CHEMICAL PROBES FOR THE LYSINE METHYLTRANSFERASES EZH2 AND G9A:
DESIGN, DEVELOPMENT, AND APPLICATION

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ABSTRACT

KYLE DAVID KONZE: Chemical Probes for the Lysine Methyltransferases EZH2 and G9a: Design, Development, and Application
(Under the direction of Jian Jin)

In eukaryotic genomes, DNA is wrapped around histone proteins to form repeating units known as nucleosomes, which are further condensed into chromosomes. This high level of structure creates a barrier to transcription, which is maintained or reversed via modifications to the N-terminal tails of histone proteins. Histone lysine methyltransferases catalyze the mono-, di-, and/or trimethylation of lysine residues within histone tails; the methylation state of histone tails has profound effects on transcription. For example, polycomb repressive complex 2 (PRC2) is responsible for regulating the methylation status of histone 3 lysine 27 (H3K27) via the catalytic subunit EZH1 or EZH2, and the lysine methyltransferase G9a catalyzes mono- and dimethylation of histone 3 lysine 9 (H3K9). These methyltransferases are of great interest, because trimethylation of H3K27 and dimethylation of H3K9 are transcriptionally repressive marks that play a key role in the progression of many diseases.

Chemical probes that selectively inhibit the methyltransferase of interest are valuable tools to drive further understanding of the biological function of these proteins and assess their potential as therapeutic targets. Here we describe the design, development, and application of chemical probes and tools.
for both EZH1 and EZH2 (UNC1999), and G9a (UNC0965). UNC1999 was the
first orally bioavailable chemical probe of EZH1 and EZH2. This discovery led to
the design of a biotinylated tool (UNC2399), which allows for selective
chemiprecipitation of EZH1 and EZH2 from cellular lysates. UNC1999 is also the
most panactive EZH1 and EZH2 chemical probe to date. We exploited this
feature to demonstrate the potential therapeutic application of small molecule
inhibition of both EZH1 and EZH2 in MLL-rearranged leukemia. Lastly, we detail
the in vitro and ex vivo use of a biotinylated G9a inhibitor (UNC0965) in a
chemical-based chromatin immunoprecipitation (chem-ChIP) assay for studying
G9a chromatin occupancy.
DEDICATION

For Mrs. Parul Kirit Bazaz. To this day I still think of your impact on my life, and how fortunate I am that you were part of it. Thank you for being the spark of science that ignited my passion for research; may you rest in peace.
ACKNOWLEDGEMENTS

I first and foremost want to thank my family. My father, Lance Edward Konze, for the influence he has had in my life. He taught me so many things that are part of the reason I am here today: never give up, don’t let anger or emotions get the best of you, and above all, “heart isn’t measured on the scoreboard.” Thank you to my mother, Jodie Kay Kulla, for her endless love and support, and continued encouragement to chase my dreams. Thanks to my step-father, Tim Kulla, and my wonderful half-but-whole, brothers and sisters, Austin Kulla, Jenna Kulla, Aaron Kulla, and Logan Konze for keeping things interesting. Thank you to my Grandfather, Loren Schulenberg, for teaching me how to grip a golf club, and to my Grandmother, Lee Schulenberg, for being the sweetest person on the face of the earth. I also want to thank my Grandmother and Grandfather Foley for always having their door open. Last, but not least, I want to thank my beautiful fiancée, Jordan Smith, for not getting too angry with me for staying up late working on this dissertation, and always being there for me after a bad day at lab. And how can I forget Tubby, the greatest dog, and best friend, a person could ask for; watching him chase down a tennis ball always helps to keep things in perspective.

In addition to my family, there were numerous teachers that had an irreplaceable impact on my life. Above all is Mrs. Parul Kirit Bazaz; she once told me to smile more, and so now, I do. I want to thank my kindergarten teacher from
Centerville Elementary, Mr. George Burr, he played an integral part in molding me into what I am today. My sixth grade teacher at Edinbrook Elementary, Mr. Dan Rice, had the largest impact on teaching me how to control and focus my anger into positive actions. I want to thank my Math teachers in both my seventh and eighth grade classes at Brooklyn Junior High for telling me that I didn’t have what it takes to move up to the higher level math classes. They were wrong, and the chip they carved into my shoulder has helped fuel me all the way to a Ph.D. I want to thank my 12th grade Pre-Calculus teacher, Mr. Marshall Thompson, and my 12th grade Statistics teacher Mr. Ed Dillon for keeping things fun, and tolerating my misbehavior from time-to-time.

I also want to thank all of my previous employers that have helped me get to where I am today. Raymond Hawk gave a 15-year old version of myself a chance to make some money washing dishes on the weekends. In the process, he taught me the value of hard work, and became one of my very best friends. Dr. Samuel Yang gave me my first science job in my second year of undergrad, and helped me get my foot in the science door. Dr. Yuk Yin Sham holds a very special place in my heart. I truly believe that I would not have applied to the Chemical Biology and Medicinal Chemistry (CBMC) program at UNC had he not offered me a chance to work on computer-aided drug design. He opened my eyes, and introduced me to a branch of science that I did not even know existed, and for that, I am forever grateful.

I also want to thank all of my friends, without you guys, I would have gone insane at this point. First my closest and best friends from Minnesota: Sam
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I next want to thank my dissertation committee for their guidance, and continued support. My advisor Dr. Jian Jin has a door that doesn’t close, and his constant feedback has taught me how to go about solving problems the correct way. I am truly grateful that Jian allowed me to join his lab three-and-a-half short years ago; the time absolutely flew by. My committee chair, Dr. Michael Jarstfer, has always provided wonderful insight into problems that have occurred throughout my research; he is a constant supply of terrific, thought-provoking questions at my committee meetings. I also owe him even more, as he was the Director of Graduate Admissions when I was applying to graduate school, and he most certainly played a large role in offering me a position at UNC, and was also a large part of the reason why I decided to attend. I want to thank Dr. Stephen Frye for setting up such a high-class environment for graduate students to work in, and allowing me to be one of those graduate students. The Center for Integrative Chemical Biology and Drug Discovery (CICBDD) truly is a unique setting, and I was very fortunate to have the chance to work within the walls. I thank Dr. Greg Wang for being an outstanding member of my committee, and a wonderful collaborator whom is a joy to work with; he has provided me with
multiple opportunities to publish papers, for that, I am very fortunate and grateful. And last, but not least, I want to thank Dr. Brian Strahl for being an approachable face in the Genomic Medicine Building, and providing wonderful insight and questions at my meetings, which really helps one figure out what they don’t know, and need to improve upon.

I want to thank all of the current and past members of the Jin lab, especially Dr. Feng Liu and Dr. Anqi Ma. Prior to joining the Jin lab, the only organic synthesis that I had performed was in organic chemistry lab. If Anqi and Feng hadn’t taken me under their wing and taught me the right way to do things, who knows what would have happened. I would also like to articulate my appreciation for all the people that I have worked with at CICBDD. This highly collaborative environment served as a catalyst for my learning. Above all, I want to thank Dr. Samantha Pattenden for her guidance in all things biology, without her I would have been banging my head on the wall far more often, and much harder. She taught me many different techniques, and underscored the importance of good notebook keeping, and honest data analysis, which are skills that I will carry with me far beyond graduate school. I also want to thank Dr. Jacqueline Norris-Drouin, Dr. Brandi Baughman, and Stephanie Cholensky for allowing me to constantly be in their space while I was working on my biological experiments.

I have truly enjoyed my time at the University of North Carolina, and my graduate experience far exceeded my expectations. I am so thankful that I had the chance to be a part of something as unique as the CBMC program. It allows
our students to see all possibilities in the world of drug discovery, and prepares us very well for the future. As such, I would like to thank the entire CBMC faculty for the work that they put into the curriculum, and the time they invest in their students. And finally, I would like to thank Dr. Fred Eshelman, the UNC Eshelman School of Pharmacy, grants awarded to Jian Jin, and the Medicinal Chemistry Division of the American Chemical Society, for their financial support, which made this research, and my graduate career, a possibility.
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<th>Ac</th>
<th>acetylation</th>
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<tr>
<td>AEBP2</td>
<td>AE Binding Protein 2</td>
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<td>AML</td>
<td>acute myeloid leukemia</td>
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<td>bone marrow</td>
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<td>base pair</td>
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<td>cyclin dependent kinase inhibitor 2a</td>
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<td>chemical inhibitor-based chromatin immunoprecipitation</td>
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<td>chromatin immunoprecipitation</td>
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<td>maximum plasma concentration</td>
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<tr>
<td>EC50</td>
<td>concentration to achieve 50% of desired effect</td>
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<td>embryonic ectoderm development</td>
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<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>green fluorescent protein</td>
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<td>GLP</td>
<td>G9a-like protein</td>
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<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>GlaxoSmithKline</td>
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<td>HPLC</td>
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<tr>
<td>IC50</td>
<td>concentration to achieve 50% inhibition</td>
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<td>ICW</td>
<td>in-cell western assay</td>
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<td>IF</td>
<td>immunofluorescence</td>
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<td>immunoglobulin G</td>
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<td>IP</td>
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<td>Jumonji AT-rich interactive domain 2A</td>
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<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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<tr>
<td>min</td>
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<td>Acronym</td>
<td>Description</td>
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<td>MLL</td>
<td>Mixed Lineage Leukemia</td>
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<td>MPN</td>
<td>myeloproliferative neoplasms</td>
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<td>MS</td>
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<tr>
<td>NET</td>
<td>Norepinephrine transporter</td>
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<td>NIMH</td>
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<td>Psychoactive Drug Screening Panel</td>
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<td>primary myelofibrosis</td>
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<td>per os</td>
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<td>Polycomb Repressive Complex 2</td>
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<td>post-translational modification</td>
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<td>S-Adenosyl methionine</td>
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<td>SET</td>
<td>Su(var)3-9 and enhancer of zeste</td>
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<td>spleen</td>
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<td>Suz12</td>
<td>suppressor of zeste 12</td>
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<td>tissue inhibitor of metalloproteinase</td>
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<td>UNC</td>
<td>University of North Carolina</td>
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<td>white blood cell</td>
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CHAPTER I: INTRODUCTION

Histone Lysine Methyltransferases

The eukaryotic genome is organized into 23 pairs of chromosomes, which are dense structures consisting of DNA and proteins. In order to package the entirety of the eukaryotic genome into an average 10 µm diameter cell, DNA and proteins are wrapped around histones for compaction. This DNA-protein amalgam is referred to as chromatin. The basic units of chromatin, nucleosomes, are comprised of DNA wrapped around 4 sets of dimeric histone proteins (H2A, H2B, H3, and H4), which have long tails protruding from their core (figure 1.1). Histone H1 is also involved in this compaction and serves as a sort of “cap” to keep the DNA wrapped around the nucleosome. DNA is wound around these proteins in ~147 bp segments, which limits the accessibility of genes contained within. As such, the dense environment of chromatin is a natural barrier to transcription, yet the genetic information encoded by DNA needs to be readily accessible for cells to respond to external and internal stimuli. Modifications to the globular domain, and tail, of histone proteins affect the state of chromatin by either promoting or deterring compaction, which makes the DNA more or less accessible to transcriptional machinery, and leads to a cascade of downstream
events [1]. Regions of high density, termed heterochromatin, are less accessible to transcriptional machinery, whereas regions that are not as compact, termed euchromatin, are more accessible, and correlate with more actively transcribed regions. Alterations in gene activity, which are not due to changes in the DNA sequence, are referred to as epigenetics (“above genetics”).

Modifications to histones are maintained via three main families of epigenetic proteins: “writers” (the enzymes responsible for producing post-translational modifications (PTMs)), “readers” (the proteins responsible for binding PTMs), and “erasers” (the enzymes responsible for removing PTMs). These proteins work in concert to dynamically regulate the chromatin landscape, controlling the temporal expression of genes in the process. One class of “writers” is protein lysine methyltransferases, which are capable of catalyzing mono-, di-, and/or trimethylation of lysine residues on histone and non-histone substrates. Most lysine methyltransferases (excluding DOT1L) catalyze the methylation of a given lysine residue by utilizing their Su(var)3-9 and enhancer of zeste (SET) domain, and the endogenous methyl donor S-Adenosyl methionine (SAM). The products of a methyl transfer from the sulfonium ion of SAM are S-Adenosyl homocysteine (SAH) and methylated lysine (Figure 1.1). Even a small chemical modification like the methylation of a lysine residue has a profound effect on transcriptional activity. For example, trimethylation of lysine 4 on histone H3 (H3K4me3) corresponds to regions of active transcription, whereas dimethylation of histone 3 lysine 9 (H3K9me2) or trimethylation of histone 3 lysine 27 (H3K27me3) correspond to regions of silenced, less transcriptionally
active chromatin. Dysregulation of these processes often occurs in many developmental diseases and maladies. Hypertrimethylation of H3K27 is present in many cancers, and usually correlates with a late-stage metastatic disease with poor prognosis. Although, lysine residues within each histone are structurally similar, specific enzymes are responsible for their maintenance.

**Polycomb Repressive Complex 2**

The methylation state of lysine residues on histone proteins is modulated by two classes of proteins: trithorax and polycomb proteins. Trithorax group proteins serve to maintain expression via methylation of specific lysines. For example, the Mixed Lineage Leukemia (MLL) (Figure 1.2, MLL-MLL4) proteins catalyze the mono-, di-, and/or trimethylation of H3K4. And H3K4me3 is associated with the 5’ regions of nearly all actively transcribed genes. Further, H3K4me3 is highly correlated with histone acetylation, high rates of transcription, and polymerase II occupancy [2, 3]. In contrast to trithorax group proteins, polycomb group proteins function to repress gene expression. For example, Polycomb Repressive Complex 2 (PRC2) catalyzes the di- and trimethylation of H3K27, which leads to a repressive chromatin state, shutting down expression of genes upstream of H3K27me3 containing promoters.

As the name implies, PRC2 is a multi-component complex that contains four essential core components for catalytic activity: enhancer of zeste homolog 1 or 2 (EZH1 or EZH2) (Figure 1.2), embryonic ectoderm development (EED), suppressor of zeste 12 (Suz12), and retinoblastoma protein-associated protein
48 and 46 (RbAp48/46). EZH1 or EZH2 are the catalytically active subunits, which utilize their evolutionarily conserved SET domain to methylate H3K27me1 or H3K27me2 to produce H3K27me2 or H3K27me3, respectively [4]. EED and RbAP46/48 contain repeats of the WD40 domain, which facilitates binding to H3K27me3 via the tryptophan (W) and aspartic acid (D) rich domains [5, 6]. Suz12 contains a zinc-finger domain necessary for DNA binding, and a VEFS domain necessary for interaction with EZH1 or EZH2; Suz12 is believed to function as a facilitator of DNA-binding for PRC2 [7, 8].

While the afore mentioned four core components of PRC2 are absolutely necessary for PRC2 function, and exhibit embryonic lethality when deleted or silenced in mouse embryos, there are several other proteins that are known to interact with PRC2 in a cell- and tissue-specific manner. These include, but are not limited to: AEBP2, which enhances PRC2 enzymatic activity at certain genes [9], PCL1, PCL2 and PCL3, which are tudor domain containing proteins that co-localize with PRC2 at specific promoters [10], and JARID2A a Jumonji family demethylase capable of demethylating H3K27 [11]. PRC2 has also been shown to interact with various other epigenetic machineries like histone deacetylases, and other histone methyltransferases, but their effect on PRC2 function is less understood [5]. Altogether, PRC2 plays an essential role in development and stem cell fate in normal healthy organisms, but dysregulation of H3K27me3 via overexpression or gain-of-function EZH2 mutations is a major occurrence in the progression of many cancers, ranging from solid tumors like breast [12, 13] and prostate [14], to blood tumors including lymphoma [15], leukemia [16], and
myeloma [17]. To further complicate matters, EZH2 has been described as both a tumor suppressor [18] and tumor promoter [16]. This paradox highlights the need for the development of chemical probes to study the mechanisms of EZH1 and EZH2 (EZH1/EZH2) catalytic activity.

**Role of EZH2 and EZH1 in Disease Progression**

As mentioned earlier, the catalytically active subunit of PRC2 is either EZH1 or EZH2. EZH1/EZH2 are highly homologous, mutually exclusive members of PRC2 that share 76% overall sequence identity, and 96% sequence identity in the catalytic SET domain [19]. Despite being highly homologous, EZH1/EZH2 are differentially expressed; EZH2 is present in poorly differentiated and actively dividing cells, whereas EZH1 is expressed in all cells [5]. In adult hematopoietic stem cells *EZH1* deletion leads to defects in self-renewal and quiescence that is not seen upon *EZH2* deletion, demonstrating that these two proteins differ functionally as well [20]. PRC2-EZH1 complexes are less catalytically active than PRC2-EZH2 complexes [21], but this lack of catalytic activity may be supplemented by the cooperative effect of PRC2 complexes containing EZH1 (PRC2-EZH1) and those containing EZH2 (PRC2-EZH2). For example, the nucleosome binding ability of PRC2-EZH1 leads to increased activity of PRC2-EZH2 complexes [22]. However, PRC2-EZH1 complexes still retain catalytic activity, so their sole purpose is not simply a scaffolding function. These observations are just the “tip of the iceberg” in terms of what we have to learn about EZH1/EZH2 biology.
Overexpression of EZH2 was first linked to the progression of prostate cancer merely twelve years ago [14]. Since that time, aberrations in both EZH2 expression and catalytic activity have been attributed to highly invasive cancers with poor prognosis. In solid tumors, overexpression of EZH2 leads to hypertrimethylation of H3K27 and subsequent repression of genes encoding proteins important for cell cycle regulation, cell-cell contacts, and cell-matrix adhesions [23, 24]. In blood tumors, like MLL-rearranged leukemia, both EZH1 and EZH2 have been shown to contribute to disease progression. MLL-rearranged leukemia result in aberrantly active proteins produced from chromosomal translocations of MLL and a host of transcription factors (e.g., AF4, AF9, ENL, PTD) [25]. These translocations lead to unnecessary recruitment of machinery responsible for transcriptional elongation and increased expression of key leukemic genes Hoxa and Meis1 [26]. The MLL-AF9 fusion complex contributes to the initiation of acute myeloid leukemia (AML), but cancer is not a one step process [27, 28], and MLL-AF9 is not the sole contributor to the AML phenotype. Recently, a MLL-AF9 AML mouse model demonstrated that EZH2−/− mice retain H3K27me3 at distinct loci [16]. This finding suggests a role for both EZH1/EZH2 in the progression of MLL-AF9 leukemia. Indeed, EZH1 has been shown to compensate for the function of EZH2 [29, 30] and plays a role as a regulator of hematopoietic neoplasms [16, 31].
Current Chemical Probes for EZH2

The identification of EZH2 overexpression in various human cancers has led the community to propose EZH2 as an attractive drug target for therapeutic intervention [25], and has inspired several pharmaceutical companies to endeavor on high-throughput screening campaigns for inhibitors of EZH2. Independent efforts by Epizyme, GlaxoSmithKline (GSK), and Novartis revealed a structurally similar, cofactor-competitive, small molecule, which potently suppresses EZH2-catalyzed H3K27me3 [32-38]. These independent hits were then optimized via traditional medicinal chemistry to enhance the drug-likeness of these compounds.

The first two published EZH2 inhibitors are EPZ005687 [33] and GSK126 [32] (Table 1.1), which potently inhibit wild type and lymphoma-associated gain-of-function EZH2 mutants. Additionally, both of these compounds are highly selective for EZH2 over a range of methyltransferases, kinases, and GPCRs. EPZ005687, from Epizyme, was not suitable for in vivo studies, but it was an essential tool compound for target validation and overall study of EZH2 biology. GSK126, from GSK, demonstrated in vivo potency via IP administration [32]. These compounds share very similar pharmacophoric features, and are fairly selective for EZH2 versus EZH1 (50-150 fold selectivity, Table 1.1), demonstrating the high specificity of these compounds. Soon after the disclosure of GSK126, GSK343 was published with similar potency against EZH2, but differs from GSK126 in that it contains an indazole core and several different substitutions such as the piperazine-substituted pyridine (Table 1.1). EI1 (Table
1.1) is a compound that was optimized from a high-throughput screening campaign hit at Novartis [34]. El1 has similar structural features and selectivity as EPZ005687 and GSK126, but did not display any in vivo activity. UNC1999 (Table 1.1) represents the first orally bioavailable inhibitor of EZH2, and is the most panactive EZH1/EZH2 inhibitor to date [35]. UNC1999 shares similarity in structure to GSK343, differing only in the substitution of the pyridine, and the capping group of the piperazine. However, this small modification has a large effect on the pharmacokinetic properties of the compound, and the EZH1/EZH2 selectivity. This EZH1/EZH2 panactivity poses a potential advantage for UNC1999 in cancer cells that rely on both EZH isoforms, such as MLL-rearranged leukemia [16, 39]. Shortly after the disclosure of UNC1999, Epizyme released an orally bioavailable derivative of EPZ005687: EPZ-6438 [36] (Table 1.1). Unlike previously published compounds that contain a bicyclic core, EPZ-6438 has a monocyclic phenyl core. The only non-pyridone containing inhibitor of EZH2 is Constellation Pharmaceuticals compound 3 (Table 1) [37]. This compound is not suitable for in vivo studies, and shows about 10-fold selectivity for EZH2 versus EZH1. All of these inhibitors demonstrate high potency and high selectivity towards wild-type EZH2 and its lymphoma-associated mutant forms [32-38] (Table 1.1).

Summary and Significance

As detailed above, the pharmaceutical industry has already begun to target EZH2 inhibition as a potential treatment for blood tumors like lymphomas
and leukemia. However, as a relatively nascent target in the field of drug discovery, there is a lack of complete knowledge of PRC2 function, and this may present a barrier to success in the clinic. For example, it is well-received that EZH2 plays a role in the progression of prostate cancer, and that overexpression of EZH2 in this context correlates with late-stage, metastatic disease [14, 40]. Contrarily, EZH2 also appears to have a tumor suppressor role, because EZH2 deletion has been observed in the development of myelodysplastic syndrome (MDS) [18, 41]. This observation is compounded by the fact that both EZH2 and EZH1 are regulators of hematopoietic stem cell function [20, 42]. Even within the realm of what is known about PRC2, the effects of complexes containing either EZH2 or EZH1 are still confounding. Suffice to say; much more research is to be conducted on PRC2 biology before it can be certain that it is a viable therapeutic target.

Until recently, the main methods used to study the role of PRC2 were genetic knockouts (KOs) or RNA interference (RNAi) of PRC2 components. These methods have many disadvantages: embryonic lethality, loss of complex integrity, residual expression, and lack of translation to animal studies. Complete removal of a protein from a biological system often results in a different physiological response than inhibition of catalytic activity with a small molecule, because many proteins have scaffolding functions apart from their catalytic activity, which are required for other biological processes. Small molecule chemical probes [43], that solely target the catalytic function of an enzyme are superior to KO and RNAi, because the protein is still produced at physiological
levels. However, early chemical probes of EZH2 were not orally bioavailable [32, 44], complicating clinical translation, and current probes do not display as potent EZH1 inhibition, which can be beneficial in certain diseases [39]. The lack of a panactive EZH1/EZH2 inhibitor further highlights the need for the development of more advanced chemical probes.

In solid tumors like ovarian and prostate cancer, it is known that EZH2, rather than both EZH1/EZH2, is the main contributor to disease progression. Therefore, a selective inhibitor of EZH2 in these cases would be more beneficial than a panactive EZH1/EZH2 compound. To establish selective EZH2 inhibition we plan to create an irreversible inhibitor of EZH2 by targeting a cysteine residue near our hypothesized binding site. Importantly, this cysteine residue is a serine residue in EZH1 which will allow for selective covalent engagement of EZH2. This finding will have profound impact on the field, as it may provide: 1) the most selective EZH2 inhibitor to date, 2) further evidence for our previously proposed binding hypothesis, and 3) the first EZH2-inhibitor co-crystal structure.
### Tables

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**Table 1.1. Summary of current EZH2 inhibitors.**

ND = no data; nM = nanomolar
Figure 1.1: Graphical representation of histones.

DNA (black) is coiled around histone proteins (large circles) for compaction. The histone proteins are composed of 4 sets of homodimeric subunits (H2A (green), H2B (yellow), H3 (red) and H4 (blue)) and an additional monomeric H1 subunit (not pictured). Lysines on histone tails (pictured on H3 tail only for simplicity) are modified by lysine methyltransferases via donation of a methyl group from SAM; trimethylated H3K27 is pictured.
Figure 1.2: Phylogenetic tree of lysine methyltransferases.

There are approximately 60 protein methyltransferases in the human genome. These are composed of both lysine and arginine methyltransferases, which have histone and non-histone substrates. The lysine methyltransferases of interest in the research presented herein are EZH1 and EZH2 (red box), G9a and GLP (blue box) and MLL1-4 (green box).
CHAPTER II: UNC1999 IS AN ORALLY BIOAVAILABLE INHIBITOR OF THE LYsINE METHYLTRANSFERASE EZH2

Introduction

Due to the impact of chromatin modifications on genetic expression, epigenetic “writers” (the enzymes responsible for producing post-translational modifications (PTMs)), “readers” (the proteins responsible for binding PTMs), and “erasers” (the enzymes responsible for removing PTMs) have gained increasing interest as an important class of drug targets [45, 46]. One “writer” of specific interest is the lysine methlytransferase EZH2, which is the catalytic component of PRC2. PRC2 is responsible for the methylation status of H3K27; H3K27me3 correlates with regions of repressed expression, and the process of monitoring this methylation state often goes awry in many cancers. As such, EZH2 is an attractive target for potential therapeutic intervention.

Recent advances in EZH2 inhibitor development have been spearheaded by Epizyme, GSK and Novartis. Specifically, independent high-throughput screening campaigns at all three of these companies identified small molecules capable of potent EZH2 inhibition. These hits were optimized via traditional medicinal chemistry methods to enhance the drug-likeness of the identified compounds, which resulted in the discovery of EPZ005687, GSK126, and El1.
(Table 1.1). These inhibitors were very potent in *in vitro* and cellular assays, but only GSK126 displayed any *in vivo* activity, and none were orally bioavailable.

Herein, we report the design and development of UNC1999, the first orally bioavailable inhibitor of EZH2. In addition to optimizing the physicochemical properties of this class of EZH2 inhibitors, we also designed several compounds that serve as additional tools for studying EZH2 biology. UNC2400 is a structurally similar, yet inactive, analog of UNC1999 that serves as a negative control. UNC2399 is a biotinylated derivative of UNC1999 that is capable of chemiprecipitating EZH2 from cell lysates. UNC2239 is a UNC1999 derivative that contains a cell penetrant dye capable of engaging EZH2 in cells. These four compounds together, help to validate our proposed binding hypothesis for UNC1999, and also provide a useful chemical toolbox for the research community to accelerate further study of EZH2 biology.

**Methods**

**Chemistry General Procedures**

HPLC spectra for all compounds were acquired using an Agilent 6110 Series system with UV detector set to 254 nm. Samples were injected (5 µL) onto an Agilent Eclipse Plus 4.6 x 50 mm, 1.8 µM, C$_{18}$ column at room temperature. Method 1: A linear gradient from 10% to 100% B (MeOH + 0.1% acetic acid) in 5.0 min was followed by pumping 100% B for another 2 minutes with A being H$_2$O + 0.1% acetic acid. Method 2: A linear gradient from 50% to 100% B (MeOH + 0.1% acetic acid) in 5.0 min was followed by pumping 100% B for another 2
minutes with A being H₂O + 0.1% acetic acid. The flow rate was 1.0 mL/min.
Mass spectra (MS) data were acquired in positive ion mode using an Agilent 6110 single quadrupole mass spectrometer with an electrospray ionization (ESI) source. Nuclear Magnetic Resonance (NMR) spectra were recorded using a Varian Mercury spectrometer with 400 MHz for proton (¹H NMR) and 100 MHz for carbon (¹³C NMR); chemical shifts are reported in ppm (δ). Preparative HPLC was performed on Agilent Prep 1200 series with UV detector set to 254 nm. Samples were injected onto a Phenomenex Luna 75 x 30 mm, 5 µM, C₁₈ column at room temperature. The flow rate was 30 mL/min. A linear gradient was used with 10% (or 50%) of MeOH (A) and 0.1 % TFA in H₂O (B) to 100% of MeOH (A). HPLC was used to establish the purity of target compounds, all compounds had > 95% purity using the HPLC methods described above unless noted otherwise. High-resolution (positive ion) mass spectrum (HRMS) for compound UNC1999 was acquired using a Thermo LTqFT mass spectrometer under FT control at 100000 resolution.
**Compound Synthesis**

Scheme 2.1. Reagents and conditions: (a) K$_2$CO$_3$, NMP (1-methyl-2-pyrrolidinone), 120°C, 50%; (b) 4,4,4',4',5,5,5',5'-octamethyl-2,2'-bi(1,3,2-dioxaborolane), KOAc, 1,4-dioxane, Pd(dppf)Cl$_2$·CH$_2$Cl$_2$, 80°C, 55%; (c) KOAc, 1,4-dioxane, H$_2$O, Pd(dppf)Cl$_2$·CH$_2$Cl$_2$, reflux, 78%; (d) HCl (aq), rt, 95%; (e) formaldehyde solution (37 wt.% in H$_2$O) / acetaldehyde / acetone, NaBH$_3$CN, AcOH, MeOH, 0°C - rt, 48-86%; (f) bis(pinacolato)diboron, KOAc, Pd(dppf)Cl$_2$·CH$_2$Cl$_2$, 1,4-dioxane, 80°C, Ar; (g) 2-bromo-5-fluoropyridine, Pd(PPh$_3$)$_4$, K$_2$CO$_3$, 1,4-dioxane, H$_2$O, 90°C, 61% (two steps); (h) K$_2$CO$_3$, (4-fluorophenyl)boronic acid, Pd(PPh$_3$)$_4$, 1,4-dioxane, H$_2$O, microwave, 150°C, 73%.

**tert-Butyl 4-(5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-2-yl)piperazine-1-carboxylate.** 5-Bromo-2-chloropyridine (10 g, 52.0 mmol), tert-
butyl piperazine-1-carboxylate (29 g, 156 mmol), and potassium carbonate (22 g, 156 mmol) were mixed in NMP (50 mL) and heated to 120°C overnight. The mixture was then cooled to room temperature and diluted with water until precipitate was observed. The solid was collected by filtration, washed with water and dried under vacuum to give tert-butyl 4-(5-bromopyridin-2-yl)piperazine-1-carboxylate as a white solid (9 g, 50% yield). tert-Butyl 4-(5-bromopyridin-2-yl)piperazine-1-carboxylate (5 g, 14.6 mmol), 4,4,4',4',5,5,5',5'-octamethyl-2,2'-bi(1,3,2-dioxaborolane) (4.46 g, 17.6 mmol), and potassium acetate (2.15 g, 22.0 mmol) were mixed with 1,4-dioxane (120 mL) in a flask. To this mixture, 1,1'-bis(diphenylphosphino)ferrocene-palladium(II)dichloride dichloromethane complex (600 mg, 0.73 mmol) was added under argon atmosphere at room temperature. The mixture was heated to 80°C for 5 h and cooled to room temperature. The resulting mixture was then diluted with EA and washed with water. The combined organic layers were dried over sodium sulfate. After concentration in vacuo, the crude product was purified by silica chromatography (Hex/EA = 5/1) to afford the title compound as a white solid (3.2 g, 55% yield). ¹H NMR (300 MHz, CDCl₃), δ: 8.56 – 8.51 (m, 1H), 7.83 (dd, J = 8.6, 1.9 Hz, 1H), 6.59 (d, J = 8.5 Hz, 1H), 3.60 (dd, J = 6.6, 3.2 Hz, 4H), 3.52 (dd, J = 6.7, 3.2 Hz, 4H), 1.48 (s, 9H), 1.31 (s, 12H).

