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Leah Kovalic, Student

Dr. R. Kip Guy, Major Professor

Dave Feola, Director of Graduate Studies

Optimization of Orally Bioavailable Inhibitors of Defective in Cullin Neddylation 1 (DCN-1)

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Pharmaceutical Science in the
College of Pharmacy
at the University of Kentucky

By

Leah Carmelle Kovalic

Lexington, Kentucky

Director: Dr. R. Kip Guy, Dean of the College of Pharmacy

Lexington, Kentucky

2023

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ABSTRACT OF THESIS

Optimization of Orally Bioavailable Inhibitors od Defective in Cullin Neddylation 1 (DCN-1)

Ubiquitin (UB) and ubiquitin-like protein (UBL) pathways have emerged as important targets for oncology drug discovery based on the success of proteasome inhibitors (bortezomib or carfilzomib), E3 inhibitors, and the NEDD8 E1 inhibitor pevonedistat (MLN42924). Chemical inhibitors have also proven to be useful probes for identifying and dissecting multifactor UB and UBL regulatory networks. Toward this end, we have pursued approaches to target NEDD8 ligation to Cullins, through developing small molecule inhibitors of DCN1 (defective in Cullin Neddylation 1). DCN1 was discovered as a potentiating RBX1-dependent NEDD8-ligation, through recognizing the acetylated N-terminal methionine of the NEDD8 E2s UBE2M and UBE2F. The DCN1 gene was also discovered to undergo frequent chromosomal amplifications and associated with poor prognosis in squamous cell carcinomas and other cancers. To date, we have conducted a high-throughput screening campaign of ~500,000 compounds and have identified three unique chemical series for optimization.

KEYWORDS: Ubiquitin-like, Oncology, DCN1, E3 inhibitors, Bioavailable

Leah Carmelle Kovalic

03/29/2023

Date

Optimization of Orally Bioavailable Inhibitors of Defective in Cullin Neddylation 1 (DCN-1)

By

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Director of Thesis

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03/29/2023

Date

DEDICATION

To Yuki, Ahsoka, Abby, and Ziva

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The following thesis, while an individual work, benefited from the insights and direction of several people. First, my Thesis Chair, Dean Guy, exemplifies the high quality scholarship to which I aspire. In addition, Dean Guy provided timely and instructive comments and evaluation at every stage of the thesis process, allowing me to complete this project on schedule. Next, I wish to thank the complete Thesis Committee, and outside reader, respectively: Jared Hammill and Vivek Rangnekar. Both provided insights that guided and challenged my thinking, substantially improving the finished product.

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CHAPTER 1. INTRODUCTION

1.1 Lung Cancer

Lung Cancer kills more people in the United States than any other type of cancer annually. The 2021 State of Lung Cancer Report (figure 1) found that the state of Kentucky was ranked as the highest rates of new lung cancer diagnosis in the United States at 89.4 per 100,000 people. Kentucky is ranked 45th in the nation for survival at a 30% survival rate at 5 years. These statistics show that there is vast room for improvement in the treatment of lung cancer.

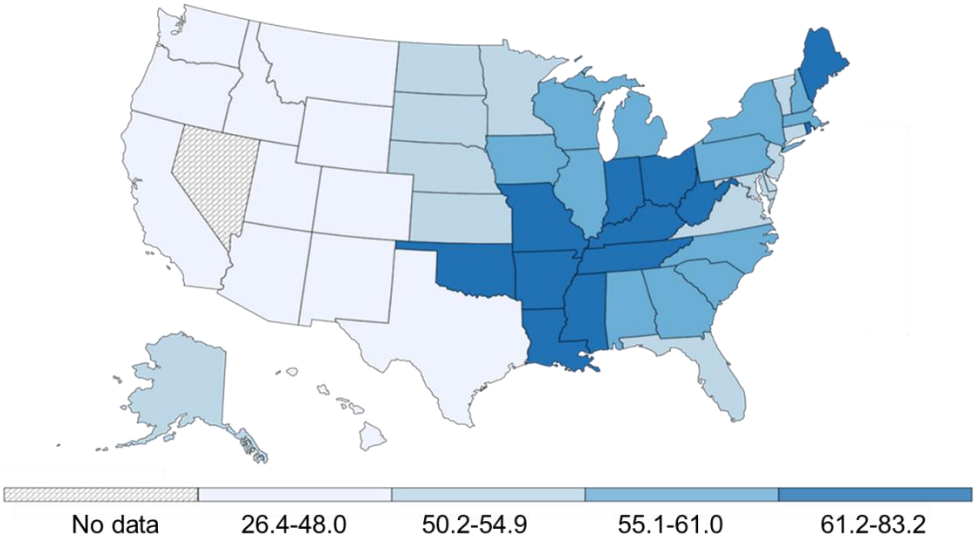

Figure 1 Map of new lung cancer diagnosis rates in the United States by State.

There are two different types of lung cancer, small cell and non-small cell. The treatment for the two different types varies. Surgery is not a treatment option for small cell cancer. Chemotherapy and radiation are the traditional treatment options for small cell. The goal of chemotherapy is to shrink tumors while the goal of radiation is to kill the cancer cells. Targeted therapy is an emerging treatment option in several different

types of cancers, not just lung. The goal of targeted therapy is to block the growth and spread of cancer cells in the body.

Targeted therapy is an exciting treatment avenue because as the name suggests, it specifically targets the cancer cells unlike traditional chemotherapy and radiation that is not tumor specific. Because chemotherapy and radiation are not tumor specific, they act on the whole body and cause a wide variety of severe adverse effects that potentially limit treatment options for patients. The goal of the DCN1 project is to develop an orally bioavailable targeted therapy option for non-small cell lung cancer that is safe and limits the severity of adverse effects that are typically associated with cancer treatment.

