Chemical Tools to Study Bromodomains in Cancer

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Chemical Tools to Study Bromodomains in Cancer

Abstract

Factors that regulate chromatin structure collaborate with transcription factors to establish and maintain cellular expression programs in both normal physiology and disease. Small molecule approaches offer a unique opportunity for direct influence on the endogenous machinery driving these processes, as previously exemplified by inhibitors of the BET coactivator family’s acetyl-lysine reader domains (bromodomains). The current work describes the development and application of chemical tools to study bromodomains containing proteins outside of this family.

Approaches to modulate ligand-bromodomain binding are discussed in Chapter I, which details structure activity relationships toward selective binders of the TAF1. This dual-bromodomain containing initiation factor is of interest for its annotated roles in gene control as the largest member of TFIID, the pioneer initiation factor in eukaryotic transcription. From a non-selective multi-target binder, structure-based design strategies are applied to guide potency and selectivity from a kinase inhibitor derived chemical scaffold.

The application of established but ineffectual bromodomain ligands for chemical degradation strategies is discussed in Chapter II, which describes the development of a selective chemical degrader for BRD9 (dBRD9) through iterative design and evaluation. As a member of the frequently BAF chromatin remodeling complex, BRD9 has been annotated to play a supportive role in acute myeloid leukemia. The latter half of this chapter thus discusses application of dBRD9 to corroborate and exploit this dependency, and provides evidence for an on-target mechanism of action through complementary chemical and genetic approaches.
The BAF complex is frequently mutated in human cancer, resulting in novel dependencies on complex members that are required for residual complexes, or that support hyperactive complex assemblies. We describe in Chapter III application of our BRD9 degradation strategy to synovial sarcoma, an aggressive early onset cancer driven by activating SS18-SSX fusion oncogene within BAF. In collaboration with the Vakoc and Armstrong laboratories, we define BRD9 inactivation as a novel vulnerability in this disease, characterize the response to acute BRD9 loss, and demonstrate efficacy for BRD9 degradation in both culture and animal models of this disease.
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Although our studies are not presented in this dissertation, I would also like to acknowledge the chemical genetics group at NIBR, who hosted me in an enormously rewarding sojourn into the world of industrial science. Most of all, Bill Forrester, for his welcoming
investment into student driven research. Bill is a pleasure to work with, and his approach to science is one I deeply admire.

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And of course, Jay. Whether on the slopes Breckenridge or the conference room whiteboard, keeping up with Jay has always been a rewarding adventure. My time in Jay’s lab opened the doors to a new world of science for me, and provided the tools and encouragement to meet success on my own research journey. There is no doubt that the vibrant culture, fearlessness, and collaborative impact of Jay’s group followed directly from his example. It has been a privilege to have Jay as a mentor.

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Lastly, thank you to my wonderful girlfriend Zoey, and to my family, Dan, Mom, and Dad.
Chapter I

Introduction: Targeting Bromodomains in Cancer

1.1 Gene Control and Chromatin
1.2 Transcriptional Vulnerabilities in Cancer
1.3 Bromodomain-Directed Tools
1.1 Gene Control and Chromatin

The regulated expression of DNA sequence information is a universal feature of life, underpinning the chemistry, structure, and behavior of the cell. A principal layer of this control occurs at the level of transcription, which is orchestrated through a convergence of regulatory factors on RNA polymerase (RNA Pol). As anticipated by Jacob and Monod’s visionary model of repressor elements in 1961, early studies of bacterial and phage operator sequences revealed gene transcription to be orchestrated by the action of trans acting protein factors on proximal cis-regulatory sequences (promoters). In Eukaryotes, cell-type-specific expression programs are established by the coordinated control of thousands of genes by distally acting stretches of regulatory ‘enhancer’ sites, which exert dramatic and long-range activation through the action of intermediary protein complexes. By collaborating with transcription start site (TSS) proximal promoter sequences, enhancers act within insulated topologies to coordinate transcription initiation and elongation in a cell-type specific manner.

A path toward understanding the coordination of this process was established by Roger Kornberg’s 1974 nucleosome hypothesis, which biochemically established the nucleosome array as the fundamental canvas for regulating gene expression. The nucleosome particle is comprised of approximately 150 bp of DNA coiled twice around a core octamer of 4 histone proteins: H2A, H2B, H3 and H4 (Fig. 1.1). In humans, approximately 3 billion base pairs of genomic DNA is assembled into nucleosomes and compacted within the nucleus of each cell to give a DNA-protein complex called chromatin. From this shared base sequence information, the cellular diversity of all human tissues is established by precise spatial and temporal regulation of gene expression. To achieve this astonishing feat of gene control, the synthesis of protein coding transcripts by RNA Pol II is directed by the combinatorial action of direct DNA-binding transcription factors (TFs), associated protein binding co-regulators, and a rich set of effectors that establish, remove, and recognize chemical modifications to nucleosomal chromatin. These modes of control, which are often heritable through cell
division, but do not involve changes in base sequence, are referred to as ‘epigenetic’.\textsuperscript{17} \textsuperscript{18}

Epigenetic control is central to differentiation of distinct cellular identities from a continuum of possible expression states.\textsuperscript{19} \textsuperscript{20} At the time of writing, new insights are continuing to evolve our understanding of chromatin regulation, including its compaction,\textsuperscript{21} its three dimensional assembly within topological domains and phase boundaries,\textsuperscript{22-25} and its modification by chromatin regulators.\textsuperscript{26} To date, well over 100 unique covalent modifications have been discovered,\textsuperscript{27} with major classes including DNA base modifications and amino acid modifications of unstructured histone tail regions (Fig. 1.1). Enzymes that catalyze histone methylation (HMTs), demethylation (HDMs), acetylation (HATs), deacetylation (HDACs), phosphorylation (HKs) and ubiquitination have been well characterized, alongside direct DNA methyltransferases (DNMTs).\textsuperscript{28-30} While DNA methylation is transcriptionally silencing, lysine acetylation is broadly associated with active chromatin at both gene bodies and regulatory elements.\textsuperscript{31} Additional histone modifications typically show greater context and site dependence.\textsuperscript{32} These nucleosome modifications enact regulation through both physiochemical action on chromatin accessibility, and by recruitment of diverse reader domains.\textsuperscript{30} Further, remodeling of the structure and positioning nucleosome themselves by complexes such as BAF (discussed in Chapters III, and IV), offers an additional layer control in transcriptional access to DNA.\textsuperscript{33}