6-Bromo-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide. This compound was synthesized according to the procedures reported previously. [47]
1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-6-((6-piperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide (2.1). 6-Bromo-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (1 g, 2.3 mmol), tert-butyl 4-(5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-2-yl)piperazine-1-carboxylate (965 mg, 2.48 mmol), and potassium acetate (680 mg, 6.90 mmol) were mixed with 1,4-dioxane (20 mL) and water (5 mL). 1,1’-bis(diphenylphosphino)ferrocene-palladium(II)dichloride dichloromethane complex (190 mg, 0.23 mmol) was added to the mixture under argon atmosphere at room temperature. The resulting mixture was heated to reflux for 5 h, then cooled and diluted with EA. The mixture was washed with water, dried over sodium sulfate and concentrated in vacuo. The crude product was purified by silica chromatography (DCM/MeOH = 40/1) to give tert-butyl 4-(5-(1-isopropyl-4-(((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)carbamoyl)-1H-indazol-6-yl)pyridin-2-yl)piperazine-1-carboxylate as a white solid (1.1 g, 78% yield). This product was then dissolved in a saturated solution of HCl in EA. The mixture was stirred at room temperature overnight. The solvent was removed in vacuo to give the title compound 2.1 as a yellow solid (1 g, 95% yield). 1H NMR (400 MHz, d4-MeOH), δ: 8.49 (d, J = 2.3 Hz, 1H), 8.35 (s, 1H), 7.93 (dd, J = 8.9, 2.6 Hz, 1H), 7.88 (s, 1H), 7.75 (d, J = 1.2 Hz, 1H), 6.84 (d, J = 8.9 Hz, 1H), 6.10 (d, J = 0.6 Hz, 1H), 5.06 (dt, J = 13.3, 6.6 Hz, 1H), 4.58 (s, 2H), 3.52 (dd, J = 6.0, 4.3 Hz, 4H), 2.91 (dd, J = 5.9, 4.3 Hz, 4H), 2.76 – 2.67 (m, 2H), 2.24 (s, 3H), 1.62 (td, J = 15.0, 7.5 Hz, 2H), 1.56 (t, J = 5.7 Hz, 6H), 1.00 (t, J = 7.3 Hz, 3H).
1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-6-(6-(4-methylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide (2.2). Compound 2.2 was prepared according to previously published procedures. [47]

6-(6-(4-Ethylpiperazin-1-yl)pyridin-3-yl)-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (2.3). Compound 2.1 (50 mg, 0.089 mmol), acetaldehyde (50 µL, 0.89 mmol) and acetic acid (~0.5 µL, 0.009 mmol) were mixed with MeOH (1 mL) at 0°C and then sodium cyanoborohydride (17 mg, 0.267 mmol) was added. The reaction was stirred at room temperature overnight and then purified by preparative HPLC (10% – 100% methanol / 0.1% TFA in H$_2$O) to afford compound 2.3 as a white solid (24 mg, 48% yield). $^1$H NMR (400 MHz, CDCl$_3$), δ: 12.66 (s, 1H), 8.48 (d, $J$ = 2.5 Hz, 1H), 8.39 (s, 1H), 8.00 (t, $J$ = 5.7 Hz, 1H), 7.76 (dd, $J$ = 8.8, 2.6 Hz, 1H), 7.71 (d, $J$ = 1.2 Hz, 1H), 7.58 (s, 1H), 6.67 (d, $J$ = 8.8 Hz, 1H), 5.93 (s, 1H), 4.87 (dp, $J$ = 13.1, 6.6 Hz, 1H), 4.67 (d, $J$ = 5.8 Hz, 2H), 3.75 – 3.58 (m, 4H), 2.75 – 2.67 (m, 2H), 2.65 (s, 4H), 2.56 (dd, $J$ = 14.2, 7.1 Hz, 2H), 2.16 (s, 3H), 1.65 (dd, $J$ = 15.2, 7.5 Hz, 2H), 1.58 (d, $J$ = 6.7 Hz, 6H), 1.18 (t, $J$ = 7.2 Hz, 3H), 1.01 (t, $J$ = 7.3 Hz, 3H).

1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (UNC1999 (2.4)). The procedure used for preparation of compound 2.3 was followed for synthesis of the title compound (UNC1999); acetone was used
instead of acetaldehyde. This compound was obtained as a white solid (21 mg, 78%). $^1$H NMR (400 MHz, $d_4$-MeOH), $\delta$: 8.48 (d, $J = 2.4$ Hz, 1H), 8.36 (s, 1H), 7.91 (dd, $J = 8.9$, 2.6 Hz, 1H), 7.87 (s, 1H), 7.75 (d, $J = 1.2$ Hz, 1H), 6.81 (d, $J = 8.9$ Hz, 1H), 6.09 (s, 1H), 5.06 (dt, $J = 13.3$, 6.6 Hz, 1H), 4.58 (s, 2H), 3.61 – 3.46 (m, 4H), 2.70 (ddd, $J = 13.1$, 8.6, 6.3 Hz, 3H), 2.63 (dd, $J = 11.2$, 6.0 Hz, 4H), 2.23 (s, 3H), 1.63 (dt, $J = 15.3$, 7.5 Hz, 2H), 1.55 (d, $J = 6.6$ Hz, 6H), 1.09 (d, $J = 6.5$ Hz, 6H), 1.00 (t, $J = 7.3$ Hz, 3H).

6-(5-Fluoropyridin-2-yl)-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (2.5). 6-Bromo-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (50 mg, 0.112 mmol), bis(pinacolato)diboron (34 mg, 0.135 mmol), and potassium acetate (16.5 mg, 0.168 mmol) were mixed with 1,4-dioxane (1.25 mL) under argon atmosphere at room temperature. The mixture was added a catalytic amount of [1,1-bis(diphenylphosphino)ferrocene]dichloropalladium(II) complex with dichloromethane (9.1 mg, 0.011 mmol), and heated to 80°C. After being stirred overnight, the resulting mixture was concentrated in vacuo, and used for the next step without further purification. To the solution of crude 1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-indazole-4-carboxamide and 2-bromo-5-fluoropyridine (158 mg, 0.896 mmol) in 1,4-dioxane and water (1.25 mL/0.25 mL) was added tetrakis(triphenylphosphine)palladium (26 mg, 0.022 mmol), and potassium
carbonate (46 mg, 0.336 mmol). After being stirred overnight at 90°C, the resulting mixture was purified by preparative HPLC (10% - 100% methanol / 0.1% TFA in H2O) to afford the title compound 2.5 as a white solid (32 mg, 61% yield). 1H NMR (400 MHz, CDCl3), δ: 12.69 (s, 1H), 8.52 (d, J = 2.2 Hz, 1H), 8.44 (s, 1H), 8.22 (s, 1H), 8.07 (s, 1H), 7.94 (s, 1H), 7.85 (dd, J = 8.6, 4.1 Hz, 1H), 7.47 (td, J = 8.4, 2.3 Hz, 1H), 6.02 (s, 1H), 4.97 (dt, J = 13.2, 6.6 Hz, 1H), 4.69 (d, J = 3.8 Hz, 2H), 2.82 – 2.68 (m, 2H), 2.21 (s, 3H), 1.70 – 1.62 (m, 2H), 1.60 (d, J = 6.6 Hz, 6H), 1.02 (t, J = 7.3 Hz, 3H).

6-(4-Fluorophenyl)-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (2.6). To a microwave vessel containing 6-bromo-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (50.6 mg, 0.112 mmol) was added potassium carbonate (46.4 mg, 0.336 mmol), (4-fluorophenyl)boronic acid (23.5 mg, 0.168 mmol), and tetrakis(triphenylphosphine)palladium (1.16 mg, 0.001 mmol). After adding 1 mL of dioxane:H2O (5:1), the vessel was placed in a microwave reactor for 20 minutes at 150°C. The contents were separated using EA and brine, extracting 3 times with EA then dried using Na2SO4. The crude product was purified using HPLC and then concentrated in vacuo to yield compound 2.6 (37.7 mg, 73%). 1H NMR (400 MHz, CDCl3), δ: 8.41 (s, 1H), 7.88 (s, 2H), 7.72 (s, 1H), 7.61 (d, J = 9.5 Hz, 3H), 7.11 (s, 2H), 6.06 (s, 1H), 4.89 (dt, J = 13.3, 6.7 Hz, 1H), 4.68 (d, J = 5.4 Hz, 2H), 2.82 – 2.71 (m, 2H), 2.19 (s, 3H), 1.72 – 1.55 (m, 8H), 1.02 (t, J = 7.3 Hz, 3H).
Scheme 2.2. Reagents and conditions: (a) DMF, K$_2$CO$_3$, CH$_3$I, rt, 68%; (b) DMF, NaH, CH$_3$I, rt, 92%; (c) K$_2$CO$_3$, Pd(PPh$_3$)$_4$, 1-(5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-2-yl)piperazine, 1,4-dioxane, H$_2$O, microwave, 150°C; (d) TFA, DCM, 88%; (e) acetone, MeOH, AcOH, NaBH$_3$CN, 0°C - rt, 83%.

6-Bromo-N-((1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-N-methyl-1H-indazole-4-carboxamide (2.7). 6-Bromo-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (50.8 mg, 0.112 mmol) and potassium carbonate (102.4 mg, 0.741 mmol) were added to a 4 mL scintillation vial, and dissolved in DMF (0.5 mL). The resulting mixture was stirred at room temperature for 5 minutes before adding iodomethane (100 µL), and stirring at room temperature for 24 hours. The resulting mixture was purified by HPLC to yield 6-bromo-N-((1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-1H-indazole-4-carboxamide (35.7 mg, 0.078 mmol, 68%). 6-Bromo-N-((1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-1H-indazole-4-carboxamide (15 mg, 0.033 mmol) and sodium hydride (2 mg, 0.050 mmol) were added to a 4 mL scintillation vial and were dissolved in DMF (0.15 mL). The mixture was stirred at
room temperature for 5 minutes before adding iodomethane (10 µL). The resulting mixture was stirred at rt for 24 hours and purified by HPLC to yield the title compound (14.4 mg, 0.030 mmol, 92%). $^1$H NMR (the major rotamer is reported here) (400 MHz, CDCl$_3$), δ: 7.93 (s, 1H), 7.64 (s, 1H), 7.25 (s, 1H), 6.12 (s, 1H), 4.85 (s, 2H), 4.81 – 4.66 (m, 1H), 3.59 (s, 3H), 2.93 (s, 3H), 2.70 (t, J = Hz, 2H), 2.38 (s, 3H), 1.76 – 1.46 (m, 8H), 1.01 (t, J = 7.3 Hz, 3H).

**N-((1,6-Dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-methyl-1H-indazole-4-carboxamide (UNC2400 (2.8)).** 6-Bromo-N-((1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-N-methyl-1H-indazole-4-carboxamide (22.6 mg, 0.034 mmol) was added to a microwave vessel with tert-butyl 4-(5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-2-yl)piperazine-1-carboxylate (27.9 mg, 0.112 mmol), potassium carbonate (23.2 mg, 0.168 mmol), and a catalytic amount of tetrakis(triphenylphosphine)palladium (1 mg, 0.001 mmol). The resulting mixture was dissolved in 1 mL of dioxane:H$_2$O (5:1) and placed in a microwave reactor at 150°C for 20 minutes. The resulting mixture was separated with EA and brine, extracted 3 times with EA, and purified by HPLC. The dried product was then dissolved in 1 mL TFA:EA (1:1), basified using NH$_3$ (1 mL), and then concentrated in vacuo to yield N-((1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-N-methyl-6-(6-(piperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide (19.9 mg, 0.030 mmol, 88%).
isopropyl-N-methyl-6-(6-(piperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide (19.9 mg, 0.030 mmol) was added to a vial containing sodium cyanoborohydride (15 mg, 0.239 mmol) on ice, and dissolved in methanol (0.2 mL). Acetone (30 µL) and acetic acid (25 µL) were then added to the mixture, the vial was removed from ice and the reaction was run at room temperature overnight. The mixture was then purified using HPLC to yield the title compound (UNC2400 (2.8)) (14.9 mg, 0.025 mmol, 83%). \(^1\)H NMR (the major rotamer is reported here) (400 MHz, CDCl\(_3\)), δ: 8.47 (s, 1H), 7.93 (s, 1H), 7.76 (d, J = 8.7 Hz, 1H), 7.50 (s, 1H), 7.32 (s, 1H), 6.73 (d, J = 8.8 Hz, 1H), 6.00 (s, 1H), 4.98 – 4.79 (m, 3H), 3.63 (s, 3H), 3.55 (s, 3H), 2.94 (s, 3H), 2.83 – 2.60 (m, 7H), 2.35 (s, 3H), 1.83 – 1.52 (m, 9H), 1.10 (d, J = 6.5 Hz, 6H), 1.02 (t, J = 7.2 Hz, 3H).

**Scheme 2.3. Reagents and conditions:** (a) hex-5-yn-1-yl 4-bromobenzenesulfonate, K\(_2\)CO\(_3\), DMF, rt, 74%; (b) azide-dye, Cat.

Cu(CH\(_3\)CN)\(_4\)PF\(_6\), DCM, rt, 90%.

6-(6-(4-(Hex-5-yn-1-yl)piperazin-1-yl)pyridin-3-yl)-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (2.9). To a solution of hex-5-yn-1-ol (2.07 g, 21.09 mmol) in DCM (30 mL) was
added 4-bromobenzenesulfonyl chloride (5.93 g, 23.20 mmol) and DABCO (2.84 g, 25.30 mmol) at 0°C. The resulting mixture was stirred for 2 hours at 0°C. 15 mL of distilled water was added. The organic phase was collected, dried with sodium sulfate, filtered, concentrated and purified by silica gel chromatography (hexanes to 15% EA in hexanes) to afford hex-5-yn-1-yl 4-bromobenzenesulfonate (6.15 g, 92%). To a solution of compound 2.1 (56 mg, 0.10 mmol) and hex-5-yn-1-yl 4-bromobenzenesulfonate (44 mg, 0.14 mmol) in 1.0 mL of DMF was added potassium carbonate (41 mg, 0.30 mmol) at room temperature, and stirred overnight. After water (2 mL) was added, the mixture was extracted with EA (10 mL × 3). The combined organic phases were dried with sodium sulfate, filtered, concentrated and purified by preparative HPLC to give the title compound as a yellow solid (44 mg, 72%). 

\(^1\)H NMR (400 MHz, CDCl\(_3\)), \(\delta\): 12.93 (s, 1H), 8.47 (dd, \(J = 2.5, 0.4\) Hz, 1H), 8.39 (s, 1H), 8.01 (t, \(J = 5.7\) Hz, 1H), 7.75 (dd, \(J = 8.8, 2.6\) Hz, 1H), 7.71 (d, \(J = 1.3\) Hz, 1H), 7.57 (s, 1H), 6.66 (d, \(J = 8.8\) Hz, 1H), 5.92 (d, \(J = 0.6\) Hz, 1H), 4.85 (dd, \(J = 13.3, 6.7\) Hz, 1H), 4.66 (d, \(J = 5.7\) Hz, 2H), 3.65 – 3.56 (m, 4H), 2.70 (dd, \(J = 8.5, 7.0\) Hz, 2H), 2.63 – 2.51 (m, 4H), 2.48 – 2.38 (m, 2H), 2.23 (td, \(J = 6.9, 2.6\) Hz, 2H), 2.15 (s, 3H), 1.96 (t, \(J = 2.6\) Hz, 1H), 1.73 – 1.52 (m, 12H), 1.00 (t, \(J = 7.3\) Hz, 3H).

\((E)\)-Acetoxymethyl 1-(3-(acetoxymethoxy)-3-oxopropyl)-2-((2\(E\),4\(E\))-4-(1-ethyl-3-(2-(4-(4-(5-(1-isopropyl-4-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)carbamoyl)-1H-indazol-6-yl)pyridin-2-yl)piperazin-1-yl)butyl)-1H-1,2,3-triazol-1-yl)ethyl)-2,4,6-
trioxotetrahydropyrimidin-5(2H)-ylidene)but-2-en-1-ylidene)-3,3-dimethylindoline-5-carboxylate (UNC2239 (2.10)). Cu(CH₃CN)₄PF₆ (0.7 mg, 0.0018 mmol) was added to a solution of 6-(6-(4-(hex-5-yn-1-yl)piperazin-1-yl)pyridin-3-yl)-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (6.3 mg, 0.0104 mmol) and the azide-dye compound (5.9 mg, 0.0087 mmol) in DCM (1.0 mL); the mixture was stirred overnight at room temperature [48]. The resulting product was concentrated and purified by preparative HPLC to give the title compound (UNC2239 (2.10)) as a dark red solid (10.1 mg, 90%). ¹H NMR (400 MHz, d₄-MeOH), δ: 8.55 (s, 1H), 8.33 (s, 1H), 8.11–8.04 (m, 1H), 8.04–7.93 (m, 3H), 7.91 (s, 1H), 7.83 (s, 1H), 7.80–7.63 (m, 3H), 7.24–7.17 (m, 1H), 7.06 (d, J = 8.9 Hz, 1H), 6.23–6.07 (m, 2H), 5.95 (d, J = 3.5 Hz, 2H), 5.68 (d, J = 8.6 Hz, 2H), 5.13–5.02 (m, 1H), 4.71–4.63 (m, 2H), 4.62 (s, 2H), 4.43–4.21 (m, 5H), 3.88–3.77 (m, 2H), 3.75–3.32 (m, 8H), 3.27–3.18 (m, 2H), 2.86 (dd, J = 14.5, 7.0 Hz, 2H), 2.82–2.73 (m, 3H), 2.27 (s, 3H), 2.11 (s, 3H), 2.00 (d, J = 3.8 Hz, 3H), 1.89–1.71 (m, 4H), 1.74–1.61 (m, 8H), 1.58 (d, J = 6.6 Hz, 6H), 1.11 (t, J = 7.0 Hz, 3H), 1.03 (t, J = 7.3 Hz, 3H).
Scheme 2.4. Reagents and conditions: (a) O-[2-(biotinylamino)ethyl]-O′-(2-carboxyethyl)undecaethylene glycol, DMF, DIPEA, HATU, rt, 41%. (b) Biotin, DMF, DIPEA, HATU, rt, 78%.

1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-6-(6-(4-(41-oxo-45-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-4,7,10,13,16,19,22,25,28,31,34,37-dodecaoxa-40-azapentatetracontanoyl)piperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide (UNC2399 (2.11)). Compound 2.1 (21.4 mg, 0.041 mmol) was added to a vial containing HATU (1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) (18.5 mg, 0.049 mmol) and O-[2-(biotinylamino)ethyl]-O′-(2-carboxyethyl)undecaethylene glycol. The two components were dissolved in DMF (0.3 mL) and DIPEA (8.5 µL) was added to the resulting mixture. The reaction was run at room temperature for 24 hours, and purified by HPLC to yield UNC2399 (22.6 mg, 0.016 mmol, 41%). 1H NMR (400 MHz, d4-MeOH), δ: 8.51 (dd, J = 9.5, 2.4 Hz, 1H), 8.43 (d, J = 2.2 Hz, 1H),
8.40 (s, 1H), 8.10 (s, 1H), 7.80 (d, J = 1.3 Hz, 1H), 7.50 (d, J = 9.5 Hz, 1H), 6.18 (d, J = 0.7 Hz, 1H), 5.13 (tt, J = 13.4, 6.6 Hz, 2H), 4.61 (s, 2H), 4.49 (dd, J = 7.9, 4.2 Hz, 1H), 4.30 (dd, J = 7.9, 4.5 Hz, 1H), 3.97 – 3.78 (m, 9H), 3.67 – 3.55 (m, 33H), 3.52 (t, J = 5.5 Hz, 2H), 3.34 (t, J = 5.5 Hz, 2H), 3.19 (ddd, J = 8.9, 5.8, 4.6 Hz, 1H), 2.92 (dd, J = 12.8, 5.0 Hz, 1H), 2.79 – 2.67 (m, 5H), 2.28 (s, 3H), 2.20 (t, J = 7.4 Hz, 2H), 1.78 – 1.52 (m, 11H), 1.48 – 1.37 (m, 2H), 1.03 (t, J = 7.3 Hz, 3H).

1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-6-(6-(4-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoyl)piperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide (UNC2398 (2.12)). The procedure for UNC2398 is the same as UNC2399 except that biotin was used instead of O-[2-(biotinylamino)ethyl]-O’-(2-carboxyethyl)undecaethylene glycol to give UNC2398 in 78% yield.

**EZH2 Homology Modeling**

An EZH2 homology model was built using the X-ray crystal structure of the lysine methyltransferase GLP (PDB: 2RFI) as a template. 3D conformation of EPZ005687 or UNC1999 was constructed and docked into the EZH2 homology model. A subsequent induced-fit minimization was conducted to further optimize the complex. The ligand interaction diagram was drawn with Maestro 9.3 (Schrodinger LLC, www.schrodinger.com).
Scintillation Proximity Assay

Methyltransferase activity assays were performed by monitoring the incorporation of a tritium-labeled methyl group from S-adenosylmethionine ($^{3}$H-SAM) to biotinylated peptide substrates using Scintillation Proximity Assay (SPA) for PRC2 trimeric complex (EZH2:EED:SUZ12), SETD7, G9a, GLP, SETDB1, SETD8, SUV420H1, SUV420H2, SUV39H2, MLL tetrameric complex (MLL:WDR5:RbBP5:ASH2L), PRMT1, PRMT3, PRMT5-MEP50 complex and SMYD2. The reaction buffer for SMYD2 and SMYD3 was 50 mM Tris pH 9.0, 5 mM DTT, 0.01% Triton X-100; for G9a, GLP and SUV39H2 was 25 mM potassium phosphate pH 8.0, 1 mM EDTA, 2 mM MgCl2 and 0.01% Triton X-100; and for other HMTs 20 mM Tris pH 8.0, 5 mM DTT, 0.01% Triton X-100. To stop the enzymatic reactions, 10 µL of 7.5 M guanidine hydrochloride was added, followed by 180 µL of buffer, mixed and transferred to a 96-well FlashPlate (Cat. # SMP103; Perkin Elmer; www.perkinelmer.com). After mixing, the reaction mixtures were incubated and the CPM counts were measured using a Topcount plate reader (Perkin Elmer, www.perkinelmer.com). The CPM counts in the absence of compound for each data set were defined as 100% activity. In the absence of the enzyme, the CPM counts in each data set were defined as background (0%). IC$_{50}$ values were determined using compound concentrations ranging from 100 nM to 100 µM. The IC$_{50}$ values were determined using SigmaPlot software. For DNMT1, the assay was performed as described above using hemi-methylated dsDNA as a substrate. The dsDNA substrate was prepared by annealing two complementary strands (biotinylated (B) forward
strand: B-GAGCCCGTAAGCCCGTTCAGGTCG and reverse strand: CGACCTGAACGGGCTTACGGGCTC), synthesized by Eurofins MWG Operon. Reaction buffer was 20 mM Tris-HCl, pH 8.0, 5 mM DTT, 0.01% Triton X-100. Methyltransferase activity assays for DOT1L were performed using filter-plates (Millipore; cat.# MSFBN6B10; www.millipore.com). Reaction mixtures in 20 mM Tris-HCl, pH 8.0, 5 mM DTT, 2 mM MgCl$_2$, and 0.01% Triton X-100 were incubated at room temperature for 1h, 100 µL 10% TCA was added, mixed and transferred to filter-plate. Plates were centrifuged at 2000 rpm for 2 min followed by 2 additional 10% TCA wash and one ethanol wash (180 µL) followed by centrifugation. Plates were dried and 100 µL MicroO was added and centrifuged. 70 µL MicroO was added and CPM were measured using Topcount plate reader.

**UNC1999 Mechanism of Action Studies**

To determine the mechanism of action of UNC1999, methyltransferase activity of PRC2-EZH2 was assessed in the presence of different concentrations of the compound (0, 1, 3 and 5 nM) at (1) fixed concentration of peptide (5 µM) and SAM concentrations from 0.625 to 10 µM and (2) fixed concentration of SAM (10 µM) and peptide concentrations from 0.3 to 5 µM. Assays were performed in triplicate, and kinetic values were obtained using Lineweaver-Burk plots (SigmaPlot, Enzyme Kinetics Module).
**In-cell Western Assay**

MCF10A cells were grown in DMEM/F12 media 5% horse serum, EGF (20 ng/mL), hydrocortisone (0.5 µg/mL), cholera toxin (100 ng/mL), and insulin (10 µg/ml) in the presence of inhibitors as stated in the figures. MCF7 cells were cultured in DMEM supplemented with 10% FBS. Fixation in 96 well black wall clear bottom plates was performed by 2% formaldehyde in PBS for 10 min. After five washes with 0.1% Triton X-100 in PBS, cells were blocked for 1 h RT, or overnight at 4°C with 3% BSA, 5% goat serum in PBS. Three replicate wells from each experimental group were incubated in primary H3K27me3 antibody, Diagenode MAb-181-050 at 1/4000 dilution in 3% BSA, 5% goat serum PBS for 18 h at 4°C. The wells were washed five times with 0.1% Tween-20 in PBS, then secondary IR800 conjugated antibody (Li-Cor) in Li-Cor blocking buffer (1:1000) and nucleic acid-intercalating dye, DRAQ5 (Cell Signaling Technologies) added for 1 h RT. After 5 washes with 0.1% Tween-20 PBS, the plates were read on an Odyssey (LiCor) scanner at 800 nm (H3K27me3 signal) and 700 nm (DRAQ5 signal). Fluorescence intensity was quantified, normalized to the background, then to the DRAQ5 signal, and expressed as a percentage of control.

**Cell Viability Assay**

Cell viability assays were performed using 0.1 mg/mL of resazurin (Sigma) in the media. Resazurin reduction was monitored with 544 nm excitation, measuring fluorescence at 590 nm.
**Kinase and GPCR studies**

Selectivity against a panel of 50 representative kinases and 46 representative GPCRs was carried out by PDSP according to their standard protocols.

**Pharmacokinetic Studies**

These studies were conducted at Sai Life Sciences according to their standard protocols. 15, 50, and 150 mg/kg IP as well as 50 mg/kg PO PK studies were performed in male Swiss albino mice (9 per dosing regimen). Each study lasted 24 hours, plasma concentrations of UNC1999 were obtained at .08, .25, .5, 1, 2, 4, 8, and 24 hr post-dosing.

**EZH2 Y641N Biochemical Assay**

EZH2 Y641N mutants were generated and assayed by BPS Bioscience. A series of dilutions of UNC1999 were prepared with 10% DMSO in HMT assay buffer (BPS #52170) and 5 µL of the dilution was added to a 50 µL reaction so that the final concentration of DMSO is 1% in all of reactions. All of the enzymatic reactions were conducted in duplicate at room temperature for 60 minutes (EZH2; BPS #51004) and 180 minutes (EZH2 Y641N; BPS #51028) in a 50 µL mixture containing HMT assay buffer, S-adenoslymethionine (BPS #52120), enzyme, and UNC1999. These 50 µL reactions were carried out in wells of a HMT substrate pre-coated plate. After enzymatic reactions, the reaction mixtures were discarded and each of the wells was washed three times with TBST buffer,
and slowly shaken with Blocking Buffer (BPS #52100) for 10 minutes. Wells were emptied, and 100 µL of diluted 1° antibody (BPS #52140F) was added. The plate was then slowly shaken for 60 minutes at room temperature. As before, the plate was emptied and washed three times, and shaken with Blocking Buffer for 10 minutes at room temperature. After discarding the Blocking Buffer, 100 µL of diluted 2° antibody (BPS #52131H) was added. The plate was then slowly shaken for 30 minutes at room temperature. As before, the plate was emptied and washed three times, and shaken with Blocking Buffer for 10 minutes at room temperature. Blocking Buffer was discarded and a mixture of the HRP chemiluminescent substrate was freshly prepared. 100 µL of this mixture was added to each empty well. Immediately, the luminescence of the samples was measured in a BioTek Synergy™ 2 microplate reader.

**DB Cell Proliferation Assay**

DB cells, a diffuse-large B-cell lymphoma cell line harboring the EZH2 Y641N mutation [49, 50] were obtained from ATCC and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, antibiotics, and various concentration of compounds (either DMSO control, UNC1999 and UNC2400). The numbers of viable cells from at least three independent experiments were measured using the TC20 automated cell counter system (Biorad). Total histones were prepared from cell nuclei using an acidic extraction protocol as previously described. [51] About 1 microgram of total histones was separated using 15% of SDS-PAGE, transferred to PVDF membranes, and probed with histone
antibodies. Antibodies used in this study are those against EZH2 (BD bioscience 612666), general H3 (Abcam ab1791), and H3K27me3 (Abcam ab6002).

Results

Discovery of UNC1999

Homology modeling of EZH2 based on the X-ray crystal structure of GLP (PDB: 2RFI), an H3K9 methyltransferase, was performed in order to establish a binding hypothesis for pyridone-containing EZH2 inhibitors. Docking of EPZ005687 into the homology model identified a low energy conformation demonstrating that the terminal morpholine group was solvent exposed, and did not appear to make contacts with the EZH2 protein surface (figure 2.1 A & B). Other interesting interactions were observed; a hydrogen bonding network present between the central amide and pyridone of EPZ005687, and asparagine 688 and histidine 689 of EZH2 (figure 2.1 A & B). On the basis of our binding hypothesis, and knowledge of key structural features of GSK126 and EPZ005687 that lead to EZH2 inhibition, we designed several compounds with the aim of endowing oral bioavailability.

We focused on modifying the morpholinomethyl region of EPZ005687 to modulate the physicochemical properties of this series, which resulted in the design and synthesis of the compounds in Table 2.1. These changes were expected to have little to no effect on potency, because that region of the scaffold was hypothesized to be solvent exposed. We maintained the pyridone indazole core of EPZ005687 to retain key hydrogen bonds and hydrophobic interactions
with the protein. Among the compounds from Table 2.1, UNC1999 was docked into the homology model, and like EPZ005687, the docking model of UNC1999 suggests that: (1) the secondary amide, and pyridone, maintain the respective key hydrogen bonds with N688 and H689, (2) the indazole core is buried in a hydrophobic pocket, and (3) the N-isopropyl piperazine does not interact with the protein (Figure 2.1 C & D).

The compounds in Table 2.1 were evaluated in an EZH2 radioactive biochemical assay which measures the direct transfer of the tritiated methyl group from the cofactor $^3$H – SAM to a peptide substrate. IC$_{50}$ values of these compounds in this assay, and their calculated partition coefficient (clogP) values, are summarized in Table 2.1. As expected, the unsubstituted and N-alkyl piperazines had high \textit{in vitro} potency for EZH2 (IC$_{50} < 10$ nM), which indicated that modifications to the N-capping group are well tolerated. These \textit{in vitro} results support our binding hypothesis: the N-alkyl piperazine does not interact with the protein. Among these four inhibitors, UNC1999 has the most desirable clogP (3.1), suggesting that this inhibitor may be well absorbed orally. In addition, UNC1999 has a more desirable clogP than either EPZ005687 (2.2) or GSK126 (2.6). Interestingly, replacing the 2-piperazinyl pyridin-5-yl moiety with the 3-fluoro pyridin-6-yl group retained high \textit{in vitro} potency (IC$_{50} = 10 \pm 1$ nM ($n = 3$)), but replacing the 3-fluoro pyridin-6-yl group with the 4-fluorophenyl group led to a 6-fold loss in potency. Based on these results, we selected UNC1999 for subsequent mechanism of action (MOA), selectivity, cellular assays, and mouse PK studies.
Discovery of UNC2400

To provide the research community with a set of useful tools, we aimed to create a structurally similar, but significantly less potent, EZH2 inhibitor as a negative control for *in vitro* and cell-based studies. The UNC1999 docking model suggests that the secondary amide and pyridone form key hydrogen bonds with N688 and H689 (Figure 2.1 C & D). So, we designed and synthesized UNC2400 (Figure 2.2 A), which contains an N-methyl group at both the secondary amide and pyridone moieties of UNC1999. We hypothesized that the addition of these two N-methyl groups would abolish the hydrogen bonds between the secondary amide and pyridone of UNC1999 with N688 and H689 of EZH2, respectively. Indeed, UNC2400 displayed an IC50 = 13,000 ± 3,000 nM (n = 3) in the EZH2 radioactive biochemical assay (Figure 2.2 B), which is more than 1000-fold less potent than UNC1999, thus supporting our *in silico* binding hypothesis for UNC1999. Importantly, UNC2400 was also inactive in a number of cell-based studies (see below). The high structural similarity and drastic potency difference between UNC1999 and UNC2400 suggest they will be excellent positive and negative control tool compounds.

UNC1999 Is a SAM-competitive, Potent, and Selective Inhibitor of EZH2 and EZH1

To determine the MOA of EZH2 inhibition by UNC1999, we generated classic Lineweaver-Burk plots [52] (Figure 2.2 C & D), which indicate that UNC1999 is competitive with cofactor SAM (Ki of 4.6 ± 0.8 nM; n = 2) and non-
competitive with the H3 peptide substrate. We also determined that increasing the H3 peptide concentration had no effect on the IC50 values of UNC1999 (Figure 2.2 E) while increasing SAM concentration dramatically affected the IC50 values of UNC1999 (Figure 2.2 F), which further supports that UNC1999 is a SAM-competitive EZH2 inhibitor.

We next determined the potency of UNC1999 against EZH2 Y641 mutants. UNC1999 was highly potent for Y641N and Y641F mutants (Figure 2.3 A, blue and green, respectively), displaying less than 5-fold more potent inhibition for the WT over the Y641N (IC50 = 24 nM) and Y641F (IC50 = 23 nM) mutant enzymes. This data is consistent with other pyridone containing EZH2 inhibitors to date [32, 34, 44]. To further determine the selectivity profile of UNC1999, we first tested UNC1999 against EZH1. Compared with EPZ005687, GSK126 and El1, which are at least 50-fold selective for EZH2 over EZH1, [32, 34, 44] UNC1999 had a higher in vitro potency for EZH1 (IC50 = 45 ± 3 nM; n = 3) (Figure 2.3 B); about 10-fold less potent for EZH1 than EZH2. UNC1999 is also very selective for EZH2 versus 15 other lysine, arginine or DNA methyltransferases (Figure 2.3 C) assayed; 10,000-fold selective for EZH2 (IC50 < 10 nM) over these methyltransferases (IC50 > 100,000 nM).

