Discovery of potent NIK PROTACs that selectively degrade NIK protein and inactivate ncNF-κB signaling in NIK-active MM and MCL cells.
Specific Aim 2: Develop Selective NIK Allosteric Inhibitors
Major Task 1: Development and biological evaluation of NIK allosteric inhibitors

Subtask 1: Determine the NIK inhibitory activities (IC_{50} values) of the 13 putative allosteric modulators identified in preliminary studies.
Subtask 2: Synthesize improved allosteric NIK analogues based on the most potent 1-2 chemotypes.
Subtask 3: Use surface plasmon resonance (SPR) assays to determine the binding affinities of the allosteric NIK modulators to human NIK.
Subtask 4: Characterize the NIK inhibitory activities of the synthesized analogues and summarize structure-activity relationship (SAR).
Subtask 5: Evaluate the cytotoxicities and ncNF-κB inactivation potencies of the allosteric NIK inhibitors in MM and MCL cells.
Major Task 2: Binding mode studies of NIK allosteric inhibitors
Subtask 1: Perform docking studies and molecular dynamics simulations with human NIK and allosteric inhibitors to predict the binding modes and guide the designs of new analogues for synthesis.
Subtask 2: Co-crystallize the best allosteric NIK inhibitors from task 2.3 with murine NIK protein.
Milestone(s) Achieved: Development of first-in-class allosteric NIK inhibitors with high selectivity and inhibitory potency towards NIK in MM and MCL cells. The development of small molecule NIK inhibitors that slow/prevent the growth of NIK-active MM and MCL cells.

What was accomplished under these goals?

Specific Aim 1: Discover Selective NIK PROTACs
Major Task 1: Discovery and characterization of selective NIK PROTACs for NIK degradation

I attempted to develop NIK-targeted PROTACs by connecting ATP-competitive NIK ligands with the cereblon (CRBN) E3 ligase ligand via variable linkers. To guide PROTAC design, previously published x-ray structures of NIK and E3 ligase CRBN were used to generate models of the putative NIK-CRBN complexes through collaboration with Dr. Rommie Amaro at the University of California, San Diego (UCSD). These models inform PROTAC design by permitting appropriate linker lengths and compositions to be considered (**Fig. 2A**). Based on these studies, several NIK PROTACs were proposed to be synthesized. However, the synthesis of such compounds was challenging and work is still underway in the laboratory to prepare those compounds. In parallel, I purchased a multiple myeloma cell line with NIK accumulation, RPMI 8226, from the American Type Culture Collection, established the culture condition of this cell line, and developed a protocol for NIK immunoblotting with a commercial antibody (**Fig. 2B**). We are now ready to evaluate NIK PROTACs once they are synthesized.

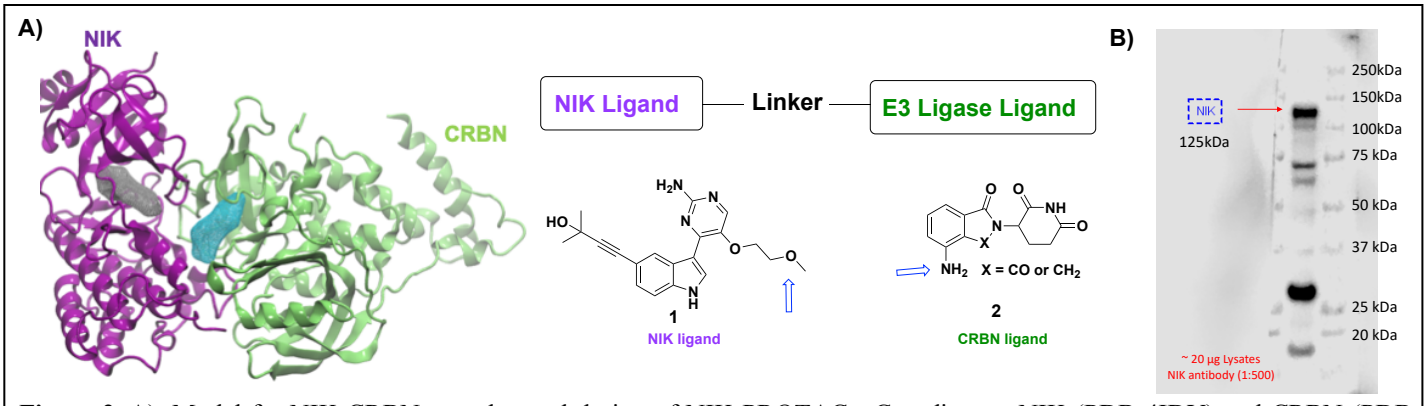


Figure 2. A). Model for NIK-CRBN complex and design of NIK PROTACs. Coordinates: NIK (PDB 4IDV) and CRBN (PDB 4CI1). Sites for PROTAC linkers marked with a blue arrow; B). Western blot image of NIK protein from RPMI-8226 cell lysates.