Chromatin regulators are guided by TF and co-regulator mediated recruitment to act on specific genomic sites.\textsuperscript{34} However, studies of directed reprogramming have revealed that TFs not only act to establish, but are also guided by, the modification state of chromatin.\textsuperscript{34-35} In turn, many chromatin modifiers are associated with reader domains, such that enzyme activity is also frequently informed by recognition of existing context, allowing for modification feedback.\textsuperscript{36} Master lineage determining TF’s also feature such autoregulatory loops, mediated by action of TF networks on their own enhancers.\textsuperscript{1,37-38} Together, these features endow switch-like behavior and signal robustness to gene regulatory networks, enforcing heritable states of cell lineage.\textsuperscript{36}
1.2 Transcriptional Vulnerabilities in Cancer

Having a central role in cell identity, modes of transcriptional and chromatin regulation are also co-opted to drive or support the dysregulated expression programs underpinning cancer pathogenesis. From a therapeutic perspective, driving mutations or amplifications involving chromatin regulators can offer transcriptional vulnerabilities, in some cases providing direct targets for intervention. The BRD4-Nut fusion is a flagship example of such an event, producing a chimeric reprogramming factor that drives de-differentiation and proliferation. Critically, the fusion’s localization is mediated by a segment of BRD4’s double bromodomain, a reader of the acetylated lysines (discussed Chapter 1.3). Enabled by this pharmacologic tractability of this domain (discussed Chapter 1.3), high quality small molecule inhibitors of BRD4 and the related BET subfamily were reported in 2010, and have now paved the way for clinical impact against aggressive BRD4-Nut driven disease.

Unfortunately, a large majority of oncogenic reprogramming events present challenges for
direct pharmacologic addressment, including alterations in pharmacologically recalcitrant TFs, inactivating mutations in chromatin regulatory tumor suppressors, and modifications to chromatin regulators without enzymatic or reader functions.\[^{42}\] However, in such cases, novel vulnerabilities are availed by the fact that while as transcriptional reprogramming may be initiated by a small number of factors, it necessarily depends on an underlying network of exiting co-regulators to direct and maintain the oncogenic transcriptional state. Accordingly, cancer etiology often features recurrent sets of mutations specific to the regulatory landscape of the originating cell type.\[^{39,46}\] For example, the SSX-SS18 BAF fusion oncogene (discussed Chapter IV) is essential to the growth and survival of synovial sarcoma, but is utterly restricted to this tumor type in both human disease and transgenic mouse models.\[^{47-48}\] Similar patterns of tissue restriction are observed in tumor suppressor and oncogene alterations across genome sequencing studies.\[^{49-50}\] This evidence implies that the hijacking of endogenous gene regulatory by cancer initiating mutations is a general feature of oncogenesis. Lacking genetic alteration, discovery of such transcriptional co-dependencies is necessarily performed through functional studies. Here, high quality chemical tools offer a definitive mode of target evaluation, and provide potential starting points for clinical therapeutic development.\[^{52}\]

1.3 Bromodomain-Directed Tools.

The bromodomain is a compact, ~110 amino acid reader domain that serves to recruit proteins to active genomic regions demarked by lysine acetylation of histones, TFs, and chromatin regulators.\[^{53-54}\] Accordingly, nearly all bromodomain containing proteins (BCPs) have been shown to contribute to chromatin regulation themselves, with predominantly activating roles. BCPs frequently contain or are associated with enzymatic domains to enact such regulation, including chromatin remodelers (eg. SMARCA2/4, BRD9), activating methyltransferases (eg. MLL, ASH1L), or acetyltransferases (eg. CBP, GCN5).\[^{55}\] Enzymes of the latter category provide a particularly compelling example of the autoregulation potential
discussed in Chapter 1.2, both recognizing and depositing the acetyl mark to reinforce active chromatin.[36] Another subset of BCPs play still more direct activating roles, through contact with the transcriptional machinery. These include the master co-activators of the BET family,[56] as well as TAF1, a component of the general initiation factor TFIID (see Chapter II).[57-58]

The bromodomain is comprised of a left-handed four-helix bundle, containing a hydrophobic cavity encircled by loop domains between the “Z-A” and “B-C” helices (Fig. 1.2 A).[55, 59] A series of 5 structural waters and conserved hydrogen bonding Asn residue line the central pocket, which offers a tractable structure for ligand discovery. The first high quality bromodomain ligands were reported for the BET family of transcriptional coactivators.[44, 60] Based on the thienodiazapine scaffold, these compounds introduced several key features frequently exploited in later discovery efforts, including hydrophobic packing within the “ZA” channel and WPF “shelf” regions, and Asn hydrogen bonding by an acetyl lysine isostere (here a 3-methyl-1,2,4 triazole) (Fig 1.2 B). A number of successful discovery efforts within and outside of the BET family have been reported,[61-62] applying screens of traditional library,[63-66] biased,[67-76] fragment,[77-86] and virtual[87-88] collections to precede elaboration by structure-based design.[89-95] These efforts have succeeded in deriving ligands for even some of the lowest predicted druggability bromodomains.[80, 94, 96] The current work discusses efforts toward selective ligands for the TAF1 2nd bromodomain in Chapter II.

Bromodomain ligands offer a number of avenues for biological investigation, foremost being endogenous domain inhibition. As observed in the first studies BET of inhibition, probes can compete acetyl-lysine binding to displace bromodomains from chromatin, often resulting in diffuse cellular localization and loss of chromatin-specific functionality.[44] In this manner inhibition of the BET coactivators has provided fundamental insights into transcriptional activation, as well as clinical candidates for disease.[61, 97-98] Currently, probes outside the BET family have fallen short of the dramatic phenotypes of BET inhibition, consistent with insights from domain-targeted CRISPR studies.[99] Although number of the non-BET bromodomain
probes cited above have given detectable displacement from chromatin, in increasing cases this action appears insufficient to completely abrogate function.\textsuperscript{100-102} In Chapter III we provide evidence that one such target, BAF complex member BRD9, achieves this resistance to inhibition through bromodomain independent contributions to chromatin recruitment.

Chemical biology approaches have also leveraged bromodomain ligands for imaging,\textsuperscript{103-104} affinity and photocapture enrichment,\textsuperscript{60, 105-106} biochemical and cellular displacement assays,\textsuperscript{107-108} protein homo-dimerization,\textsuperscript{109} generation of bioorthogonal ligand transgene ("bump-hole") pairs,\textsuperscript{110} and measurements of genome-wide probe localization.\textsuperscript{111} Recently, probes have also been applied as anchors for targeted protein degradation, applying small-molecule advances to the bifunctional degron strategy originally pioneered by Sakamoto et al.\textsuperscript{112-113} In this approach, ligands are functionalized with appendages for E3 ubiquitin ligase recruitment, resulting in the adoption of bound targets as neosubstrates for ubiquitin-proteasome mediated degradation. As demonstrated within the BRD9 degradation work presented in Chapters II & III, this strategy offers to extend the scope of bromodomain probes to rapid and complete ablation of protein function; a new modality for functional and therapeutic investigation of BCPs.