We also evaluated the selectivity of UNC1999 over a broad range of non-epigenetic targets. UNC1999 is competitive with SAM, an adenine nucleoside, so we tested it against a panel of 50 representative kinases (Table 2.2). UNC1999 showed no appreciable inhibition (no more than 20% inhibition at 10,000 nM) against these kinases. Further, UNC1999 possesses an N-isopropyl piperazine
moiety, which is a potential pharmacophore for aminergic G protein-coupled receptor (GPCR) binding, so we tested it in the National Institute of Mental Health (NIMH) Psychoactive Drug Screen Program (PDSP) Selectivity Panel. This panel consists of 44 representative GPCRs, transporters, and ion channels (Table 2.3); UNC1999 was found to show no more than 50% inhibition at concentrations as high as 10,000 nM against 40 targets, and > 50% inhibition at 10,000 nM against 4 targets in the panel. Ki determinations in the radioligand binding assay for each of the 4 interacting targets was subsequently performed (Table 2.4). UNC1999 had Ki values of 4,700 nM, 65 nM, 300 nM, and 1,500 nM for sigma1, sigma2, histamine H3, and NET (norepinephrine transporter), respectively. Although we could not evaluate UNC1999 in sigma2 functional assays, because they do not exist, we tested UNC1999 in histamine H3 functional assays and found that it did not display any agonist or antagonist activities at concentrations up to 1,000 nM. Therefore, with the exception of sigma2, UNC1999 was more than 200-fold selective for EZH2 over a broad range of kinases, GPCRs, transporters, and ion channels.

**UNC1999 Potently Reduces H3K27me3 in Cells and Selectively Kills EZH2-mutant DLBCL Cells**

To assess the cellular potency of UNC1999, we employed an H3K27me3 antibody cell immunofluorescence In-Cell Western (ICW) assay. This assay allows for rapid processing of multiple samples for H3K27me3 immunofluorescence signal and normalization to cell number via the use of the
nucleic acid dye, DRAQ5. We characterized UNC1999 and UNC2400 in MCF10A cells, which bear the WT EZH2 enzyme. UNC1999 (72 h exposure) exhibited concentration-dependent reductions in H3K27me3 with an IC50 of 124 ± 11 nM (n = 3) (Figure 2.4 A, blue dots). On the other hand, UNC2400 (negative control) showed little or no activity in this ICW assay (Figure 2.4 B, red dots), which is consistent with its poor in vitro potency. In addition, the treatment of MCF7 cells with UNC1999 at 5,000 nM for 72 h almost completely removed the H3K27me3 mark but did not have significant effects on cellular levels of EZH2 (Figure 2.4 C).

One of the most desirable characteristics of a high quality chemical probe is low toxicity due to off-target effects. Both UNC1999 (EC50 = 19,200 ± 1,200 nM; n = 3) and UNC2400 (EC50 = 27,500 ± 1,300; n = 3) had low cellular toxicity in a standard resazurin (Alamar Blue) reduction assay (Figure 2.4 A & B). Taken together, UNC1999 had an excellent separation of cellular potency and toxicity with a function/toxicity ratio of more than 150 (Figure 2.4 A, blue vs grey dots).

We next investigated whether DB cells, a DLBCL cell line harboring the EZH2 Y641N mutant [49, 50], are more sensitive to UNC1999 treatment. UNC1999 (8 day exposure) displayed robust, concentration-dependent inhibition of cell proliferation (EC50 = 633 ± 101 nM; n = 3) (Figure 2.5 A), which is slightly more potent than GSK126 [32]. At 5,000 nM, UNC1999 (8 day exposure) completely killed DB cells (Figure 2.5 A, blue line). Interestingly, we observed a delayed onset of activity for UNC1999; the 3 day treatment with this inhibitor did not have significant effects on cell proliferation at all tested concentrations.
(Figure 2.5 A) as was also seen with EPZ005687 and GSK126 [32, 44]. We also compared the effects of the negative control UNC2400 on cell proliferation to UNC1999. UNC1999 (3,000 nM, 8 day treatment) significantly inhibited DB cell proliferation, while UNC2400 (3,000 nM, 8 day treatment) had negligible effects (Figure 2.5 B, blue bar vs red bar). Furthermore, UNC1999 (3,000 nM, 3 day treatment) significantly reduced the H3K27me3 mark but did not significantly change EZH2 levels in DB cells (Figure 2.5 C, well 2). On the other hand, treatment with UNC2400 at 3,000 nM for 3 days did not result in a significant reduction in the H3K27me3 mark, nor EZH2 levels in DB cells (Figure 2.5 C, well 3). Combining the observed low toxicity in MCF10A cells and high sensitivity in DB cells, we provide evidence that UNC1999 selectively kills DLBCL cells heterozygous for Y641 point mutations. In addition, we showed that UNC1999 has robust on-target activities in cells and UNC2400 is an excellent negative control for cell-based studies.

**Discovery of UNC2399 and UNC2239**

Our UNC1999 binding hypothesis predicts that the N-terminal nitrogen of the piperazine of UNC1999 is solvent exposed. To test this possibility, and create functionalized chemical tools, we appended both a PEG-biotin group (UNC2399) and a cell permeable dye (UNC2239) to the terminal piperazine nitrogen of UNC1999.

To synthesize UNC2399, we used a simple amide coupling between the free piperazine of UNC1999 (compound 2.1) and O-[2-(biotinylamino)ethyl]-O’-[2-
carboxyethyl)undecaethylene glycol (Scheme 2.4), which yielded a 12 PEG linker between the UNC1999 moiety, and biotin handle (Figure 2.6 A). We implemented a lengthy linker to ensure that the biotin handle would be sufficiently solvent exposed for capture by streptavidin-coated beads. Indeed, UNC2399 displayed high \textit{in vitro} potency (IC50 = 17 ± 2 nM; n = 3) in the EZH2 radioactive biochemical assay (Figure 2.6 B), which provides further evidence for our \textit{in silico} binding hypothesis of UNC1999.

To perform EZH2 chemiprecipitation, we first conjugated UNC2399 to streptavidin-coated beads. The compound-conjugated beads were used to capture EZH2 protein from HEK293T (human embryonic kidney 293T) cell lysates. We were pleased to find that UNC2399 – streptavidin conjugated beads (Figure 2.6 C, well 3) enriched EZH2 from cell lysates as compared to unconjugated streptavidin beads (DMSO control, Figure 2.6 C, well 2). To control for non-specific interactions, we pretreated the cell lysates with a soluble competitor, UNC1999 or UNC2400, before pull-down. As expected, pretreatment of lysate with UNC1999 (100 \(\mu\)M) antagonized the enrichment of EZH2 by UNC2399 – streptavidin beads (Figure 2.6 C, well 4 versus well 3). On the other hand, pretreatment with UNC2400 (100 \(\mu\)M) resulted in EZH2 levels nearly identical to the untreated lysates (Figure 2.6 C, well 5 versus well 3). It is important to note that a direct conjugation of biotin to the free piperazine of UNC1999, yielding UNC2398 (Figure 2.6 D), was as potent as UNC2399 in the \textit{in vitro} scintillation proximity assay (Figure 2.6 E), but was unable to
chemiprecipitate EZH2 (Figure 2.6 F), so a linker is imperative for efficient chemiprecipitation of EZH2.

Next, we synthesized UNC2239 (Figure 2.7 A) by utilizing click chemistry between a UNC1999 analog containing a terminal alkyne (compound 2.9) and a membrane permeable merocyanine dye with an azide side chain (Scheme 2.3). As expected, UNC2239 displayed high in vitro potency (IC50 = 21 ± 1 nM; n = 3) in the EZH2 radioactive biochemical assay, despite the addition of the fluorophore (Figure 2.7 B). Mouse embryonic fibroblast (MEF) cells stably expressing yellow fluorescent protein (YFP) were treated with the fluorescent EZH2 probe UNC2239 or with only the dye component of the probe as a control (Figure 2.7 C). The dye is known to distribute uniformly in MEF cells without evidence of any compartmental or organelle specific interactions. YFP serves as a uniformly distributed volume control fluorophore. When examining the localization of the probe, the ratio of dye intensity to YFP intensity was normalized for effects arising from varying cell thickness and uneven illumination intensity. Because the concentration of the probe and YFP varied from cell-to-cell, ratio images were normalized by setting the lowest 5% of pixels within each cell equal to 1. Cells treated with UNC2239 showed significantly higher fluorescence intensity in the nucleus relative to the cytoplasm than cells treated with the dye alone (Figure 2.7 C, p < 0.05, n = 6). Perinuclear localization of the probe was also observed, potentially due to autophagocytic uptake of the probe. The observed localization of UNC2239 is consistent with the nuclear localization
of GFP – tagged EZH2 as seen in transfected HEK 293 cells (Figure 2.7 D), suggesting that UNC2239 co-localizes with EZH2.

**UNC1999 Is Orally Bioavailable in Mice**

We next evaluated the *in vivo* PK properties of UNC1999. A single intraperitoneal (IP) injection of UNC1999 at 15, 50, or 150 mg/kg achieved high Cmax (9,700 – 11,800 nM) and exhibited dose linearity in male Swiss albino mice (Figure 2.8 A). Both the 150 and 50 mg/kg IP doses resulted in plasma concentrations of UNC1999 above its cellular IC50 over the entire 24 h period, and the 15 mg/kg IP dose led to the plasma concentrations of UNC1999 above its cellular IC50 for approximately 12 h. We next examined whether UNC1999 is orally bioavailable and were pleased to find that a single 50 mg/kg oral dose of UNC1999 achieved high Cmax (4,700 nM) and good exposure levels in male Swiss albino mice (Figure 2.8 B). The plasma concentrations of UNC1999 were maintained above its cellular IC50 for approximately 20 h following this single oral dose. It is worth noting that all doses including the 150 mg/kg IP dose were well tolerated by all test mice, and no adverse effects were observed.

**Discussion**

The use of homology modeling helped to establish a putative binding hypothesis for pyridone containing inhibitors of EZH2. We then tested this hypothesis by synthesizing various compounds, and in the process, discovered several key compounds, which are of current and future use to the biological
community as a set of chemical tools that will help accelerate our understanding of PRC2 function, and how this function becomes dysregulated in cancers and developmental diseases.

In addition to our chemical probe UNC1999, we discovered UNC2400, a structurally similar analog that is almost completely inactive in both *in vitro* and cellular assays. The use of this compound provided evidence for the presence of our hypothesized hydrogen bonding network between the pyridone and acyclic amides of UNC1999, and N688 and H689 of EZH2. Further, the fact that UNC2400 and UNC1999 display low, yet nearly equal, cytotoxicity in cell viability assays, demonstrates that potential toxicity of pyridone containing compounds is most likely not due to inhibition of EZH2. This data is exciting, as it provides a positive prognosis for the development of EZH2 inhibitors as therapeutics going forward.

Our UNC1999 binding hypothesis also predicted the N-terminal piperazine to be solvent exposed, which provided a useful handle to functionalize this site. First, PEG-biotinylation led to the discovery of UNC2399. UNC2399 selectively chemiprecipitates EZH2 from cell lysates, which is especially useful for complexes like PRC2. While the core components of PRC2 (EZH2 or EZH1, SUZ12 and EED) are always present, many other proteins (DNMT1, JARID2A, etc) which possess strictly different functions are present in a cell- and developmental-specific context. So, a small molecule capable of chemiprecipitating the complex will be extremely useful for determining the composition of complexes in malignant cells versus benign cells going forward.
The chemoproteomics applications of UNC2399 are very promising, and will surely play a role in the understanding of PRC2 function in malignant transformation. We also developed UNC2239, which contains a cell penetrant dye conjugated to the piperazine of UNC1999. This compound will have many applications going forward, one of which is to provide an alternative to traditional immunofluorescence (IF). A cell permeable compound which engages and identifies the target is more advantageous than traditional antibody-based immunofluorescence for intracellular targets, which involves permeation of the membrane.

UNC1999 is also the most active inhibitor of EZH1 to date, which may have potential benefit in treating specific cancers [16]. The exact reasoning for this selectivity switch between the scaffolds is unfounded, as the key interactions between UNC1999 and EPZ005687 and EZH1/EZH2 appear identical in homology models that were constructed. It is possible that this change in selectivity may be due to issues beyond direct ligand – protein interactions; UNC1999 may affect how EZH1/EZH2 interacts with other PRC2 components, but this is purely speculation. The dual inhibition of EZH2 and EZH1 by UNC1999 might result in higher efficacy in cell-based and animal models where the methylation of H3K27 by PRC2-EZH2 is compensated by PRC2-EZH1, but in the very least provides another avenue for exploration in the nascent field of PRC2 small molecule drug discovery.

Compared with existing EZH2 inhibitors such as EPZ005687 and EI1, which were reported to not have sufficient in vivo PK properties [34, 44] and
GSK126, which was only used via IP injection [32], UNC1999 displayed a more attractive cLogP, and is the first orally bioavailable inhibitor of EZH2 and EZH1. An orally bioavailable inhibitor makes chronic animal studies more practical and convenient, because the compound can easily be administered in the food or water of test mice. Contrarily, chronic daily IP injections to test mice could lead to infections, which might complicate long-term animal studies. Therefore, the intrinsic oral bioavailability of UNC1999 is a valuable attribute for the biomedical research community to assess long-term therapeutic benefit(s) and potential toxicity resulting from pharmacological inhibition of EZH2 and EZH1 in mouse models.
Table 2.1: *in vitro* activity of EZH2 inhibitors.

*a* IC$_{50}$ determination experiments were performed in triplicate. *b* The IC$_{50}$ limit of the EZH2 radioactive biochemical assay is 10 nM because the concentration of EZH2 used in this assay is 20 nM. GSK126 and EPZ005687 values were taken from [32] and [44], respectively.

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Table 2.2: Selectivity of UNC1999 versus representative kinase panel.
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<td>13</td>
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Table 2.3: Selectivity of UNC1999 versus NIMH PDSP panel.
Table 2.4: UNC1999 binding affinity and potency.

(0 = no agonist or antagonist activity at concentrations up to 1 μM, NT = not tested, NA = not available).
Figures

Figure 2.1: EZH2 homology model: UNC1999 and EPZ005687 have similar modes of binding.

A homology model of EZH2 was constructed using GLP as a template (PDB: 2RFI). (A) EPZ005687 was docked into the SET domain; the ligand (green) orients such that the morpholine group is solvent exposed and does not interact with EZH2 (purple). Additionally, there are three key hydrogen bonds that appear to be requisite for activity (dotted red lines). (B) Proposed ligand interactions of EPZ005687 display a hydrogen bond between the middle amide and N688, and two hydrogen bonds between the pyridone and H689. (C) A docking model of UNC1999 shows a similar hydrogen bonding network as it binds to EZH2 similar to EPZ005687 and its N-isopropyl piperazine is solvent exposed. (D) Proposed
ligand interactions of UNC1999 display the same key hydrogen bonds and hydrophobic interactions as EPZ005687.
Figure 2.2: Characterization of UNC1999 and UNC2400 in biochemical assays.

(A) Structures of UNC1999 and negative control UNC2400. (B) UNC2400 displayed > 1000-fold decrease in IC$_{50}$ as compared to UNC1999 in the *in vitro* EZH2 radioactive assay. (C) – (D) Lineweaver-Burk plots demonstrated that
UNC1999 is non-competitive with the histone H3 substrate (C), and competitive with the co-factor SAM (D). (E) – (F) Further evidence demonstrating that UNC1999 is SAM competitive; increasing concentration of H3 has no effect on UNC1999 IC$_{50}$ (E), but increasing concentration of SAM leads to a higher IC$_{50}$ (F).
Figure 2.3: Characterization of UNC1999 versus EZH2 mutants, EZH1, and other methyltransferases.

(A) UNC1999 was less than 5-fold more potent for WT EZH2 versus Y641N EZH2 and Y641F EZH2. (B) UNC1999 displayed high potency ($IC_{50} = 45 \pm 3$ nM) for EZH1. (C) UNC1999 was selective for EZH2 and EZH1 over 15 other lysine, arginine, and DNA methyltransferases assayed.
Figure 2.4: UNC1999 potently reduces H3K27me3 levels in cells and shows low cell toxicity.

(A) UNC1999 reduced H3K27me3 levels in MCF10A cells ($IC_{50} = 124 \pm 11 \text{ nM}; n = 3$) as determined by the ICW assay (blue), and displayed low cell toxicity ($EC_{50} = 19,200 \pm 1,200 \text{ nM}; n = 3$) in the resazurin assay (grey). (B) UNC2400 displayed negligible inhibition of H3K27me3 levels (red) in MCF10A cells while displaying similar toxicity as UNC1999 ($EC_{50} = 27,500 \pm 1,300 \text{ nM}; n = 3$) in the resazurin assay (grey). (C) Immunofluorescence staining showed that treatment with UNC1999 (5 µM, 3 days) effectively reduced the H3K27me3 mark but did not affect EZH2 levels in MCF7 cells. Scale bar represents 10 µm.
Figure 2.5: UNC1999 selectively kills DB cells, a DLBCL cell line with the EZH2 Y641N mutation.

(A) UNC1999 displayed a concentration- and time-dependent inhibition of DB cell proliferation ($EC_{50} = 633 \pm 101$ nM; n = 3). (B) UNC2400 (3,000 nM, 8 days) did not significantly inhibit DB cell proliferation in contrast to UNC1999. (C) Western blotting of EZH2, H3K27me3, and total H3 following the treatment of DB cells with UNC1999 or UNC2400 at 3,000 nM for 3 days. UNC1999 decreased H3K27me3, but not EZH2 levels, while UNC2400 did not significantly reduce H3K27me3 or EZH2 levels in DB cells.
Figure 2.6: Biotinylated UNC1999 (UNC2399) enriches EZH2 from HEK 293T cell lysates.

(A) Structure of UNC2399, a biotinylated UNC1999 analog. (B) UNC2399 displayed high in vitro potency ($IC_{50} = 17 \pm 2$ nM) for EZH2. (C) In UNC2399 pull-down experiments, EZH2 levels were markedly enriched (well 3) as compared to
the DMSO control (well 2). The ability to pull-down EZH2 out of cell lysates was abolished by the pretreatment with 100 µM UNC1999 (well 4), but was not affected by the pretreatment with 100 µM UNC2400 (well 5). This blot is representative of three biological replicates. (D) Structure of UNC2398. (E) UNC2398 retains high in vitro potency for EZH2. (F) UNC2398 pull-down (well 5) does not effectively enrich for EZH2 versus DMSO treated beads (well 3).
Figure 2.7: Live cell imaging with UNC1999-dye conjugate (UNC2239).

(A) Structure of UNC2239, a fluorescent dye conjugate of UNC1999. (B) UNC2239 displayed high in vitro potency (IC$_{50}$ = 21 ± 1 nM) for EZH2. (C) HEK 293T cells transfected with EZH2-GFP and stained with nuclear marker Hoechst 33342 showed nuclear localization of EZH2-GFP. (D) Ratio images showing localization of the EZH2 probe in MEF cells. The ratio of dye fluorescence intensity over YFP volume control fluorophore intensity was determined. Cells were normalized by setting the lowest 5% of cytoplasmic ratio values = 1.
Figure 2.8: Pharmacokinetic profiles of UNC1999 in male swiss albino mice.

(A) Plasma concentrations of UNC1999 following a single IP injection (15, 50, or 150 mg/kg) over the 24 h period. (B) Plasma concentrations of UNC1999 following a single oral dose (50 mg/kg) over the 24 h period. The dashed black line indicates the cellular IC$_{50}$ of UNC1999.
CHAPTER III: DUAL INHIBITION OF EZH2 AND EZH1 IS A VIABLE THERAPEUTIC OPTION FOR MLL-REARRANGED LEUKEMIA

Introduction

PRC2 complexes containing either EZH2 or EZH1 play an integral role in hematopoietic gene silencing at various stages of hematopoiesis [20, 21]. As such, there is extensive evidence demonstrating that deregulation of PRC2 function plays a role in malignant hematopoiesis [53, 54]. This includes, but is not limited to, recurrent gain-of-function EZH2 mutations observed in germinal center B-cell lymphoma patients [50, 55], and constitutive expression of wild-type or mutant EZH2 in lymphomagenesis [56] and myeloproliferative diseases in murine models [57]. Interestingly, in myeloid neoplasms, EZH1 has been shown to compensate for the functions of EZH2, and contributes to malignant growth [19, 21]. To date, small molecule inhibitor development has mostly focused on selective inhibition of EZH2 for treating lymphomas bearing EZH2 gain-of-function mutants [32, 34, 37, 44]. However, dual inhibition of EZH2 and EZH1 may be a viable therapeutic intervention for malignancies dependent upon both isoforms, like MLL-rearranged leukemia [16, 31].
MLL is a gene encoding a histone 3 lysine 4 (H3K4) specific methyltransferase; methylation of H3K4 at gene promoters is highly correlated with regions of active transcription. Genetic products resulting from MLL rearrangement inappropriately recruit other epigenetic factors and/or transcriptional elongation machinery to abnormal sites, which results in aberrant gene expression and potential malignant transformation [25, 58]. Rearrangements of the MLL gene are responsible for ~70% of acute myeloid or lymphoid leukemia in infants, and ~10% of cases in adults [59]. This genetic predisposition leads to poor prognosis and low survival rates, which underscores the need for novel viable therapeutics [60]. Recently, studies have demonstrated that PRC2 acts in parallel with MLL rearrangements to maintain an oncogenic signature essential for sustaining leukemogenicity [61].

Specifically, EZH2 and EZH1 compensate one another to promote MLL-AF9 AML. Genetic ablation of EZH2 in MLL-AF9 cells does not lead to complete loss of H3K27me3, but genetic ablation of EED leads to a global loss of H3K27me3 [16]. Due to the fact that EED is absolutely required for PRC2 function, the prevailing thought in the field is that PRC2-EZH1 complexes maintain H3K27me3 at specific loci in the absence of EZH2. Therefore, development of a pan EZH2/EZH1 chemical probe will aid in determining whether dual inhibition is a viable option for therapy in specific maladies.

To date, UNC1999 is the most panactive EZH2/EZH1 chemical probe available [62]. Herein, we demonstrate the use of UNC1999 as an anti-leukemic agent capable of reducing H3K27me3 levels in MLL-rearranged cells. GSK126,
which is more selective for EZH2, is not effective in killing cells displaying this MLL-rearrangement. Further, we demonstrate a survival advantage of mice treated with UNC1999 in a murine model of MLL-AF9 AML. Lastly, we discuss the potential for further development of EZH2/EZH1 panactive chemical probes.

**Methods**

**Synthesis of N-methylated derivatives**

![Scheme 3.1. Reagents and Conditions:](image)

(a) Pd(PPh₃)₄, K₂CO₃, H₂O:Dioxane, µ-wave, 150 °C, 60%; (b) TFA:DCM, rt; (c) Acetone, acetic acid, NaBH₃CN, MeOH, 0 °C → rt, 58%.

**tert-butyl 4-(5-(4-(((1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)carbamoyl)-1-isopropyl-1H-indazol-6-yl)pyridin-2-yl)piperazine-1-carboxylate (3.1).** 6-bromo-N-((1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-1H-indazole-4-carboxamide was prepared as previously reported [62] (.096 g, .209 mmol) and then dissolved in 2 mL H₂O:Dioxane (1:5) in a microwave vessel. tert-butyl 4-(5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-2-yl)piperazine-1-carboxylate, prepared as previously reported [62] (.1
g, .256 mmol) was added, and then potassium carbonate (.088 g, .639 mmol), and Tetrakis(triphenylphosphine)palladium(0) (.05 eq, ~2 mg) were added. The mixture was then placed in a microwave reactor for 20 minutes at 150 \(^\circ\)C. The crude product was separated using Brine:EA, washing 3x with EA. The organic layers were combined, dried with Na\(_2\)SO\(_4\), concentrated in vacuo, and purified using HPLC to yield tert-butyl 4-(5-(4-(1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)carbamoyl)-1-isopropyl-1H-indazol-6-yl)pyridin-2-yl)piperazine-1-carboxylate as a white powder (.082 g, .128 mmol, 60.1%). \(^1\)H NMR (400 MHz, \(d_4\)-MeOH), \(\delta\): 8.46 – 8.30 (m, 1H), 8.09 – 7.98 (m, 1H), 7.84 – 7.69 (m, 1H), 7.45 – 7.30 (m, 1H), 6.32 – 6.18 (m, 1H), 5.19 – 4.97 (m, 1H), 4.61 (s, 1H), 3.85 – 3.63 (m, 4H), 3.57 (s, 2H), 2.81 – 2.67 (m, 1H), 2.41 (s, 2H), 1.70 – 1.54 (m, 4H), 1.51 (s, 4H), 1.02 (t, \(J = 7.3\) Hz, 2H).

**N-((1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide (UNC3142 (3.2)).** tert-butyl 4-(5-(4-(1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)carbamoyl)-1-isopropyl-1H-indazol-6-yl)pyridin-2-yl)piperazine-1-carboxylate (.040 g, .062 mmol) was dissolved in DCM (0.25 mL) and added TFA (0.25 mL). The reaction was stirred for ~2 h until complete. Then, the contents were concentrated in vacuo, dissolved in MeOH (0.40 mL), and placed in an ice bath. Acetone (.058 mL, 0.79 mmol) and acetic acid (.045 mL, 0.79 mmol) were then added to the reaction. After cooling to 0 \(^\circ\)C, NaBH\(_3\)CN (.025 g, 0.40 mmol) was added and the reaction was stirred for 15 minutes. Then
the ice bath was removed and the contents were allowed to warm to rt overnight. After completion, the MeOH was removed *in vacuo* and the crude product was isolated by separating with EA:Brine, 3x EA washes. The organic layers were combined, dried with Na$_2$SO$_4$, and purified using HPLC to give N-((1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide as a brown oil (.021 g, .036 mmol, 58.1%). $^1$H NMR (400 MHz, d-CHCl$_3$), δ: 8.49 (d, $J = 2.2$ Hz, 1H), 8.39 (s, 1H), 7.80 (td, $J = 8.8, 4.2$ Hz, 1H), 7.69 (s, 1H), 7.58 (s, 1H), 6.73 (d, $J = 8.8$ Hz, 1H), 5.97 (s, 1H), 4.88 (s, 1H), 4.65 (d, $J = 5.9$ Hz, 1H), 3.68 (s, 2H), 3.53 (s, 2H), 3.04 – 2.45 (m, 4H), 2.32 (s, 2H), 1.72 – 1.52 (m, 5H), 1.25 (s, 3H), 1.14 (d, $J = 6.0$ Hz, 3H), 1.02 (s, 2H).

Scheme 3.2. Reagents and Conditions. (a) Di-*tert*-butyl dicarbonate, THF, rt, 97%; (b) LAH, THF, reflux, 67%; (c) EDCI, HOBT, TEA, DMF, rt, 44%; (d) Pd(dppf)Cl$_2$·DCM, K$_2$CO$_3$, H$_2$O:Dioxane, reflux, 76%; (e) TFA:DCM, reflux; (f) Acetone, AcOH, NaBH$_3$CN, MeOH, 0°C → rt, 53%.
tert-butyl ((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)carbamate (3.3). 3-(aminomethyl)-6-methyl-4-propylpyridin-2(1H)-one (1.00 g, 5.60 mmol) was dissolved in THF (20.0 mL) and added di-tert-butyl dicarbonate (1.22 g, 5.60 mmol). The mixture was then stirred at rt for 4 h. After completion, the contents were separated with EA (3X) and brine. The organic layers were combined, dried with Na₂SO₄, and concentrated in vacuo. The crude product was then used for the next step without further characterization (1.50 g, 97%).

6-methyl-3-((methylamino)methyl)-4-propylpyridin-2(1H)-one (3.4). tert-butyl ((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)carbamate (0.70 g, 2.50 mmol) was dissolved in THF (50.0 mL) and a solution of LAH (0.95 mg, 25.0 mmol) in THF (50.0 mL) was added to the mixture dropwise; the mixture was heated to reflux for 4 h. After completion, the contents were cooled to 0 °C, added 0.5 N NaOH, and extracted with EA 3x. The organic layers were combined, dried with Na₂SO₄, and combined to give 6-methyl-3-((methylamino)methyl)-4-propylpyridin-2(1H)-one (.332 g, 67% yield) as a yellow oil. 1H NMR (300 MHz, d-CHCl₃), δ: 5.891 (s, 1H), 3.659 (s, 2H), 2.520-2.494 (m, 2H), 2.418 (s, 3H, ), 2.256(s, 3H), 1.565-1.514 (m, 2H), 0.980-0.931 (t, 3H, J=7.2 Hz).

6-bromo-1-isopropyl-N-methyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (3.5). 6-methyl-3-
((methylamino)methyl)-4-propylpyridin-2(1H)-one (.232 g, 1.18 mmol) was dissolved in DMF (10.0 mL) and added 6-bromo-1-isopropyl-1H-indazole-4-carboxylic acid (.330 g, 1.18 mmol), prepared as previously reported, EDCI (.338 g, 1.77 mmol), HOBt (.239 g, 1.77 mmol) and TEA (0.49 mL). The reaction was then stirred at rt overnight. The contents were diluted with H$_2$O, stirred for 1 h, and then filtered to collect a yellow solid. The product was then washed with H$_2$O and DEE to give 6-bromo-1-isopropyl-N-methyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (.230 g, 44%) as a white solid. $^1$H NMR (300 MHz, d-$CHCl_3$), δ: 13.137 (m, 1H), (8.072, 7.945) (s, 1H), (7.634, 7.489) (s, 1H), 7.275-7.270 (m, 1H), 5.999 (s, 1H), 4.766-4.743 (m, 2H), 2.905 (s, 3H), 2.712-2.661 (t, 2H, $J$=7.6 Hz), 2.15 (s, 3H), 1.552 (m, 8H), 1.042-0.993 (t, 3H, $J$=7.4 Hz).

tert-butyl 4-(5-(1-isopropyl-4-(methyl)((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)carbamoyl)-1H-indazol-6-yl)pyridin-2-yl)piperazine-1-carboxylate (3.6). 6-bromo-1-isopropyl-N-methyl-N-((6-methyl-2-oxo-4-propyl-1,2-di hydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (.152 g, 0.33 mmol) was dissolved in a mixture of 5 mL H$_2$O:Dioxane (1:5) and then tert-butyl 4-(5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-2-yl)piperazine-1-carboxylate (.142 g, .364 mmol), prepared as previously reported$^{[2]}$, and KOAc (.098 g, 1.00 mmol) were added to the flask. The mixture was purged and placed under argon atmosphere and added 1,1'-Bis(diphenylphosphino)ferrocene-palladium(II)dichloride dichloromethane
complex (.027 g, .033 mmol), and the reaction was heated to reflux for 5 h. After completion, the contents were cooled, diluted with EA, washed with H₂O, and dried over Na₂SO₄. The crude product was then concentrated and prepared for silica chromatography with (DCM/MeOH = 40/1) to give tert-butyl 4-(5-(1-isopropyl-4-(methyl((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)carbamoyl)-1H-indazol-6-yl)pyridin-2-yl)piperazine-1-carboxylate (.175 g, 76%) as a white solid. ¹H NMR (300 MHz, d-CHCl₃), (Rotamers), δ: 8.48 (s, 1H), (8.106, 7.944) (s, 1H), 7.803-7.775(m, 1H), 7.508-7.389(m, 3H), 6.752-6.723(d, 1H, J=8.7 Hz), (5.990, 5.819) (s, 1H), 4.907(s, 2H), 3.590-3.582(m, 7H), (3.066, 2.935) (s, 3H), 2.712-2.685 (m, 1H), 2.289(s. 4H), 1.601-1.580(m, 8H), 1.489(s, 9H), 1.042-0.995 (t, 3H J=7.0 Hz).

1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-methyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (UNC1756 (3.7)). tert-butyl 4-(5-(1-isopropyl-4-(methyl((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)carbamoyl)-1H-indazol-6-yl)pyridin-2-yl)piperazine-1-carboxylate (.173 g, 2.96 mmol) was dissolved in DCM (2.0 mL) and added an excess of TFA. The product (.042 g, .010 mmol) was concentrated in vacuo, dissolved in MeOH (5.0 mL), and placed in an ice bath. Acetone (1.0 mL, 13.6 mmol) and acetic acid (.065 mL, 1.10 mmol) were then added to the reaction. After cooling to 0 °C, NaBH₃CN (.034 g, 0.54 mmol) was added and the reaction was stirred for 15 minutes. Then the ice bath was removed and the contents were allowed to warm to rt overnight. After completion, the MeOH was
removed *in vacuo* and the crude product was isolated by separating with DCM:Brine, 3x DCM washes. The organic layers were combined, dried with Na$_2$SO$_4$, and purified using HPLC to give 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-methyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide as a brown oil (0.023 g, 0.393 mmol, 53%).