I applied similar principles to develop selective degraders targeting another kinase, Aurora kinase A (Aurora-A), which is also commonly overexpressed in hematologic cancers, such as multiple myeloma and acute myeloid leukemia. Several Aurora-A inhibitors are currently under clinical investigation for these diseases. In addition, a recent study demonstrated that Aurora-A inhibition promotes NIK protein stabilization, which in turn restrains the efficacy of Aurora-A inhibitors in multiple myeloma, suggesting that dual inhibition of Aurora-A and NIK may confer a more effective therapy (*Haematologica*, **2019**, *104*, 2465). To explore this idea, a series of potent Aurora-A chemical degraders was successfully developed. First, I modified the CDK4/6 selective inhibitor ribociclib for Aurora-A binding through extensive chemical modifications. The selective Aurora A degrader, HLB-0532259, was then developed by connecting the ribociclib analogue (HLB-0532261) to the CRBN ligand through a hexyloxyl chemical linker (**Fig. 3A**). Immunoblotting experiments indicated that HLB-0532259 potently induced the degradation of Aurora-A in MCF-7 breast cancer cells after 4 hours, while no obvious degradation of CDK4 was observed. The degrader also showed a lower degradation potency for Aurora-A at a high concentration, indicating the characteristic "hook effect" feature of a PROTAC molecule. The calculated DC_{50} value for Aurora-A is 20 nM, and the D_{max} (maximum degradation efficacy) is >92% (**Fig. 3B**). Tandem mass tag (TMT) proteomics profiling was also performed to validate the high selectivity of HLB-0532259 for degrading Aurora-A at the proteome level (**Fig. 3C**). Pharmacokinetics (PK) profiling of HLB-0532259 was also conducted. The results showed that HLB-0532259 achieved a favorable PK properties after a 10 mg/kg intraperitoneal injection into CD-1 male mice, including a long half-life ($T_{1/2}$, 14.5 hrs), a good residence time (MRT, 16.1 hrs) and a high exposure (AUC, 1993 ng.h/mL, **Fig. 3D**). These PK parameters allow and will guide *in vivo* efficacy studies of HLB-0532259 in mouse xenograft tumor models in the future. Combining the Aurora-A degrader with known NIK inhibitors in multiple myeloma cellular models are currently ongoing with an expectation to observe synergistic effects as well as an improved therapeutic outcomes. HLB-0532259 and related compounds have been covered in a PCT patent application from the University of Minnesota (Harki, D. A.; Tang, J. *et. al.*, PCT/US2020/035977; filed June 3rd, 2020).