In under a decade, the combined effort of both academic and industrial science has resulted in selective probes for the majority of human bromodomain families. Continued development and inventive application of these tools offers a toward definitive investigation of this rich family of transcriptional regulators, promising to further evolve our understanding of gene control, and to reveal new opportunities for intervention in human disease.
Figure 1.2. A) Cartoon representation of JQ1(black-CPK) bound to BRD4 (colored by helix/loop), showing structural waters (spheres) and h-bonding (dashes) to conserved Asn (gray-CPK). (3MXF)
B) Surface representation of above structure highlighting key structural features.
Chapter II

Pursuing TAF1 Selective Bromodomain Activity from a Kinase Derived Scaffold

Contributors: David Remillard, Dennis L. Buckley, Hyuk-Soo Seo, Sirano Dhe-Paganon, James E. Bradner, and Nathanael S. Gray.
Featured within 41 human proteins, the evolutionarily conserved bromodomain mediates protein-protein interactions at acetylated lysine residues.\[^{55}\] With vetted roles in active chromatin recognition, the bromodomains of the BET family of transcriptional co-activators have provided a point of proximal pharmacologic intervention in human gene control,\[^{44, 98}\] yielding novel insights in disease etiology and disruption of cancer cell identity,\[^{114}\] \[^{97, 115-117}\] and providing new therapeutic agents under clinical investigation.\[^{45, 61}\] The successes of competitive bromodomain probe JQ1 and accompanied BET inhibitor development has also ensued a wealth of probe development for bromodomains outside this subfamily.\[^{62}\] Increasingly however, evidence supports that BET proteins may occupy a unique minority that depend sensitively on acetyl lysine recognition for their function, with bromodomain inhibition often failing to recapitulate genetic knockdown for non-BET targets.\[^{100-102}\] Recent successes in repurposing poorly functional bromodomain ligands as anchors for E3 recruiting chemical degraders provides a renewed development incentive for bromodomain ligands, offering to extend the scope of bromodomain probes to rapid and complete ablation of protein function; a new modality for investigating transcriptional roles of epigenetic regulators.\[^{101-102}\]

Transcription initiation factor TFIID subunit 1 is the largest of approximately 13 subunits that assemble with TATA binding protein to comprise TFIID,\[^{57}\] the pioneer factor for general eukaryotic transcription.\[^{118-120}\] In-vitro and TS-mutant experiments have implied TAF1 is dispensable for basal transcription,\[^{121-122}\] but directly mediates transcriptional regulation downstream of RB,\[^{123}\] B-Myb,\[^{124}\] and C-Jun,\[^{125}\] and is critical to transcription downstream of key cell cycle drivers.\[^{126-127}\] Recent work has further suggested that TAF1 may also collaborate with BRD4 to control cancer cell proliferation.\[^{128}\] As such, TAF1 is of interest as a potential point of intervention in the dysregulated transcriptional state orchestrated by tumor oncogenes.\[^{42}\] Although several TAF1 lead compounds have been published,\[^{66, 69, 128-129}\] to date none have provided sufficiently selective probes for TAF1 specific investigation, and exploration of novel TAF1 lead scaffolds offers a path toward this objective.
In the course of our studies on bromodomain ligand development, we reported the first small molecule ligand for TAF1 (compound 32), a 530nM (DiscoverX) binder accessed through biased multicomponent library synthesis.[69] Further exploration of the parent dimethylisoxazole class revealed however a strong bias toward BRD4 activity, and proved this group a challenging chemotype for further elaborating TAF1 affinity. We therefore sought to profile other reported bromodomain binders for TAF1 activity, with an interest in scaffolds outside the BET-biased diazapine class. Among novel chemotypes recently described were several kinase inhibitor scaffolds, which feature diverse recognition motifs.[130-131] Using the DiscoverX platform, we identified that the PLK1 kinase inhibitor BI-2536[132] yielded potent IC50 on the second bromodomain of TAF1 (TAF1(2)) (170nM) in addition to BRD4 (24 nM).

To investigate structure activity relationships (SAR) of this scaffold, we developed an in-house TAF1 AlphaScreen assay using a biotin functionalized chemical derivative (Synthetic procedures App. A). Guided by previously published BRD4 cocrystal structures, we sought to determine the influence of substituents buried within the acetyl-lysine recognition pocket, as well as those positioned within the ZA channel, a known hotspot for bromodomain selectivity (Fig. 2.1). As expected, the key imbedded methyl amide acetyl lysine mimetic showed high sensitivity to modification for both TAF1 and BRD4 (Table 2.1). Modifications of the ZA alkoxy substituent were better accommodated, with the des-methoxy analog offering the best TAF1 selectivity, and larger alkyl substituents providing improved potency. Truncation of the buried asymmetric ethyl group to a methyl substituent also improved potency. Substitution of the solvent projected 4-amino-methylpipiridine solubilizing group was well tolerated (Table S2.1).

We next considered that the potent cytostatic activity arising from the PLK1 kinase activity of this scaffold would preclude biological studies of bromodomain inhibition. We therefore sought to alter the H-bonding capabilities of the imbedded aminopyrimidine hinge-binding motif, a known approach for disrupting kinase binding (Fig. 2.2, Table 2.2).
Figure 2.1. A) Chemical structure of BI-2536. B) Surface representation of BI-2536-BRD4(1) cocystal structure (4O74).[^3] C) Cartoon cutaway of view as in (B) showing conserved waters (spheres), key residues (sticks) and H-bonding (dashed) discussed in this manuscript.

[^3]: [Reference Number]
Table 2.1. BI-2536 methyl amide substitutions (R₁), ZA-channel projecting alkoxy substituent (R₂), and asymmetric methyl (R₃)

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>TAF1(2)a (µM)</th>
<th>BRD4(1)a (µM)</th>
<th>Ratiob</th>
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<tr>
<td>1 BI-2536</td>
<td>Me</td>
<td>OMe</td>
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<tr>
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</tr>
<tr>
<td>5</td>
<td>Me</td>
<td>O-iPr</td>
<td>Et</td>
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<td>0.063</td>
<td>0.29</td>
</tr>
<tr>
<td>6</td>
<td>Me</td>
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<td>0.017</td>
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</tr>
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a AlphaScreen IC₅₀s from quadruplicate measurement.
b Fold selectivity toward TAF1 given by IC₅₀ ratio: BRD4(1)/TAF1(2).