$^1$H NMR (300 MHz, d-$CHCl_3$), (Rotamers), $\delta$: 8.48 (s, 1H), (8.106, 7.944) (s, 1H), 7.803-7.775 (m, 1H), 7.508-7.389 (m, 3H), 6.752-6.723 (d, 1H, $J$=8.7 Hz), (5.990, 5.819) (s, 1H), 4.907 (s, 2H), 3.590-3.582 (m, 7H), (3.066, 2.935) (s, 3H), 2.712-2.685 (m, 1H), 2.289 (s, 4H), 1.601-1.580 (m, 8H), 1.489 (s, 9H), 1.042-0.995 (t, 3H $J$=7.0 Hz).

**Synthesis of compounds for EZH1/EZH2 selectivity**

![Scheme 3.3. Reagents and Conditions.](image)

(a) Acetone, Morpholinium-TFA, reflux, o/n; (b) cyanoacetamide, t-BuOK, DMSO, 4-62%; (c) H$_2$, Raney-Ni, MeOH; (d) (6-
(4-isopropylpiperazin-1-yl)pyridin-3-yl)boronic acid, Pd(dppf)Cl₂·DCM, AcOK, H₂O:Dioxane, reflux, 85%; (e) NaOH, rt, o/n, 95%; (f) TBTU, DIPEA, DMF, rt, 16-72%.

4-ethyl-6-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile. Propionaldehyde (5.0 g, 86.5 mmol) and morpholinium trifluoroacetate (1.5 g, 17.3 mmol) were added to acetone (50 mL) and heated to reflux overnight. The reaction was monitored via TLC, organic solvents were removed in vacuo and the crude product was used for the next step without characterization. Cyanoacetamide (1.13 g, 13.5 mmol) and potassium tert-butoxide (1.51 g, 13.5 mmol) were dissolved in DMSO (15 mL) and stirred at room temperature under N₂ for 15 minutes before adding crude (E)-hex-3-en-2-one. The contents were kept under N₂ for 3 h before the addition of more potassium tert-butoxide (4.08 g, 4.08 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H₂O, and added HCl to precipitate the desired product in a two-step yield of 16% (.342 g, 2.11 mmol). ¹H NMR (400 MHz, d₆-CHCl₃), δ: 5.61 (s, 1H), 2.52 (q, 2H), 2.31 (s, 3H), 1.11 (t, 3H).
4-isobutyl-6-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile.

Isovaleraldehyde (2.0 g, 23.2 mmol) and morpholinium trifluoroacetate (.4 g, 4.62 mmol) were added to acetone (14 mL) and heated to reflux overnight. The reaction was monitored via TLC, organic solvents were removed in vacuo and the crude product was used for the next step without characterization.

Cyanoacetamide (.733 g, 8.72 mmol) and potassium tert-butoxide (.889 g, 8.72 mmol) were dissolved in DMSO (15 mL) and stirred at room temperature under N₂ for 15 minutes before adding (E)-6-methylhept-3-en-2-one (1.00 g, 7.92 mmol). The contents were kept under N₂ for 3 h before the addition of more potassium tert-butoxide (2.64 g, 23.5 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H₂O, and added HCl to precipitate the desired product with a two-step yield of 4% (.158 g, .830 mmol). ¹H NMR (400 MHz, d-CHCl₃), δ: 5.61 (s, 1H), 2.44 (d, 2H), 2.31 (s, 3H), 1.89 (m, 1H), 0.98 (d, 6H).

4-cyclopentyl-6-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile.

Cyclopentanecarbaldehyde (1.0 g, 10.3 mmol) and morpholinium trifluoroacetate
(0.2 g, 2.1 mmol) were added to acetone (6 mL) and heated to reflux overnight. The reaction was monitored via TLC, organic solvents were removed \textit{in vacuo} and the crude product was used for the next step without characterization.

Cyanoacetamide (1.4 g, 16.4 mmol) and potassium tert-butoxide (1.8 g, 16.4 mmol) were dissolved in DMSO (19 mL) and stirred at room temperature under N$_2$ for 15 minutes before adding crude (E)-4-cyclopentylbut-3-en-2-one. The contents were kept under N$_2$ for 3 h before the addition of more potassium tert-butoxide (5.0 g, 44.4 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H$_2$O, and added HCl to precipitate the desired product in a two-step yield of 8% (.157 g, .776 mmol). $^1$H NMR (400 MHz, d$_2$-CHCl$_3$), δ: 5.61 (s, 1H), 2.31 (s, 3H), 1.97 (m, 2H), 1.78 (m, 2H), 1.66 (m, 2H), 1.56 (m, 2H).

2-oxo-4-propyl-1,2-dihydropyridine-3-carbonitrile. Cyanoacetamide (.471 g, 5.60 mmol) and potassium tert-butoxide (.629 g, 5.60 mmol) were dissolved in DMSO (6 mL) and stirred at room temperature under N$_2$ for 15 minutes before adding (E)-hex-2-enal (.500 g, 5.09 mmol). The contents were kept under N$_2$ for 3 h before the addition of more potassium tert-butoxide (1.70 g, 15.1 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H$_2$O, and added HCl to precipitate the desired product in 50% yield (1.24 g, 7.64 mmol). $^1$H
NMR (400 MHz, $d$-CHCl$_3$), $\delta$: 7.67 (d, 1H), 6.32 (d, 1H), 2.54 (t, 2H), 1.62 (m, 2H), 0.92 (t, 3H).

4-ethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile. Cyanoacetamide (1.65 g, 19.6 mmol) and potassium tert-butoxide (2.20 g, 19.6 mmol) were dissolved in DMSO (22 mL) and stirred at room temperature under N$_2$ for 15 minutes before adding (E)-pent-2-enal (1.50 g, 17.8 mmol). The contents were kept under N$_2$ for 3 h before the addition of more potassium tert-butoxide (5.94 g, 53.0 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H$_2$O, and added HCl to precipitate the desired product in 21% yield (.545 g, 3.68 mmol). $^1$H NMR (400 MHz, $d$-CHCl$_3$), $\delta$: 7.47 (d, 1H), 6.28 (d, 1H), 2.52 (q, 2H), 1.11 (t, 3H).

4,5-dimethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile. Cyanoacetamide (1.65 g, 19.6 mmol) and potassium tert-butoxide (2.20 g, 19.6 mmol) were dissolved in DMSO (22 mL) and stirred at room temperature under N$_2$ for 15 minutes before adding (E)-2-methylbut-2-enal (1.50 g, 17.8 mmol). The contents were kept under N$_2$ for 3 hours before the addition of more potassium tert-butoxide (5.94 g,
53.0 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H$_2$O, and added HCl to precipitate the desired product in 4% yield (.093 g, .628 mmol). $^1$H NMR (400 MHz, d-CHCl$_3$), δ: 7.94 (s, 1H), 2.50 (s, 3H), 1.98 (s, 3H).

4-isopropyl-6-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile.

Cyanoacetamide (1.24 g, 14.7 mmol) and potassium tert-butoxide (1.65 g, 14.7 mmol) were dissolved in DMSO (17 mL) and stirred at room temperature under N$_2$ for 15 minutes before adding (E)-5-methylhex-3-en-2-one (1.50 g, 13.4 mmol). The contents were kept under N$_2$ for 3 h before the addition of more potassium tert-butoxide (4.46 g, 39.7 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H$_2$O, and added HCl to precipitate the desired product in 29% yield (.676 g, 3.84 mmol). $^1$H NMR (400 MHz, d-CHCl$_3$), δ: 6.27 (s, 1H), 3.02 (m, 1H), 2.25 (s, 3H), 1.17 (d, 6H).
4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile. Cyanoacetamide (.660 g, 7.85 mmol) and potassium tert-butoxide (.881 g, 7.85 mmol) were dissolved in DMSO (9 mL) and stirred at room temperature under N\textsubscript{2} for 15 minutes before adding crotonaldehyde (.500 g, 7.13 mmol). The contents were kept under N\textsubscript{2} for 3 h before the addition of more potassium tert-butoxide (2.38 g, 21.2 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H\textsubscript{2}O, and added HCl to precipitate the desired product in 62% yield (.592 g, 4.41 mmol). \textsuperscript{1}H NMR (400 MHz, d\textsubscript{-}CHCl\textsubscript{3}), \textsuperscript{δ}: 7.64 (d, 1H), 6.3 (d, 1H), 2.35 (s, 3H).

4-butyl-2-oxo-1,2-dihydropyridine-3-carbonitrile. Cyanoacetamide (.412 g, 4.46 mmol) and potassium tert-butoxide (.550 g, 4.90 mmol) were dissolved in DMSO (6 mL) and stirred at room temperature under N\textsubscript{2} for 15 minutes before adding (E)-hept-2-enal (.500 g, 4.46 mmol). The contents were kept under N\textsubscript{2} for 3 hours before the addition of more potassium tert-butoxide (1.49 g, 13.2 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H\textsubscript{2}O, and added HCl to precipitate the desired product in 38% yield (.903 g,
5.10 mmol). $^1$H NMR (400 MHz, $d$-CHCl$_3$), δ: 7.68 (d, 1H), 6.32 (d, 1H), 2.63 (t, 2H), 1.56 (m, 2H), 1.31 (m, 2H), 0.90 (t, 3H).

1-methyl-3-oxo-3,5,6,7-tetrahydro-2H-cyclopenta[c]pyridine-4-carbonitrile.
Cyanoacetamide (.420 g, 4.99 mmol) and potassium tert-butoxide (.560 g, 4.99 mmol) were dissolved in DMSO (6 mL) and stirred at room temperature under N$_2$ for 15 minutes before adding 1-(cyclopent-1-en-1-yl)ethan-1-one (.500, 4.54 mmol). The contents were kept under N$_2$ for 3 hours before the addition of more potassium tert-butoxide (1.51 g, 13.5 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H$_2$O, and added HCl to precipitate the desired product in 44% yield (1.03 g, 5.91 mmol). $^1$H NMR (400 MHz, $d$-CHCl$_3$), δ: 2.89 (t, 2H), 2.64 (t, 2H), 2.19 (s, 3H), 2.01 (m, 2H).

4,6-dimethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile. Cyanoacetamide (1.65 g, 19.6 mmol) and potassium tert-butoxide (2.20 g, 19.6 mmol) were dissolved in DMSO (22 mL) and stirred at room temperature under N$_2$ for 15 minutes before adding (E)-pent-3-en-2-one (1.50 g, 17.8 mmol). The contents were kept under
N₂ for 3 hours before the addition of more potassium tert-butoxide (5.94 g, 53.0 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H₂O, and added HCl to precipitate the desired product in 45% yield (1.18 g, 7.96 mmol). ¹H NMR (400 MHz, d-CHCl₃), δ: 6.16 (s, 1H), 2.30 (s, 3H), 2.22 (s, 3H).

6-methyl-2-oxo-4-pentyl-1,2-dihydropyridine-3-carbonitrile. Cyanoacetamide (.660 g, 7.84 mmol) and potassium tert-butoxide (.860 g, 7.84 mmol) were dissolved in DMSO (9 mL) and stirred at room temperature under N₂ for 15 minutes before adding (E)-non-3-en-2-one (1.00 g, 7.13 mmol). The contents were kept under N₂ for 3 hours before the addition of more potassium tert-butoxide (2.36 g, 21.2 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H₂O, and added HCl to precipitate the desired product in 55% yield (.803 g, 3.93 mmol). ¹H NMR (400 MHz, d-CHCl₃), δ: 6.26 (s, 1H), 2.42 (t, 2H), 2.20 (s, 3H), 1.29 (m, 2H), 1.23 (m, 2H), 1.23 (m, 2H), 1.02 (m, 3H).
6-ethyl-4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile. Cyanoacetamide (.942 g, 11.2 mmol) and potassium tert-butoxide (1.26 g, 11.2 mmol) were dissolved in DMSO (13 mL) and stirred at room temperature under N$_2$ for 15 minutes before adding (E)-hex-4-en-3-one (1.00 g, 10.2 mmol). The contents were kept under N$_2$ for 3 hours before the addition of more potassium tert-butoxide (3.40 g, 30.3 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H$_2$O, and added HCl to precipitate the desired product in 32% yield (.525 g, 3.24 mmol). $^1$H NMR (400 MHz, $d_6$-DMSO), $\delta$: 6.19 (s, 1H), 2.5 (q, 2H), 2.32 (s, 3H), 1.17 (t, 3H).

1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinoline-4-carbonitrile.
Cyanoacetamide (.745 g, 8.86 mmol) and potassium tert-butoxide (.994 g, 8.86 mmol) were dissolved in DMSO (10 mL) and stirred at room temperature under N$_2$ for 15 minutes before adding 1-(cyclohex-1-en-1-yl)ethan-1-one (1.00 g, 8.05 mmol). The contents were kept under N$_2$ for 3 hours before the addition of more potassium tert-butoxide (2.68 g, 23.9 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via
TLC, and after completion, diluted with H$_2$O, and added HCl to precipitate the desired product in 57% yield (.861 g, 4.57 mmol). $^1$H NMR (400 MHz, $d$-CHCl$_3$), δ: 2.72 (m, 2H), 2.37 (m, 2H), 2.21 (s, 3H), 1.68 (m, 4H).

4,5,6-trimethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile. Cyanoacetamide (.942 g, 11.2 mmol) and potassium tert-butoxide (1.26 g, 11.2 mmol) were dissolved in DMSO (13 mL) and stirred at room temperature under N$_2$ for 15 minutes before adding (E)-hex-3-en-2-one (1.00 g, 10.2 mmol). The contents were kept under N$_2$ for 3 hours before the addition of more potassium tert-butoxide (3.40 g, 30.0 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H$_2$O, and added HCl to precipitate the desired product in 38% yield (.626 g, 3.86 mmol). $^1$H NMR (400 MHz, $d$-CHCl$_3$), δ: 2.32 (s, 3H), 2.25 (s, 3H), 1.93 (s, 3H).

5-ethyl-2-oxo-4-propyl-1,2-dihydropyridine-3-carbonitrile. Cyanoacetamide (.811 g, 9.5 mmol) and potassium tert-butoxide (1.04 g, 16.4 mmol) were dissolved in DMSO (20 mL) and stirred at room temperature under N$_2$ for 15
minutes before adding 3(E)-2-ethylhex-2-enal (1.0 g, 7.92 mmol). The contents were kept under N₂ for 3 hours before the addition of more potassium tert-butoxide (2.90 g, 28.5 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H₂O, and added HCl to precipitate the desired product in a two-step yield of 11% (.166 g, .871 mmol). ¹H NMR (400 MHz, d-CHCl₃), δ: 7.50 (s, 1H), 2.55 (q, 2H), 2.46 (t, 2H), 1.44 (m, 2H), 1.10 (t, 3H), 0.96 (t, 3H).

6-(sec-butyl)-4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile.

Cyanoacetamide (1.5 g, 17.7 mmol) and potassium tert-butoxide (2.0 g, 17.7 mmol) were dissolved in DMSO (20 mL) and stirred at room temperature under N₂ for 15 minutes before adding (E)-5-methylhept-2-en-4-one (2.0 g, 16.1 mmol). The contents were kept under N₂ for 3 hours before the addition of more potassium tert-butoxide (5.4 g, 47.7 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H₂O, and added HCl to precipitate the desired product in 38% yield (.590 g, 3.10 mmol). ¹H NMR (400 MHz, d-CHCl₃), δ: 6.22 (s, 1H), 2.56 (m, 1H), 2.33 (s, 3H), 1.60 (m, 1H), 1.52 (m, 1H), 1.16 (d, 3H), 0.78 (t, 3H).
5-ethyl-4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile. Cyanoacetamide (1.3 g, 15.7 mmol) and potassium tert-butoxide (1.8 g, 15.7 mmol) were dissolved in DMSO (18 mL) and stirred at room temperature under N₂ for 15 minutes before adding (E)-2-ethylbut-2-enal (2.0 g, 14.3 mmol). The contents were kept under N₂ for 3 hours before the addition of more potassium tert-butoxide (1.8 g, 42.5 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion, diluted with H₂O, and added HCl to precipitate the desired product in 12% yield (.271 g, 1.67 mmol). ¹H NMR (400 MHz, d-CHCl₃), δ: 7.55 (s, 1H), 2.64 (q, 2H), 2.06 (s, 3H), 1.13 (t, 3H).

6-butyl-4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile. Cyanoacetamide (1.5 g, 17.8 mmol) and potassium tert-butoxide (2.0 g, 17.8 mmol) were dissolved in DMSO (20 mL) and stirred at room temperature under N₂ for 15 minutes before adding (E)-oct-2-en-4-one (2.0 g, 16.2 mmol). The contents were kept under N₂ for 3 hours before the addition of more potassium tert-butoxide (5.4 g, 48.0 mmol) and exposure to air. The contents were then stirred overnight at room temperature. The reaction was monitored via TLC, and after completion,
diluted with H₂O, and added HCl to precipitate the desired product in 12% yield (.180 g, .946 mmol). ¹H NMR (400 MHz, d-CHCl₃), δ: 6.19 (s, 1H), 2.49 (t, 2H), 2.31 (s, 3H), 1.54 (m, 2H), 1.28 (m, 2H), 0.87 (t, 3H).

N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide. 4,6-dimethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (.625 g, 4.22 mmol) was dissolved in MeOH (10 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under H₂ overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated in vacuo and used for the next step without further characterization. Crude 3-(aminomethyl)-4,6-dimethylpyridin-2(1H)-one (10.5 mg, .054 mmol), TBTU (23.6 mg, .073 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (20.0 mg, .049 mmol) were dissolved in DMF (.33 mL) prior to the addition of DIPEA (20 uL). The contents were stirred at room temperature overnight, monitored by LC/MS and purified using HPLC. The desired product was obtained with a two-step yield of 16% (4.6 mg, .008 mmol). ¹H NMR (400 MHz, Methanol-d₄), δ: 8.58 (dd, J = 2.5, 0.7 Hz, 1H), 8.37 (s, 1H), 8.18 (dd, J =
1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((4-methyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide. 4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (0.592 g, 4.41 mmol) was dissolved in MeOH (9 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under H₂ overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated in vacuo and used for the next step without further characterization. Crude 3-(aminomethyl)-4-methylpyridin-2(1H)-one (7.4 mg, 0.054 mmol), TBTU (23.6 mg, 0.073 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (20.0 mg, 0.049 mmol) were dissolved in DMF (0.34 mL) prior to the addition of DIPEA (20 μL). The contents were stirred at room temperature overnight, monitored by TLC and purified using HPLC. The desired product was obtained with a two-step yield of 40% (10.4 mg, 0.020 mmol). ¹H NMR (400 MHz, d₄-MeOH), δ: 8.58 (t, J = 2.6 Hz,
2H), 8.45 (s, 1H), 8.37 (s, 1H), 8.12 (d, J = 1.4 Hz, 1H), 8.07 (s, 1H), 7.96 (s, 1H), 7.78 (s, 1H), 7.31 (d, J = 6.7 Hz, 1H), 6.34 (d, J = 6.7 Hz, 1H), 5.10 (dt, J = 12.1, 6.6 Hz, 1H), 4.61 (s, 2H), 3.61 (dt, J = 13.4, 6.6 Hz, 2H), 2.49 (s, 3H), 1.58 (t, J = 7.1 Hz, 6H), 1.43 (d, J = 6.7 Hz, 6H).

1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide. 2-oxo-4-propyl-1,2-dihydropyridine-3-carbonitrile (.600 g, 3.70 mmol) was dissolved in MeOH (14 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under H₂ overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated in vacuo and used for the next step without further characterization. Crude 3-(aminomethyl)-4-propylpyridin-2(1H)-one (9.7 mg, .054 mmol), TBTU (23.6 mg, .073 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (20.0 mg, .049 mmol) were dissolved in DMF (.33 mL) prior to the addition of DIPEA (20 uL). The contents were stirred at room temperature overnight, monitored by TLC and purified using HPLC. The desired product was obtained with a two-step yield of 41% yield (11.1 mg, .020 mmol). ¹H NMR (400 MHz,
Methanol-\(d_4\), \(\delta\): 8.57 (dd, \(J = 2.5, 0.7\) Hz, 1H), 8.36 (s, 1H), 8.20 (m, 2H), 7.96 (t, \(J = 1.1\) Hz, 1H), 7.77 (d, \(J = 1.3\) Hz, 1H), 7.34 (d, \(J = 6.8\) Hz, 1H), 7.15 (td, \(J = 9.0, 0.8\) Hz, 1H), 6.36 (d, \(J = 6.8\) Hz, 1H), 5.09 (dt, \(J = 13.2, 6.4\) Hz, 1H), 4.63 (s, 2H), 3.56 (m, 2H), 2.76 (m, 2H), 1.61 (m, 2H), 1.57 (d, \(J = 6.5\) Hz, 6H), 1.43 (dd, \(J = 6.7, 1.1\) Hz, 6H), 1.03 (t, \(J = 7.3\) Hz, 3H).

\[\text{N-}((4\text{-ethyl-2-oxo-1,2-dihydropyridin-3-yl})\text{methyl}-1\text{-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)}\text{pyridin-3-yl)-1H-indazole-4-carboxamide}.\]

4-ethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (0.550 g, 3.68 mmol) was dissolved in MeOH (8 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under \(H_2\) overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated \textit{in vacuo} and used for the next step without further characterization. Crude 3-(aminomethyl)-4-ethylpyridin-2(1H)-one (8.2 mg, 0.054 mmol), TBTU (23.6 mg, 0.073 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (20.0 mg, 0.049 mmol) were dissolved in DMF (0.33 mL) prior to the addition of DIPEA (20 uL). The contents were stirred at room temperature overnight, monitored by LC/MS and purified using HPLC. The desired product was obtained with a two-
step yield of 32% yield (8.5 mg, .016 mmol). \(^1\)H NMR (400 MHz, Methanol-\textit{d}_4), \(\delta:\)

8.57 (d, \(J = 2.4\) Hz, 1H), 8.36 (s, 1H), 8.16 (dd, \(J = 9.0,\) 2.5 Hz, 1H), 7.96 (t, \(J = 1.1\) Hz, 1H), 7.77 (d, \(J = 1.2\) Hz, 1H), 7.36 (d, \(J = 6.8\) Hz, 1H), 7.14 (d, \(J = 9.0\) Hz, 1H), 6.38 (d, \(J = 6.8\) Hz, 1H), 5.09 (h, \(J = 6.6\) Hz, 1H), 4.63 (s, 2H), 3.61 (hept, \(J = 6.7\) Hz, 4H), 3.42 (s, 1H), 2.86 (q, \(J = 7.6\) Hz, 2H), 1.57 (d, \(J = 6.6\) Hz, 6H), 1.43 (d, \(J = 6.6\) Hz, 6H), 1.25 (t, \(J = 7.6\) Hz, 3H).

\[ \text{1-isopropyl-N-((4-isopropyl-6-methyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide.} \]

4-isopropyl-6-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (.550 g, 3.68 mmol) was dissolved in MeOH (8 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under H\(_2\) overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated \textit{in vacuo} and used for the next step without further characterization. 3-(aminomethyl)-4-isopropyl-6-methylpyridin-2(1H)-one (16.3 mg, .090 mmol), TBTU (39.5 mg, .073 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (33.5 mg, .082 mmol) were dissolved in DMF (.55 mL) prior to the addition of DIPEA (30 uL). The contents were stirred at room temperature.
overnight, monitored by LC/MS and purified using HPLC. The desired product was obtained with a two-step yield of 55% yield (28.5 mg, .050 mmol). \(^1\)H NMR (400 MHz, Chloroform-\(d\)), \(\delta\): 12.60 (s, 1H), 8.48 (d, \(J = 2.5\) Hz, 1H), 7.89 (t, \(J = 5.8\) Hz, 1H), 7.76 (dd, \(J = 8.8, 2.6\) Hz, 1H), 7.70 (d, \(J = 1.4\) Hz, 1H), 7.57 (t, \(J = 1.1\) Hz, 1H), 6.69 (d, \(J = 8.8\) Hz, 1H), 6.06 ? 6.01 (m, 1H), 4.86 (hept, \(J = 6.7\) Hz, 1H), 4.71 (d, \(J = 5.8\) Hz, 2H), 3.59 (t, \(J = 5.1\) Hz, 4H), 3.52 (q, \(J = 6.8\) Hz, 1H), 2.80 (broad, 4H) 2.59 (m, 5H), 2.19 (s, 3H), 1.58 (d, \(J = 6.7\) Hz, 6H), 1.21 (d, \(J = 6.8\) Hz, 6H), 1.08 (d, \(J = 6.5\) Hz, 6H).

\[\text{N-((4-butyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide.}\]

4-butyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (.550 g, 3.68 mmol) was dissolved in MeOH (8 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under \(H_2\) overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated \emph{in vacuo} and used for the next step without further characterization. 3-(aminomethyl)-4-butylpyridin-2(1H)-one (10.5 mg, .054 mmol), TBTU (23.6 mg, .073 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (20.0 mg, .049
mmol) were dissolved in DMF (.33 mL) prior to the addition of DIPEA (20 uL). The contents were stirred at room temperature overnight, monitored by LC/MS and purified using HPLC. The desired product was obtained with a two-step yield of 32% yield (5.1 mg, .010 mmol). $^1$H NMR (400 MHz, Methanol-$d_4$), $\delta$: 8.58 (dd, $J = 2.5$, 0.7 Hz, 1H), 8.36 (s, 1H), 8.13 (dd, $J = 9.0$, 2.5 Hz, 1H), 7.95 (t, $J = 1.1$ Hz, 1H), 7.77 (d, $J = 1.3$ Hz, 1H), 7.33 (d, $J = 6.8$ Hz, 1H), 7.10 (dd, $J = 9.0$, 0.8 Hz, 1H), 6.35 (d, $J = 6.7$ Hz, 1H), 5.08 (hept, $J = 6.5$ Hz, 1H), 4.63 (s, 2H), 3.61 (hept, $J = 6.6$ Hz, 4H), 2.86 (broad, 4H), 2.77 (m, 2H), 1.54 (m, 6H), 1.38 (m, 6H), 0.93 (t, $J = 7.3$ Hz, 3H).

\[\text{1-isopropyl-6-}(6\text{-}(4\text{-isopropylpiperazin-1-yl})\text{-pyridin-3-yl})\text{-N-}((1\text{-methyl-3-oxo-3,5,6,7-tetrahydro-2H-cyclopenta}[c]\text{-pyridin-4-yl})\text{methyl})\text{-1H-indazole-4-carboxamide.}\]

1-methyl-3-oxo-3,5,6,7-tetrahydro-2H-cyclopenta[c]pyridine-4-carbonitrile (.550 g, 3.68 mmol) was dissolved in MeOH (8 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under H$_2$ overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated in vacuo and used for the next step without further characterization. Crude 4-(aminomethyl)-1-methyl-2,5,6,7-tetrahydro-3H-
cyclopenta[c]pyridin-3-one (9.6 mg, .054 mmol), TBTU (23.6 mg, .073 mmol),
and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-
carboxylic acid (20.0 mg, .049 mmol) were dissolved in DMF (.33 mL) prior to the
addition of DIPEA (20 uL). The contents were stirred at room temperature
overnight, monitored by LC/MS and purified using HPLC. The desired product
was obtained with a two-step yield of 42% yield (11.8 mg, .021 mmol). \(^1\)H NMR
(400 MHz, Methanol-\(d_4\)), \(\delta:\) 8.54 (m, 1H), 8.38 (s, 1H), 8.17 (dd, \(J = 9.0, 2.5\) Hz,
1H), 7.98 (t, \(J = 1.1\) Hz, 1H), 7.81 (d, \(J = 1.3\) Hz, 1H), 7.19 ? 7.11 (m, 1H), 5.09
(hept, \(J = 6.2\) Hz, 1H), 4.54 (s, 2H), 3.62 (hept, \(J = 6.7\) Hz, 2H), 3.13 (t, \(J = 7.6\)
Hz, 2H), 2.77 (t, \(J = 7.3\) Hz, 2H), 2.27 (s, 3H), 2.13 (q, \(J = 7.5\) Hz, 2H), 1.57 (d, \(J =
6.6\) Hz, 6H), 1.43 (d, \(J = 6.7\) Hz, 6H).

\[ N-((4,5\text{-dimethyl-2-oxo-1,2-dihydropyridin-3-yl})\text{methyl})-1\text{-isopropyl-6-(6-(4-}
\text{isopropylpiperazin-1-yl)pyridin-3-yl})-1\text{H-indazole-4-carboxamide}. \] 
4,5-
dimethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (.093 g, .630 mmol) was
dissolved in MeOH (10 mL) and added a catalytic amount of Raney-Ni. The
contents were purged and kept under \(H_2\) overnight. The reaction was monitored
via TLC. After completion, the contents were filtered, concentrated \textit{in vacuo} and
used for the next step without further characterization. Crude 3-(aminomethyl)-
4,5-dimethylpyridin-2(1H)-one (7.4 mg, .054 mmol), TBTU (23.6 mg, .073 mmol),
and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-
carboxylic acid (20.0 mg, .049 mmol) were dissolved in DMF (.33 mL) prior to the
addition of DIPEA (20 uL). The contents were stirred at room temperature
overnight, monitored by LC/MS and purified using HPLC. The desired product
was obtained with a two-step yield of 20% yield (5.1 mg, .010 mmol). 1H NMR
(400 MHz, Methanol-d₄), δ: 8.59 (dd, J = 2.5, 0.7 Hz, 1H), 8.39 (s, 1H), 8.19 (dd,
J = 9.0, 2.5 Hz, 1H), 7.99 (t, J = 1.1 Hz, 1H), 7.84 (d, J = 1.3 Hz, 1H), 7.51 (s,
1H), 7.12 (m, 1H), 5.09 (hept, J = 6.7 Hz, 2H), 4.48 (s, 2H), 3.62 (hept, J = 6.7
Hz, 3H), 2.26 (s, 3H), 2.08 (s, 3H), 1.58 (d, J = 6.6 Hz, 6H), 1.43 (d, J = 6.6 Hz,
6H).

1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((4,5,6-trimethyl-
2-oxo-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide. 4,5,6-
trimethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (.500 g,3.08 mmol) was
dissolved in MeOH (6 mL) and added a catalytic amount of Raney-Ni. The
contents were purged and kept under H₂ overnight. The reaction was monitored
via TLC. After completion, the contents were filtered, concentrated in vacuo and used for the next step without further characterization. 3-(aminomethyl)-4,5,6-trimethylpyridin-2(1H)-one (10.0 mg, .060 mmol), TBTU (29.0 mg, .090 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (27.0 mg, .066 mmol) were dissolved in DMF (.60 mL) prior to the addition of DIPEA (30 uL). The contents were stirred at room temperature overnight, monitored by LC/MS and purified using HPLC. The desired product was obtained with a two-step yield of 56% (18.7 mg, .034 mmol). ¹H NMR (400 MHz, Chloroform-d), δ: 8.47 (d, J = 2.4 Hz, 1H), 8.35 (s, 1H), 7.97 (dd, J = 8.9, 2.3 Hz, 1H), 7.62 (s, 1H), 7.57 (s, 1H), 7.42 (s, 1H), 6.87 (d, J = 8.9 Hz, 1H), 4.88 (hept, J = 6.7 Hz, 3H), 4.72 (d, J = 5.4 Hz, 2H), 3.56 (h, J = 6.7 Hz, 2H), 2.60 (s, 3H), 2.45 (s, 3H), 2.15 (s, 3H), 1.59 (s, 6H), 1.38 (d, J = 6.6 Hz, 6H).

N-((6-ethyl-4-methyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide. 6-ethyl-4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (.530 g, 3.24 mmol) was dissolved in MeOH (7 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under H₂ overnight. The reaction was monitored
via TLC. After completion, the contents were filtered, concentrated in vacuo and used for the next step without further characterization. Crude 3-(aminomethyl)-6-ethyl-4-methylpyridin-2(1H)-one (10.0 mg, .060 mmol), TBTU (29.0 mg, .090 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (27.0 mg, .066 mmol) were dissolved in DMF (.60 mL) prior to the addition of DIPEA (30 μL). The contents were stirred at room temperature overnight, monitored by LC/MS and purified using HPLC. The desired product was obtained with a two-step yield of 61% (20.3 mg, .037 mmol).