The first line pharmacotherapy for NSCLC is Chemotherapy and radiation. The chemotherapy regimen usually consists of multiple medications, each with their own adverse effects ranging from severe nausea to a risk of new cancer later in life.

1.2 Background of Project

Our collaborator, Dr. Singh at Memorial Sloan Kettering Cancer Institute, identified a gene loci (3q) that was amplified in his cancer patients. Amplification 3q has been seen in multiple tumor types. However, it does seem to be observed in squamous cell carcinoma that are mucosal in origin, including lung tumors that have been associated tumor progression and aggressive clinical course. He was able to identify what he called Squamous Cell Carcinoma Related Oncogene (later renamed Defective in Cullin Neddylation 1).[1] He saw that DCN1 was upregulated in around 50% of his patients, and he noticed that patients that had the DCN1 gene, had worse clinical outcomes (figure 2).

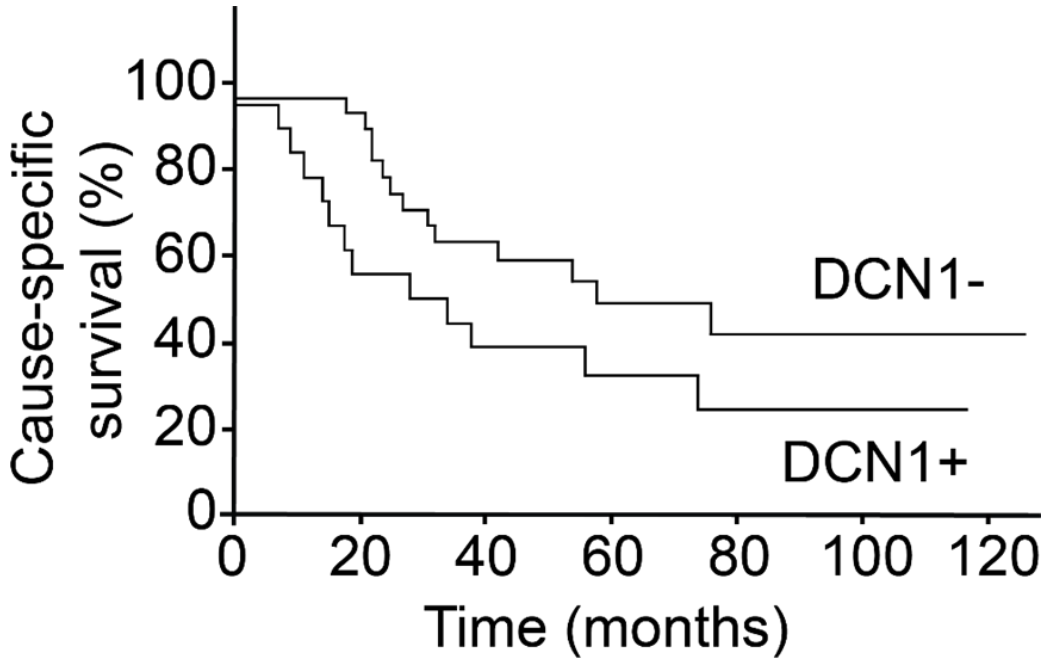

Figure 2 Survival time of patients with and without DCN1

After determining that patients with DCN1 upregulation had worse clinical outcomes, we needed to determine what role, if any, DCN1 played in the patient's tumor. A series of knock out studies were performed, and it was determined that DCN1 facilitated tumor growth (figure 3, reproduced from Squamous cell carcinoma related oncogene/DCUN1D1 is highly conserved and activated by amplification in squamous cell carcinomas[1]).

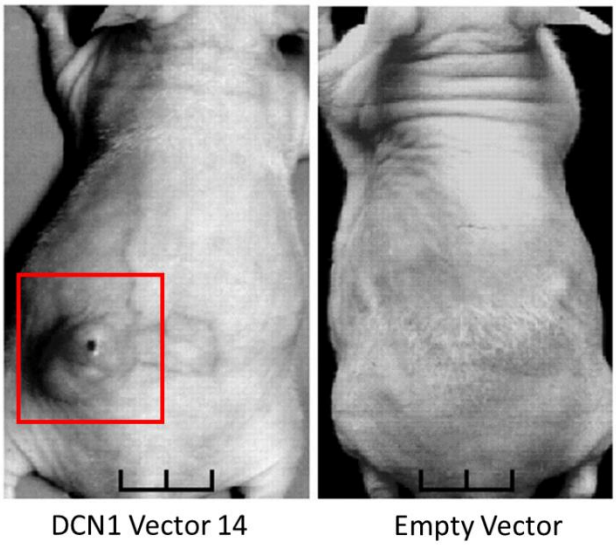

Figure 3 Naked mouse on the left was injected with a vector containing amplified levels of DCN1 while the mouse of the right was injected with a control vector. The box outlines the tumor growth that the mouse experienced.[1]

These experiments shown in figure 3 were performed in BALB-c nude mice that were injected with DCN1-transfected NIH-3T3 cells or an empty vector. BALB-c mice do not produce t-cells and are immunocompromised at baseline. Six mice were infected with DCN1 cells and six were injected with the empty vector. All 6 mice that were injected with DCN1 transfected cells grew tumors within 8 weeks. The mice that were injected with empty vector showed no tumor growth, even as far out as 12 weeks after injection. This further confirmed that DCN1 could be a viable target for a drug.[1]

1.2.1 How DCN1 Works

Defective in Cullin Neddylation 1 is an important component of the E3 complex for the process called neddylation (figure 4).[2] Neddylation regulates biological processes such as cell proliferation and metabolism. Neddylation is a process in which Cullin RING Ligases (CRLs) are activated by modification of a specific lysine on the cullin subunit with the ubiquitin-like protein NEDD8. Neddylation influences CRL activity through multiple mechanisms. Cullin neddylation helps to recruit the ubiquitin E2 enzyme to the

ligase. NEDD8 has the ability to selectively bind to the ubiquitin-charged E2 which allows for the recruitment of the charged E2.