Figure 2.2. Cartoon cutaway of BI-2536 bound to PLK1 (2RKU) showing hinge binding H-bonding (dashed) to key Cys residue backbone (sticks).
Replacement of key NH with NMe as we previously reported,\textsuperscript{108} or with O,\textsuperscript{135} largely abolished kinase activity, relative to BRD4, but resulted in both cases in dramatic and preferential loss of TAF1 bromodomain activity, presumably due to greater contribution of the expected water-mediated H-bonding to TAF1 affinity. We thus applied an alternative strategy, removing H-bond accepting to the pyrimidine core through N to CH substitution,\textsuperscript{136} and were pleased to find that this approach successfully achieved kinase selectivity while better retaining TAF1 affinity.

We next sought to assess further avenues toward potency on TAF1, alongside selectivity against BRD4. Introducing the favored asymmetric methyl substitution into the kinase-selective pyridopiperazone scaffold resulted in a potent dual inhibitor of TAF1 (16 nM) and BRD4 (37 nM) (Table 2.3). Unexpectedly however, previously examined substitutions of the methoxy ZA substituent, particularly H replacement, were no longer favored, potentially reflecting a rearrangement toward water mediated H-bonding by this substituent, as observed in BRD4(1).\textsuperscript{137} To better understand the binding mode of this series, we obtained an X-ray cocrystal of 15 bound to TAF1(2). Intriguingly, the resolved structure contains two protein molecules per unit cell, with 15 showing a conserved core binding mode, but exhibiting two dissimilar poses of the substituted phenylamino tail (Fig. 2.3).

To reconcile the ZA SAR sensitivity with the apparent crystallographic flexibility, we undertook to study the role of tail group positioning in TAF1(2) binding (Table 2.4). We thus examined substituents that would preclude ligand planarity (17), alternative tail compositions including those which might engage Asp 1524 if positioned as in orientation (B) 18-22, inclusion of sulfonamide or methylene spacers which might allow shelf access as in related efforts 23-25,\textsuperscript{73} or direct aryl tail appendage 26,27. The unanimously diminished affinity of these analogs appears best rationalized with structural interpretation based on pose (A), in which offset pi-stacking and backbone H-bonding to Phe1536 are positioned to contribute considerably to affinity (Fig 2.4). Consistently, further structural examination revealed pose (B) to be heavily influenced by crystal packing contacts (Fig. S2.2).
Table 2.2. Aminopyrimidine hinge-binding motif replacement.

![Chemical structure](image)

<table>
<thead>
<tr>
<th>CMPD</th>
<th>X</th>
<th>Y</th>
<th>TAF1(2)</th>
<th>BRD4(1)</th>
<th>Ratio</th>
<th>PLK1</th>
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<td>O</td>
<td>N</td>
<td>&gt;10 uM</td>
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<tr>
<td>12</td>
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<td>CH</td>
<td>0.45</td>
<td>0.15</td>
<td>0.33</td>
<td>&gt;10 uM</td>
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</table>

*a AlphaScreen IC\textsubscript{50} s from quadruplicate measurement.

*b Fold selectivity toward TAF1 given by IC\textsubscript{50} ratio: BRD4(1)/TAF1(2).

*c Z-Lyte assays (Invitrogen) with [ATP] of K\textsubscript{M} for each kinase, duplicate measurement.

Table 2.3. Alkoxy substituent SAR for kinase selective dihydropyridopyrazinone scaffold.

![Chemical structure](image)

<table>
<thead>
<tr>
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<th>R</th>
<th>TAF1(2)</th>
<th>BRD4(1)</th>
<th>Ratio</th>
</tr>
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<td>0.037</td>
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<td>14</td>
<td>H</td>
<td>0.34</td>
<td>0.19</td>
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</tr>
<tr>
<td>15</td>
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<td>O-cycloPent</td>
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<td>0.048</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*a AlphaScreen IC\textsubscript{50} s from quadruplicate measurement.

*b Fold selectivity toward TAF1 given by IC\textsubscript{50} ratio: BRD4(1)/TAF1(2).
We further sought to examine SAR within the dihydropyridopyrazone warhead, with a particular interest in discriminating BRD4 bromodomain activity. We anticipated that TAF1’s Val1547 might better accommodate larger cyclopentane replacements relative to BRD4’s equivalently placed Leu 94. We observe that six membered rings modestly improve TAF1 bias at this site, with tetrahydropyran substitution producing further selectivity, potentially due to polar contacts to the domain’s proximal Tyr “gatekeeper” (Table 2.5). Further considering that BRD4 Leu packs between the cyclopentyl and asymmetric ethyl substituents of BI-2536, we envisioned an alternative steric discrimination of BRD4 could be achieved by linking these positions through this space via 4-carbon bridgehead. Gratifyingly, the resulting tricyclic warhead containing 34 gave a marked 66 fold improvement (vs. parent 28) in measured selectivity at 210 nM TAF1 potency.
Table 2.4. Exploration of tail group SAR.

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{Cmpd} & \text{R} & \text{TAF1(2)}^{a} \hspace{1cm} \text{BRD4(1)}^{a} \hspace{1cm} \text{Ratio}\hspace{1cm}^{b} \\
\hline
17 & & >10 \text{ uM} & 0.35 & 0.031 \\
18 & & 0.055 & 0.080 & 1.5 \\
19 & & 0.27 & 0.15 & 0.54 \\
20 & & 0.17 & 0.14 & 0.85 \\
21 & & 0.16 & 0.056 & 0.35 \\
22 & & 0.38 & 0.40 & 1.1 \\
23 & & >10 \text{ uM} & 0.49 & < 0.049 \\
24 & & >10 \text{ uM} & 1.4 & < 0.14 \\
25 & & >10 \text{ uM} & 0.85 & < 0.085 \\
26 & & 1.2 & 0.63 & 0.53 \\
27 & & 2.6 & 0.86 & 0.33 \\
\hline
\end{array}
\]

\(^{a}\) AlphaScreen IC\textsubscript{50}s from quadruplicate measurement.

\(^{b}\) Fold selectivity toward TAF1 given by IC\textsubscript{50} ratio: BRD4(1)/TAF1(2).
Concurrently, we also investigated substitutions to the warhead’s pyridine ring, with as well as replacement of the embedded methyl amide with extended acyl mimetics following the approach of Crawford et al (Table 2.6).\textsuperscript{[129]} Of this series, the 7-methoxy analog 38 proved most promising, showing largely abrogated BRD4 activity alongside only minor losses in TAF1 affinity relative to its parent analog (35). This SAR is consistent with the observations of Bennett et al,\textsuperscript{[73]} who found that analogous derivatization of a related bicyclic TRIM24 inhibitor diminished BRD4 binding. The extended 1-butenyl analog 41 also appeared to improve selectivity; as discussed previously, we expect this permissiveness relates to the ability of TAF1 to favorably accommodate displacement of water 4,\textsuperscript{[129]} in line with its ability to recognize lysine butyrlation and crotonylation.\textsuperscript{[138]}

The current work presents a new lead for TAF1 probe development leveraging a previously unappreciated TAF1(2) activity of the dual PLK1-BET inhibitor BI-2536.
Table 2.5. KAc pocket cycloalkyl substituent SAR.