1H NMR (400 MHz, Chloroform-d), δ: 8.49 (d, J = 2.4 Hz, 1H), 7.89 (dd, J = 8.8, 2.3 Hz, 1H), 7.61 (s, 1H), 7.59 (s, 1H), 7.37 (s, 1H), 6.80 (d, J = 8.8 Hz, 1H), 6.45 (s, 1H), 4.89 (p, J = 6.7 Hz, 1H), 4.68 (broad, 4H), 4.62 (m, 2H), 4.40 (s, 1H), 3.56 (p, J = 6.7 Hz, 3H), 3.25 (s, 4H), 2.69 (q, J = 7.6 Hz, 3H), 2.63 (s, 3H), 1.60 (d, J = 6.6 Hz, 7H), 1.38 (d, J = 6.6 Hz, 6H), 1.29 (t, J = 7.6 Hz, 3H).

1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinolin-4-yl)methyl)-1H-indazole-4-carboxamide.

1-methyl-3-oxo-2,3,5,6,7,8-hexahydroisoquinoline-4-carbonitrile (.500 g, 2.66 mmol) was dissolved in MeOH (5 mL) and added a catalytic amount of Raney-Ni.
The contents were purged and kept under H₂ overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated \textit{in vacuo} and used for the next step without further characterization. Crude 4-(aminomethyl)-1-methyl-5,6,7,8-tetrahydroisoquinolin-3(2H)-one (10.0 mg, .052 mmol), TBTU (25.0 mg mg, .078 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (23.3 mg, .057 mmol) were dissolved in DMF (.52 mL) prior to the addition of DIPEA (30 uL). The contents were stirred at room temperature overnight, monitored by LC/MS and purified using HPLC. The desired product was obtained with a two-step yield of 42% (12.8 mg, .022 mmol). \(^1\)H NMR (400 MHz, Chloroform-\(d\)), \(\delta\): 8.48 (s, 1H), 8.36 (s, 1H), 8.04 ? 7.97 (m, 1H), 7.63 (s, 1H), 7.56 (s, 1H), 7.41 (s, 1H), 6.90 (d, \(J = 8.9 \text{ Hz, 1H}\)), 4.89 (p, \(J = 6.6 \text{ Hz, 3H}\)), 4.68 (d, \(J = 5.4 \text{ Hz, 3H}\)), 3.56 (p, \(J = 6.7 \text{ Hz, 2H}\)), 3.19 (t, \(J = 6.1 \text{ Hz, 3H}\)), 2.56 (t, \(J = 6.1 \text{ Hz, 2H}\)), 2.40 (s, 2H), 1.81 (d, \(J = 11.8 \text{ Hz, 4H}\)), 1.59 (d, \(J = 6.6 \text{ Hz, 6H}\)), 1.38 (d, \(J = 6.6 \text{ Hz, 6H}\)).

\[\text{1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-pentyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide.}\]

6-methyl-2-oxo-4-pentyl-1,2-dihydropyridine-3-carbonitrile (.500 g, 2.46 mmol) was
dissolved in MeOH (5 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under H₂ overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated in vacuo and used for the next step without further characterization. Crude 3-(aminomethyl)-6-methyl-4-pentyldipyridin-2(1H)-one (10.0 mg, .048 mmol), TBTU (23.1 mg, .072 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (21.5 mg, .053 mmol) were dissolved in DMF (.48 mL) prior to the addition of DIPEA (25 μL). The contents were stirred at room temperature overnight, monitored by LC/MS and purified using HPLC. The desired product was obtained with a two-step yield of 46% (13.2 mg, .022 mmol). ¹H NMR (400 MHz, Chloroform-d), δ: 8.49 (s, 1H), 8.35 (s, 1H), 7.93 (dd, J = 8.7, 2.3 Hz, 1H), 7.62 (s, 1H), 7.58 (s, 1H), 7.39 (s, 1H), 6.83 (d, J = 8.8 Hz, 1H), 6.47 (s, 1H), 4.88 (q, J = 6.6 Hz, 2H), 4.70 (broad, 4H), 4.63 (m, 2H), 3.56 (p, J = 6.7 Hz, 8H), 2.88 (m, 3H), 2.43 (s, 3H), 1.54 (m, 12H), 1.39 (dd, J = 6.7, 2.2 Hz, 12H), 0.81 (m, 3H).

N-((4-ethyl-6-methyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide.
6-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (.340 g, 2.11 mmol) was dissolved in MeOH (5 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under H₂ overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated in vacuo and used for the next step without further characterization. Crude 3-(aminomethyl)-4-ethyl-6-methylpyridin-2(1H)-one (13.0 mg, .078 mmol), TBTU (34.2 mg, .107 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (29.0 mg, .071 mmol) were dissolved in DMF (.71 mL) prior to the addition of DIPEA (40 uL). The contents were stirred at room temperature overnight, monitored by LC/MS and purified using HPLC. The desired product was obtained with a two-step yield of 22% yield (9.4 mg, .017 mmol). ¹H NMR (600 MHz, Chloroform-d), δ: 8.47 (d, J = 2.5 Hz, 1H), 8.39 (s, 1H), 7.83 (t, J = 5.8 Hz, 1H), 7.76 (dd, J = 8.6, 2.5 Hz, 1H), 7.68 (s, 1H), 7.57 (s, 1H), 6.67 (d, J = 8.7 Hz, 1H), 5.91 (s, 1H), 4.87 (hept, J = 6.7 Hz, 1H), 4.64 (d, J = 5.9 Hz, 2H), 3.66 (s, 4H), 2.71 (s, 4H), 2.43 (s, 4H), 1.58 (d, J = 6.6 Hz, 8H), 1.47 (dp, J = 14.1, 7.2 Hz, 1H), 1.14 (d, J = 6.7 Hz, 6H), 0.72 (t, J = 7.3 Hz, 3H).
**N-((4-isobutyl-6-methyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide.**

4-isobutyl-6-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (.020 g, .120 mmol) was dissolved in MeOH (.5 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under H₂ overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated *in vacuo* and used for the next step without further characterization. 3-(aminomethyl)-4-isobutyl-6-methylpyridin-2(1H)-one (16.4 mg, .084 mmol), TBTU (37.0 mg, .115 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (31.3 mg, .077 mmol) were dissolved in DMF (.77 mL) prior to the addition of DIPEA (40 uL). The contents were stirred at room temperature overnight, monitored by LC/MS and purified using HPLC. The desired product was obtained with a two-step yield of 62% yield (30.4 mg, .052 mmol).

**¹H NMR (600 MHz, Chloroform-d), δ:** 8.48 (s, 1H), 8.38 (s, 1H), 8.04 (s, 1H), 7.76 (d, J = 8.6 Hz, 1H), 7.72 (s, 1H), 7.57 (s, 1H), 6.67 (d, J = 8.8 Hz, 1H), 5.90 (s, 1H), 4.92 – 4.80 (m, 1H), 4.66 (d, J = 5.3 Hz, 2H), 3.59 (s, 4H), 2.78 – 2.68 (m, 1H), 2.64 (s, 4H), 2.61 (d, J = 7.2 Hz, 2H), 2.14 (s, 3H), 1.94 – 1.81 (m, 1H), 1.57 (d, J = 6.5 Hz, 6H), 1.08 (d, J = 6.4 Hz, 6H), 0.97 (d, J = 6.5 Hz, 6H).
N-((6-(sec-butyl)-4-methyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide. 6-(sec-butyl)-4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (.100 g, .526 mmol) was dissolved in MeOH (5 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under H₂ overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated in vacuo and used for the next step without further characterization. Crude 3-(aminomethyl)-6-(sec-butyl)-4-methylpyridin-2(1H)-one (13.0 mg, .067 mmol), TBTU (29.3 mg, .091 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (24.8 mg, .061 mmol) were dissolved in DMF (.61 mL) prior to the addition of DIPEA (30 uL). The contents were stirred at room temperature overnight, monitored by LC/MS and purified using HPLC. The desired product was obtained with a two-step yield of 63% (24.5 mg, .042 mmol). ¹H NMR (600 MHz, Chloroform-d), δ: 8.39 (s, 1H), 7.83 (t, \( J = 5.8 \) Hz, 1H), 7.76 (dd, \( J = 8.6, 2.5 \) Hz, 1H), 7.68 (s, 1H), 7.57 (s, 1H), 6.67 (d, \( J = 8.7 \) Hz, 1H), 5.91 (s, 1H), 4.87 (hept, \( J = 6.7 \) Hz, 1H), 4.64 (d, \( J = 5.9 \) Hz, 2H), 3.66 (s, 4H), 3.48 (s, 2H), 2.71 (s, 4H), 2.43 (s, 4H), 1.58 (d, \( J = 6.6 \) Hz, 8H), 1.47
(dp, $J = 14.1, 7.2$ Hz, 1H), 1.34 – 1.18 (m, 2H), 1.14 (d, $J = 6.7$ Hz, 6H), 0.72 (t, $J = 7.3$ Hz, 3H).

**N-((5-ethyl-4-methyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide.** 5-ethyl-4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (.100 g, .617 mmol) was dissolved in MeOH (13 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under H$_2$ overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated *in vacuo* and used for the next step without further characterization. Crude 3-(aminomethyl)-5-ethyl-4-methylpyridin-2(1H)-one (13.6 mg, .082 mmol), TBTU (35.8 mg, .112 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (30.3 mg, .074 mmol) were dissolved in DMF (.74 mL) prior to the addition of DIPEA (40 uL). The contents were stirred at room temperature overnight, monitored by LC/MS and purified using HPLC. The desired product was obtained with a two-step yield of 72% (32.9 mg, .059 mmol). $^1$H NMR (600 MHz, Methanol-$d_4$), $\delta$: 8.61 (d, $J = 2.4$ Hz, 1H), 8.40 (s, 1H), 8.19 (dd, $J = 9.2, 2.4$ Hz, 1H), 8.00 (s, 1H), 7.81 (s, 1H), 7.27 (s, 1H), 7.17 (d, $J = 8.8$ Hz, 1H), 5.12 (p,
\[ J = 6.6 \text{ Hz, 1H}), 4.69 \text{ (s, 2H), 4.61 (broad, 2H), 3.64 (hept, } J = 6.9 \text{ Hz, 1H), 3.60 (broad, 2H), 2.93 (q, } J = 7.7 \text{ Hz, 2H), 2.18 (s, 3H), 1.60 (d, } J = 6.4 \text{ Hz, 8H), 1.45 (d, } J = 6.5 \text{ Hz, 8H), 1.24 (t, } J = 7.6 \text{ Hz, 3H).} \]

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\text{N-((4-cyclopentyl-6-methyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide. 4-cyclopentyl-6-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (.157 g, .776 mmol) was dissolved in MeOH (16 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under H}_2 \text{ overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated } in \text{ vacuo and used for the next step without further characterization. Crude 3-(aminomethyl)-4-cyclopentyl-6-methylpyridin-2(1H)-one (16.0 mg, .078 mmol), TBTU (34.0 mg, .106 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (28.7 mg, .071 mmol) were dissolved in DMF (.71 mL) prior to the addition of DIPEA (40 uL). The contents were stirred at room temperature overnight, monitored by LC/MS and purified using HPLC. The desired product was obtained with a two-step yield of 56% (26.0 mg, .043 mmol). \text{^1H NMR (600 MHz, Chloroform-d), } \delta: 8.42 \text{ (s, 1H), 8.39 (s,} \]
1H), 8.00 (s, 1H), 7.73 (d, J = 7.4 Hz, 1H), 7.64 (s, 1H), 7.55 (s, 1H), 6.56 (d, J = 8.7 Hz, 1H), 6.04 (s, 1H), 4.92 – 4.80 (m, 1H), 4.69 (d, 2H), 3.87 (broad, J = 91.1, 63.2 Hz, 2H), 3.54 (m, 1H), 3.38 (d, J = 40.8 Hz, 2H), 3.06 (broad, 4H), 2.20 (s, 3H), 2.04 (m, 2H), 1.78 (m, J = 18.9 Hz, 4H), 1.57 (d, J = 6.6 Hz, 6H), 1.52 (m, 2H), 1.32 (d, J = 6.4 Hz, 6H).

N-((6-butyl-4-methyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide. 6-butyl-4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (.181 g, .951 mmol) was dissolved in MeOH (19 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under H₂ overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated in vacuo and used for the next step without further characterization. Crude 3-(aminomethyl)-6-butyl-4-methylpyridin-2(1H)-one (17.5 , .090 mmol), TBTU (39.4 mg, .123 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (33.4 mg, .082 mmol) were dissolved in DMF (.82 mL) prior to the addition of DIPEA (40 uL). The contents were stirred at room temperature overnight, monitored by LC/MS and purified using HPLC. The desired product
was obtained with a two-step yield of 66% (34.7 mg, 0.059 mmol). \(^1\)H NMR (600 MHz, Methanol-\(d_4\)), \(\delta\): 8.56 (d, \(J = 2.4\) Hz, 1H), 8.39 (s, 1H), 8.07 (dd, \(J = 8.8, 2.5\) Hz, 1H), 7.95 (d, \(J = 5.5\) Hz, 1H), 7.78 (s, 1H), 7.03 (d, \(J = 8.9\) Hz, 1H), 6.17 (s, 1H), 5.10 (p, \(J = 6.6\) Hz, 1H), 4.60 (s, 2H), 3.65 – 3.59 (m, 5H), 3.33 (m, \(J = 3.2, 1.6\) Hz, 1H), 3.30 (broad, 4H), 2.55 – 2.51 (m, 1H), 2.46 (s, 3H), 2.17 (m, 2H), 1.64 – 1.57 (d, 6H), 1.45 (d, \(J = 6.6, 3.8\) Hz, 6H), 1.31 (d, 4H), 0.94 (t, \(J = 7.4\) Hz, 3H).

**N-((5-ethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide.** 5-ethyl-2-oxo-4-propyl-1,2-dihydropyridine-3-carbonitrile (.166 g, .871 mmol) was dissolved in MeOH (20 mL) and added a catalytic amount of Raney-Ni. The contents were purged and kept under \(H_2\) overnight. The reaction was monitored via TLC. After completion, the contents were filtered, concentrated in vacuo and used for the next step without further characterization. Crude 3-(aminomethyl)-5-ethyl-4-propylpyridin-2(1H)-one (9.0 mg, 0.046 mmol), TBTU (23.1 mg, 0.072 mmol), and 1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxylic acid (21.5 mg, 0.053 mmol) were dissolved in DMF (.46 mL) prior to
the addition of DIPEA (25 uL). The contents were stirred at room temperature overnight, monitored by LC/MS and purified using HPLC. The desired product was obtained with a two-step yield of 18% (4.7 mg, .008 mmol). 

$^1$H NMR (400 MHz, Chloroform-d), δ: 8.49 (s, 1H), 8.36 (s, 1H), 7.86 (d, $J = 8.7$ Hz, 1H), 7.61 (s, 1H), 7.47 (s, 1H), 7.32 (s, 1H), 6.77 (d, $J = 8.7$ Hz, 1H), 4.89 (p, $J = 6.7$ Hz, 1H), 4.73 (d, $J = 5.4$ Hz, 2H), 4.41 (s, 2H), 3.56 (p, $J = 6.6$ Hz, 4H), 2.99 (s, 2H), 2.59 (q, $J = 7.5$ Hz, 4H), 1.59 (d, $J = 6.7$ Hz, 8H), 1.38 (d, $J = 6.6$ Hz, 7H), 1.22 (t, $J = 7.4$ Hz, 4H), 1.12 (t, $J = 7.3$ Hz, 3H).

**In vitro activity of N-methylated UNC1999 derivatives**

EZH2 and IC50’s were calculated as described in Chapter 2 Methods.

**Mass spectrometry–based quantification**

Total histones were prepared and subject to mass spectrometry analysis as previously described. [63]

**Antibodies**

Antibodies used in immunoblotting include those against H3K27me3 (Millipore 07-449 and Abcam 6002, 1:5,000), H3K27me2 (Millipore 07-452, 1:5,000), H3K27me1 (Active Motif 39377, 1:5000), H3K27ac (Abcam 4729, 1:2,000), H3K4me3 (Abcam 8580 and Active Motif 39159, 1:5,000), H3K36me3 (Abcam 9095, 1:5,000), H3K36me1 (ab9048, 1:1,000); H3K36me2 (Active Motif 39255, 1:1,000), general H3 (Abcam 1791, 1:10,000), EZH2 (BD Biosciences 612666,
1:1,000), SUZ12 (Abcam 12073, 1:1,000), and Tubulin (Sigma, 1:10,000). The antibodies used for ChIP-Seq are H3K27me3 (Millipore 07-449), H3K27ac (Abcam 4729) and SUZ12 (Abcam 12073).

**Assays of Cell Proliferation, Wright-Giemsa Staining and Apoptosis.** All human cell lines were purchased from the ATCC or DSMZ Cell Bank and cultured as recommended. Approximately 20,000 cells were plated in triplet in the presence of various concentrations of compounds at day 1, and the numbers of viable cells were counted using a TC20 automated cell counter (BioRad) every 3-4 days. Medium containing the compounds was refreshed every 2-3 days, and the cell concentration was kept under 1-2 million per mL as recommended. Wright-Giemsa staining was performed as previously described [64]. Cell apoptotic assays were performed using the Annexin V-FITC Early Apoptosis Detection Kit (Cell Signaling). Images of cell staining were captured with an EVOS-XL Cell Imaging System (Life Technologies).

**Colony Formation Unit (CFU) Assay.** Approximately 200-500 leukemia progenitor cells were initially plated in replicates in the methylcellulose medium (MethoCult™ GF M3434; StemCell Technologies) with either DMSO or compound present. For serial replating, approximately 5,000-10,000 cells isolated from colonies in the previous plating were seeded again in the same semi-solid medium. CFUs were scored by direct quantification every 7-9 days.
post seeding. Identity of each colony was defined by morphology according to manufacturer’s specification.

**Cell Cycle Profiling.** Exponentially dividing cells were seeded in the medium containing either DMSO or compounds. 10^6 of mock-treated versus compound-treated cells were washed with ice-cold PBS and fixed in pre-chilled methanol (80%), followed by staining with PBS added with 20 μg/ml propidium iodide (Sigma), 0.1% Triton-X100, and 200 μg/ml RNase A (Roche). DNA contents of cells were then detected with a CyAn ADP flow cytometer (Beckman-Coulter), and then analyzed by ModFit Software (Verity Software House).

**Gene Knockdown.** miR30-based shRNAs targeting *EED* (clone 1820) and *Renilla* (control) were kindly provided by Dr. Chris Vakoc [31] and then sub-cloned into sites of XhoI and EcoRI of an inducible shRNA expression system, TRMVP-IR [65] (pSIN-TREdsRed-miR30-PGK-Venus-IRES-rtTA3, Addgene 27994). Cells were infected with TRMPVIR virus and sorted based on Venus+ signals, followed by treatment with doxycycline (Sigma-Aldrich) at a final concentration of 2 μg/ml to induce shRNA expression. Gene knockdown efficiency was determined by real-time PCR.

**Real-Time PCR.** Reverse transcription was performed with random primers and the cDNA Reverse Transcription kit according to the manufacturers protocols (Invitrogen and Biorad). PCR amplicons (usually 80–150 bp) were designed to
span over introns, and quantitative PCR was performed in triplicate using the SYBR Green master mix reagent on an ABI 7900 qPCR system (Applied Biosystems). The real-time PCR signals are typically examined in triplicates and normalized to those of the β-Actin or Gapdh gene.

**In vivo Leukemia Assay and Compound Treatment Studies.** The murine model of MLL-rearranged leukemia was generated by retroviral delivery of MLL-AF9-IRES-GFP into lineage-negative bone marrow stem/progenitor cells, followed by bone marrow transplantation as previously described [66, 67]. To generate the secondary leukemia, 2x10^6 leukemia cells isolated from bone marrow were suspended in 200 μl of sterile PBS and intravenously introduced to the sub-lethally irradiated (300 Rads) syngeneic Balb/c mice (12-week-old) by tail-vein injection. Leukemic development was assessed by complete blood counting (CBC) of peripheral blood (HemaVet ® 950 LV CBC, Drew Scientific Group) and treatment with UNC1999 was commenced on the day when the counts in white blood cells (WBC) were found significantly elevated. The powder of UNC1999 (verified by HPLC and mass spectrometry) was slowly dissolved and incorporated into vehicle (0.5% of sodium carboxymethylcellulose [NaCMC] and 0.1% of Tween-80 in sterile water) with continuous trituration by a pestle and mortar. After transfer to a conical tube, the UNC1999-vehicle mixture was subject to vigorous vortex and brief sonication to achieve a homogenous suspension at a formulation concentration of 20 mg/mL. UNC1999 or vehicle control was administered orally twice daily [BID] at a dose of 50 mg/kg. Mice were inspected
daily, and complete blood counts measured twice per week; mice exhibiting a
terminal leukemia phenotype (lethargy, splenomegaly and limb paralysis) were
sacrificed, and cells isolated from leukemic tissues were subjected to further
pathological analysis as described before [66, 67]. All procedures pertaining to
animal handling, care, and treatment were performed according to guidelines
approved by the Institutional Animal Care and Use Committee (IACUC) of the
University of North Carolina at Chapel Hill.

Results

**UNC1999 Reduces H3K27me2 and H3K27me3 Levels in MLL-rearranged Leukemia**

Previously, UNC1999 and UNC2400 were characterized as a positive and
negative control of EZH2 inhibition [62]. In addition to UNC2400, we created two
monomethyl compounds to observe the effects of only a single methylation on
EZH2 activity, which resulted in UNC1756 and UNC3142 (Table 3.1). Both
monomethyl compounds retained a significant amount of activity for EZH2 (IC$_{50}$
$\sim$100 nM), which demonstrated that methylation of both amides is requisite to
abolish the potency of UNC1999. We next sought to observe the effects of
UNC1999 and UNC2400 on the methylation state of histones in cells treated with
both compounds.

Specifically, MLL-ENL cells were treated with UNC1999 or UNC2400, and
then subjected to mass spectrometry to identify modified histone peptides. 55
unique histone peptides bearing a single or combinatorial modification were
detected, and only those covering the H3 residues 27-40 were altered > 2-fold upon UNC1999 treatment (Figure 3.1 A). As expected, the greatest decrease was observed in peptides containing either H3K27me3 or H3K27me2. Peptides bearing both H3K27me3 and H3K36me2 also displayed a significant decrease in H3K27me3 levels when treated with UNC1999. Interestingly, the relative abundance of peptides containing the H3K36me1/2 mark was increased in cells treated with UNC1999. This phenomenon is due to the “demethylation” effect of UNC1999 treatment, and has previously been observed in cells lacking Suz12 [68]. Overall, H3K27me3 was reduced from 8.5% to 1.3% of total H3 in mock versus UNC1999 treated cells, and H3K27me2 was reduced from 30.9% to 7.1% of total H3 in mock versus UNC1999 treated cells (Figure 3.1 B). Importantly, UNC2400 displayed no significant changes versus mock treated cells, and consistent with the antagonistic relationship between H3K27me3 and H3K27ac, we observed a large increase in H3K27ac after UNC1999 treatment (Figure 3.1 C). The effects of UNC1999 did not alter PRC2 levels, the assembly of the PRC2 complex (Figure 3.1 D), or the amount of chromatin bound EZH2 and EZH1 (Figure 3.1 E). Taken together, these results demonstrate cell permeability, PRC2 engagement, and direct inhibition of the methyltransferase subunit of PRC2 in MLL-rearranged leukemia cells.

**EZH2 and EZH1 dual inhibition is required for MLL-AF9 cell death**

Based on recent findings that both EZH1 and EZH2 are required for MLL-rearranged leukemic growth [16], we hypothesized that a more panactive
inhibitor of EZH2 and EZH1 would be advantageous over an EZH2-specific inhibitor. So we compared the results of UNC1999 treatment (5-fold selective for EZH2) with that of GSK126 (150-fold selective for EZH2) in two distinct cellular scenarios: DB Cells, a form of diffuse large B cell lymphoma where EZH1 plays a minimal role, and MLL-AF9, an *MLL*-rearranged leukemia, where EZH1 is believed to play a larger role in disease progression [16]. In DB Cells, both UNC1999 and GSK126 potently reduce the H3K27me3 mark (Figure 3.2 A), and halt cellular proliferation (Figure 3.2 B). However, when treating MLL-AF9 cells with the same compounds, a different phenomenon is observed. UNC1999 is much more efficient at both reducing the H3K27me3 mark (Figure 3.2 A), and inhibiting cell proliferation, compared to that of GSK126 (Figure 3.2 C).

Importantly, we confirmed that MLL-AF9 cells do co-express EZH2 and EZH1 (data not shown). These results indicate that the additional EZH1 inhibition of UNC1999 is an advantage over other more EZH2-selective chemical probes in this context.

We next applied UNC1999 treatment to various other leukemic cell lines, 10 of which bear *MLL*-rearrangements. All of the 10 *MLL*-rearranged leukemia cell lines responded to UNC1999 treatment with EC50 ranging from 102 nM to 1.96 µM, and several cell lines demonstrated comparable sensitivity as DB Cell (Figure 3.3). LOUCY cells, a T-cell acute lymphoblastic leukemia cell line that does not possess EZH2 nor display the H3K27me3 hypertrimethylation phenotype, were not affected by UNC1999 treatment, which indicated that the previous results were not due to off-target cytotoxicity. Further, K562 cells, a Bcr-
Abl-bearing myeloid leukemia cell line, did not respond to UNC1999. This data demonstrates the robust effect of UNC1999 treatment in MLL-rearranged leukemia.

**UNC1999 Treatment Reactivates CDKN2a**

To identify the mechanism of leukemic death upon UNC1999 treatment, microarray analysis in cells bearing the MLL-AF9 translocation was performed. This analysis revealed that expression of several hundred transcripts are altered after UNC1999 treatment, and consistent with PRC2 function, the majority of transcripts that elicited a change were upregulated as opposed to downregulated; UNC2400 had no effect (Figure 3.4 A). As expected, RNA silencing of EED (shEED) had a different effect on the number and type of upregulated and downregulated genes (Figure 3.4 A & B). One gene of specific interest that was upregulated in the presence of UNC1999 treatment was Cdkn2a.

Cdkn2a is a gene that encodes two crucial cell cycle regulators, p16Ink4a and p19Arf. UNC1999 treatment induced reactivation of these two proteins in a dose- and time-dependent manner; after seven days of treatment >150-fold and >60-fold upregulation of p16Ink4a and p19Arf were observed, respectively (Figure 3.4 C). This increase in expression of the Cdkn2a gene products resulted in time-dependent cell cycle arrest at the G1-to-S transition (Figure 3.4 D). Cells treated with UNC2400 demonstrated no concomitant increase in Cdkn2a gene products, or cell cycle arrest when compared to DMSO-treated cells.
To examine whether the UNC1999-induced phenotypes were dependent on Cdkn2a expression, we generated p16Ink4a and p19Arf double knockout cells and compared their response to UNC1999 treatment with that of their wild-type counterparts. Unlike wild-type cells, p16Ink4a<sup>−/−</sup>/p19Arf<sup>−/−</sup> cells elicited no response to UNC1999 treatment; there was no detectable change in proliferation (Figure 3.4 E), or apoptosis (Figure 3.4 F). These studies demonstrate that inhibition of MLL-AF9 cell growth is in part due to derepression of key cell cycle regulating genes (p16Ink4a & p19ARF), and confirm that the Cdkn2a products are key downstream mediators of UNC1999-induced inhibition.

**UNC1999 Provides a Survival Advantage in a MLL-AF9 Murine Model**

To determine if UNC1999 provides an advantage at the organismal level, we generated a murine model of MLL-AF9 by transplanting primary bone marrow tumors to syngenic recipients, which developed aggressive leukemia with a consistent latency of 20 to 35 days. Mice were treated with vehicle or 50 mg/kg UNC1999 via oral gavage 7 days post-transplantation. Leukemic progression was then monitored via periodic assessment of peripheral blood. Although there was steady accumulation of white blood cells (WBCs) in both the UNC1999 and vehicle-treated cohorts, UNC1999-treated mice displayed significantly less accumulation of WBC, which is consistent with delayed leukemic progression (Figure 3.5 A). UNC1999 did not alter red blood cell or platelet counts (Figure 3.5 B & C). Further, UNC1999-treated mice exhibited a significant increase in survival (36.6 ± 11.7 days) when compared with vehicle treated mice (24 ± 6.7
days) (Figure 3.5 D). Analysis of cells isolated from bone marrow and spleens of moribund mice demonstrated that UNC1999-treatment significantly reduced H3K27me3 levels (Figure 3.5 E), elevated p16ink4a and p19arf expression (Figure 3.5 F), and reduced the size of lymph nodes (Figure 3.5 G) when compared to vehicle-treated mice; splenomegaly was observed in both cohorts.

In summary, oral administration of UNC1999 delays the onset of MLL-AF9 leukemia in mice, and EZH2/EZH1 dual inhibition provides a new therapeutic option for MLL-rearranged leukemia.

**Analogs of UNC1999 Demonstrate Capability for Panactive EZH2/EZH1 Inhibitors**

While UNC1999 is the most panactive EZH2/EZH1 inhibitor to date, we aimed to explore the potential for increased EZH1 activity in order to discover a more panactive inhibitor, which we presume would provide a greater survival advantage in mice afflicted with MLL-AF9. Previously, GSK released a publication which detailed the change in EZH2/EZH1 selectivity among a class of inhibitors very similar in structure to UNC1999 [47]. Specifically, modulation of the 4-position on the pyridone ring appeared to affect this selectivity; substituting the methyl group for an n-propyl group led to a 5-fold increase in EZH2 affinity and a 25-fold increase in affinity for EZH1 (Table 3.3). Further, simply changing the substitution of the pyridine ring from the indazole core affected the activity of EZH1, albeit reducing it compared to the prior substitution (Table 3.3). To date, these three compounds are the only published structure activity relationship
(SAR) for pyridone containing compounds against both EZH1 and EZH2. Therefore, we aimed to explore the tolerability of modifications to the pyridone ring of UNC1999 (Figure 3.6) with the goal of increasing EZH1 inhibition.

Initially, we aimed to install various substituents at the C4 position of the pyridone (Figure 3.6, R1), which resulted in the compounds displayed in Table 3.4. This set of nine compounds led to two main conclusions. First, the methyl group at the C6 position of the pyridone (R3 in Figure 3.6) is imperative for potent inhibition of both EZH2 and EZH1; compare UNC3855 vs UNC3860, UNC3857 vs kdk7-058, and UNC3856 vs UNC1999 (Table 3.4). Second, like what was observed in the set of compounds from GSK, larger hydrophobic substituents at the C4 position of the pyridone (Figure 3.6, R1) appear to increase the affinity of the compounds for both EZH2 and EZH1; compare UNC3855 vs UNC3857 vs UNC3856 vs UNC3859 and UNC3860 vs kdk7-058 vs UNC1999 vs UNC3862 vs kdk215-059. This trend does appear to have a ceiling, as kdk215-146 was less potent for both EZH2 and EZH1 than UNC3862, UNC1999 and kdk215-059 (Table 3.4).

Next, we aimed to determine if any substituents were tolerated at the C5 position of the pyridone (Figure 3.6, R2); no EZH2 or EZH1 inhibition data of compounds with substitutions here are available in the public domain. This resulted in the compounds pictured in Table 3.3. While substituents at C5 were largely tolerated for inhibition of EZH2, essentially any substituent at the C5 position resulted in next-to-no inhibition of EZH1 activity. This is most easily demonstrated by comparing UNC3861 with kdk215-060. UNC3861 contains a
cyclopentyl group attached to the pyridone ring at C4 and C5, and is still relatively potent for EZH2 (IC50 = 80 nM), but is essentially inactive against EZH1 (IC50 = 5000 nM). However, if that cyclopentyl ring is pulled off of C5, such that there is a cyclopentyl substituent at the C4 position and a hydrogen atom at C5, a large amount of EZH1 activity is restored (IC50 = 126 nM). This data demonstrates that no hydrophobic substituents are tolerated at C5 for EZH1 inhibition.