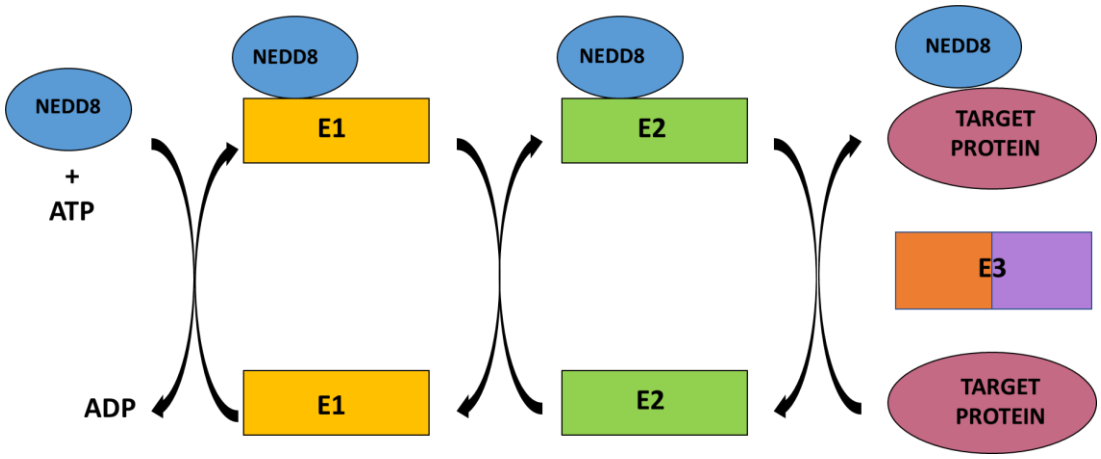

Figure 4 Neddylation pathway[2]

Post-translational neddylation modification is a process in which ubiquitin like protein NEDD8 is transfigured to targeted substrates via the enzymatic E1-E2-E3 reaction leading to functional changes to the targeted substrates.[3] DCN1 is part of a hierarchical E1-E2-E3 enzymatic cascade that covalently attaches the UBL NEDD8 to target proteins. The E3 complex is responsible for recruiting the NEDD8 to the target protein. There are two main classes of E3 that are characterized by the presence of either HECT domain or a RING domain. The HECT domain harbors an active-site cysteine, which forms a thioester intermediate with ubiquitin before its transfer onto the substrate. The RING domain serves as an inert scaffold that facilitates the direct transfer of ubiquitin from the E2 on the substrate without forming an E3-ubiquitin intermediate.[4]

Figure 5 shows that DCN1 is not necessary *in vitro* for neddylation to occur. However, neddylation happens much faster in the presence of DCN1.

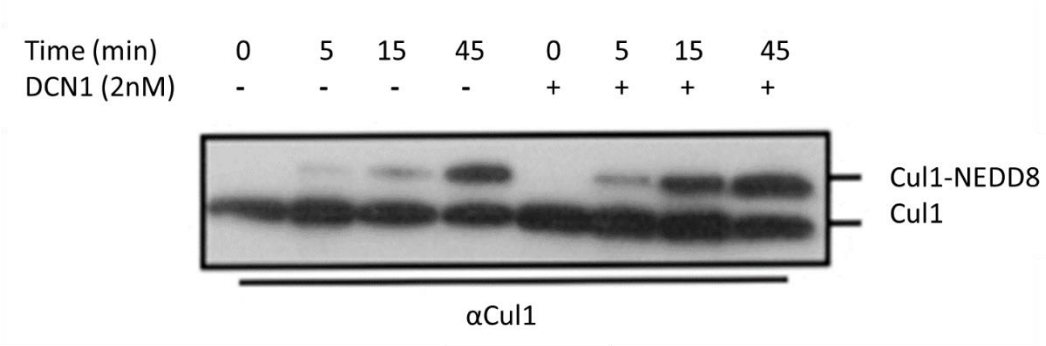

Figure 5 The effect DCN1 has on neddylation. Western blot for Cul1 for a time course with and without DCN1.

Neddylation has been proven to be an effective target for treating lung cancer. A drug called pevonedistat (MLN42924) made it to phase 3 clinical trials as a treatment for NSCLC. This drug targeted the NAE (E1), the first step in neddylation therefore completely obliterated the neddylation pathway. While it was effective at shrinking the tumors, it failed phase 3 trials due to its side effect profile.[5, 6] DCN1 is part of the E3 complex which is more downstream than NAE and neddylation still occurs in the absence of DCN1. We also know that DCN1 is only upregulated in the cancer cells. All of this together gives way to our hypothesis that DCN1 will be a clinically effective treatment for NSCLC while also accomplishing our goal of a less toxic treatment.

The group was able to show that selective small molecule probes can competitively inhibit the protein-protein interactions mediated by N-terminal acetylation. This confirmed the groups therapy that DCN1 would be an effective target.[7]

1.2.2 Previous SAR

The initial High throughput screening library consisted of over 600,000 compounds. Pictured in figure 6 is the structure that the scaffold of this project is based

on, NAcM-HIT. Crystal structures of NAcM-HIT bound to DCN1 were obtained and the structure was further optimized to NAcM-OPT.