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>R1</th>
<th>TAF1(2)(^a) (µM)</th>
<th>BRD4(1)(^a) (µM)</th>
<th>Ratio(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>*</td>
<td>0.093</td>
<td>0.047</td>
<td>0.51</td>
</tr>
<tr>
<td>29</td>
<td>*</td>
<td>0.30</td>
<td>0.14</td>
<td>0.47</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>0.072</td>
<td>0.15</td>
<td>2.0</td>
</tr>
<tr>
<td>31</td>
<td>*</td>
<td>0.038</td>
<td>0.14</td>
<td>3.8</td>
</tr>
<tr>
<td>32</td>
<td></td>
<td>0.24</td>
<td>0.22</td>
<td>0.94</td>
</tr>
<tr>
<td>33</td>
<td>*</td>
<td>0.72</td>
<td>0.24</td>
<td>0.33</td>
</tr>
<tr>
<td>34</td>
<td></td>
<td>0.21</td>
<td>7.2</td>
<td>34</td>
</tr>
</tbody>
</table>

\(^a\)AlphaScreen IC\(_{50}\)s from quadruplicate measurement.

\(^b\)Fold selectivity toward TAF1 given by IC\(_{50}\) ratio: BRD4(1)/TAF1(2).
Table 2.6. Bicyclic core exploration

![Chemical structure]

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>R1</th>
<th>TAF1(2)$^a$ (µM)</th>
<th>BRD4(1)$^a$ (µM)</th>
<th>Ratio</th>
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</thead>
<tbody>
<tr>
<td>35</td>
<td>![Compund 35 structure]</td>
<td>0.58</td>
<td>0.11</td>
<td>0.20</td>
</tr>
<tr>
<td>36</td>
<td>![Compund 36 structure]</td>
<td>&gt;10 µM</td>
<td>2.1</td>
<td>&lt; 0.21</td>
</tr>
<tr>
<td>37</td>
<td>![Compund 37 structure]</td>
<td>0.98</td>
<td>&gt;10 µM</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>38</td>
<td>![Compund 38 structure]</td>
<td>0.75</td>
<td>7.3</td>
<td>9.7</td>
</tr>
<tr>
<td>39</td>
<td>![Compund 39 structure]</td>
<td>4.6</td>
<td>0.68</td>
<td>0.15</td>
</tr>
<tr>
<td>40</td>
<td>![Compund 40 structure]</td>
<td>1.7</td>
<td>4.5</td>
<td>2.7</td>
</tr>
<tr>
<td>41</td>
<td>![Compund 41 structure]</td>
<td>0.57</td>
<td>4.4</td>
<td>7.6</td>
</tr>
</tbody>
</table>

$^a$AlphaScreen IC$_{50}$s from quadruplicate measurement.

$^b$Fold selectivity toward TAF1 given by IC$_{50}$ ratio: BRD4(1)/TAF1(2).
We describe implementation of this probe to generate a robust TAF1 AlphaScreen displacement Assay, report a strategy to preserve activity on this bromodomain while reducing PLK1 kinase binding, and present structure-based approaches toward discrimination of BRD4 activity by TAF1 lead compounds. Three independent approaches emerged as most promising in the latter endeavor; 3 to 4-position cyclization (34), 7-position methoxy projection within the ZA channel (38), and the reported methyl to 1-butenyl amide substitution as an extended acyl-lysine mimetic strategy (41). Conversely, we find the NMe analog 10 to be over 150 fold selective for BRD4 over TAF1, offering a starting point toward improving the selectivity of BET inhibitors based on this scaffold.

To date, no chemical mater has been reported with sufficient selectivity to be implemented for TAF1-specific biology. The fundamental role in transcription initiation, marked effects on cell cycle, and potentially selective effects on amplified gene expression, make TAF1 an attractive target for both basic and translational research. Further, the multiple purported enzymatic activities make TAF1 an excellent candidate for targeted degradation approaches.[139-141] Such tools might also help to reconcile the broad effects of TAF1 loss observed recent yeast studies with selective defects of previous TS-mutant based approaches.[122, 142-143] To this end, the compounds disclosed in this study offer new and advanced starting points for TAF1 probe development. In addition, previous studies of TAF1-BRD4 crosstalk have also outlined functional synergy of BRD4 and TAF1 inhibition in MYC driven cancers.[128] Accordingly, the potent dual BRD4 / TAF1 probes reported herein may offer single agent efficacy in these contexts, and merit further biological investigation in this regard.
Materials and Methods

TAF1(2) Protein Purification

A construct containing His-TEV tagged TAF1 residues 1501-1635 (Uniprot P21675-1) within the pNIC28-Bsa4 construct (SGC, Addgene 39118) was transformed into BL21 (DE3) (New England Biosciences) and expression optimized through serial colony selection as described by Sivashanmugam et al.\cite{144} Cells were grown at 37°C to an OD of 1.0, induced overnight at 17°C with 500 µM isopropyl-1-thio-D-galactopyranoside, collected by centrifugation, and stored at -80°C. Pellets were microfluidized at 18000 psi in buffer A (50mM NaPO4(7.4), 500mM NaCl, 10% glycerol, 10mM Imidazole, and 14mM BME, 0.2% IGEPAL: 150mL per liter ON culture), and the resulting lysate was centrifuged at 30,000xg for 30 min at 4°C. Ni-NTA beads (Qiagen) were mixed with lysate supernatant for 30 min before beads were transferred to an FPLC-compatible column and the bound protein was washed with 15% buffer B (20mM NaPO4(7.5), 200mM NaCl, 10% glycerol, 300mM Imidazole, and 14mM BME) and eluted with 100% buffer B. Concentrated fractions were gel-filtered through a Superdex-200 10/300 column (GE Healthcare) in buffer C (20mM HEPES(7.5), 500mM NaCl, 5% glycerol, and 1mM DTT). For AlphaScreen, eluted fractions were concentrated and frozen for direct use. For crystallization, aliquots were incubated at 4°C overnight with TEV, re-purified by SEC as above, and ion-exchanged through a MonoQ 5/50GL (Sigma-Aldrich) in a similar buffer using a 0-1M NaCl linear gradient. Fractions were pooled, concentrated, and frozen at -80°C.