As the last part of our initial exploration into substituted pyridones, we explored additional groups at the C6 position (Figure 3.6, R3). Like the C5 position, there is a lack of compounds reported in the literature containing anything other than a methyl group at the C6 position. Our initial efforts resulted in the compounds contained in Table 3.4. It appears that inhibition of EZH2 tolerates substitutions at the C6 position much more than EZH1 inhibition; addition of just a single carbon onto the C6 methyl group of UNC1999 results in a compound that is ineffective at inhibiting EZH1 catalytic activity (kdk215-143, Table 3.6). We also experimented with two larger groups at C6, an isobutyl group (kdk7-057) and an n-butyl group (kdk215-061), but both of these compounds also lost all EZH1 inhibition displayed by UNC1999. This small set of compounds suggests that inhibition of EZH1 catalytic activity is antagonized by the addition of substituents at the C6 position of the pyridone.
Discussion

This study represents the first oral delivery of a dual EZH1/EZH2-specific compound as a means to inhibit MLL-rearranged leukemia. This is especially relevant because MLL-rearranged leukemia demonstrate a poor prognosis, so extremely toxic therapies (daunorubicin and cytarabine) are implemented in an attempt to eradicate the disease. Prior to our study, 3-deazaneplanocin (DZNep) was claimed to be an inhibitor of EZH2, and demonstrated tumor suppressive roles in MLL-rearranged leukemia [69], but DZNep operates through non-specific mechanisms; it is a SAH-hydrolase inhibitor, and depletes the global pool of SAH [70, 71]. Contrarily, inhibition of EZH2 activity in MLL-AF9 AML cells was demonstrated via use of a stapled peptide that inhibited EED-EZH2 binding, and led to PRC2 degradation [72]. While this research was integral in demonstrating a proof of concept for EZH2 inhibition, one could argue that this method is as effective as shEED or shEZH2, and will have serious issues being translated into patients bearing MLL-rearranged leukemia. Removal of a protein from a biological system is not equal to catalytic inhibition of said protein. This is especially true in the case of EZH2, because it is a single component of a much larger complex that recruits other silencing factors. For example, our data demonstrated that silencing of EED (shEED) had a differing effect on the number and types of upregulated and downregulated genes than catalytic inhibition of EZH2 with UNC1999. Small molecules that inhibit the catalytic activity of EZH2 will present far fewer issues when translating into the clinic.
Our data demonstrate that UNC1999 treatment effectively reduces H3K27me3 levels in cells, and at the organismal level. Mechanistically, UNC1999 treatment leads to concurrent increases in expression of *Cdkn2a* gene products (p16Ink4a and p19ARF), and accordingly, UNC1999 is ineffective at inhibiting the proliferation of cells bearing p16Ink4a and p19ARF double knockouts. While UNC1999 does prolong the survival of mice afflicted with MLL-AF9 AML, the effects are much more modest than those seen at the cellular level. A similar effect has been seen with the development of Brd4 [73] and DOT1L [74] inhibitors. In short, much more effort needs to be done to optimize the potency, selectivity, and pharmacokinetic properties of these compounds in an *in vivo* setting. In the case of MLL-AF9 AML, additional EZH1 activity may be an avenue that will provide additional benefits.

To date, there is a dearth of information in the literature surrounding the EZH2/EZH1 selectivity of the current classes of EZH2 inhibitors. We aimed to increase the activity of UNC1999 analogs against EZH1 in order to create a more panactive chemical probe of both EZH1 and EZH2; such a compound may be of use in treating *MLL*-rearranged leukemia. While we have been unsuccessful in creating such a probe to date, we did learn that additional hydrophobicity at the C4 position of the pyridone increases the activity for EZH1 and EZH2. However, all alkyl substituents appended to either the C5 or C6 position of the pyridone resulted in nearly complete loss of EZH1 inhibition. Going forward, we plan to continue to explore the potential for substituents at the C4 position.
EZH1 and EZH2 have high sequence similarity (96%) in their SET domain, but one residue that differs in a sequence alignment of EZH1 and EZH2 is M743, which is a threonine in EZH1 (T744). A recent computational study observed the ligand-residue contacts of various pyridone-containing EZH2 inhibitors. This group created a larger EZH2 complex using the apo SET domain crystal structure as a foundation, and added additional residues in an attempt to create a closer to full-length structure. Docking of compounds, followed by simulations and alanine-scanning for important residues determined that M743 makes significant contacts with the pyridone of bound ligands during the course of an MD simulation [75]. We are curious if we can take advantage of T744 of EZH1, by introducing moieties that may engage in hydrogen bonds with the threonine of EZH1 (i.e., alcohols, carbonyls, etc.). This may lead to greater affinity for EZH1, and hopefully maintain the EZH2 activity. Nonetheless, it remains to be seen if increased in vitro EZH1 activity translates to increased efficacy at the cellular and/or organismal level.
### Table 3.1: N-methylated derivatives of UNC1999.

H = hydrogen, Me = methyl.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>EC50</th>
<th>Genetic Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB Cell</td>
<td>.227</td>
<td>EZH2&lt;sup&gt;Y641N&lt;/sup&gt;</td>
</tr>
<tr>
<td>EOL-1</td>
<td>.102</td>
<td>MLL-PTD; FIP1L1-PDGFR</td>
</tr>
<tr>
<td>K562</td>
<td>&gt;250</td>
<td>BCR-ABL</td>
</tr>
<tr>
<td>LOUCY</td>
<td>&gt;200</td>
<td>EZH2 deletion</td>
</tr>
<tr>
<td>m-MLL-AF9</td>
<td>.404 – .621</td>
<td>MLL-AF9</td>
</tr>
<tr>
<td>m-MLL-ENL</td>
<td>.547 – .636</td>
<td>MLL-ENL</td>
</tr>
<tr>
<td>MV4;11</td>
<td>.523</td>
<td>MLL-AF4</td>
</tr>
<tr>
<td>OCI-AML2</td>
<td>1.50</td>
<td>MLL-AF9</td>
</tr>
<tr>
<td>RS4;11</td>
<td>1.96</td>
<td>MLL-AF4</td>
</tr>
</tbody>
</table>

Table 3.2: UNC1999 activity in leukemic cell lines of differing genetic backgrounds.
<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>EZH2 $IC_{50}$ (nM)</th>
<th>EZH1 $IC_{50}$ (nM)</th>
<th>Fold Selectivity</th>
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</thead>
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<tr>
<td>GSK926</td>
<td>Me</td>
<td></td>
<td>20</td>
<td>2500</td>
<td>125</td>
</tr>
<tr>
<td>4</td>
<td>$n$-Pr</td>
<td></td>
<td>4</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>GSK343</td>
<td>$n$-Pr</td>
<td></td>
<td>4</td>
<td>240</td>
<td>60</td>
</tr>
<tr>
<td>UNC1999</td>
<td>$n$-Pr</td>
<td></td>
<td>&lt;10</td>
<td>45</td>
<td>≥5</td>
</tr>
</tbody>
</table>

Table 3.3: EZH2/EZH1 selectivity of GSK reported compounds [47] and UNC1999.

Me = methyl, $n$-Pr = n-propyl.
<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>EZH2 IC$_{50}$ (nM)</th>
<th>EZH1 IC$_{50}$ (nM)</th>
<th>Fold Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNC3855</td>
<td>Me</td>
<td>H</td>
<td>790</td>
<td>NI</td>
<td>N/A</td>
</tr>
<tr>
<td>UNC3857</td>
<td>Et</td>
<td>H</td>
<td>560</td>
<td>5000</td>
<td>9</td>
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<td>UNC3856</td>
<td>$n$-Pr</td>
<td>H</td>
<td>150</td>
<td>3500</td>
<td>23</td>
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<tr>
<td>UNC3859</td>
<td>$n$-Bu</td>
<td>H</td>
<td>35</td>
<td>2000</td>
<td>57</td>
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<tr>
<td>UNC3860</td>
<td>Me</td>
<td>Me</td>
<td>50</td>
<td>2500</td>
<td>50</td>
</tr>
<tr>
<td>kdk7-58</td>
<td>Et</td>
<td>Me</td>
<td>17</td>
<td>1160</td>
<td>68</td>
</tr>
<tr>
<td>UNC3862</td>
<td>$i$-Pr</td>
<td>Me</td>
<td>&lt;10</td>
<td>320</td>
<td>≥32</td>
</tr>
<tr>
<td>kdk215-59</td>
<td>$i$-Bu</td>
<td>Me</td>
<td>&lt;10</td>
<td>141</td>
<td>≥14</td>
</tr>
<tr>
<td>kdk215-146</td>
<td>$n$-heptyl</td>
<td>Me</td>
<td>18</td>
<td>488</td>
<td>27</td>
</tr>
<tr>
<td>UNC1999</td>
<td>$n$-Pr</td>
<td>Me</td>
<td>&lt;10</td>
<td>45</td>
<td>≥5</td>
</tr>
</tbody>
</table>

Table 3.4: EZH2/EZH1 selectivity of UNC1999 analogs: exploration of C4 ($R_1$) and C6 ($R_2$) of pyridone.

H = hydrogen, Me = methyl, Et = ethyl, $n$-Pr = propyl, $i$-Pr = isopropyl, $i$-Bu = isobutyl, $n$-heptyl = heptane. NI = no inhibition, N/A = not applicable.
<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>R&lt;sub&gt;3&lt;/sub&gt;</th>
<th>n</th>
<th>EZH2 IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>EZH1 IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Fold Selectivity</th>
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<tbody>
<tr>
<td>kdk215-142</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
<td>0</td>
<td>325</td>
<td>&gt;5000</td>
<td>≤15</td>
</tr>
<tr>
<td>UNC3858</td>
<td>Me</td>
<td>Me</td>
<td>H</td>
<td>0</td>
<td>5000</td>
<td>5000</td>
<td>1</td>
</tr>
<tr>
<td>kdk7-56</td>
<td>Et</td>
<td>Me</td>
<td>H</td>
<td>0</td>
<td>1156</td>
<td>&gt;5000</td>
<td>≥4</td>
</tr>
<tr>
<td>kdk215-60</td>
<td>c-pentyl</td>
<td>H</td>
<td>Me</td>
<td>0</td>
<td>&lt;10</td>
<td>126</td>
<td>≥13</td>
</tr>
<tr>
<td>UNC3861</td>
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<td>Me</td>
<td>Me</td>
<td>1</td>
<td>80</td>
<td>5000</td>
<td>40</td>
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<tr>
<td>kdk215-144</td>
<td>Me</td>
<td>Me</td>
<td>Me</td>
<td>2</td>
<td>&gt;2500</td>
<td>&gt;5000</td>
<td>2</td>
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<tr>
<td>kdk215-147</td>
<td>n-Pr</td>
<td>Et</td>
<td>H</td>
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<td>152</td>
<td>&gt;5000</td>
<td>≤33</td>
</tr>
<tr>
<td>UNC1999</td>
<td>n-Pr</td>
<td>H</td>
<td>Me</td>
<td>0</td>
<td>&lt;10</td>
<td>45</td>
<td>≥5</td>
</tr>
</tbody>
</table>

**Table 3.5: EZH2/EZH1 selectivity of UNC1999 analogs: exploration of C5 (R<sub>2</sub>) of pyridone.**

H = hydrogen, Me = methyl, Et = ethyl, n-Pr = propyl, c-Pen = cyclopentane.
<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>EZH2 IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>EZH1 IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Fold Selectivity</th>
</tr>
</thead>
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<tr>
<td>kdk215-143</td>
<td>Me</td>
<td>Et</td>
<td>107</td>
<td>&gt;5000</td>
<td>≤47</td>
</tr>
<tr>
<td>kdk7-57</td>
<td>Me</td>
<td>i-bu</td>
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<tr>
<td>kdk215-61</td>
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<td>493</td>
<td>&gt;5000</td>
<td>≤10</td>
</tr>
<tr>
<td>UNC1999</td>
<td>n-Pr</td>
<td>Me</td>
<td>&lt;10</td>
<td>45</td>
<td>≥10</td>
</tr>
</tbody>
</table>

Table 3.6: EZH2/EZH1 selectivity of UNC1999 analogs: exploration of C6 (R<sub>2</sub>) of pyridone.

Me = methyl, Et = ethyl, n-Pr = propyl, i-Pr = isopropyl, i-Bu = isobutyl.
Figure 3.1: Activity of UNC1999 and UNC2400 in MLL-rearranged cells.

(A) Quantitative mass spectrometry analysis of H3 peptides covering histone H3 (a.a. 27-40) after treatment with 3 µM UNC1999 (blue) or UNC2400 (red) for 4 days. (B) Overall percentages of unmodified, monomethylated, dimethylated, or trimethylated H3K27 (H3K27me1/2/3) following DMSO, UNC2400, or UNC1999 treatment. (C) Immunoblot of the indicated histone modifications in MLL-AF9-transformed leukemia progenitor cells after treatment with DMSO, UNC2400 or UNC1999 (3 µM). (D) Co-IP of PRC2 components following Flag IP with extracts.
of a Flag-PHF1 stable expression cell line [76] in the presence of DMSO, UNC2400, or UNC1999 (2 µM). (E) Immunoblots detecting the chromatin-bound and nucleoplasmic fraction of EZH2 or EZH1 after treatment with DMSO, UNC2400, or UNC1999 (2 µM) for 5 days. ac = acetylation, co-IP = coimmunoprecipitation, Ig = immunoglobulin, IP = immunoprecipitation, me1/2/3 = mono/di/trimethylation.
Figure 3.2: UNC1999 is more effective than GSK126 at suppressing H3K27me3 and inhibiting MLL-rearranged leukemic cell growth.

(A) Immunoblots of global H3K27me3 level after treatment of DB lymphoma cells (top) or MLL-AF9–transformed murine leukemia progenitors (bottom) with DMSO, GSK126, UNC1999 or UNC2400 (2 µM) for 5 days. Total H3 serves as control. (B) Relative proliferation of DB cells treated with a range of concentrations of GSK126 (top) or UNC1999 (bottom) for the indicated duration. (C) Relative proliferation of MLL-AF9–transformed leukemia progenitors treated with a range of concentrations of GSK126 (top) or UNC1999 (bottom) for the indicated duration.
Figure 3.3: UNC1999 activity in leukemic cell lines.
Dose-response curves of various MLL-rearranged leukemic cell lines (EOL-1, m-MLL-AF9, m-MLL-ENL, MV4;11, RS4;11), Diffuse large B-cell lymphoma cells (DB), T-cell lymphoma (LOUCY), and Bcr-Abl CML (K562) in the presence of UNC1999 after 16 days.
Figure 3.4: UNC1999 derepresses PRC2 gene targets.

(A) upregulated (blue) and downregulated (red) transcripts in 2 independent MLL-AF9–transformed leukemia lines after a 5-day treatment with 3 µM of compounds or after knockdown of EED vs Renilla, as identified by microarray analysis. (B) RT-qPCR detects relative expression levels of the indicated genes in MLL-AF9–transformed leukemia cells following treatment with 3 µM of compounds or EED knockdown (shEED) for 5 days; normalized to GAPDH. (C) RT-qPCR shows time-dependent derepression of p16Ink4a and p19Arf by UNC1999 in a leukemia line derived from MLL-AF9–induced primary tumors. *P < .05; **P < .01; ***P < .001. (D) Summary of cell-cycle status of MLL-AF9–transformed murine leukemia progenitors following 2-day or 7-day treatment with DMSO, UNC2400 or UNC1999 (3 µM). (E) Relative proliferation of MLL-AF9–transformed murine leukemia cells, either wild-type (red) or p16Ink4a−/−/p19Arf−/− (purple), after treatment with various concentrations of UNC1999 for 12 days. (F) Summary of apoptotic induction in MLL-AF9–transformed murine leukemia
progenitors, either wild-type (WT) or p16Ink4a<sup>−/−</sup>/p19Arf<sup>−/−</sup>, following a 6-day treatment with 3 µM of compounds as assayed by propidium iodide staining.
Figure 3.5: UNC1999 treatment prolongs survival of mice with MLL-AF9.

(A-C) Summary of counts of the WBCs (A), neutrophils (B), and RBCs (C) in the peripheral blood of vehicle- (white) or UNC1999-treated (black) leukemic mice at the indicated date post transplantation. *P < .05; **P < .01; ***P < .001. (D) Kaplan-Meier curve showing leukemia kinetics after transplantation of MLL-AF9–induced primary murine leukemia into syngeneic mice. Starting from day 7 post transplantation, mice received oral administration of either vehicle (blue) or 50 mg/kg UNC1999 (red) twice per day. Black lines (top) represent non-transplanted normal mice treated with vehicle or UNC1999. Cohort size = 6 to 7 mice. (E) Summary of H3K27me3 levels in cells isolated from bone marrow or spleen of vehicle- (LV) and UNC1999-treated (LT) leukemic mice as quantified by flow cytometry. *P < .05; **P < .01. (F) Fold-change in p16Ink4a and p19Arf gene
expression in cells isolated from bone marrow or spleen of the UNC1999-treated (black) leukemic mice in comparison with vehicle-treated (white). *P < .05; **P < .01. (G) Comparison of sizes of lymph nodes isolated from the MLL-AF9–induced leukemic mice after treatment with either vehicle or 50 mg/kg UNC1999. WBC = white blood cell; BM = bone marrow; GFP = green fluorescent protein; RBC = red blood cell; SP = spleen.
Figure 3.6: Location of pyridone substitutions of EZH1/EZH2 selectivity.

C4 carbon = R₁, C5 carbon = R₂, and C6 carbon = R₃.
CHAPTER IV: IRREVERSIBLE INHIBITORS AS PUTATIVE EZH2-SPECIFIC CHEMICAL PROBES

Introduction

While both EZH2 and EZH1 activity contribute to the progression of MLL-rearranged leukemia, EZH2 is most often the main antagonist in the progression of solid tumors. In ovarian cancer, overexpression of EZH2 leads to poor prognosis and is associated with drug resistance [77, 78]. Specifically, following cisplatin treatment, resistant stem cell-like side populations persist and have been shown to overexpress EZH2 [79, 80]. EZH2 has also been shown to promote proliferation and invasiveness of prostate cancer cells by repressing the expression of TIMP2 and TIMP3, which allows for dysregulated matrix metalloproteinase activity [14, 80]. In these instances, it would be advantageous to maintain basal EZH1 activity, due to its imperative endogenous role in hematopoietic stem cell maintenance [20]. To date, the most selective chemical probe for EZH2 is GSK126, which is reported to be 150-fold more potent for EZH2 over EZH1 [32]. This is remarkable considering the high overall identity (76%) and SET domain identity (96%) between the two proteins, and it is uncertain if current scaffolds can attain more selectivity. We plan to develop the first irreversible inhibitor for EZH2 which will provide: 1) the most potent EZH2
inhibitor to date, 2) the most selective EZH2 inhibitor to date, and 3) direct confirmation of our binding hypothesis.

One issue hampering further design of small molecules to inhibit EZH2 is the lack of an EZH2-inhibitor co-crystal structure. To date, the only solved PRC2-related crystal structures are EED in complex with a small peptide from the EZH2 binding interface [81], and the apo structure of the EZH2 SET domain [82, 83]. The EED-EZH2 crystal structure proved useful, because it was instrumental in the development of an EZH2 stapled peptide, which inhibits PRC2 activity by triggering EZH2 degradation [72]. The utility of the previously published EZH2 apo crystal structures remains to be seen, and considering the high degree of complexity of PRC2 (i.e. multiple subunits present in tissue-specific contexts) one would expect that a crystallized conformation of the apo SET domain of EZH2 may not reflect the true architecture of a functional SET domain contained within the entire complex. Nonetheless, in an attempt to demonstrate the utility of this crystal structure, we aim to design and synthesize irreversible inhibitors of EZH2. Our goal is to exploit a cysteine residue that is hypothesized to be in close proximity to our predicted UNC1999 binding site.

Methods

In vitro activity of irreversible inhibitors

EZH2 and EZH1 IC50’s were calculated as described in Chapter 2 Methods.
Synthesis of Irreversible Inhibitors

**Scheme 4.1. Reagents and Conditions.** (a) CuCN, NMP, μ-wave (200 \(^\circ\)C), 25 min, 99%; (b) LiOH, MeOH, rt, o/n, 95%; (c) NaH, epichlorohydrin, DMF, rt, o/n, 98%; (d) 3-(aminomethyl)-4,6-dimethylpyridin-2(1H)-one, HOAt, EDCI, DMSO, NMM, rt, 5 h, 17%.

**methyl 6-bromo-1H-indole-4-carboxylate.** Synthesized according to previously published procedures [47].

**methyl 6-cyano-1H-indole-4-carboxylate (4.1).** methyl 6-bromo-1H-indole-4-carboxylate (200 mg, .790 mmol) and CuCN (141 mg, 1.57 mmol) were dissolved in NMP (3.00 mL) and placed into a microwave reactor for 25 min at 200 \(^\circ\)C. The contents were then quenched with H2O, extracted with EA, dried over MgSO4, filtered, concentrated *in vacuo* and purified by ISCO (EA:Hexane, 1:10). The desired product was obtained at 99.6% yield (158 mg, .789 mmol).

**6-cyano-1H-indole-4-carboxylic acid (4.2).** methyl 6-cyano-1H-indole-4-carboxylate (170 mg, .849 mmol) was dissolved in MeOH (5 mL) and then LiOH (107 mg, 2.55 mmol) dissolved in H2O (1 mL) was added. The reaction was stirred at room temperature overnight. The next morning, TLC showed
disappearance of starting material, so the reaction was quenched with 5% HCl, and purified by ISCO to obtain the desired product at 94.5% yield (150 mg, .802 mmol).

6-cyano-1-(oxiran-2-ylmethyl)-1H-indole-4-carboxylic acid (4.3). 6-cyano-1H-indole-4-carboxylic acid (140 mg, .752 mmol) was dissolved in DMF (10 mL) and then NaH (75.0 mg, 1.88 mmol) was slowly added and the resulting mixture was stirred at room temperature for 15 minutes. Then epichlorohydrin (.117 mL, 1.504 mmol) was added and the reaction was stirred at room temperature overnight. Product formation was confirmed by LC/MS and then the contents were purified by ISCO (Hex:EA; 1:1) in 98% yield (180 mg, .743 mmol).

6-cyano-N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-(oxiran-2-ylmethyl)-1H-indole-4-carboxamide (xy224-020 (4.4)). 6-cyano-1-(oxiran-2-ylmethyl)-1H-indole-4-carboxylic acid (.185 g, .764 mmol), 3-(aminomethyl)-4,6-dimethylpyridin-2(1H)-one (.128 g, .840 mmol), and HOAt (.156 g, 1.15 mmol) were dissolved in DMSO (5 mL) and stirred at room temperature for 15 minutes prior to addition of EDCI (.219 g, 1.15 mmol) and NMM (0.25 mL, 2.29 mmol) for 5 hours at room temperature. LC/MS demonstrated formation of the desired product, the mixture was then purified using ISCO (DCM:MeOH; 1:1) to give the title compound in 17% yield (.049 g, .130 mmol). $^1$H NMR (400 MHz, Methanol-$d_4$), δ: 8.11 (s, 1H), 7.67 (d, $J = 1.3$ Hz, 1H), 7.62 (d, $J = 3.2$ Hz, 1H), 6.97 (dd, $J = 3.2, 0.9$ Hz, 1H), 6.11 (s, 1H), 4.72 (dd, $J = 15.4, 2.5$ Hz, 1H), 4.54 (s, 2H), 4.23
(dd, $J = 15.4, 6.1$ Hz, 1H), 3.30 (p, $J = 1.7$ Hz, 8H), 2.80 (dd, $J = 4.8, 4.0$ Hz, 1H),
2.47 (dd, $J = 4.8, 2.6$ Hz, 1H), 2.40 (s, 3H), 2.24 (d, $J = 0.9$ Hz, 3H).

**Scheme 4.2. Reagents and Conditions.** (a) NaH, 3-bromopropanoate, DMF, rt, o/n, 63%; (b) 3-(aminomethyl)-4,6-dimethylpyridin-2(1H)-one, HOAt, EDCI, DMSO, NMM, rt, 2 h, 87%; (c) LiOH, THF, rt, o/n, 96%; (d) diphenyl phosphorazidate, TEA, toluene, dioxane, rt, o/n, 100%; (e) acrylic acid, HOAt, EDCI, DMSO, NMM, rt, 2 h, 88%; (f) 2-fluoroacrylic acid, HOAt, EDCI, DMSO, NMM, rt, 2 h, 73%.

**6-cyano-1-(3-methoxy-3-oxopropyl)-1H-indole-4-carboxylic acid (4.5).** 6-cyano-1H-indole-4-carboxylic acid (300 mg, 1.61 mmol) was dissolved in DMF (10 mL) and NaH (148 mg, 3.71 mmol) was slowly added and stirred at room
temperature for 15 minutes before adding methyl 3-bromopropanoate (0.35 mL, 3.22 mmol). The reaction was stirred at room temperature overnight. The next morning, LC/MS showed that the reaction was complete, the contents were then quenched with 5% HCl, extracted with EA, dried over MgSO₄, filtered, concentrated in vacuo and purified via ISCO (Hexane:EA, 100% → 40%) to give the desired product at 63% yield (370 mg, 1.36 mmol).

**methyl 3-(6-cyano-4-(((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)carbamoyl)-1H-indol-1-yl)propanoate (4.6).** 6-cyano-1-(3-methoxy-3-oxopropyl)-1H-indole-4-carboxylic acid (370 mg, 1.36 mmol), 3-(aminomethyl)-4,6-dimethylpyridin-2(1H)-one (248 mg, 1.63 mmol), and HOAt (277 mg, 2.04 mmol) were dissolved in DMSO (5.00 mL) and stirred at room temperature for 15 minutes prior to addition of EDCI (391 mg, 2.04 mmol) and NMM (0.45 mL, 4.08 mmol) for 2 hours at room temperature. LC/MS demonstrated formation of product, the mixture was then quenched with H₂O, and purified using HPLC to give the desired product in 87% yield (480 mg, 1.22 mmol).

**3-(6-cyano-4-(((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)carbamoyl)-1H-indol-1-yl)propanoic acid (4.7).** methyl 3-(6-cyano-4-(((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)carbamoyl)-1H-indol-1-yl)propanoate (200 mg, .493 mmol) was dissolved in THF (10 mL) prior to addition of LiOH (51.0 mg, 1.23 mmol) dissolved in H₂O (2 mL). The reaction was stirred overnight at room temperature and monitored by TLC the next
morning. After completion, the contents were precipitated with addition of HCl, the observed precipitate was filtered to give the desired product in 96% yield (186 mg, .473 mmol).

1-(2-aminoethyl)-6-cyano-N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1H-indole-4-carboxamide (4.8). 3-(6-cyano-4-(((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)carbamoyl)-1H-indol-1-yl)propanoic acid (.050 g, .127 mmol) was dissolved in toluene (15 mL) and dioxane (15 mL) and added diphenyl phosphorazidate (.042 g, .153 mmol) before addition of TEA (.05 mL, .382 mmol). The contents were stirred overnight at room temperature, then concentrated in vacuo, and THF (10 mL) and LiOH (3 eq) dissolved in H2O were added and the contents were stirred overnight at room temperature before purification by HPLC to afford the desired product in quantitative yield (46.2 mg, .127 mmol).

1-(2-acrylamidoethyl)-6-cyano-N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1H-indole-4-carboxamide (4.9). 1-(2-aminoethyl)-6-cyano-N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1H-indole-4-carboxamide (46.2 mg, .127 mmol), acrylic acid (11.0 mg, .153 mmol), and HOAt (26.0 mg, .191 mmol) were dissolved in DMSO (2.00 mL) and stirred for 15 minutes prior to the addition of EDCI (37.0 mg, .191 mmol) and NMM (.05 mL, .454 mmol), then the contents were stirred at room temperature for 2 hours. The reaction was monitored by LC/MS and purified by HPLC after completion to give the title
compound in 88% yield (46.8 mg, .112 mmol). $^1$H NMR (400 MHz, Methanol-$d_4$), δ: 8.04 (s, 1H), 7.66 (d, J = 1.3 Hz, 1H), 7.57 (d, J = 3.2 Hz, 1H), 6.92 (m, 1H), 6.17 (d, J = 2.0 Hz, 1H), 6.11 (m, 2H), 6.06 (dd, J = 17.1, 9.9 Hz, 2H), 5.62 (dd, J = 9.9, 2.1 Hz, 2H), 4.54 (s, 2H), 4.44 (t, J = 6.0 Hz, 3H), 3.63 (t, J = 6.0 Hz, 3H), 2.40 (s, 3H), 2.25 (d, J = 0.8 Hz, 3H).

6-cyano-N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-(2-(2-fluoroacrylamido)ethyl)-1H-indole-4-carboxamide (4.10). 1-(2-aminoethyl)-6-cyano-N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1H-indole-4-carboxamide (15.0 mg, .041 mmol), 2-fluoroacrylic acid (4.50 mg, .042 mmol), and HOAt (8.40 mg, .062 mmol) were dissolved in DMSO (3 mL) and stirred for 15 minutes prior to the addition of EDCI (11.8 mg, .062 mmol) and NMM (.01 mL, .124 mmol), then the contents were stirred at room temperature for 2 hours. The reaction was monitored by LC/MS and purified by HPLC after completion to give the desired product in 73% yield (13.1 mg, .030 mmol). $^1$H NMR (400 MHz, Methanol-$d_4$), δ: 8.58 (s, 1H), 8.07 (s, 1H), 7.66 (d, J = 1.3 Hz, 1H), 7.58 (d, J = 3.1 Hz, 1H), 6.95 (dd, J = 3.2, 0.9 Hz, 1H), 6.13 (s, 1H), 5.42 (d, J = 3.4 Hz, 1H), 5.12 (dd, J = 15.3, 3.4 Hz, 1H), 4.54 (s, 2H), 4.45 (t, J = 6.1 Hz, 2H), 3.66 (q, J = 6.1 Hz, 2H), 2.40 (s, 3H), 2.25 (d, J = 0.8 Hz, 3H).
Scheme 4.3. Reagents and Conditions. (a) LiOH, THF, MeOH, NaH, rt, o/n, 95%; (b) NaH, bromoacetonitrile, DMF, rt, o/n, 97%; (c) 3-(aminomethyl)-4,6-dimethylpyridin-2(1H)-one, HOAt, EDCI, DMSO, NMM, rt, 2 h, 75%; (d) 6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)boronic acid, Pd(dppf)Cl$_2$·DCM, AcOK, H$_2$O:Dioxane, reflux, 3 h, 97%; (e) H$_2$, Raney-Ni, rt, 3 h; (f) acrylic acid, HOAt, EDCI, DMSO, NMM, rt, 2 h, 91%; (g) 2-fluoroacrylic acid, HOAt, EDCI, DMSO, NMM, rt, 2 h, 89%.

6-bromo-1H-indole-4-carboxylic acid (4.11). methyl 6-bromo-1H-indole-4-carboxylate (1.00 g, 3.94 mmol) was dissolved in MeOH (20 mL) and THF (5 mL) and then LiOH (469 mg, 11.2 mmol) dissolved in H$_2$O (5 mL) was added. The contents were stirred overnight at room temperature. The next morning, TLC showed the reaction was complete, the organic solvents were removed in vacuo, and then HCl was added. Precipitate was observed, filtered, and collected to afford the desired product at 95% yield (900 mg, 3.75 mmol).
**6-bromo-1-(cyanomethyl)-1H-indole-4-carboxylic acid (4.12).** 6-bromo-1H-indole-4-carboxylic acid (400 mg, 1.67 mmol) was dissolved in DMF (10 mL) and NaH (167 mg, 4.17 mmol) was slowly added and stirred at room temperature for 15 minutes before adding bromoacetonitrile (238 mg mL, 2.00 mmol). The reaction was stirred at room temperature overnight. The next morning, LC/MS showed that the reaction was complete, the contents were then quenched with H2O, extracted with EA, washed with NaCl, dried over MgSO4, filtered, concentrated in vacuo and purified via ISCO (Hexane:EA, 100% → 70%) to give the desired product at 97% yield (260 mg, .932 mmol).

**6-bromo-1-(cyanomethyl)-N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1H-indole-4-carboxamide (4.13).** 6-bromo-1-(cyanomethyl)-1H-indole-4-carboxylic acid (.260 g, .932 mmol), 3-(aminomethyl)-4,6-dimethylpyridin-2(1H)-one (.170 g, 1.12 mmol), and HOAt (.190 g, 1.40 mmol) were dissolved in DMSO (10 mL) and stirred at room temperature for 15 minutes prior to addition of EDCI (.268 g, 1.40 mmol) and NMM (0.31 mL, 2.80 mmol) for 2 hours at room temperature. LC/MS demonstrated formation of product, the mixture was then quenched with H2O, which precipitated the desired product, which was obtained after filtering in 75% yield (.290 g, .703 mmol).

**1-(cyanomethyl)-N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indole-4-carboxamide (4.14).** 6-bromo-1-(cyanomethyl)-N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-
1H-indole-4-carboxamide (.290 g, .701 mmol), (6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)boronic acid (.192 g, .772 mmol), potassium acetate (.206 g, 2.11 mmol), and 1,1'-Bis(diphenylphosphino)ferrocene]dichloropalladium(II) (.029 g, .035 mmol) were dissolved in dioxane (30 mL) and H2O (6 mL). The mixture was heated to reflux for 3 hours, and monitored by LC/MS for product formation. The contents were then extracted using EA and purified using ISCO (DCM:MeOH; 5:1) to give the desired product in 97% yield (.369 g, .680 mmol).

1-(cyanomethyl)-N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indole-4-carboxamide (4.15).