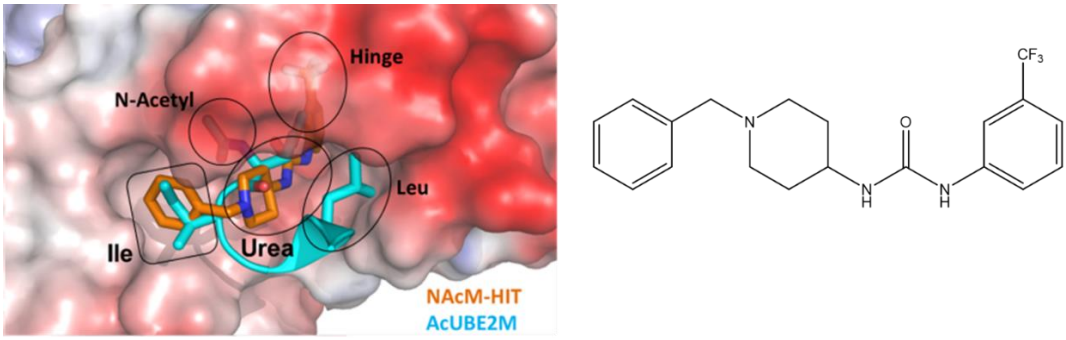

Figure 6 NAcM-HIT bound to DCN1

Through a series of standard structure activity relationship experiments, the group was able to determine what part of NAcM-OPTs structure was important for binding in the DCN1 pocket (Figure 7). This work was completed previously in the Guy lab.[8]

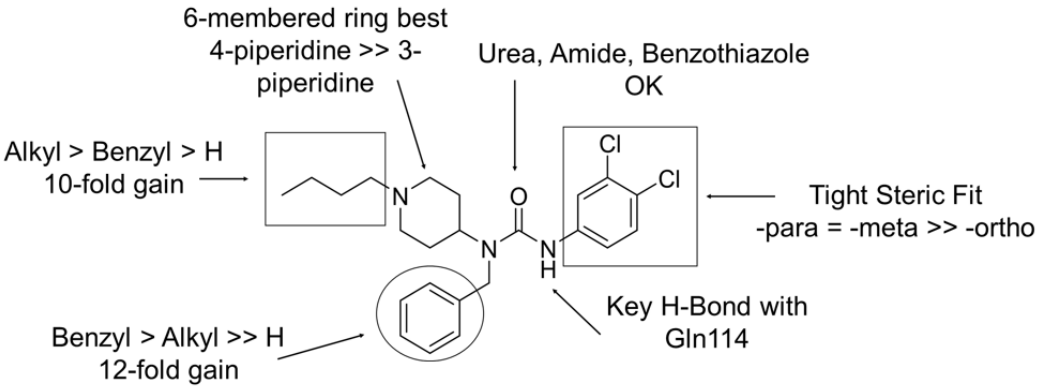

Figure 7 SAR of optimized structure NAcM-OPT

1.2.3 TR-FRET

In order to test the compounds that the lab group made, one of our members designed a screening assay, Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) (figure 8). In the assay, DCN1 is tagged with streptavidin and terbium and UBE2M is tagged with alexafluor. Terbium and alexafluor emit light at different wave lengths when excited. The terbium is excited with a pulse of light, and the energy is transferred to alexafluor. Without the presence of inhibitor, DCN1 and UBE2M are able to interact with each other and the energy can be transferred. When an inhibitor is present, DCN1 and UBE2M are not close enough for the energy to get transferred and the alexafluor is not excited and does not emit light. Because they emit light at different wavelengths, the difference can be measured. With no inhibitor, a high FRET is measured and with inhibitor, a low FRET is measured. When this assay is run, we are looking for a low FRET outcome.

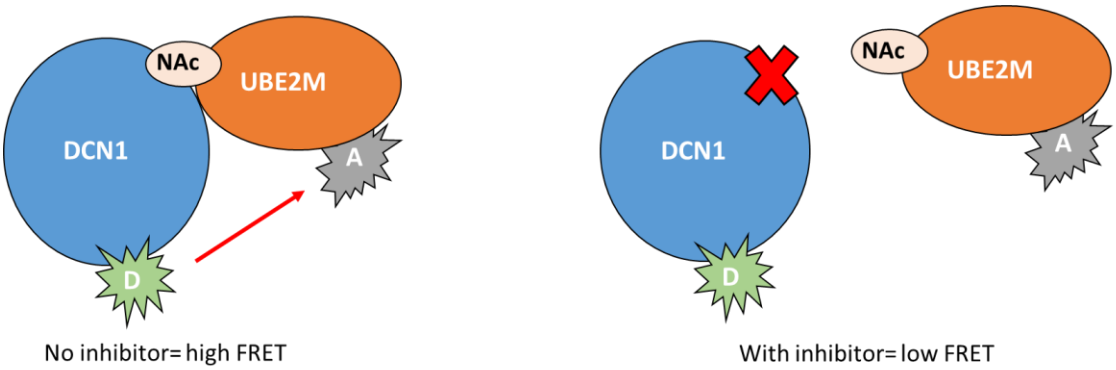

Figure 8 DCN1-UBE2M interaction with and without inhibitor

1.2.4 Problems with NAcM-OPT

NAcM-OPT had great potency to DCN1 with an IC_{50} value of 60nM. However, NAcM-OPT also had a strong binding affinity or hERG with a Ki of 245nM. Human Ether-a-go-go Related Gene (hERG) is a potassium channel that is expressed in the heart and other areas of the body. When drugs bind to hERG, they elongate the heart rhythm and can eventually lead to a fatal arrhythmia called torsade's de pointe. In order to achieve the group's goal of having a safe treatment option, the next steps needed to be to find a way of decreasing binding affinity to hERG while maintaining potency at DCN1. The group used two well-known medicinal chemistry tactics in order to achieve this, the basicity of the nitrogen needed to be reduced and an isoquinoline group was added in place of the benzyl ring to reduce lipophilicity of the molecule (figure 9).[9]