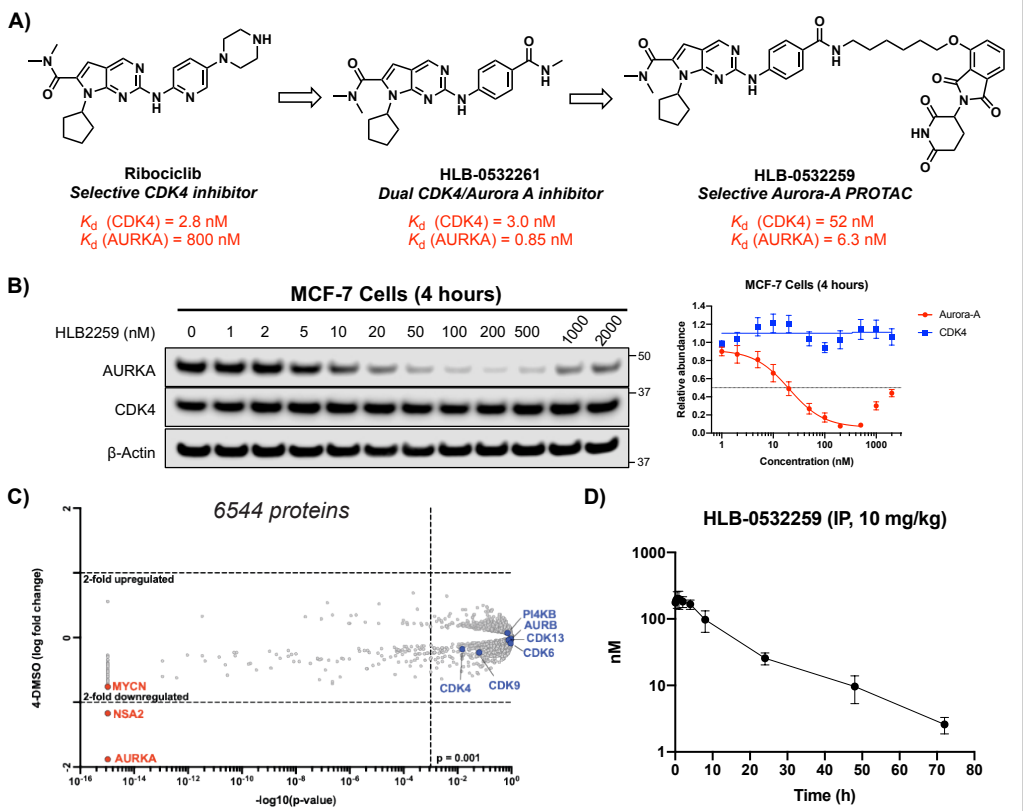


Figure 3. A). Development of the selective Aurora-A PROTAC HLB-0532259; B). Degradation of Aurora-A in MCF-7 cells with HLB-0532259. AURKA: Aurora-A; C). TMT proteoteomics profiling of HLB-0532259 in SK-N-BE(2) cells; D). PK data of HLB-0532259 in CD-1 male mice.

Specific Aim 2: Develop Selective NIK Allosteric Inhibitors
Major Task 1: Development and biological evaluation of NIK allosteric inhibitors
Major Task 2: Binding mode studies of NIK allosteric inhibitors

Molecular dynamics (MD) simulations of the NIK structure (PDB 4IDT and 4IDV) conducted by the Amaro lab resulted in the identification of two potential NIK allosteric sites, one pocket near Arg509 and another surrounding Phe535, that may be amenable to small-molecule modulation (**Fig. 4A**). Subsequent virtual screening to target these two putative allosteric pockets was performed, and following the prioritization of docking scores and ligand efficiencies, 120 potential hit compounds were identified as potential NIK allosteric modulators. The 120 compounds were then tested in the Harki lab using an ADP-Glo assay and 13 compounds were confirmed with >90% inhibition at 100 µM concentration against NIK kinase activity. However, at the outset of this project, we realized that the ADP-Glo assay for evaluating NIK inhibitory activity by our compounds was inconsistent. We also determined that commercial sources of recombinant NIK recombinant protein were too expensive for purchasing enough protein to enable large screening efforts.

To address these issues, human NIK kinase domain (a.a. 340-694) was successfully expressed in a baculovirus-infected insect cell expression system by contract to Genescript. 21 mg of hNIK (340-694) was produced with > 85% purity and its enzymatic activity has been confirmed by our group (**Fig. 4B**). In parallel, 17 mg of mouse NIK kinase domain (a.a. 329-675) has been produced with 90% purity, which is being used in NIK crystallography studies by our collaborator (Dr. Hideki Aihara, University of Minnesota). To address the