1-(2-aminoethyl)-N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indole-4-carboxamide (.032 g, .059 mmol), acrylic acid (.005 g, .071 mmol), and HOAt (.012 g, .089 mmol) were dissolved in DMSO (2 mL) and stirred for 15 minutes
prior to the addition of EDCI (0.017 g, 0.089 mmol) and NMM (0.02 mL, 0.177 mmol), then the contents were stirred at room temperature for 2 hours. The reaction was monitored by LC/MS and purified by HPLC after completion to give the title compound in 91% yield (32.0 mg, 0.054 mmol). $^1$H NMR (400 MHz, Methanol-$d_4$), δ: 8.47 (d, $J = 2.4$ Hz, 1H), 7.93 (dd, $J = 8.8$, 2.5 Hz, 1H), 7.75 (t, $J = 1.1$ Hz, 1H), 7.61 (d, $J = 1.4$ Hz, 1H), 7.30 (d, $J = 3.2$ Hz, 1H), 6.93 (d, $J = 8.8$ Hz, 1H), 6.80 (d, $J = 3.2$ Hz, 1H), 6.00 (m, 4H), 5.54 (dd, $J = 8.4$, 3.5 Hz, 1H), 4.91 (s, 1H), 4.54 (s, 2H), 4.38 (t, $J = 6.2$ Hz, 3H), 3.48 (m, 5H), 3.20 (s, 1H), 2.37 (s, 3H), 2.20 (s, 3H), 1.38 (d, $J = 6.7$ Hz, 6H).

N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-(2-(2-fluoroacrylamido)ethyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indole-4-carboxamide (xy203-156 (4.17)). 1-(2-aminoethyl)-N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-1H-indole-4-carboxamide (20.0 mg, 0.037 mmol), 2-fluoroacrylic acid (4.00 mg, 0.071 mmol), and HOAt (7.50 mg, 0.055 mmol) were dissolved in DMSO (2 mL) and stirred for 15 minutes prior to the addition of EDCI (10.5 mg, 0.055 mmol) and NMM (0.015 mL, 0.111 mmol), then the contents were stirred at room temperature for 2 hours. The reaction was monitored by LC/MS and purified by HPLC after completion to give the title compound in 89% yield (7.07 mg, 0.033 mmol). $^1$H NMR (400 MHz, Methanol-$d_4$), δ: 8.44 (m, 1H), 7.95 (dd, $J = 8.9$, 2.6 Hz, 1H), 7.78 (d, $J = 1.2$ Hz, 1H), 7.65 (d, $J = 1.5$ Hz, 1H), 7.31 (d, $J = 3.3$ Hz, 1H), 6.88 (m, 2H), 6.79 (m, 1H), 6.09 (s, 1H), 5.10 (d, $J = 3.4$ Hz, 1H), 5.06 (d, $J = 3.4$ Hz,
1H), 4.90 (m, 75H), 4.62 (s, 2H), 4.43 (t, $J = 6.2$ Hz, 2H), 3.67 (t, $J = 6.2$ Hz, 2H), 3.55 (m, 4H), 2.63 (m, 6H), 2.33 (s, 3H), 2.20 (s, 3H), 1.13 (d, $J = 6.5$ Hz, 6H).

Scheme 4.4. Reagents and Conditions. (a) NaH, 2-bromopropane, DMF, rt, o/n, 25%; (b) 3-(aminomethyl)-4,6-dimethylpyridin-2(1H)-one, HOAt, EDCI, DMSO, NMM, rt, 2 h, 96%; (C) 6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)boronic acid, Pd(dppf)Cl$_2$·DCM, AcOK, H$_2$O:Dioxane, reflux, 3 h, 84%.

6-bromo-1-isopropyl-1H-indole-4-carboxylic acid (4.18). 6-bromo-1H-indole-4-carboxylic acid (.500 g, 2.08 mmol) was dissolved in DMF (10 mL) and NaH (.208 g, 5.20 mmol) was slowly added to the mixture, and stirred at room temperature for 15 minutes prior to the addition of 2-bromopropane (.312 mL, 3.12 mmol). The contents were then stirred overnight at room temperature. The reaction was monitored via TLC. After completion, the reaction was quenched with H$_2$O, extracted with DCM, washed with NaCl, the solvent was removed in vacuo and purified by ISCO (Hex:EA; 5:3) to afford the desired product in 25% yield (.147 g, .521 mmol).
6-bromo-N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-1H-indole-4-carboxamide (4.19). 6-bromo-1-isopropyl-1H-indole-4-carboxylic acid (.147 g, .521 mmol), 3-(aminomethyl)-4,6-dimethylpyridin-2(1H)-one (.095 g, .625 mmol), and HOAt (.106 g, .782 mmol) were dissolved in DMSO (10 mL) and stirred at room temperature for 15 minutes prior to addition of EDCI (.150 g, .782 mmol) and NMM (0.17 mL, 1.56 mmol) for 2 hours at room temperature. LC/MS demonstrated formation of product, the mixture was then quenched with H2O, which precipitated the desired product, which was obtained after filtering in 96% yield (.210 g, .504 mmol).

N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-isopropympiperazin-1-yl)pyridin-3-yl)-1H-indole-4-carboxamide (xy224-065 (4.20)). 6-bromo-N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-1H-indole-4-carboxamide (.210 g, .504 mmol), 6-(6-isopropympiperazin-1-yl)pyridin-3-yl)boronic acid (.150 g, .605 mmol), potassium acetate (.148 g, 1.51 mmol), and 1,1'-Bis(diphenylphosphino)ferrocene)dichloropalladium(II) (~20 mg) were dissolved in dioxane (50 mL) and H2O (10 mL). The mixture was heated to reflux for 3 hours, and monitored by LC/MS for product formation. The contents were then extracted using EA and purified using ISCO (DCM:MeOH, 5:1) to give the title compound in 84% yield (.229 g, .423 mmol). $^1$H NMR (400 MHz, Methanol-$d_4$), δ: 8.43 (m, 1H), 7.93 (dd, $J = 8.9, 2.5$ Hz, 2H), 7.74 (s, 2H), 7.62 (d, $J = 1.5$ Hz, 1H), 7.48 (d, $J = 3.3$ Hz, 1H), 6.91 (d, $J = 8.9$ Hz, 2H), 6.80 (m, 1H), 6.10 (s, 2H), 4.55 (d, $J = 5.0$ Hz, 6H), 3.58 (t, $J = 5.1$ Hz, 5H), 2.70 (s,
4H), 2.41 (s, 3H), 2.23 (d, J = 0.8 Hz, 3H), 1.53 (d, J = 6.6 Hz, 6H), 1.12 (d, J = 6.5 Hz, 6H).

Scheme 4.5. Reagents and Conditions. (a) LiOH, THF, MeOH, NaH, rt, o/n, 92%; (b) NaH, bromoacetonitrile, DMF, rt, o/n, 48%; (c) 3-(aminomethyl)-6-methyl-4-propylpyridin-2(1H)-one, HOAt, EDCI, DMSO, NMM, rt, 2 h, 20%; (d) 6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)boronic acid, Pd(dppf)Cl₂-DCM, AcOK, H₂O:Dioxane, reflux, 3 h, 74%; (e) H₂, Raney-Ni, rt, 3 h; (f) acrylic acid, HOAt, EDCI, DMSO, NMM, rt, 2 h, 85%; (g) 2-fluoroacrylic acid, HOAt, EDCI, DMSO, NMM, rt, 2 h, 83%.

6-bromo-1H-indazole-4-carboxylic acid (4.21). Methyl 6-bromo-1H-indazole-4-carboxylate (1.00 g, 3.92 mmol) was dissolved in THF (50 mL) and LiOH (.313 g, 7.84 mmol) dissolved in H₂O (10 mL) was added. The contents were stirred at room temperature overnight and the reaction was monitored by TLC to observe the disappearance of starting material. After completion, the organic solvents
were removed in vacuo, and HCl was added to precipitate the desired product in 92% yield (.870 g, 3.61 mmol).

6-bromo-1-(cyanomethyl)-1H-indazole-4-carboxylic acid (4.22). 6-bromo-1H-indazole-4-carboxylic acid (.870 g, 3.61 mmol) was dissolved in DMF (30 mL) and NaH (.360 g, 9.02 mmol) was slowly added. The mixture was then stirred at room temperature for 15 minutes. Then bromoacetonitrile (.325 mL, 4.69 mmol) was added to the mixture and the contents were stirred overnight at room temperature. The reaction was monitored by LC/MS, and after completion the contents were quenched with H2O, separated with EA and purified by ISCO (Hex:EA; 3:1) to afford the desired product in 48% yield (.560 g, 1.90 mmol).

6-bromo-1-(cyanomethyl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (4.23). 6-bromo-1-(cyanomethyl)-1H-indazole-4-carboxylic acid (.100 g, .357 mmol), 3-(aminomethyl)-6-methyl-4-propylpyridin-2(1H)-one (.077 g, .428 mmol), and HOAt (.073 mg, .536 mmol) were dissolved in DMSO (10 mL) and stirred at room temperature for 15 minutes prior to addition of EDCI (.103 g, .536 mmol) and NMM (0.078 mL, .714 mmol) for 2 hours at room temperature. LC/MS demonstrated formation of product, the mixture was then purified using ISCO (DCM:MeOH; 10:1) to give the desired product in 20% yield (.032 g, .072 mmol).
1-(cyanomethyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (4.24). 6-bromo-1-(cyanomethyl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (.032 g, .072 mmol), (6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)boronic acid (.022 g, .087 mmol), potassium acetate (.021 g, .217 mmol), and 1,1'-Bis(diphenylphosphino)ferrocene dichloropalladium(II) (~10 mg) were dissolved in dioxane (20 mL) and H2O (4 mL). The mixture was heated to reflux for 3 hours, and monitored by LC/MS for product formation. The contents were then extracted using EA and purified using ISCO (DCM:MeOH, 10:1) to give the desired product in 74% yield (.030 g, .053 mmol).

1-(2-aminoethyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (4.25). 1-(cyanomethyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (.030 g, .053 mmol) was dissolved in MeOH (50 mL) and Raney-Ni (~50 mg) was added to the solution. The solution was stirred overnight at room temperature and monitored via LC/MS for product formation. The contents were filtered, concentrated in vacuo and used for the next step without any further characterization.
1-(2-acrylamidoethyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (xy3-083 (4.26)). 1-(2-aminoethyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (.015 g, .026 mmol), acrylic acid (.009 g, .040 mmol), and HOAt (.014 g, .053 mmol) were dissolved in DMSO (5 mL) and stirred for 15 minutes prior to the addition of EDCI (.020 g, .053 mmol) and NMM (.1 mL, .454 mmol), then the contents were stirred at room temperature for 2 hours. The reaction was monitored by LC/MS and purified by HPLC after completion to give the title compound in 85% yield (.014 g, .022 mmol). ^H NMR (600 MHz, Methanol-d₄), δ: 8.50 (d, J = 2.5 Hz, 1H), 8.37 (s, 1H), 7.96 (dd, J = 8.9, 2.6 Hz, 1H), 7.82 (d, J = 1.4 Hz, 1H), 7.76 (d, J = 1.4 Hz, 1H), 6.93 (d, J = 8.8 Hz, 1H), 6.15 (s, 1H), 6.10 (dd, J = 17.2, 1.7 Hz, 2H), 5.46 (m, 4H), 4.63 (m, 3H), 4.59 (s, 3H), 3.72 (t, J = 5.8 Hz, 3H), 3.62 (s, 4H), 3.57 (s, 2H), 3.34 (d, J = 2.8 Hz, 3H), 2.69 (m, 10H), 2.30 (d, J = 15.3 Hz, 1H), 2.26 (s, 3H), 1.58 (m, 4H), 1.29 (s, 5H), 1.14 (dd, J = 6.5, 3.8 Hz, 10H), 1.02 (t, J = 7.4 Hz, 4H), 0.85 (m, 4H).

1-(2-(2-fluoroacrylamido)ethyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (xy3-096 (4.27)). 1-(2-aminoethyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (.065 g, .114 mmol), 2-fluoracrylic acid (.015 g, .171 mmol), and HOAt (.031 g, .228 mmol) were dissolved in DMSO (5 mL) and
stirred for 15 minutes prior to the addition of EDCI (.044 g, .228 mmol) and NMM (.1 mL, .454 mmol), then the contents were stirred at room temperature for 2 hours. The reaction was monitored by LC/MS and purified by HPLC after completion to give the title compound in 83% yield (.061 g, .095 mmol). \(^1\)H NMR (600 MHz, Methanol-\(d_4\)), \(\delta\): 8.50 (d, \(J = 2.5\) Hz, 1H), 8.36 (d, \(J = 0.8\) Hz, 1H), 7.96 (dd, \(J = 8.9, 2.6\) Hz, 1H), 7.86 (t, \(J = 1.2\) Hz, 1H), 7.73 (m, 1H), 6.87 (m, 2H), 6.14 (s, 1H), 5.44 (m, 1H), 5.39 (d, \(J = 3.5\) Hz, 1H), 5.05 (dd, \(J = 15.3, 3.4\) Hz, 1H), 4.67 (t, \(J = 5.9\) Hz, 3H), 4.59 (s, 3H), 3.74 (t, \(J = 5.9\) Hz, 3H), 3.54 (m, 6H), 3.43 (m, 1H), 3.31 (m, 1H), 2.66 (m, 11H), 2.26 (s, 4H), 1.57 (m, 3H), 1.28 (s, 1H), 1.05 (m, 10H), 1.01 (t, \(J = 7.3\) Hz, 4H).

**Scheme 4.6. Reagents and Conditions.** (a) NaH, 1-chloro-5-iodopentane, DMF, rt, o/n; (b) NaOH, MeOH, rt, o/n, 47%; (c) 3-(aminomethyl)-6-methyl-4-propylpyridin-2(1H)-one, HOAt, EDCI, DMSO, NMM, rt, 2 h, 72%; (d) 6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)boronic acid, Pd(dpdpf)Cl\(_2\)-DCM, AcOK,
H$_2$O:Dioxane, reflux, 3 h, 97%; (e) NaN$_3$, DMF, 80 C, 2 h; (f) MeOH, Pd/C, H$_2$, rt, 2 h; (g) acrylic acid, HOAt, EDCI, DMSO, NMM, rt, 2 h, 76%; (h) 2-fluoroacrylic acid, HOAt, EDCI, DMSO, NMM, rt, 2 h, 82%.

6-bromo-1-(5-chloropentyl)-1H-indazole-4-carboxylic acid (4.28). methyl 6-bromo-1H-indazole-4-carboxylate (.500 g, 1.96 mmol) was dissolved in DMF (20 mL) and NaH (.117 g, 2.94 mmol) was slowly added, the mixture was stirred at room temperature for 15 minutes prior to the addition of 1-chloro-5-iodopentane (.683 g, 2.94 mmol). The contents were then stirred at room temperature overnight. The reaction was monitored by LC/MS and concentrated in vacuo after completion. Then the contents were dissolved in MeOH (5 mL) and NaOH (.024 g, 5.88 mmol) dissolved in H$_2$O (5 mL) was added; the contents were stirred at room temperature overnight. The reaction was monitored via TLC, and after disappearance of the starting material, the organic solvents were removed in vacuo, the contents were extracted with EA and purified by ISCO (Hexane:EA; 4:1) to afford the desired product in 47% yield over 2 steps (.320 g, .926 mmol).

6-bromo-1-(5-chloropentyl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (4.29). 6-bromo-1-(5-chloropentyl)-1H-indazole-4-carboxylic acid (330 mg, .926 mmol), 3-(aminomethyl)-4,6-dimethylpyridin-2(1H)-one (183 mg, 1.02 mmol), and HOAt (189 mg, 1.39 mmol) were dissolved in DMSO (15 mL) and stirred at room temperature for 15 minutes prior to addition of EDCI (266 mg, 1.39 mmol) and NMM (0.31 mL, 2.77 mmol)
for 2 hours at room temperature. LC/MS demonstrated formation of product, the mixture was then quenched with H2O, separated with EA, and purified using ISCO (Hexane:EA; 5:1) to give the desired product in 72% yield (340 mg, .669 mmol).

1-(5-chloropentyl)-6-(6-(4-isopropypiperazin-1-yl)pyridin-3-yl)N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (4.30). 6-bromo-1-(5-chloropentyl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (.340 g, .669 mmol), (6-(4-isopropypiperazin-1-yl)pyridin-3-yl)boronic acid (.167 g, .669 mmol), potassium acetate (206 mg, 2.11 mmol), and 1,1′-Bis(diphenylphosphino)ferrocene)dichloropalladium(II) (~5 mg) were dissolved in dioxane (10 mL) and H2O (2 mL). The mixture was heated to reflux for 3 hours, and monitored by LC/MS for product formation. The contents were then extracted using EA and purified using ISCO (DCM:MeOH; 5:1) to give the desired product in 97% yield (.286 g, .452 mmol).

1-(5-azidopentyl)-6-(6-(4-isopropypiperazin-1-yl)pyridin-3-yl)N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (4.31). 1-(5-chloropentyl)-6-(6-(4-isopropypiperazin-1-yl)pyridin-3-yl)N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (.090 g, .142 mmol) and sodium azide (.046 g, .711 mmol) were dissolved in DMF (5 mL), the reaction was heated to 80 °C for 2 hours. The
reaction was monitored via LC/MS, quenched with H2O after completion and extracted with DCM. The crude product was used for the next step without further characterization.

1-(5-aminopentyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (4.32). 1-(5-azidopentyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (.030 g, .047 mmol) was dissolved in MeOH (4 mL) and afforded a catalytic amount of Pd/C and placed under Hydrogen at room temperature for 2 hours. The reaction was monitored by LC/MS, concentrated in vacuo, and used for the next step without further characterization.

1-(5-acrylamidopentyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (xy3-104 (4.33)). 1-(5-aminopentyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (.030 g, .049 mmol), acrylic acid (.007 g, .073 mmol), and HOAt (.014 g, .098 mmol) were dissolved in DMSO (5 mL) and stirred for 15 minutes prior to the addition of EDCI (.020 g, .098 mmol) and NMM (.1 mL, .454 mmol), then the contents were stirred at room temperature for 2 hours. The reaction was monitored by LC/MS and purified by HPLC after completion to give the title compound in 76% yield (.025 g, .037 mmol). $^1$H NMR (600 MHz,
Methanol-$d_4$, $\delta$: 8.52 (d, $J = 2.5$ Hz, 1H), 8.34 (d, $J = 0.8$ Hz, 1H), 7.99 (dd, $J = 8.9$, 2.7 Hz, 1H), 7.89 (d, $J = 1.5$ Hz, 1H), 7.77 (d, $J = 1.3$ Hz, 1H), 6.92 (d, $J = 8.9$ Hz, 1H), 6.10 (m, 2H), 5.58 (dd, $J = 7.8$, 4.2 Hz, 1H), 4.59 (s, 2H), 4.50 (t, $J = 6.9$ Hz, 2H), 3.61 (t, $J = 5.2$ Hz, 4H), 3.19 (t, $J = 7.0$ Hz, 2H), 2.71 (m, 3H), 2.69 (t, $J = 5.2$ Hz, 4H), 2.26 (s, 3H), 1.95 (p, $J = 7.0$ Hz, 2H), 1.59 (m, 2H), 1.55 (p, $J = 7.2$ Hz, 2H), 1.27 (m, 3H), 1.08 (m, 6H), 1.01 (t, $J = 7.4$ Hz, 3H).

1-(5-(2-fluoroacrylamido)pentyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (xy3-103 (4.34)). 1-(5-acrylamidopentyl)-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (.012 g, .020 mmol), 2-fluoroacrylic acid (.005 g, .070 mmol), and HOAt (.013 g, .094 mmol) were dissolved in DMSO (5 mL) and stirred for 15 minutes prior to the addition of EDCI (.018 g, .094 mmol) and NMM (.1 mL, .454 mmol), then the contents were stirred at room temperature for 2 hours. The reaction was monitored by LC/MS and purified by HPLC after completion to give the desired product in 82% yield (.011 g, .016 mmol). $^1$H NMR (600 MHz, Methanol-$d_4$), $\delta$: 8.52 (d, $J = 2.5$ Hz, 1H), 8.34 (d, $J = 0.9$ Hz, 1H), 7.99 (dd, $J = 8.9$, 2.6 Hz, 1H), 7.90 (s, 1H), 7.77 (d, $J = 1.3$ Hz, 1H), 6.92 (d, $J = 8.9$ Hz, 1H), 6.14 (s, 1H), 5.11 (dd, $J = 15.3$, 3.3 Hz, 1H), 4.84 (m, 4H), 4.59 (s, 2H), 4.50 (t, $J = 6.9$ Hz, 2H), 3.61 (t, $J = 5.2$ Hz, 4H), 3.29 (d, $J = 3.9$ Hz, 6H), 3.22 (t, $J = 7.1$ Hz, 2H), 2.67 (m, 7H), 2.25 (d, $J = 2.8$ Hz,
3H), 1.96 (p, J = 7.1 Hz, 2H), 1.63 (m, 2H), 1.52 (m, 3H), 1.30 (m, 2H), 1.27 (m, 1H), 1.13 (d, J = 6.5 Hz, 7H), 1.01 (t, J = 7.4 Hz, 3H).

Results

Homology Modeling and EZH2 SET Domain Crystal Structure Guide EZH2

Irreversible Design

Prior to the release of the EZH2 apo crystal structure (PDB: 4MI0 and PDB: 4MI5) [82, 83] we built a homology model of EZH2 using the GLP crystal structure (PDB: 3RFI) as a template [62]. Our initial binding hypothesis was generated using this model, so we were eager to see if the predicted mode of binding generated with our homology model was recapitulated in the crystal structure. Indeed, our homology model superimposes well onto the crystal structure (blue vs purple in figure 4.1 A) yielding an RMSD of 2.2 Å. Further, docking of UNC1999 into the crystal structure results in a very similar pose as the homology model. Our binding hypothesis predicts that cysteine 663 (C663) resides approximately 5.2 Å away from the isopropyl substituent of the indazole core of UNC1999 (Figure 4.1 A). This is especially advantageous as the indazole nitrogen can easily be modified to install reactive moieties capable of irreversible inhibition. Further, this cysteine residue is a serine residue in EZH1 (Figure 4.1 B), so we believe that we can take advantage of the more nucleophilic properties of cysteine to develop a selective, irreversible inhibitor of EZH2, which will give direct confirmation of our proposed UNC1999 binding hypothesis.
**In vitro Activity of First Generation UNC1999 Irreversible Analogs**

Our goal in designing the first EZH2 irreversible inhibitors was to tease out nearly all EZH1 inhibition, thereby creating an EZH2-specific chemical probe. As such, we decided to adopt the indole core of GSK126 instead of the indazole core of UNC1999, and the dimethyl pyridone of GSK126, EPZ005687 and El1. We believed these features would negate a significant amount of the reversible binding to EZH1, while maintaining it for EZH2; after initial reversible binding the cysteine would be able to attack the reactive moiety. We also varied the substituent at the 6 position of the indole, and decided to synthesize compounds bearing the smaller nitrile in addition to the piperazine substituted pyridine. While the piperazine substituted pyridine is believed to contribute to the potency of the inhibitors, we felt that the nitrile would allow for additional movement of the compound that may be needed to allow for C663 to engage the irreversible moieties. We began with an epoxide, acrylamide, and α-fluoroacrylamide as reactive moieties, installing them on the indole nitrogen. Our binding hypothesis predicted that these moieties would be tolerated in the active site.

The synthesis of the aforementioned compounds can be found in Scheme 4.1, and the corresponding structures with *in vitro* activities are displayed in Table 4.1. Unexpectedly, almost all of the compounds synthesized with this design in mind were inactive against both EZH1 and EZH2. All of the nitrile derivatives displayed >5 µM IC50s against both enzymes (Table 4.1, xy203-020, xy203-117, and xy203-157). Of the compounds bearing the piperazine-substituted pyridine, only xy224-014 displayed an IC50 < 2 µM against EZH2.
Due to the change in scaffold from our previous EZH2 inhibitors, we synthesized xy224-065 as a positive control. Xy224-065 displayed potent inhibition of EZH2 (IC50 = 24 nM), and as hoped with this scaffold, was significantly less potent for EZH1 (IC50 = 1.95 µM).

**In vitro Activity of Second Generation UNC1999 Irreversible Analogs**

The results from the set of compounds in Table 4.1 made it clear that the switch to the indole and dimethylpyridone led to a significant loss in reversible binding. Further, maintaining the piperazine-substituted pyridine may be essential for potency, so we revisited our initial UNC1999 scaffold, and decided to append the same irreversible moieties to the indazole. Prior to synthesizing the set of compounds in Table 4.2, we studied the binding of UNC1999 derivatives bearing irreversible moieties *in silico*. Indeed, our UNC1999 binding hypothesis is recapitulated when appending an acrylamide to the indazole nitrogen (Figure 4.2), so we moved forward with acrylamides, and α-fluoroacrylamides. We also decided to increase the linker length from the indazole to the reactive group.

The set of compounds pictured in Table 4.2 yielded much more positive results than those of Table 4.1; all had IC50s in the nanomolar range for EZH2. However, we did pick up additional EZH1 activity in switching back to the 4-methyl-6-propyl pyridone indazole scaffold; xy3-103 and xy3-104 demonstrated fairly potent inhibition of EZH1. While none of these compounds were as effective as UNC1999, we hoped that we may be able to observe an EZH2-inhibitor
adduct via MS, but unfortunately, none of the compounds produced said adduct, and were therefore determined not to be irreversible inhibitors.

**Discussion**

Irreversible inhibitors have proved to be very useful tools in the study of protein function, which is demonstrated in the success of activity-based protein profiling [84]. Not only do these probes lend themselves as tools to aid in the discovery of additional inhibitors, but they also serve to help characterize enzyme active sites [85, 86]. One issue hampering further discovery and understanding of EZH2 inhibitors is the lack of direct evidence for a mode of binding, which in part, is due to the lack of an EZH2-ligand co-crystal structure. An irreversible inhibitor of EZH2 may facilitate said structure.

Homology modeling of the EZH2 SET domain led to the hypothesis that a cysteine residue resides near the presumed binding site of UNC1999. After release of the EZH2 SET domain apo structure, we were delighted to discover that our homology model superimposed well onto the crystal structure, and that the cysteine of interest (C663) was also present in the same vicinity as C663 in the homology model. Additionally, this cysteine is a serine in EZH1 (S664), so we aimed to create an EZH2-selective, irreversible inhibitor by exploiting the increased nucleophilicity of the cysteine versus the serine. However, our efforts were initially thwarted as none of the 4,6-dimethyl pyridone indole compounds displayed promising results *in vitro*. Yet, switching back to the 4-propyl-6-methyl pyridone indazole scaffold did provide compounds that were nanomolar potent,
which demonstrated that substituents are tolerated at this position. The exact reasoning for this change in tolerability of the reactive moieties with the indazole versus the indole is unfounded. Further, the longer linker (n=2 vs n=5) resulted in more potent compounds, but none of the designed irreversible inhibitors were able to create an EZH2-ligand adduct, as detected by MS.

It is very possible that the full-length EZH2 architecture does not mimic the EZH2 SET domain apo structure. This could mean that C663 is buried deep within the complex, and is not available for attack of the reactive moiety. It is also possible, that if the cysteine is solvent exposed, as our truncated model suggests, that the local environment is influencing the pKa of C663, making it less reactive [87]. Acrylamides are fairly slow reacting irreversible motifs, which is beneficial, because there is less potential for off-target effects. However, going forward, we plan to install more reactive irreversible moieties (such as α-fluoro- and α-chloroketones) in order to try and engage C663 as a proof of concept for EZH2 irreversible inhibition.
### Table 4.1: First generation of EZH2 irreversible inhibitors.

|i-Pr = isopropyl. CN = cyano.|

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>EZH2 IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>EZH1 IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
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<tbody>
<tr>
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<td>-CN</td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td>&gt;5000</td>
<td>&gt;5000</td>
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<tr>
<td>xy203-117</td>
<td>-CN</td>
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</tr>
<tr>
<td>xy203-157</td>
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Table 4.2: Second generation of EZH2 irreversible inhibitors.

$i$-Pr = isopropyl.
Figure 4.1: Rationale for EZH2 irreversible inhibitor design.

(A) Superimposition of EZH2 homology model (blue) and apo X-ray co-crystal structure of the EZH2 SET domain (purple). UNC1999 (green) is hypothesized to occupy the SET domain with a pose that orients the isopropyl substituent of the indazole towards a nearby (5.2 Å) cysteine residue (C663). (B) C663 in EZH2 is a serine residue (S664) in EZH1.
Figure 4.2: xy3-083 binding hypothesis.

Docking of xy3-083 (green) into the EZH2 apo crystal structure (purple) predicts that the compound binds in a way that orients the reactive moiety (acrylamide pictured here) towards C663.
CHAPTER V: CHEMICAL PROBE FUNCTIONALIZATION PROVIDES A NOVEL METHOD FOR DETECTION OF GENETIC OCCUPANCY

Introduction

Chemical probes that demonstrate sufficient cellular potency and target selectivity can greatly accelerate understanding of protein function [43, 45]. This is especially important in relatively nascent fields like epigenetic drug discovery; chemical probes allow for extensive target validation, and can be functionalized to create chemical tools from the parent chemical probe [62, 88, 89]. G9a and EZH2 are two very interesting potential drug targets, and several highly potent small molecule inhibitors are widely available for both. So, we focused our efforts on functionalizing the small molecule inhibitor of G9a, as we previously did with EZH2 (Chapter 1 & [62]), into a tool that will allow the research community to further advance the understanding of these two interesting targets.

Like EZH2, G9a is a lysine methyltransferase, but differs in substrate specificity; G9a catalyzes the mono- and dimethylation of histone H3 lysine 9 (H3K9) [90-95]. G9a activity has an effect on multiple biological pathways and human diseases including cancer (reviewed in [96]), and is overexpressed in a
number of tumors [97]; specifically, it is associated with increased metastasis and invasion of tumor cells in lung cancer [98]. Surprisingly, loss of G9a-dependent histone 3 lysine 9 dimethylation (H3K9me2) has also been observed in various cancer cell lines [99]. In parallel with EZH2, the discrepancy of G9a activity in different cancer types underscores the importance of further research into the biological role of G9a, and chemical tools will provide an immeasurable amount of assistance in teasing out these observed discrepancies.

Herein, we describe the discovery of UNC0965, a biotinylated tool of the G9a chemical probe UNC0638 [100]. UNC0965 displays low nanomolar in vitro potency, reduces the H3K9me2 mark in cellular assays, and selectively chemiprecipitates G9a from whole cell lysates. UNC2399 is a biotinylated analog of UNC1999 [62] that displays low nanomolar in vitro potency, and selectively chemiprecipitates EZH2 from whole cell lysates. We also show that UNC0965 is a useful tool for exploring the localization of G9a on chromatin both in vitro, and in vivo, and provide rationale for the development of the same application for UNC2399.
Methods

UNC0965 Synthesis

Scheme 5.1. Reagents and Conditions: (a) 1-Chloro-3-iodopropane, K$_2$CO$_3$, CH$_3$CN, reflux; (b) HNO$_3$, Ac$_2$O, 0 °C to RT, 75% over two steps; (c) pyrrolidine, K$_2$CO$_3$, Nal, cat. tetrabutylammonium iodide, CH$_3$CN, reflux, 81%; (d) Fe dust, NH$_3$OAc, EA-H$_2$O, reflux, 63%; (e) 4N HCl, dioxane, cyclohexanecarbonitrile; (f) N,N-diethylaniline, POCl$_3$, reflux, 47% over two steps; (g) hex-5-yn-1-yl 4-methylbenzenesulfonate, K$_2$CO$_3$, DMF, 60 °C, 88%; (h) TFA, DCM, rt, 99%; (i) 1-(hex-5-yn-1-yl)piperidin-4-amine-2TFA (5.8), K$_2$CO$_3$, DMF, 60 °C, 81%; (j) biotin-PEG$_3$-azide, cat. Cu(CH$_3$CN)$_4$PF$_6$, DCM, rt, 92%.