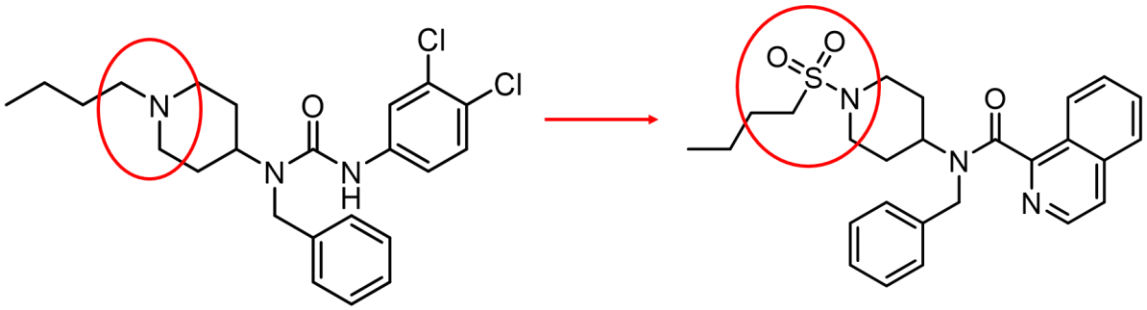

Figure 9 Structural changes made to NAcM-OPT.

The new scaffold was successful in decreasing binding affinity to hERG by reducing the Ki to 8985 nM from 245 nM. However, unfortunately, the new structure also lost potency to bind to DCN1 reducing the IC_{50} value to 1,790 nM from 60 nM. This is where the work discussed in this thesis project starts, with the goal of optimizing binding to DCN1 of the new sulfonamide scaffold while maintaining the low binding affinity to hERG.

The work was split up into three sections based on the different binding pockets. My lab mates, Tucker and Kristen took the leucine and isoleucine pockets respectively. My pocket is the hinge pocket. It is a very small, hydrophobic pocket. My work consists of testing the limits of sterics and electronics of what we can put in the hinge pocket and still have high binding capabilities.

CHAPTER 2. EXPERIMENTAL

2.1 Chemistry

General. All NMR data were collected at room temperature in CDCl3 or MeOD on a 600 Bruker instrument. Solvents and reagents were used directly as obtained from commercial sources unless otherwise specified. Chemical shifts (δ) are reported in parts per million (ppm) with internal CHCl3 (δ 7.26 ppm for 1H and 77.0 ppm for 13C), internal DMSO (δ 2.50 ppm for 1H and 39.5 ppm for 13C), or internal TMS (δ 0.0 ppm for 1H and 0.0 ppm for 13C) as the reference. 1H NMR data are reported as follows: chemical shift, multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, p = pentet, sext = sextet, sep = septet, m = multiplet, dd = doublet of doublets, ddd = doublet of doublet of doublets, dt = doublet of triplets, td = triplet of doublets, tt = triplet of triplets, qd = quartet of doublets), coupling constant(s) (J) in Hertz (Hz), and integration. Flash column chromatography was performed using a Biotage Isolera One and Biotage KP-SIL SNAP cartridges. Purity was assessed by LC/MS/UV using a Waters Acquity UPLC-MS and by NMR spectroscopy. All compounds were confirmed to ≥95% purity prior to testing. Compounds that proved critical to our SAR analysis were further characterized using 1H NMR and HRMS/LRMS.

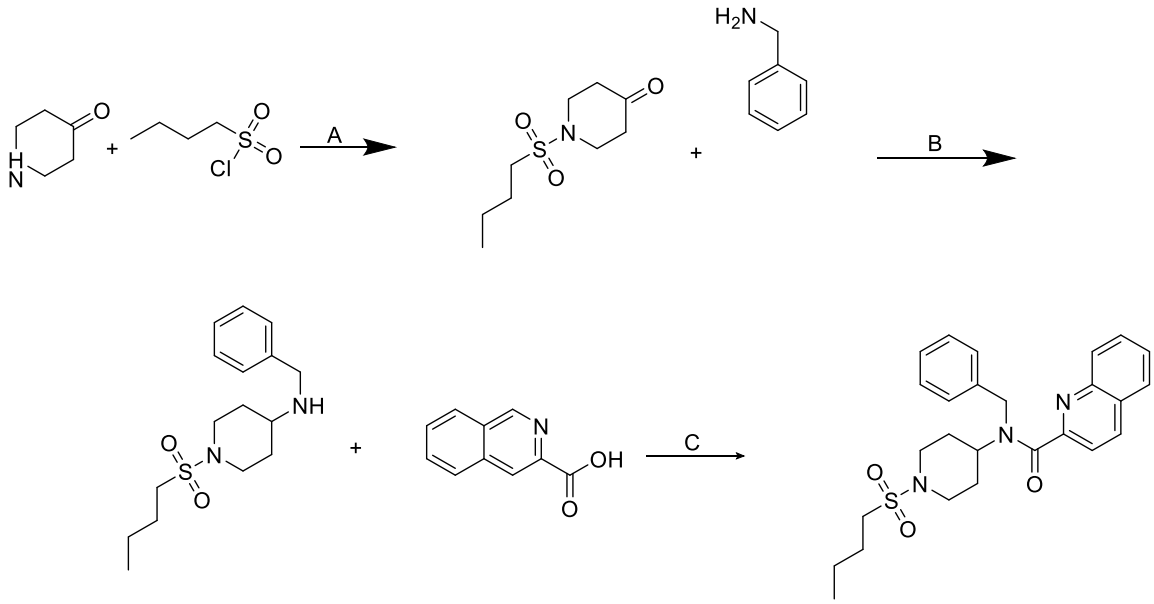

Figure 10 Reaction scheme. A. DIPEA, DCM at room temperature. B. NaBH(OAc)3, AcOH, DCM at room temperature. C. EDCI, HOBt, DIPEA, DMF at 30°C.