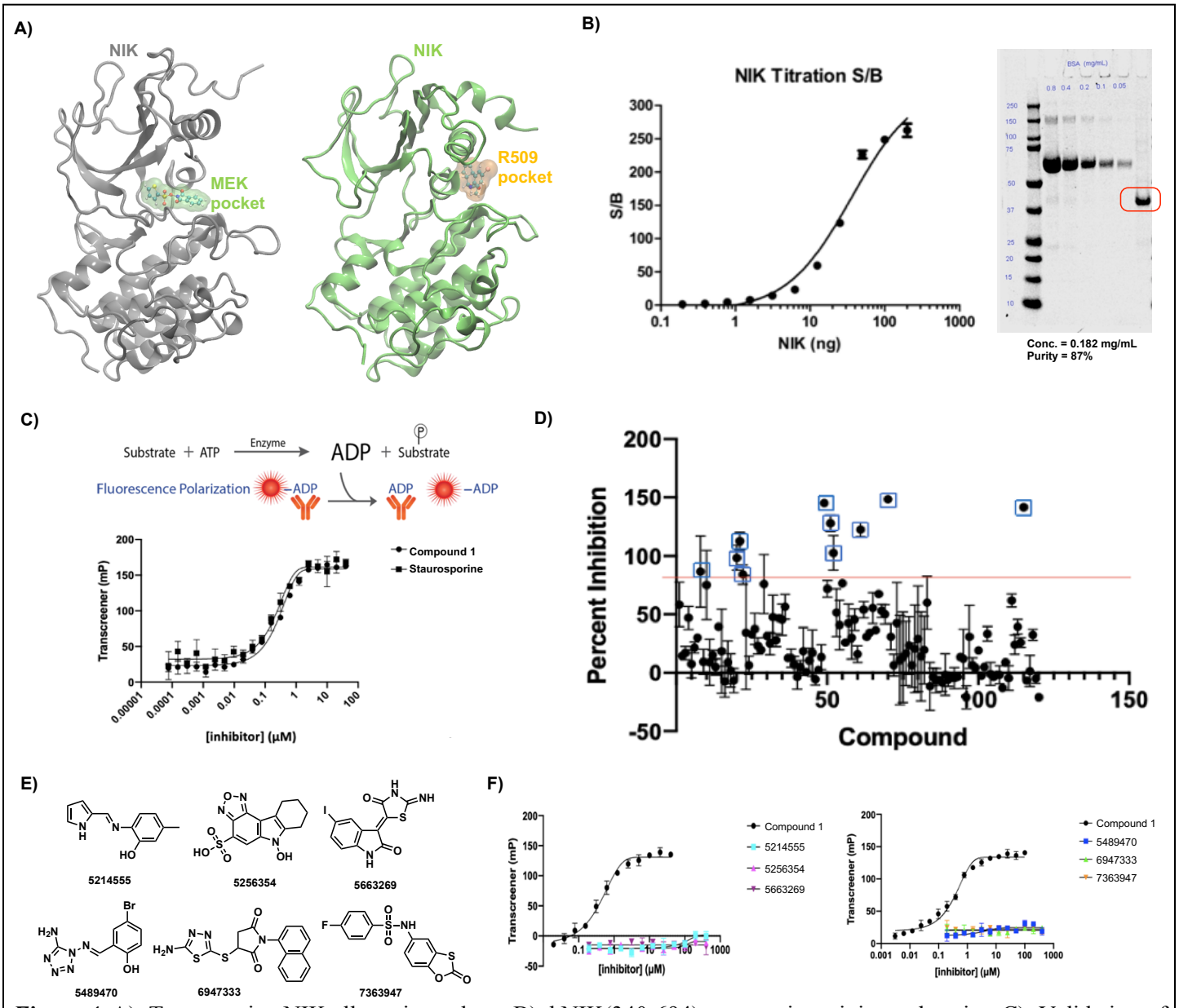


Figure 4. A). Two putative NIK allosteric pockets; B). hNIK(340-694) enzymatic activity and purity; C). Validation of NIK Transcreener ADP^2 FP assay; D). Screening results of the 120 compound for NIK inhibition; E). Chemical structures of the six primary hit compounds; F). Dose-response inhibitory activities of the six repurchased compounds.

reproducibility issue of ADP-Glo assay, a more robust Transcreener ADP^2 Fluorescence Polarization (FP) assay was established by another member of the Harki laboratory. This assay was validated using two known kinase inhibitors, compound **1** and the pan-kinase inhibitor Staurosporine (**Fig. 4C**). This FP assay has been proven to produce more consistent results. We next turned our attention to the putative allosteric NIK modulators identified in the computational screen. Evaluation of the 120 compounds from the virtual screening in the NIK FP assay at 200 µM yielded 10 compounds that exhibited greater than 80% inhibition (**Fig. 4D**). After PAINS filtering, six compounds were repurchased and purified by HPLC (**Fig. 4E**). Next, we validated their structures with mass spectrometry and all compounds were obtained with >95% HPLC purity. Unfortunately, the six primary hit compounds did not display any inhibitory activity against NIK (**Fig. 4F**). The false positivity was presumably caused by the impurities in the original compound stocks. These hits from the computational screen are no longer being pursued. Other screening approaches are currently underway in the Harki laboratory to identify allosteric NIK binders.

I also developed a competition differential scanning fluorimetry (DSF) assay to validate hits as *bona fide* allosteric inhibitors. In this assay, we expect to see a second thermal shift of the melting temperature (T_m) of the hNIK protein that is saturated by an orthosteric/ATP-competitive ligand (e.g. AMP-PNP). In the pilot experiments, we have determined that the pan-kinase inhibitor Staurosporine, as well as the reported NIK inhibitor compound **1** could stabilize hNIK protein under the optimized assay condition, and exhibited a T_m shift (ΔT_m) of +4.10 °C and +8.58 °C, respectively (**Fig. 5C**). Moreover, to evaluate these allosteric inhibitors for *in cellulo* target engagement on endogenous NIK, I developed a NIK cellular thermal shift assay (CETSA). In this assay, ligand-bound protein has a higher thermal stability upon heat challenge, and soluble protein content is visualized by immunoblotting. I have generated the melting curve of NIK in RPMI-8226 multiple myeloma cells, in which NIK shows a T_{agg} value of 46.5 °C (**Fig. 5D**). These assays will be utilized to evaluate our NIK allosteric inhibitors once they are identified from screening.