TAF1 Crystallization:

A sample of 1mM protein and 1.2mM 15 was crystallized by sitting-drop vapor diffusion at 20°C. Crystals were transferred briefly into crystallization buffer containing 25% glycerol prior to flash-freezing in liquid nitrogen. Diffraction data were collected at beamline 24ID-C of the NE-CAT at
the Advanced Photon Source (Argonne National Laboratory). Data sets were integrated and scaled using XDS.[145] Structures were solved by molecular replacement using the program Phaser[146] and the search model PDB entry 3UV5. The ligand was positioned and preliminarily refined using Buster and Rhofit.[147] Iterative manual model building and refinement using Phenix[148] and Coot led to a model with excellent statistics.

**TAF1(2) & BRD4(1) Ligand-Displacement AlphaScreen**

Probe displacement assays were performed with minimal modifications from the manufacturer's protocol (PerkinElmer, USA). All reagents were diluted in 50 mM HEPES, 150 mM NaCl, 0.1% w/v BSA, 0.01% w/v Tween20, pH 7.5. A 2x solution of TAF1(2) (final assay concentration 20 nM) (see protein purification), and biotin-BI-2536 (10 nM final) (see synthetic procedures) was added at 10μl/well to 384-well plates (Proxiplate-384 White, PerkinElmer, USA). Plates were spun down at 150x g, and 100 nL of DMSO compound stock was added from pre-diluted plates by pin transfer using a Janus Workstation (PerkinElmer, USA). Under low light, a 2x suspension of Ni-coated Acceptor Beads and Streptavidin Donor Beads, was then added at 10μl/well to a final concentration of 5 µg/ml by EL406 automated liquid handler (Biotek, USA). The plates were spun down again at 150g, at room temperature for 1 hour and then read on an Envision 2104 (PerkinElmer, USA) using the manufacturer's protocol. (BRD4 assays were performed exactly as above, substituting protein and biotin-probe components for BRD4(1) protein at 20nM final, and Biotin-JQ1 at 20nM final as previously described.[107] Data were analyzed using GraphPad PRISM v7.
Table S2.1. Substitution of 4-amino-methylpipridine solubilizing group.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Cmpd</th>
<th>TAF1(2)(^a) (µM)</th>
<th>BRD4(1)(^a) (µM)</th>
<th>Ratio(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure S1" /></td>
<td>S1</td>
<td>0.15</td>
<td>0.063</td>
<td>0.43</td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure S2" /></td>
<td>S2</td>
<td>0.035</td>
<td>0.017</td>
<td>0.49</td>
</tr>
</tbody>
</table>

\(^a\) AlphaScreen IC\(_{50}\)s from quadruplicate measurement.

\(^b\) Fold selectivity toward TAF1 given by IC\(_{50}\) ratio: BRD4(1)/TAF1(2).
Figure S2.2. A) Cocrystal structure of 15 (CPK) and TAF1(2) pose (B) showing ligand-protein crystal packing contacts (cartoon, magenta sticks). B) Surface representation crystal contacts within above structure.
Acknowledgements
The authors would like to thank Minoru Tanaka for technical discussions. D.L.B. is a Merck Fellow of the Damon Runyon Cancer Research Foundation (DRG-2196-14). This work was supported by the Claudia Adams Barr Program for Innovative Cancer Research (to D.L.B.). J.E.B. is now an executive and shareholder in Novartis AG.

Author Contributions
D.R. performed compound design and synthesis, protein expression, and AlphaScreen. D.L.B. contributed to compound design and synthesis. H.S.S. and S.D.P. produced protein and performed crystallography. J.E.B. and N.S.G. contributed direction and supported the study. D.R. wrote the manuscript.
Chapter III

Degradation of the BAF Complex Factor BRD9 by Heterobifunctional Ligands

Contributors: David Remillard, Dennis L. Buckley, Joshiawa Paulk, Gerard L. Brien, Matthew Sonnett, Hyuk-Soo Seo, Shiva Dastierdi, Martin Wühr, Sirano Dhe-Paganon, Scott A. Armstrong, and James E. Bradner*
Small-molecule inhibitors of BRD4 established the feasibility of inhibiting acetyl-lysine recognition domains (bromodomains).\textsuperscript{[44]} The broad use of the chemical probe JQ1 and other inhibitors of the bromodomain and extra-terminal domain (BET) subfamily of transcriptional coactivators has contributed an enhanced mechanistic understanding of gene control and has availed new therapeutic opportunities in cancer.\textsuperscript{[149-150]} Indeed, multiple BET inhibitor candidates from several groups are now undergoing clinical trials for diverse indications in and outside of oncology.\textsuperscript{[61, 151-152]} Further, significant research effort has contributed a number of high quality chemical probes for bromodomain-containing proteins beyond the BET family.\textsuperscript{[153-154]}

The bromodomain-containing protein BRD9 has garnered particular attention as a component of the human ATP-dependent chromatin remodeling BAF complex (also known as SWI/SNF). Meta-analyses of whole-genome sequencing efforts have recently identified a high frequency of recurrent somatic mutations in BAF factors in diverse human cancers.\textsuperscript{[155]} Within several subsets of these genetically defined malignancies, components of the BAF complex have emerged as context-specific dependencies, either supporting growth within a residual complex following loss of function mutation, or as novel oncogenes such as the SS18-SSX fusion.\textsuperscript{[156]} These observations have generated interest in therapeutic strategies to target BAF.

Beyond its presence in the BAF complex, a lack of functional annotation for BRD9 has provided incentive for development of BRD9 selective inhibitors to interrogate its biological role and to assess any therapeutic potential. Several BRD9-directed efforts in discovery chemistry have been reported,\textsuperscript{[71, 90, 93, 157-158]} developing chemotypes for BRD9-specific engagement. Further, a recent study implicated BRD9 as a dependency in acute myeloid leukemia (AML), where bromodomain inhibition prompted a cytostatic response.\textsuperscript{[159]} Beyond the bromodomain, the function of BRD9 remains unclear, and chemical tools to study other functions of BRD9 are not available. We therefore undertook to create first chemical probes that destabilize BRD9, anticipating that the study of acute BRD9 loss would offer a powerful approach to interrogate BAF complex function.
Recently, we reported a strategy to direct protein-specific degradation using bifunctional molecules to recruit the cereblon (CRBN) ubiquitin ligase complex to non-physiologic protein substrates, \(^{112}\) providing an all-chemical solution to prior efforts using peptides to bridge E3 ligases and ligand targets (PROTACs).\(^ {113, 160}\) In our prior research, we directed the degradation of BET family proteins by appending CRBN ligands to JQ1, resulting in rapid and potent degradation of BRD2, BRD3, and BRD4. These findings have been well validated, suggesting a broader utility of this strategy.\(^ {161-163}\)

Toward the elaboration of BRD9-directed degraders, we initially evaluated putative pharmacophores from reported bromodomain probes LP99 and I-BRD9, and subsequently expanded our study to a third probe, BI7273, reported during the course of this research (Fig. 3.1 A).\(^ {71, 90, 93}\) To explore the potential of bifunctional derivatives to induce BRD9 degradation, we initially selected as our starting point a close chemical analog of I-BRD9 described by GlaxoSmithKline (GSK-39).\(^ {90}\) This ligand was attractive in that it offered high binding affinity (IC50 = 7.9 nM) as well as a solvent exposed methoxy substituent amenable to chemical derivatization. In our initial design strategy, we adapted this ligand by installing an ether-linked acetyl moiety as a handle for E3 ligand attachment, as exemplified by compounds 1–3 (Fig. 3.1 B). Using this approach, we prepared a series of analogs that differ in linker length and composition, and explored varied attachment chemistries to CRBN or von Hippel–Lindau (VHL) E3 ligase ligands (Table S3.1 in the Supporting Information).