LCK_NB01_001: To a stirred solution of Piperidin-4-one hydrochloride (0.250 g, 1.8 mmol, 1 eqiuv.) and DIEA (0.697 mL, 4.0 mmol, 2.2 equiv.) at 0˚ C, 1-butansulfonyl chloride (0.233 mL, 1.8 mmol, 1 equiv.) in DCM (8 mL) was added and stirred at room temperature for 4 h. Saturated sodium bicarbonate solution (equal parts) was added to the reaction mixture. The reaction mixture was extracted with DCM twice and washed with 0.1M HCl, then dried over MgSO4. The residue was used in the next step without purification (figure 10, reaction A).

LCK_NB01_002: A mixture of LCK_NB01_001 (0.417 g, 1.9 mmol, 1 equiv.), benzylamine (0.208 mL, 1.9 mmol, 1 equiv.), and AcOH (0.146 mL, 2.28 mmol, 1.2 equiv.) in DCM (7.0 mL) was stirred at rt for 1 hour, then NaBH(OAc)3 (0.805 g, 3.8 mmol, 2 equiv.) was added, and stirred at room temperature overnight. The reaction mixture was diluted with DCM and washed with 1 N NaOH (aq.), brine, dried over

MgSO4. The residue was used in the next step without purification (figure 10, reaction B).

LCK_NB01_003 (coupling reaction): To a mixture of LCK_NB01_002 (0.590 g, 1.9 mmol, 1. Equiv.), isoquinoline carboxylic acid (0.329 g, 1.9 mmol, 1 equiv.), HOBt (0.349 g, 2.28 mmol, 1.2 equiv.), and DIEA (0.496 mL, 2.857 mmol, 1.5 equiv.) in DMF (9 mL) was added EDCI (0.437 g, 2.28 mmol, 1.2 equiv.) and stirred at 30 °C for 24 h. The mixture was diluted with water, extracted with ethyl acetate, washed with water and brine, dried over MgSO4, and concentrated. MPLC was attempted with ethyl acetate and hexane (12.5-100%) to purify product (figure 10, reaction C).

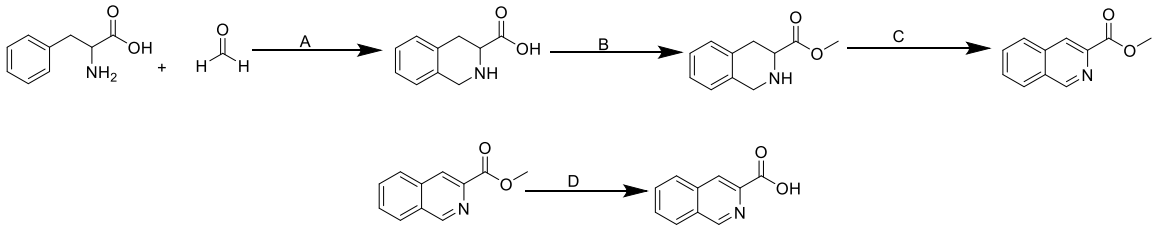

Figure 11 Acid reaction scheme. A. HCl at 90ºC. B. SOCl2, MeOH at reflux. C. Pd/C, DMF, Xylene at reflux. D. 2M LiOH, MeOH at 0ºC, then room temperature.

LCK_NB01_004: To a mixture of phenylalanine (2.00 g, 12.11 mmol, 1 equiv.) and formaldehyde solution (10 mL) was added concentrated hydrochloric acid (20 mL) slowly. The reaction mixture was stirred at 95 C overnight. The reaction mixture was cooled to room temperature and the solid was collected by filtration and washed with water and acetone. The product was then washed with benzene and concentrated two times and washed with anhydrous toluene once to remove water (figure 11, reaction A).

LCK_NB01_005: To a solution of LCK_NB01_004 (0.200 g, 1.13 mmol, 1 equiv.) in methanol (10 mL) was added thionyl chloride (0.247 mL, 3.39 mmol, 3 equiv.) slowly at 0 C under argon. The reaction mixture was allowed to come to room temperature and

was stirred for 24 hrs. The reaction was quenched with water. Dichloromethane was added and saturated sodium bicarbonate solution was used during the extraction, it was washed by brine, dried over magnesium sulfate, filtered, and concentrated. The solid was used in the next step without purification (figure 11, reaction B).

LCK_NB01_010: To a solution of LCK_NB01_007 (0.216 g, 1.13 mmol, 1 equiv.) in DMF (1.106 mL) and xylene (5.501 mL) was palladium on activated charcoal (0.158 g). The reaction mixture was refluxed at 130 ˚C for 48 hours. The reaction mixture was cooled to room temperature and was filtered over celite and concentrated under vacuum. MPLC was used with ethyl acetate: hexanes (12.5-100%) then solvent was switched to methanol: dichloromethane (1-20%) to purify the product (figure 11, reaction C).

LCK_NB01_019: To a solution of LCK_NB01_015 (0.050 g, 0.267 mmol) in methanol (1.67 mL) was added 2M LiOH (1.67 mL). The reaction mixture was stirred at 0 C for 4 hours and then at room temperature overnight. The pH was adjusted to pH 6 using 1 M HCl. The product was extracted with dichloromethane, dried over magnesium sulfate, and concentrated (figure 11, reaction D).