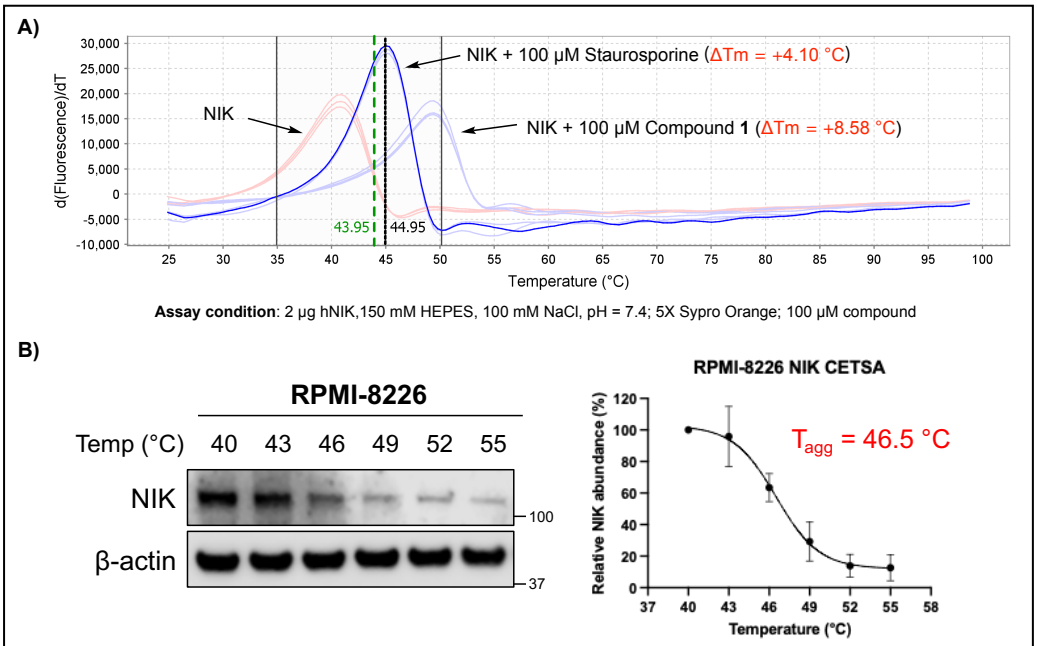


Figure 5. A). DSF assay with human NIK protein; B). CETSA data of endougerous NIK in RPMI-8226 cells.

What opportunities for training and professional development has the project provided?

I have benefited significantly from the resources and opportunities provided by this grant. First, to improve my interdisciplinary research experience, I have been trained to use synthetic organic and medicinal chemistry to synthesize novel small molecule probes, and I have acquired techniques in cell and molecular biology to evaluate these compounds. I also paid an on-site visit to the Amaro group at UCSD in August 2019 to review data, design experiments and get training on computational chemistry techniques. I have been frequently communicating with the Aihara group at UMN to discuss structural biology studies of NIK with chemical probes. Second, to get more in-depth training in cancer biology, I have been attending the weekly Masonic Cancer Center seminar series from 2019 to early 2020. I also audited the graduate course *Biology of Cancer* (MICa 8004) in the Spring 2020 semester. I also took a four-day course on Proteomics provided by the Cold Spring Harbor Laboratory in August, 2020, which was supported by funds from this grant. In terms of professional development, I attended the Fall 2019 ACS National Meeting & Exposition and presented a poster about the NIK allosteric inhibitors project. I also

attended the 58th Annual MIKIW Medicinal Chemistry meeting and presented a poster about the Aurora-A PROTAC project, where I won a third-place presentation award (See **Section 9, Appendices**). I have also presented at the annual Chemistry-Biology Interface Training Grant Symposium at UMN in both May 2020 and May 2021.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