To characterize these compounds biochemically, we developed competitive ligand binding assays to the BRD9 bromodomain and the co-purified CRBN-DDB1 complex. High BRD9 affinity was retained across all compounds of this series relative to the parental bromodomain ligand, as exemplified by IC50 values for compounds 1–3 that closely approximate the published IC50 for GSK-39 (7.9 nM; Fig. 3.1 C, Table S3.1).
Figure 3.1. Design and characterization of thienopyridinone BRD9-targeted degraders. A) Structures of select BRD9 bromodomain probes. B) Schematic representation of degrader design. C) Vehicle-normalized BRD9(bd) displacement (AlphaScreen quadruplicate means +/- SEM). D) Compound-induced ternary complex formation of recombinant BRD9(bd) and CRBN–DDB1 (AlphaScreen quadruplicate means +/- SEM). E) Cocrystal structure of 3 with BRD9(bd) (PDB 5TWX). F) Docking of (E) into the published CRBN–DDB1 structure (4CI3).
Moderate differences were observed in CRBN–DDB1 affinity among compounds with divergently linked phthalimides (Table S3.1). For example, the direct alkyl ether phthalimide linkage of 3 showed slightly improved binding over acetamide ethers 1 and 2. Interestingly, measured affinities of all compounds exceeded that of unmodified thalidomide, perhaps reflecting positive affinity contributions of the pendant linker substituents.

To elicit protein degradation, bifunctional molecules must be able to efficiently associate the E3 ligase with the target. To measure this activity, we developed a homogenous luminescence assay to report on compound-induced proximity of BRD9 and CRBN. All of the bifunctional compounds in our initial series were able to significantly induce proximity of the BRD9 bromodomain and CRBN-DDB1 relative to unmodified thalidomide; an activity subsequently referred to simply as “dimerization” (Fig. 3.1 D, Table S3.1). This ternary interaction exhibited a characteristic auto-inhibitory concentration dependence consistent with bimolecular interactions dominating at saturating ligand concentrations.\[164]\n
Across a range of concentrations, the intermediate length alkyl ether analog 3 afforded robust dimerization. To understand BRD9 recognition by this compound, we solved a cocrystal structure with the BRD9 bromodomain. The pose adopted by the bromodomain warhead confirmed a conserved binding mode relative to the free probe, with the derivatized methoxy position projected to solvent as envisioned (Fig. 3.1 E, Fig. S3.1). In-silico modeling of the ternary assembly including CRBN–DDB1 demonstrated the steric feasibility of ternary formation, with the two ligand-binding domains brought into close assembly by compound 3 (Fig. 3.1 F).\[165]\n
To evaluate the ability of these compounds to degrade BRD9 in a cellular context, we treated a human AML cell line (MOLM-13) for 4 hours at varied concentrations and assessed BRD9 protein levels by immunoblot. While 1 and the extended PEG-linked S1 (see supplement) had little effect on BRD9 protein abundance, marked BRD9 loss was observed with the more potent biochemical dimerizers 2 and 3 (Fig. 3.2 A, Table S3.1).
Figure 3.2. Performance of thienopyrinnone degraders. A) Immunoblot for BRD9 and actin after 4-hour treatment of MOLM-13 with indicated concentrations of 1, 2, and 3. B) Chemical structures of 4 and 5. C) Vehicle-normalized CRBN-DDB1 displacement (AlphaScreen quadruplicate means +/- SEM). D) Immunoblot for BRD9 and actin after 4-hour treatment of MOLM-13 with indicated concentrations of 4 and 5.
Encouraged by this activity, we prepared an additional focused set of analogs exploring various molecular features. We examined the effect of liker rigidity by installing a conformationally constrained bipiperidine linker in compound S2. This molecule showed significant improvement in both dimerization and cellular potency, possibly by enforcement of an extended ternary-competent linker conformation (Table S3.1). To pursue degradation by alternate E3 ligases, we prepared the VHL-ligand conjugates S3 and S4; however, these were found to be ineffective (Table S3.1).

Additional analogs explored substitution of the phenolic attachment as found in 3 for the amine type linkages found in compounds S5, 4, and 5 (Fig. 3.2 B, Table S3.1). These compounds tightly bound CRBN, and effectively induced degradation of BRD9 (Fig. 3.2 C,D). The lenalidomide-based analog 5 showed the best overall performance, effectively downregulating BRD9 protein over a broad range of concentrations. We therefore selected this molecule for further characterization.

To evaluate the kinetics of BRD9 degradation, we exposed MOLM-13 cells to 5 at a fixed concentration (100nM) and assessed BRD9 abundance over time by immunoblot. Near complete BRD9 loss was observed within 1 hour, with no detectable return observed for the duration of the 24-hour treatment period (Fig. 3.3 A). This profile is appropriate to enable study of primary consequences of acute BRD9 loss, as well as viability defects manifested over longer periods.

To interrogate the mechanism of degradation by compound 5 in a cellular context, we assessed the requirement for target binding, proteasome activity, and activated cullin E3 ligases via chemical and genetic perturbations (Fig. 3.3 B,C). Pretreatment with excess I-BRD9 or lenalidomide competed with 5 for binding to BRD9 or CRBN, respectively, and prevented degradation, consistent with a requirement for intracellular engagement of both targets (Figure 3.3 B).
Figure 3.3. Temporal and mechanistic characterization of BRD9 degradation by 5. A) Immunoblot for BRD9 and actin after treatment of MOLM-13 Cells with 100 nM 5 for the indicated times. B) Immunoblot for BRD9 and actin after a 4 h pre-treatment of MM.1S cells with vehicle, I-BRD9, Lenalidomide, Carfilzomib[*] or MLN-4924, followed by a 2-hour treatment with 5 (100 nM). [*] Car pretreatment 30 min. C) Immunoblot for BRD9 and actin after 4 hour treatment with 5 at the indicated doses in MM.1S^{wt} or MM.1S^{CRBN^{-/-}} cells.
Degradation was abolished by the co-treatment with the proteasome inhibitor carfilzomib, confirming a requirement for proteasome function. Pretreatment using a mechanism-based inhibitor of neddylation also rescued BRD9 levels, as expected given the requirement for neddylation of CRL E3 ligases for activity.\textsuperscript{166-167} We further established a requirement for CRBN by examining the effects of compound 5 treatment in cells rendered CRBN deficient by CRISPR/Cas9 (CRBN\textsuperscript{−/−}). While treatment of wild type MM.1S cells resulted in marked dose-dependent BRD9 loss, treatment of the paired MM.1S CRBN\textsuperscript{−/−} line failed to induce BRD9 degradation (Fig. 3.3 C).\textsuperscript{168} These data support CRBN- and proteasome-dependent degradation of BRD9 by 5.