2.2 TR-FRET

The TR-FRET assay was carried out in black 384-well microtiter plates at a final volume of 20 μL per well. To screen compounds, the assay cocktail was prepared as a mixture of 50 nM Biotin-DCN1, 20 nM Ac-UBE2M12-AlexaFluor488, 2.5 nM Tb-Streptavidin (ThermoFisher) in assay buffer (25 mM HEPES, 100 mM NaCl, 0.1% Triton X-100, 0.5 mM DTT, pH 7.5). The assay cocktail was then incubated for 1 h at room temperature and distributed using a WellMate instrument (Matrix). Compounds to

be screened were added to assay plates from DMSO stock solutions by pin transfer using 50SS pins (V&P Scientific). The assay mixture was incubated for 1 h at room temperature prior to measuring the TR-FRET signal with a PHERAstar FS plate reader (BMG Labtech) equipped with excitation modules at 337 nm and emissions at 490 and 520 nm. We set the integration start to 100 μs and the integration time to 200 μs. The number of flashes was set to 100. The ratio of 520:490 was used as TR-FRET signal in calculations. Assay endpoints were normalized from 0% (DMSO only) to 100% inhibition (unlabeled competitor peptide) for hit selection and curve fitting. All compounds were tested in triplicate or more.

CHAPTER 3. RESULTS AND DISCUSSION

3.1 Synthesized and Tested Compounds

Over the course of my time in the lab, I was able to make a total of six molecules (figure 12). To date, only two compounds have been tested, LCK_NB01_013 and LCK_NB01_026 (figure 12). Neither molecule had improved potency over NAcM-OPT. We believe that this is due to the chlorine atom that has been added to the ring. We think that it is too big to fit properly in the hinge binding pocket.

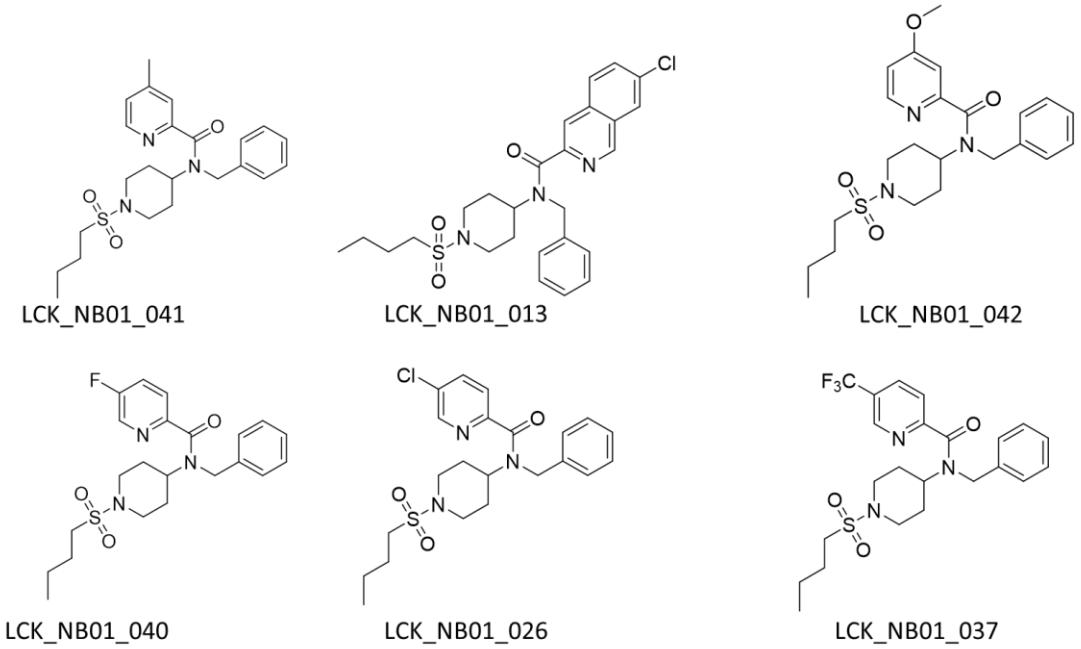

Figure 12 Synthesized compounds.

The molecules above were designed to test the sterics and electronics of the hinge binding pocket. The molecules LCK_NB01_013, LCK_NB01_040, LCK_NB01_026, and LCK_NB01_037 were all designed to improve the metabolism of the molecule. The overall scaffold is metabolized by a cytochrome P450 enzyme which means it goes through a radical intermediate. Radical intermediates are destabilized by adding electron withdrawing groups to the area where the molecules are being acted on by the CYP

enzyme. By adding electronegative atoms, like fluorine and chlorine, we will achieve that goal. Because we were unable to test most of those molecules in the TR-FRET assay, we don't know if they are as potent at DCN1 as we need them to be. The molecules LCK_NB01_041 and LCK_NB01_042 were designed as a test against our theory for the electron withdrawing group. Both methyl and methoxy groups are electron donating, so we believe that the metabolism will be worse. The methoxy group is significantly larger than the methyl group. This allows us to compare the binding of the molecules and form better conclusions about the size limitations of the substitutions on the ring in the hinge pocket. However, LCK_NB01_041 was also designed with the knowledge that a methyl group will make the molecule more hydrophobic. We believe that the addition of a hydrophobic substituent will allow the molecule to fit better and bind tighter in the hydrophobic hinge pocket.

3.2 Picolinic Acids

Because of the slow progress with the isoquinolinic acids, a new direction to the project was approached. The necessity of the isoquinoline group is unknown. In order to test its importance in binding, the group was replaced with a picolinic acid. Those molecules were commercially available and in theory, easy to make. The picolinic acids that were synthesized in figure 12, were very difficult to purify. We believe this is due to the increased lipophilicity of the molecule due to having one less aromatic ring than the isoquinoline. In the future, we believe that recrystallization or reverse phase chromatography would be a viable option to try to better purify these molecules.

3.3 Future Direction

Looking at the crystal structure in figure 13, we can start to think about molecules that we want to test in the future. In the hinge pocket, you can see the key hydrogen bond that we believe is needed for activity. We know that in the place of the nitrogen, we can also place a hydrogen bond donor and still have activity from our previous work. We want to test the limits of where we can place that hydrogen bond donor or acceptor on the molecule.