There are currently no cures for aggressive blood cancers such as multiple myeloma (MM) and mantle cell lymphoma (MCL). Therefore, targeting the cellular vulnerabilities of these malignancies to develop new, effective therapies is urgently needed. Studies have demonstrated that depletion of NF-κB-inducing kinase (NIK), an enzyme that is aberrantly activated in many aggressive hematologic cancers, results in rapid induction of apoptosis and yields anti-proliferative activity, which validates NIK as a promising target for therapeutic development. In this project, we took two different approaches to develop selective NIK inhibitors as potential therapeutics for hematologic cancers. In addition, during the course of this project, I developed orthogonal biochemical and biophysical assays that enable studies of NIK inhibitors and degraders *in vitro* and *in cellulo*. With these assays in-hand, I helped to triage a screening campaign for discovering NIK allosteric modulators. I also successfully developed potent chemical degraders targeting a related kinase, Aurora-A. These efforts not only allow us to explore the synergistic effects of dual inhibition of NIK and Aurora-A in multiple myeloma cells, but also yield a platform to efficiently characterize PROTAC molecules, which will facilitate our ongoing work to develop NIK PROTACs.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

A PCT patent application has been filed by the University of Minnesota for the Aurora-A degraders (Harki, D. A.; Tang, J. *et. al*., PCT/US2020/035977; filed June 3rd, 2020).

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

We have stopped using the ADP-Glo assay to characterize NIK inhibitors and degraders due to the aforementioned variabilities (see **Section 3. Accomplishments**). Instead, we established a more robust Transcreener ADP2 FP assay for the same purpose. Furthermore, based on the interesting discovery published recently (*Haematologica*, **2019**, *104*, 2465), I developed Aurora-A degraders with the goal of evaluating a combined treatment by inhibiting both Aurora-A and NIK to improve the therapeutic outcomes for multiple myeloma patients. I also developed DSF and CETSA assays for use in evaluating NIK modulators for target engagement *in vitro* and *in cellulo*, respectively.

6. PRODUCTS:

Jian Tang, Ramkumar Moorthy, Zachary D. Baker, Ozlem Demir, Katherine F. M. Jones, Ella S. Haefner, Rommie E. Amaro, Nicholas M. Levinson, Daniel A. Harki, Targeting N-Myc in Neuroblastoma Cells with Selective Aurora Kinase A Degraders. 58th Annual MIKIW Medicinal Chemistry Meeting, **2021**. (Poster presentation)

Jian Tang, Ozlem Demir, Garrett Chan, Alex M. Ayoub, Rommie E. Amaro, Daniel A. Harki, Discovery of Allosteric Inhibitors of NF-κB Inducing Kinase (NIK), American Chemical Society **Fall 2019** National Meeting & Exposition, ID: 3207441. (Poster presentation)

Daniel A. Harki, Jian Tang, Ramkumar Moorthy, Rommie E. Amaro, Ozlem Demir, Compounds that Degrade Kinases and Uses Thereof, PCT/US2020/035977. (Patent application; filed June 3rd, 2020)

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Jian Tang
Project Role:	Principal investigator
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	24
Contribution to Project:	Mr. Tang has conceived this project and performed most of the experiments, including organic synthesis, assay development, and in cellular assays. Mr. Tang also analyzed data, wrote scientific documents and presented data at scientific meetings.
Funding Support:	DoD PRCRP Horizon Award

Name:	Daniel A. Harki
Project Role:	Mentor
Researcher Identifier (e.g. ORCID ID):	0000-0001-5950-931X
Nearest person month worked:	0.5 (no-cost)
Contribution to Project:	Dr. Harki has conceived and supervised this project. He designed the study, analyzed data and wrote scientific documents.
Funding Support:	Various sources of external funding and institutional support