2-Cyclohexyl-N-(1-(hex-5-yn-1-yl)piperidin-4-yl)-6-methoxy-7-(3-(pyrrolidin-1-yl)propoxy)quinazolin-4-amine (5.9). Compound 5.6 was prepared from the commercially available starting material (5.1) according to the synthetic route developed previously [100-103]. Compound 5.8 was synthesized from
commercially available tert-butyl piperidin-4-ylcarbamate (5.7) in two straightforward steps. A mixture of tert-butyl piperidin-4-ylcarbamate (5.7) (0.735 g, 3.67 mmol), hex-5-yn-1-yl 4-methylbenzenesulfonate (0.926 g, 3.67 mmol) and K$_2$CO$_3$ (0.760 g, 5.50 mmol) in DMF (5.5 mL) was stirred overnight at 60°C. The resulting mixture was cooled to rt, added H$_2$O (11 mL) and then extracted with CH$_2$Cl$_2$ (20 mL × 3). The combined organic phases were dried over Na$_2$SO$_4$, concentrated and purified by flash column chromatography on silica gel (0 – 6% MeOH / CH$_2$Cl$_2$) to afford the intermediate tert-butyl (1-(hex-5-yn-1-yl)piperidin-4-yl)carbamate (0.906 g, 88%). Trifluoroacetic acid (2.0 mL) was added to a solution of this intermediate (0.906 g, 3.22 mmol) in CH$_2$Cl$_2$ (10 mL) for 6 h at rt. The mixture was concentrated to afford 1-(hex-5-yn-1-yl)piperidin-4-amine·TFA (5.8) as a slurry oil. MS (ESI): 181 [M + H]$^+$. Compound 5.6 (0.210 g, 0.52 mmol) (prepared according to the previously reported methods [104]) was added to a suspension of compound 5.8 (~ 1.1 mmol) and K$_2$CO$_3$ (1.1 g, 8 mmol) in DMF (4.0 mL). The resulting mixture was stirred overnight at 60 °C, cooled to rt, added H$_2$O (10 mL), and extracted with CH$_2$Cl$_2$ (3 x 20 mL). The combined organic layers were dried over Na$_2$SO$_4$, concentrated, and purified by flash column chromatography on silica gel (0 – 10% MeOH / CH$_2$Cl$_2$) to afford the title compound 5.9 as a yellowish solid (0.231 g, 81% yield). $^1$H NMR (400 MHz, CDCl$_3$), δ: 7.13 (s, 1H), 6.86 (s, 1H), 5.32 (d, $J = 7.2$ Hz, 1H), 4.27–4.15 (m, 1H), 4.12 (t, $J = 6.7$ Hz, 2H), 3.86 (s, 3H), 2.97–2.85 (m, 2H), 2.68 (tt, $J = 11.7$, 3.4 Hz, 1H), 2.61–2.52 (m, 2H), 2.52–2.41 (m, 4H), 2.40–2.31 (m, 2H), 2.24–2.10 (m,
6H), 2.10–2.00 (m, 2H), 1.99–1.89 (m, 3H), 1.86–1.47 (m, 15H), 1.44–1.21 (m, 3H). MS (ESI): 548 [M + H]+. HPLC: 97%, RT: 2.81 min.

\[N-(2-(2-(4-(4-(2-Cyclohexyl-6-methoxy-7-(3-pyrrolidin-1-yl)propoxy)quinazolin-4-yl)amino)piperidin-1-yl)butyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (5.10). \]

Cu(CH₃CN)₄PF₆ (2.1 mg, 0.0054 mmol) was added to a solution of compound 5.9 (17 mg, 0.0312 mmol) and biotin-PEG₃-azide (14 mg, 0.0312 mmol) in DCM (3.0 mL). The resulting mixture was stirred overnight at rt, concentrated and purified by preparative HPLC to give the title compound 5.10 as a white solid (28 mg, 92%). \(^1\)H NMR (400 MHz, CDCl₃), δ: 7.45 (s, 1H), 7.15 (s, 1H), 6.98 (s, 1H), 6.69 (t, J = 5.6 Hz, 1H), 6.35 (s, 1H), 5.55 (d, J = 7.2 Hz, 1H), 5.43 (s, 1H), 4.49 (t, J = 5.2 Hz, 2H), 4.36-4.43 (m, 1H), 4.28–4.21 (m, 2H), 4.17 (t, J = 6.8 Hz, 2H), 3.92 (s, 3H), 3.86 (t, J = 5.2 Hz, 2H), 3.62–3.56 (m, 8H), 3.53 (t, J = 5.2 Hz, 2H), 3.43–3.39 (m, 2H), 3.12–3.07 (m, 1H), 2.94–2.91 (m, 2H), 2.85 (AB, J_AB =12.8 5.2 Hz, 1H), 2.75–2.66 (m, 4H), 2.6261 (t,J = 7.2 Hz, 2H), 2.52–2.47 (m, 5H), 2.40 (t, J = 7.6 Hz, 2H), 2.23–2.12 (m, 6H), 2.09 (t, J =7.2 Hz, 2H), 1.98–1.95 (m, 2H), 1.84–1.54 (m, 18H), 1.45–1.23 (m, 5H). MS (ESI): 992 [M + H]^+. HPLC: 99%, RT: 3.19 min. HRMS (ESI) calcd for C₅₁H₈₂N₁₁O₇S [M + H]^+: 992.6119. Found:992.6143.
Cell Lines

HEK 293T cells were grown at 37°C/5% CO₂ on 15 cm plates in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum. MDA-MB-231 cells were grown as described in [100].

Western Blotting

Western blotting was performed as described previously [62], except that blotting was performed with rabbit anti-G9a (Cell Signalling #3306), and goat anti-rabbit IgG(H+L) IR Dye 800 CW (LI-COR #926-32211). Imaging was performed on the LI-COR Odyssey.

Antibody ChIP

HEK 293T cells were grown to 90% confluency on four 15 cm plates. Each ChIP contained chromatin from approximately 1x10⁷ cells. Chromatin preparation and ChIP assay were performed using the Active Motif ChIP-IT® High Sensitivity kit #53040 according to manufacturer’s guidelines. Antibodies used included: G9a, Abcam #ab40542, and rabbit IgG whole molecule, Jackson Immunoresearch #011-000-003.

In vitro chem-ChIP

Chromatin was prepared from HEK 293T cells as described for the antibody ChIP. Each ChIP contained chromatin from approximately 8x10⁶ cells. Streptavidin-conjugated magnetic beads (Dynabeads® M-280 streptavidin, Life
Technologies #11205D, 250 µg per chemiprecipitation) were washed 3 times with 500 µL TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20), diluted in 100 µL TBST, and 4 µL of 10 mM UNC0965 was added, followed by rotation on an end-over-end rotator for 1 hour at room temperature to saturate the beads. After binding, the beads were washed with TBST (500 µL x 3) to remove excess unbound compound. Chromatin was then added to the beads and the mixture was placed on an end-over-end rotator at 4°C overnight. The next morning, tubes were removed from the rotator, and supernatant was aspirated from the beads. The beads were washed (500 µL x 3) with ChIP wash buffer (0.1% SDS, 1% TritonX-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris, pH=8.0) followed by one wash with 500 µL of ChIP final wash buffer (1% SDS, 1% TritonX-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0). During each wash, the sample was rotated at room temperature for 3 minutes. The washed beads were resuspended in 200 µL TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) with 20 µg proteinase K, and the contents were transferred into a PCR tube. The mixture was incubated at 55°C, for 30 minutes followed by 2 hours at 80°C to reverse the crosslinks. The DNA in the collected supernatant was then purified as described in Active Motif ChIP-IT® High Sensitivity kit #53040.

**In vivo chem-ChIP**

HEK 293T cells were grown to 80% confluency on 15 cm plates as described. Cells were treated with 5 µM UNC0965 for 15 minutes prior to
formaldehyde crosslinking. Chromatin was prepared as described for the antibody ChIP. Each chem-ChIP represented approximately $8 \times 10^6$ cells. Streptavidin-conjugated magnetic beads (Dynabeads® M-280 streptavidin, Life Technologies #11205D, 500 µg per chemiprecipitation) were washed 3 times with 500 µL TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20). Chromatin was then added to the beads and the mixture was placed on a rotator at 4°C overnight. Wash and DNA purification steps were performed as described in the method for \textit{in vitro} chem-ChIP, above.

\textbf{Quantitative PCR}

Antibody ChIP and \textit{in vivo} chem-ChIP DNA was prepared by diluting input samples 1:1000 and ChIP/chem-ChIP samples 1:10 in water. \textit{In vitro} chem-ChIP DNA was prepared by diluting input samples 1:1000 and ChIP/chem-ChIP samples 1:3 in water. Two microliters of each diluted sample was subjected to relative quantification qPCR in duplicate on the ABI 7900HT using FastStart SYBR Green Master Mix ROX (Roche) in a 10 µL final volume. Percent input was determined using the ΔCt method [105] followed by normalization to a genomic region near the \textit{GAPDH} gene that is not occupied by G9a.

\textbf{In-cell Western and Cell Toxicity assays}

In-cell western and cell toxicity assays were performed in MDA-MB-231 cells as previously reported [104].
Results

Discovery of UNC0965

Previously, our lab reported the discovery of UNC0638 (Figure 5.1 A) [100] and UNC1152 (Figure 5.1 B) [106] the first chemical probe, and a structurally similar negative control of G9a, respectively. The discovery of UNC0638 also led to an X-ray co-crystal structure of the G9a-UNC0638-SAH complex [100]. In this structure, UNC0638 adopted a conformation that buried the 7-(3-pyrrolidin-1-yl)-propoxy side chain in the lysine binding channel, and the isopropyl group of the 4-(N-isopropylpiperidin-4-ylamino) moiety was solvent exposed. We took advantage of this solvent exposed chemical handle, and biotinylated the piperidine of UNC0638 yielding UNC0965; a functionalized chemical tool for biological applications (Figure 5.1 C). We hypothesized that a relatively long linker (~20 atoms from the piperidine nitrogen to the biotin carbonyl) would ensure that the biotin was at a sufficient distance from the surface of G9a to permit binding to streptavidin-conjugated magnetic beads while keeping the G9a warhead available for binding to G9a. This hypothesis was supported by docking studies using the G9a X-ray structure. As expected, UNC0965 docked in a nearly identical orientation to UNC0638 (Figure 5.2); with the 7-(3-pyrrolidin-1-yl)propoxy side chain embedded in the lysine binding channel, and the biotin solvent exposed, well away from the surface of the protein.

We next confirmed that UNC0965 binds G9a and inhibits its catalytic activity. As with EZH2, we used a scintillation proximity assay [104] to determine
the *in vitro* IC50 of UNC0965 (Figure 5.3 A). This assay is a direct measurement of the G9a catalyzed transfer of a tritiated methyl group from $^3$H-SAM to the histone peptide substrate. UNC0965 showed potent inhibition, with an IC50 value of < 2.5 nM. This strong inhibition of G9a catalytic activity implied that UNC0965 would bind the protein tightly enough to efficiently chemiprecipitate G9a from cell lysates.

**UNC0965 Selectively Chemiprecipitates G9a**

UNC0965 was coupled to streptavidin-conjugated magnetic beads in order to chemiprecipitate G9a from HEK 293T whole cell extracts (Figure 5.3 B). Beads pre-treated with DMSO (1%) did not pull down G9a (Figure 5.3 B, lane 2). UNC0965-coated streptavidin-conjugated beads effectively pulled down G9a, while pre-incubation of the lysate with UNC0638 blocked this interaction (Figure 5.3 B, lanes 3 and 4). Additionally, pre-treatment of lysates with UNC1152, a negative control of UNC0638 that does not significantly inhibit G9a catalytic activity (compound 26a in [104]), was unable to block the ability of UNC0965-coated beads to chemiprecipitate G9a (Figure 5.3 B, lanes 4 and 5). UNC0965 is capable of chemiprecipitating G9a from cell lysates, which further confirms that the biotin moiety of UNC0965 does not interfere with compound binding to G9a.

**UNC0965 Chemiprecipitates G9a Crosslinked to Chromatin *in vitro***

Using traditional antibody ChIP, we identified four chromosomal regions in HEK 293T cells (Table 5.1) that showed a significant fold change in G9a
occupancy over background when compared to a negative control region near the GAPDH gene (Figure 5.4 A). We performed in vitro chem-ChIP using the same protocol used for antibody ChIP. Instead of the G9a antibody and IgG control, we used streptavidin-conjugated magnetic beads pre-incubated with UNC0965 and DMSO, respectively. We then examined the identified G9a occupied chromosomal regions by qPCR (Figure 5.4 B). The UNC0965 in vitro chem-ChIP showed a greater than 5-fold increase over DMSO at each chromosomal region. The difference between the G9a antibody ChIP signal and the IgG signal was less significant (p<0.5 to p<0.01) (Figure 5.4 A), than the signal for UNC0965 in vitro chem-ChIP compared to DMSO (p<0.01 to p<0.001). Thus, UNC0965 effectively captures G9a in an in vitro ChIP assay (termed in vitro chem-ChIP), and in this case with a better signal over background with G9a than the traditional antibody ChIP.

**UNC0965 is Cell Permeable and Engages Chromatin Bound G9a in Cells**

Unlike antibodies, small-molecule tools capable of permeating the cell membrane can be used to engage the target in cells prior to crosslinking. We assessed the cellular activity of UNC0965 using an in-cell western assay as reported previously [13]. Treatment of cells for 48 hours with increasing concentrations of UNC0965 resulted in a decrease in H3K9me2 (IC50 = 3.3 ± 1.2 µM), with low cell toxicity (EC50 > 50 µM), indicating that UNC0965 is indeed cell permeable (Figure 5.5 A).
For the in vivo chem-ChIP assay, we aimed to minimize any effects of G9a inhibition on its chromatin localization by quickly treating the cells with compound, rather than treating for several days. Therefore, we grew HEK 293T cells to 80% confluency, and then exposed the cells to 5 µM UNC0965 or 0.05% (volume equivalent) DMSO for 15 minutes prior to formaldehyde crosslinking. We then performed the standard antibody ChIP protocol, but used streptavidin-conjugated magnetic beads for the precipitation step. Cells treated with UNC0965 showed a significant average change in G9a occupancy over DMSO-treated cells (p<0.01) (Figure 5.5 B), which was very comparable to the in vitro chem-ChIP results (Figure 5.4 B). These data demonstrate that even with compound pre-treatment of the cells, the biotin moiety of UNC0965 remains solvent exposed after formaldehyde crosslinking, confirming that UNC0965 is a useful chemical tool for what we call “in vivo chem-ChIP” and could be used in additional cellular applications.

Putative Applications of UNC2399

The success of UNC0965 with G9a in both the in vitro and in vivo chem-ChIP assays inspired us to try the same technique with UNC2399 and EZH2. We began by conducting a traditional EZH2 ChIP assay in PC3 cells (Figure 5.6 A). Table 5.2 details the regions tested, and about half of the regions tested displayed significant (> 2-fold change) amplification versus IgG control (1, 2, 4, 8, and 10, Table 5.1 for details), while the other half were amplified but < 2-fold versus IgG control (3, 5, 6, 7, 9, Table 5.1 for details); primer pair 11 is GAPDH
and was used as a negative control. We next aimed to recapitulate the results from Figure 5.6 A using the *in vitro* chem-ChIP method, but to our surprise, we observed no significant amplification with UNC2399 versus UNC2398 control (Figure 5.6 B); DMSO treated beads (not pictured) resulted in a similar profile. We believe that the failure of UNC2399 in the *in vitro* chem-ChIP assay may be due to masking of the SET domain after crosslinking of the chromatin. So, *in vivo* chem-ChIP may be the best route for determination of EZH2 genetic occupancy using UNC2399.

Unfortunately, UNC2399 is inactive in the EZH2 ICW, so we aimed to create a UNC2399 analog that is capable of both chemiprecipitating EZH2 and permeating the cell membrane. It is probable that the 12 PEG linker of UNC2399 contributes to the low cell permeability, so we first aimed to determine the shortest linker possible that maintains the ability to chemiprecipitate EZH2 via titration of the linker length with various PEG-biotin groups (Figure 5.6 C). This data suggests that kdk215-090 (6 PEG groups) is fully capable of chemiprecipitating EZH2, which cuts the PEG12 linker in half. Next, we tested kdk215-090 in the ICW assay (Figure 5.6 D), and noticed modest inhibition at high concentrations of compound, suggesting a high dose of kdk215-090 may be able to engage EZH2 in the cellular environment. We are currently in the process of testing this compound in the *in vivo* chem-ChIP assay.
Discussion

Our success with UNC0965 and G9a highlights methods to convert highly selective chemical probes into extremely valuable tools that may aid in understanding biological questions. Use of a functionalized chemical tool for chromatin precipitation has distinct advantages over an antibody. First, such chemical tools can be easily synthesized at low cost, and eliminate the batch-to-batch variability common with antibody reagents. Second, a biotin tag can be designed to be solvent exposed for chemical tools that have a well-defined mode of binding. This design has a potential added advantage over traditional ChIP; crosslinking can prevent access of an antibody to the protein of interest. Third, chemical tools can be cell permeable, which allows cell treatment prior to crosslinking. This *in vivo* chem-ChIP method would be particularly well suited for proteins that are difficult to precipitate after crosslinking due to antibody epitope masking. The *in vivo* chem-ChIP method provides an additional advantage in that it allows the user to engage the target of interest prior to crosslinking, cell lysing, and fragmentation of the chromatin; potentially providing a more physiologically relevant readout.

While both the *in vitro* and *in vivo* chem-ChIP technique proved to be a viable alternative method for G9a, the same was not observed for UNC2399 and EZH2. Unexpectedly, UNC2399 failed to chemiprecipitate EZH2 crosslinked to chromatin in PC3 (Figure 5.6 A), MLL-AF9, and HEK 293T cells (MLL-AF9 & HEK 293T data not shown). To date, only the *in vitro* chem-ChIP method has been attempted with EZH2, because UNC2399 is not active in the EZH2 ICW
assay. We hypothesize that this is due to a similar masking effect as is occasionally seen with antibodies. The *in vitro* chem-ChIP technique does not attempt to chemiprecipitate the target until after crosslinking, lysing, and fragmenting the chromatin, and we believe that this process may block access of the UNC2399 binding site in the SET domain of EZH2. Further complicating the issue is the fact that EZH2 is one subunit of a much larger complex, so this site may be more challenging to access than in the case of UNC0965 and G9a. We presume that part of the inactivity of UNC2399 in the ICW is due to the very long hydrophilic PEG linker, which is four times longer in UNC2399 than UNC0965. We determined that we could cut this linker in half (PEG 6 from PEG12) while maintaining the ability to chemiprecipitate EZH2; kdk215-090 demonstrated modest activity in the ICW. Going forward, we will attempt the *in vivo* chem-ChIP assay with kdk215-090 by exposing cells to a large dose of kdk215-090 (5-10 \( \mu \text{M} \)). We hope this will alleviate any masking of the binding site that may be present with formaldehyde crosslinking. Nonetheless, more work needs to be done to optimize the cellular activity of UNC2399 derivatives in order to engage EZH2 prior to crosslinking.

Future applications of this chemiprecipitation technique could include proteomic analysis of precipitated proteins to identify G9a-binding partners. Further, it would be interesting to compare genome-wide occupancy of G9a between all three methods (ChIP, *in vitro*, and *in vivo* chem-ChIP) to determine how well the overall signal for UNC0965 chem-ChIP compares to that of traditional ChIP.
### Tables

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                          | R - GGCAGTCTGTCTAGTACAGACCAGT            |
| CR2         | hg19_chr7:154861842-154861989  | F - CAGCACCACGACGACGACCACATGT  
                          | R - CTACGTAAGCCATCAGCTGATAAAGG            |
| CR3         | hg19_chr9:126872846-126872928  | F - CTTGTCTGCTGTATGCCAGGCACCA  
                          | R - CCTATGGCAATTGCATTGGGACAC            |
| CR4         | hg19_chr3:38847557-38847667  | F - GCTTGGTAATGGATCTGTTTTG  
                          | R - CTTGAATTACGCTCTCTTCCCTTT          |
| GAPDH       | hg19_chr12:6643539-6643704   | F - TACTAGCGGGTTTACGAGGCG  
                          | R - TCGAACAGGAGGAGCAGAGCAGGA            |

Table 5.1: Primer sequences for qPCR of G9a ChIP and chem-ChIP.  
F = forward, R = reverse.
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<td>6</td>
<td>hINK4A TSS</td>
<td>F - CGACTTCAGGGTGCCACATTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R - TCTTTCCAGGCAAGGGGAGC</td>
</tr>
<tr>
<td>7</td>
<td>hINK4A TSS</td>
<td>F - CTCAGTGTAGCATATTGCCTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R - GGTTCGTAGCGCGCATTTGAGGATA</td>
</tr>
<tr>
<td>8</td>
<td>hARF(p14) TSS</td>
<td>F - GAGAGTGAAGGACCTCTCAAACAAAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R - GAGTATTATCTTTCTCATAAT</td>
</tr>
<tr>
<td>9</td>
<td>hARF1 TSS</td>
<td>F - CGCCGTGTCAGATGTGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R - TGAATGTCAGTTTTGAATAAAGCCCG</td>
</tr>
<tr>
<td>10</td>
<td>hINK4B exon</td>
<td>F - GCTTGACAGGTCCAGACGAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R - GCACTGGCGAGCCACCTAAGGAG</td>
</tr>
<tr>
<td>11</td>
<td>GAPDH</td>
<td>F - TACTAGCGGTTTTCAGGGCG</td>
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<tr>
<td></td>
<td></td>
<td>R - TCGAACAGGGAGCAGAGGAGCAGA</td>
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Table 5.2: Primer sequences used for EZH2 qPCR.
F = forward, R = reverse.
Figure 5.1: G9a chemical toolbox.

(A) Structure of UNC0638. (B) Structure of UNC1152. (C) Structure of UNC0965.
Figure 5.2: Docking model of UNC0965.

UNC0965 (green) is predicted to bind to G9a (purple) in a nearly identical fashion as UNC0638, and the biotin moiety is solvent exposed allowing for capture by streptavidin-conjugated magnetic beads.
Figure 5.3: *in vitro* characterization of UNC0965.

(A) UNC0965 shows potent inhibition (IC50 < 2.5 nM) of G9a catalytic activity in an *in vitro* scintillation proximity assay. Experiments were performed in duplicate.

(B) Streptavidin-conjugated magnetic beads pre-coated with UNC0965 selectively chemiprecipitate G9a from HEK 293T cell lysates. G9a western blot indicates that UNC0965-treated beads (lane 3) chemiprecipitate G9a compared to DMSO-treated beads (lane 2). This interaction can be blocked or retained, by pretreating lysate with UNC0638 (lane 4) or its negative control UNC1152 (lane 5), respectively. These data are representative of three biological replicates.
Figure 5.4: G9a occupancy can be determined using antibody ChIP or *in vitro* chem-ChIP with UNC0965.

(A) ChIP using G9a antibody or IgG control followed by qPCR. (B) *In vitro* chem-ChIP with UNC0965-coated streptavidin-conjugated magnetic beads followed by qPCR. PCR primer sets correspond to chromosomal regions (CR) occupied by G9a as well as a GAPDH control region that shows no G9a occupancy. Chromosomal regions assayed include: CR1 (blue), CR2 (purple), CR3 (green), and CR4 (red), and GAPDH (grey). Error bars represent the standard deviation of three biological replicates. Statistical significance between DMSO and UNC0965 chem-ChIP for each region was determined by an unpaired t-test and indicated by asterisks (* = p<.05, ** = p<.01, *** = p<.001).
Figure 5.5: UNC0965 is cell penetrant and can be used for *in vivo* chem-ChIP assays.

(A) In-cell western assay of MDA-MB-231 cells treated with UNC0965 and assayed for total cellular H3K9me2 levels. UNC0965 reduces cellular levels of H3K9me2 (blue squares; IC50 = 3.3 ± 1.2 µM) and is not toxic in the compound concentration range tested (grey triangles; EC50 > 50 µM). Error bars represent the standard deviation of 3 biological replicates. These data confirm that UNC0965 is cell penetrant. (B) *In vivo* chem-ChIP. HEK 293T cells were treated with UNC0965 (5 µM) or DMSO (0.05%) for 15 minutes, crosslinked with formaldehyde, and then chromatin was isolated. ChIP was performed using streptavidin-conjugated magnetic beads followed by qPCR. PCR primer sets correspond to chromosomal regions (CR) occupied by G9a as well as a GAPDH
control region that shows no G9a occupancy. Chromosomal regions assayed include: CR1 (blue), CR2 (purple), CR3 (green), and CR4 (red), and GAPDH (grey). Error bars represent the standard deviation of three biological replicates. Statistical significance between DMSO and UNC0965 chem-ChIP for each region was determined by an unpaired t-test and indicated by asterisks (** = p<.01, *** = p<.001).
Figure 5.6: EZH2 ChIP and \textit{in vitro} chem-ChIP of UNC2399 in PC3 cells.

(A) ChIP using EZH2 antibody or IgG control in PC3 cells followed by qPCR. (B) \textit{In vitro} chem-ChIP with UNC2399-coated streptavidin-conjugated magnetic beads in PC3 cells followed by qPCR. PCR primer sets correspond to chromosomal regions occupied by EZH2 as well as a GAPDH control region that shows no EZH2 occupancy. Chromosomal regions assayed are detailed in Table 5.2. (C) UNC2399 linker titration. Results are representative of three biological replicates. (D) In-cell western assay of kdk215-090. The assay was performed in triplicate and error bars represent the standard deviation.
Chapter VI: CONCLUSION

Key Findings

The research conducted throughout the course of this dissertation has undoubtedly progressed chemical probe development for EZH1, EZH2, and G9a. The discovery of UNC1999 afforded the biological community the first orally bioavailable chemical probe of both lysine methyltransferases EZH2 and EZH1 [62]; prior inhibitors were not active in animal models [34, 44], or required administration via IP injection [32]. In addition to UNC1999, we also discovered UNC2400, UNC2239, and UNC2399. UNC2400 provides a structurally similar UNC1999 analog that is inactive against EZH2, providing the perfect negative control for in vitro and cellular assays. UNC2239 is a cell permeable UNC1999-dye conjugate that can be of use when traditional IF methods do not work. UNC2399 is a biotinylated UNC1999 analog that selectively chemiprecipitates EZH1/EZH2 from cellular lysates, which is a very desirable attribute for a variety of applications. Not only did this set of compounds provide a full chemical toolbox, but the compounds also provided evidence for the prevailing binding hypothesis for pyridone-containing EZH2 inhibitors.
Previous EZH2 inhibitors were also 50-150-fold selective for EZH2 when compared with EZH1, but UNC1999 is < 5-fold selective for EZH2 versus EZH1, which makes it the most panactive EZH1/EZH2 chemical probe to date. This is a potential advantage for UNC1999 as there are certain maladies where EZH1 has demonstrated compensation of EZH2 functions, like \textit{MLL}-rearranged leukemia [16]. Indeed, UNC1999 displayed potent inhibition of an \textit{MLL}-rearranged leukemic cell-line, MLL-AF9, but GSK126 (150-fold selective for EZH2) did not [39]. Further, UNC1999 displayed a survival advantage in mice bearing MLL-AF9 AML, which represents the first oral delivery of a dual EZH1/EZH2-specific compound as a means to inhibit \textit{MLL}-rearranged leukemia. An additional aim of this research was to increase the activity of UNC1999 for EZH1, effectively making a more panactive EZH1/EZH2 inhibitor. While this objective was not achieved, it was determined that modifications to the C5 or C6 carbon of the pyridone abolished all EZH1 activity, and that bulkier hydrophobic substituents at the C4 of the pyridone may lead to a more potent EZH1 inhibitor.

In contrast to dual EZH1/EZH2 inhibition as a potential therapy, many solid tumors appear to rely solely on EZH2, so an EZH2-specific chemical probe would be more advantageous in this scenario. We attempted to take advantage of a cysteine residue near the hypothesized binding site of UNC1999, but unfortunately, none of the compounds synthesized were able to covalently modify the cysteine residue of interest. However, we did determine that the indazole nitrogen bearing the isopropyl group of UNC1999 can be modified with
reactive functionalities and still retain a greater level of potency for EZH2 versus EZH1.

Prior to the start of this research, UNC0638, the first chemical probe for G9a [100] had been discovered. To functionalize this compound into a useful chemical tool, we appended a biotin tag to UNC0638 and discovered UNC0965. This compound retained *in vitro* activity for G9a, selectively chemiprecipitated G9a from cell lysates, and was also able to chemiprecipitate G9a from crosslinked chromatin, both *in vitro* and *ex vivo*. This proof of concept for what we termed the chem-ChIP assay is a significant advancement in the field of epigenetic drug discovery, because the method can be applied to a variety of targets for which there are probes available. Treating cells with a small molecule instead of an chemical antibody lowers costs, allows for target engagement in a more physiologically relevant environment, and aids in scenarios where quality ChIP-grade antibodies are not available due to epitope masking of crosslinked chromatin. To our dismay, UNC2399 was unable to chemiprecipitate EZH2 after crosslinking to chromatin. We believe that this may be due to masking of the SET domain after crosslinking. Use of the *in vivo* chem-ChIP assay may alleviate these issues, because the target is engaged prior to crosslinking, but UNC2399 is inactive in cellular assays, likely due to the long hydrophilic PEG12 linker, so we cannot perform the *in vivo* chem-ChIP method in the cellular environment. However, we did determine that only 6 PEG groups (kdk215-090) need to be present in order to maintain the ability to chemiprecipitate EZH2, and kdk215-090
does display modest inhibition of H3K27me3 in cellular assays, suggesting it may be more cell permeable than UNC2399.

**Future Directions**

Continued exploration into panactive EZH1/EZH2 probes, as well as EZH2-specific irreversible probes is of extreme interest, because both have applications. Going forward, derivatives of UNC1999 with larger hydrophobic groups at the C4 of the pyridone may increase the activity for EZH1. Additionally, introducing substituents with hydrogen bond donors and acceptors at pyridone C4 may lead to increased EZH1 potency, because of the hypothesis that M743 of EZH2 interacts with the pyridone moiety, and this residue is a threonine (T744) in EZH1. In an attempt to discover EZH2-specific, irreversible inhibitors, additional reactive functionalities will be installed on UNC1999 derivatives with the goal of engaging the cysteine of EZH2 (C663); C663 is a serine in EZH1.

Finally, due to the inability of UNC2399 to chemiprecipitate EZH2 crosslinked to chromatin, we will try to perform the cellular chem-ChIP method using kdk215-090, because it displayed notable inhibition in cellular assays. This suggests that we can treat cells with kdk215-090, engage the target prior to crosslinking, and then chemiprecipitate the crosslinked chromatin-ligand complex. In parallel with kdk215-090, we also plan to try to use a UNC1999 alkyne derivative to enter the cell prior to crosslinking, and then use azide beads to perform a click reaction after crosslinking. The advantage here is that the
smaller azide group should be more adept at navigating through chromatin in order to engage the SET domain of EZH2.

**Potential Adverse Effects of PRC2 Inhibition**

It is well-received that overexpression of EZH2 plays a role in the progression of metastatic prostate cancer [14, 40], and that overactive PRC2 and/or gain-of-function EZH2 mutants play a role in a variety of hematological maladies. However, EZH2 also appears to have a tumor suppressor role. EZH2 deletion has been observed in the development of myelodysplastic syndrome (MDS) [18, 41], and homozygous frame-shift or truncation mutations that disrupt EZH2, frequently occur in T-ALL [107-109] and myeloid leukemia such as primary myelofibrosis (PMF), myeloproliferative neoplasms (MPN), and chronic myelomonocytic leukemia (CMML) [110]. This observation is compounded by the fact that both EZH2 and EZH1 are regulators of hematopoietic stem cell function [20, 42], and EZH1 activity is crucial for maintaining self-renewal capacity of adult HSCs, by protecting cells from senescence [20]. The collective evidence suggests that there exists a dichotomous role of PRC2 in different biological contexts, which raises concerns over PRC2 and/or EZH2 inhibition in clinical treatments. Moreover, PRC2 is important in normal development, so EZH2/EZH1 inhibitors may also cause toxicity by inhibiting the normal functions of the PRC2.

These facts further highlight the need for high quality chemical probes, and functionalized chemical tools. Far too often, research is performed with a non-specific compound that has been stated to be an EZH2 inhibitor; DZNep, for
example. UNC1999, UNC2399, UNC0638, and UNC0965 are freely available to
the research community with no restrictions on their use. We can only hope that
as a community we continue to study the effects of PRC2 inhibition with the
correct tools, and hopefully, further validate EZH2 as a therapeutic target.
APPENDIX: PERSONAL CONTRIBUTIONS TO DISSERTATION CHAPTERS

All *in vitro* scintillation proximity assays, and in-cell western assays to assess the potency of synthesized compounds in any chapter were performed by collaborators at the Structural Genomics Consortium (SGC). Compounds from Chapter 2 were synthesized by the author with additional support from Dr. Anqi Ma and Dr. Feng Liu. The cellular studies and synthesis of the dye portion of UNC2239 were completed by Dr. Chris MacNevin of Dr. Klaus Hahn’s laboratory. All UNC2239 chemiprecipitations were performed by the author, in addition to homology modeling, docking studies, and compound design. All biological work reported in Chapter 3 was conducted in the laboratory of Dr. Greg Wang. The author synthesized UNC1999 N-methylated derivative UNC3142, while UNC1756 was synthesized at Changchun Discovery Sciences (CDS). The entirety of the final products and intermediates of compounds synthesized to assess EZH1/EZH2 selectivity in Chapter 3 were performed by the author. The docking and design of irreversible inhibitors of EZH2 from Chapter 4 were completed by the author, while all compounds were synthesized by Dr. Xiaobao Yang. Synthesis of UNC0965 from Chapter 5 was performed by Dr. Feng Liu. The author performed all of the chemiprecipitations of UNC0965, antibody ChIP, and chem-ChIP; Dr. Samantha Pattenden was an integral part in preparation of chromatin samples. UNC2399 chem-ChIP, EZH2 ChIP, and synthesis of compounds for truncated UNC2399 derivatives and linker titration chemiprecipitations were performed by the author, and Dr. Samantha Pattenden prepared chromatin for the *in vitro* UNC2399 chem-ChIP.
REFERENCES