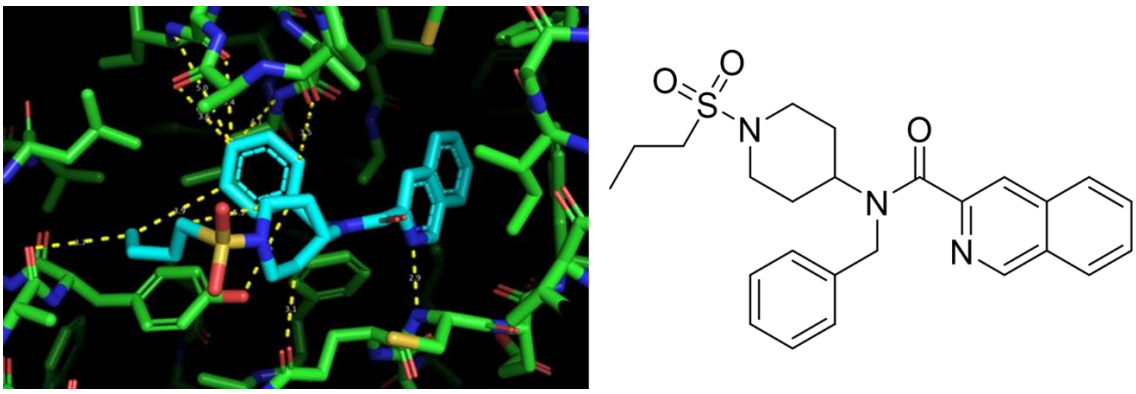

Figure 13 Crystal structure of molecule bound to DCN1

Figure 14 shows proposed molecules to be synthesized in the future to test our hypothesis. We believe that if we can place a hydrogen bond donor and acceptor pair in the right orientation to the binding pocket, it will greatly increase the activity of the inhibitor.

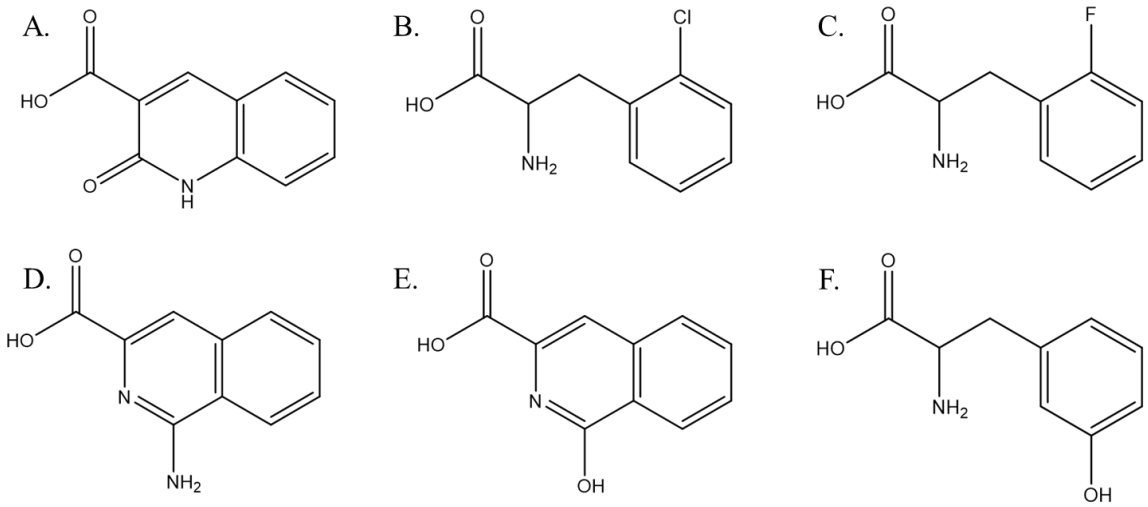

Figure 14 Proposed future molecules

In molecules A, D, and E, there are atoms that can act as both hydrogen bond donors and acceptors. We believe that those molecules have the potential to add an additional hydrogen bond, increasing the potency of the molecule by ten-fold. Molecules B and C we have placed the electronegative atoms on the ring in a way that when then molecule is bound to DCN1, they will be oriented toward the top of binding pocket. We believe that this will allow for better binding because the atoms on the ring will not bump against the bottom of the hinge pocket. Molecule F was designed in order to further test the size of the hinge pocket. The alcohol was chosen as the substituent because of its electron donating nature in order to see how the metabolism of the molecule is affected.

Work has already been started to synthesize the new molecules. The starting materials have been ordered, and the lab group is currently trying to optimize the reaction scheme in figure 11 in order to synthesize the new molecules. We are also working on a more efficient way to purify the picolinic acids. Once we are able to get the compounds more than 95% pure, we will be able to test them using the TR-FRET assay.

As mentioned previously, other members of the lab group are working on different parts of the scaffold in order optimize those binding pockets. The ultimate goal is to combine the different modifications that have been made to the molecule that have improved binding affinity to DCN1 in order to optimize all binding pockets. Once we have optimized the scaffold to the best of our abilities, we will start moving forward with clinical trials.

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VITA

Leah Carmelle Kovalic was born in Lexington, Kentucky and was raised in Winchester, KY. She attended Northern Kentucky University where she earned her Bachelor of Science in Chemistry with a minor in Biology in May of 2019. She began her journey at the University of Kentucky College of Pharmacy in August of 2019 where she decided to pursue a dual degree and obtain a PharmD and Master's of Pharmaceutical Science. Leah will graduate in May of 2023 with her Doctor of Pharmacy and Master of Pharmaceutical Science. She will continue her pharmacy education as a Pharmacy Resident at the Medical University of South Carolina starting in July of 2023.