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

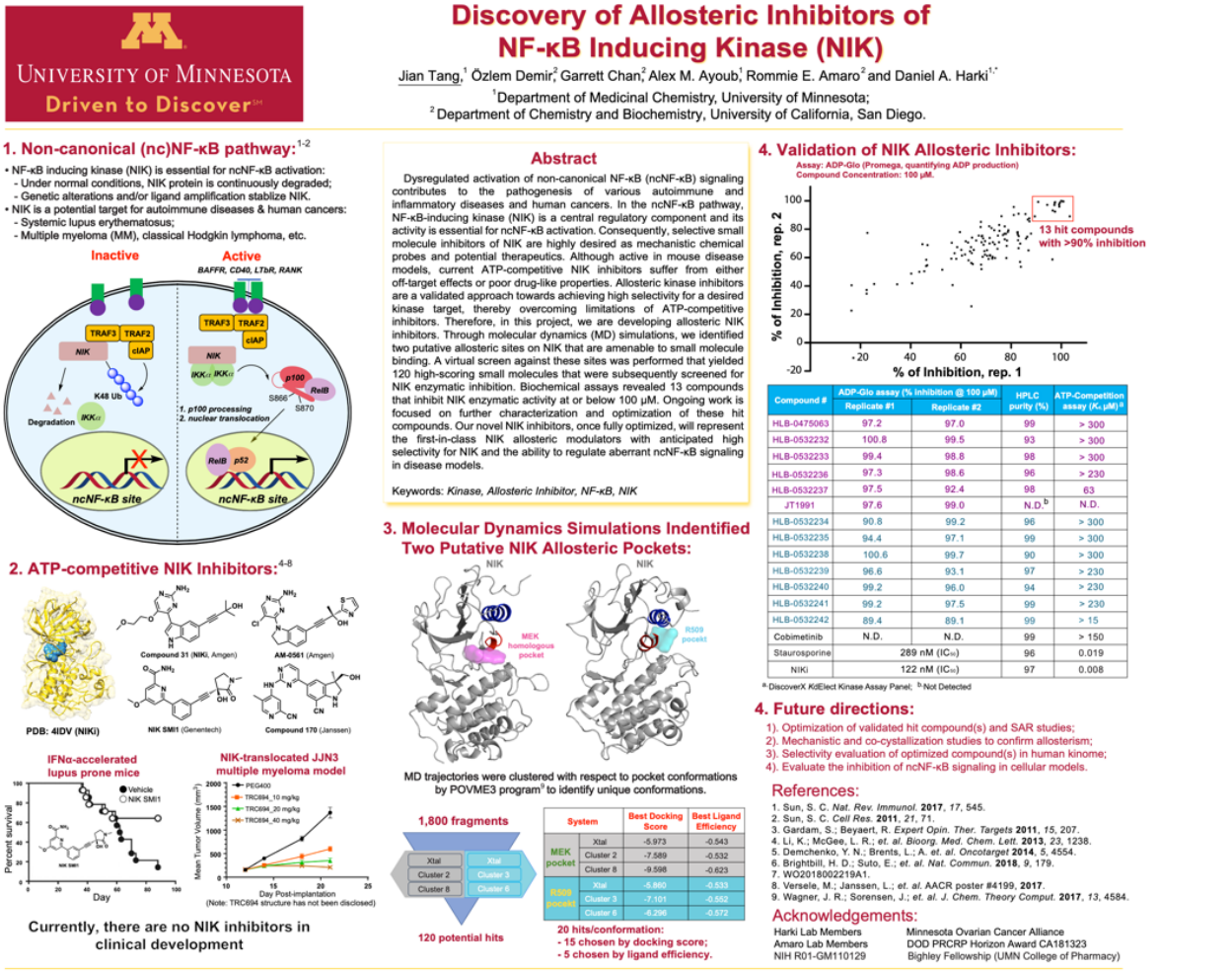
8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

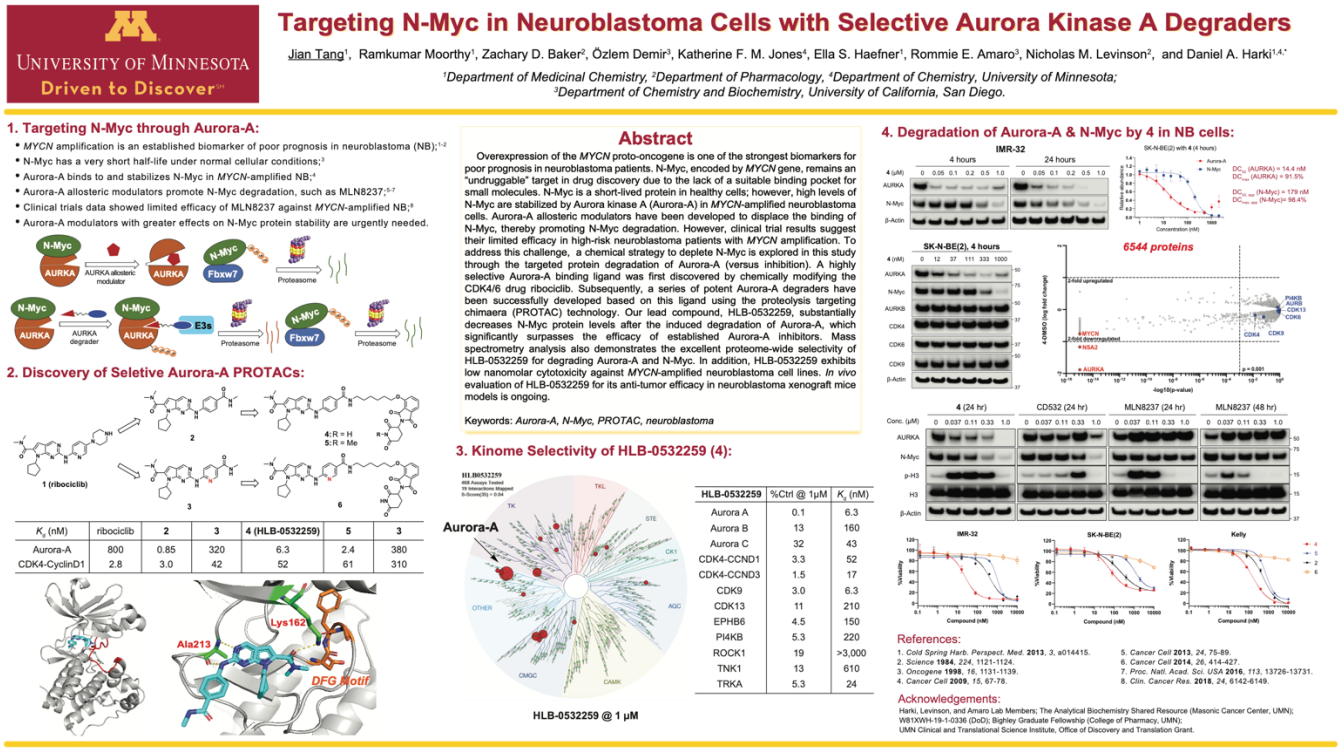
9. APPENDICES:

See next page.

A). American Chemical Society Fall 2019 National Meeting & Exposition Poster Presentation:



B). 58th Annual MIKIW Medicinal Chemistry Meeting, 2021. Poster Presentation:



C). Patent application (PCT/US2020/035977, UMN).

SLW 600.A66WO1 (U of M Docket No.:2019-323)

COMPOUNDS THAT DEGRADE KINASES AND USES THEREOF

BACKGROUND

[0001] N-Myc is a member of the Myc family of transcription factors encoded by the *MYCN* proto-oncogene. Enhanced and deregulated expression of N-Myc drives the development of a number of human cancers, including neuroblastoma, medulloblastoma, neuroendocrine prostate cancer, and small-cell lung cancer, etc. For instance, in neuroblastoma *MYCN* amplification is the strongest indicator of poor prognosis. 5-year survival rates for low- or moderate-risk patients are 80-95%; for high-risk patients that typically have increased N-Myc levels, 5-year survival is only 50% although with more aggressive treatments. Despite recognition of its critical roles in neuroblastoma, N-Myc, like its homolog C-Myc, remains a challenge for drug discovery scientists. To date, there are no N-Myc targeted therapies available for clinical use. A critical need remains unmet for the development of N-Myc modulators that could potentially yield a more effective therapeutic regimen for N-Myc driven cancers.

[0002] Ovarian cancer is the second most common and the most lethal gynecologic malignancy in the western world. In the United States alone, 22,240 new cases of ovarian cancer and 14,070 ovarian cancer deaths were estimated for 2018. Due to the lack of effective early detection methods, about 70% of ovarian cancer patients are diagnosed at an advanced stage, which is incurable in the majority of cases. The standard treatment for ovarian cancer is debulking surgery followed by platinum-based chemotherapy. However, many patients do not receive chemotherapy due to the high risk of side effects. Moreover, recurrence following initial therapy is very common in ovarian cancer and those tumors are frequently resistant to platinum-based chemotherapy. Accordingly, new and innovative therapeutic strategies for regulating the proliferation of ovarian cancer cells are needed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0003] **FIG. 1** is a cartoon showing G1-to-S phase transition and the roles of cyclin D-CDK4/6 complexes.

[0004] **FIG. 2** are chemical strutures of three CDK4/6 inhibitors.

[0005] **FIG. 3A** is the chemical structure of compound **22**.

[0006] **FIG. 3B** are Western blot experiments showing dose-dependent and time-dependent degradation of cyclin D-CDK4 complexes in MCF-7 cells.

[0007] **FIG. 3C** is a gel showing degradation of cyclin D-CDK4/6 complexes in MCF-7 cells is dependent on the ubiquitin proteasome system (UPS).

[0008] **FIG. 3D** is a plot showing anti-proliferative activites in ovarian cancer cell line SK-OV-3. Compound concentrations: 10 µM, 1 µM, 100 nM and 10 nM. “Poma” and “Ribo” represent pomalidomide and ribociclib, respectively.

[0009] **FIG. 4** shows plots of percent cell viability as a function of compound concentration in ovarian and breast cancer cell lines.