We aimed to further characterize 5 by establishing the biochemical selectivity profile among 32 representative members of the human bromodomain family. While the results of this analysis confirmed potent engagement of BRD9, we also observed substantial off-target binding activity, notably including BET bromodomains (Figure 3.4 A). Because of the confounding transcriptional and anti-proliferative effects associated with BET inhibition or loss, we felt selectivity over this family to be an important concern. At this stage of research, the concurrent publication of BI-7273, a highly selective BRD9 probe from Boehringer Ingelheim,\textsuperscript{93,159} inspired exploration of a novel chemical series of bifunctional degraders (Table S3.2).

Compound 6 (dBRD9), a PEG-linked pomalidomide conjugate, was found to prompt rapid BRD9 degradation over a broad range of concentrations (Fig. 3.4 B,C). Gratifyingly, dBRD9 also showed an improved bromodomain engagement profile, with reduced binding activity across the BET family (Fig. 3.4 D). A comparison of biochemical affinity of 5 and dBRD9 for the BET bromodomain, BRD4(1), by competitive ligand displacement confirmed this result (Fig. S3.2). Moreover, while 5 was able to effectively induce biochemical association of CRBN-DDB1 with either BRD9 or BRD4, dBRD9 lost all ability to dimerize BRD4 with CRBN-DDB1 above background levels, but retained robust dimerization of BRD9 (Figure 3.4 E,F).
Figure 3.4. Naphthiridinone degrader 6 (dBRD9) offers improved biochemical and cellular selectivity. A) Selectivity of phage-displayed bromodomain displacement by 5 (Bromoscan). B) Chemical structure of dBRD9. C) Immunoblot of BRD9 and actin after 4 h treatment of MOLM-13 cells with indicated concentrations of dBRD9. D) Selectivity of phage-displayed bromodomain displacement by dBRD9 (Bromoscan). E) Compound-induced ternary complex formation of recombinant BRD9(bd) and CRBN–DDB1 (AlphaScreen quadruplicate means +/- SEM). F) Compound-induced ternary complex formation of recombinant BRD4(1) and CRBN–DDB1 as in (E). G) Immunoblot for BRD4 and actin after 4 h treatment of MOLM-13 cells with indicated concentrations of 5 or dBRD9. H) Immunoblot for BRD7 following treatment as in (G).
Consistent with this result, dBRD9 showed improved cellular selectivity. Off-target degradation activity on BRD4 and BRD7, observed at high concentrations of 5, was not detectable by western blot following dBRD9 treatment (Fig. 3.5 G,H).

To assess the cellular selectivity for BRD9 degradation in an unbiased, quantitative manner, we measured effects of dBRD9 (100 nM for 2 hours) versus vehicle (DMSO) on all cellular proteins in MOLM-13 cells detected by isobaric tagging and mass spectrometry. Strikingly, of the 7326 proteins quantified in this experiment, BRD9 was the singular protein showing a marked and statistically significant difference in abundance, showing a median 5.5 fold lower abundance in dBRD9 treated samples (FDR corrected q-value < 0.01) (Fig. 3.5). Levels of other proteins were remarkably static between treatments with 99% of proteins differing less than 0.30-fold. Consistent with quantification by western blot, no significant change in BRD4 or BRD7 levels was observed.
Figure 3.6. Impact of BRD9 degradation on cultured human leukemia lines. **A)** Viability of EOL-1 and MOML-13 cell lines treated for 7 days with the indicated compounds (ATP-Lite quadruplicate means +/- SEM). **B)** Viability of MOLM-13 AML treated with dBRD9 and measured as in (A) following transduction with recombinant BRD9 alleles or vector control.
Having characterized two potent and pharmacologically distinct degraders of BRD9, we next sought to evaluate the anti-proliferative activity of these molecules in comparison to the parental bromodomain inhibitors. In the context of human AML lines (EOL-1, MOLM-13, MV4;11), compound 5 and dBRD9 both exerted a potent anti-proliferative effect, exceeding non-degrading probe potencies in excesses of 10- to 100-fold (Fig. 3.6A, Fig. S3.3). Interestingly, although these two compounds exhibited comparable low nanomolar half-maximal anti-proliferative concentrations, the maximal effect (Emax) of 5 exceeded that of dBRD9, likely owing to the polymmpharmacology associated with 5, particularly activity on BRD4, a well-described AML dependency. These data argue that polypharmacologic degraders are a viable chemical strategy, and further demonstrate the ability of cereblon ligand conjugation to reveal relevant cellular off-target activities of chemical probes.

To further scrutinize the conclusion that the observed growth defect results from on-target activity of dBRD9, we extended the bromodomain-swap strategy of the Vakoc laboratory, who found that substitution of BRD4 or BRD7 bromodomains for the endogenous BRD9 domain produced recombinant BRD9 alleles that lost affinity to BI-7273, but could functionally substitute for the wild type protein. We therefore stably transduced MOLM-13 cells with wild type BRD9, bromodomain substituted BRD9 alleles, or a GFP vector control, and re-evaluated sensitivity to dBRD9 in the presence of each transgene. In both lines expressing domain-swap alleles, the antiproliferative affect of dBRD9 was dramatically rescued relative to vector control. Viral expression of exogenous wild type BRD9 also shifted sensitivity, but to an intermediate degree, consistent with retained susceptibility to dBRD9-induced degradation. These responses are fully congruent with the observed activity of dBRD9 in each line by western blot (Fig. S3.4A). Consistent with its polypharmacology, the activity of compound 5 was only partially shifted by domain-swapped alleles, while the BRD9 independent activity of BET inhibitor JQ1 was wholly unaffected (Fig. S3.4B). Together with expression proteomics, these data provide extensive support that the observed sensitivity in MOLM-13 is a BRD9-specific effect.
In the course of our on-target validation work, we prepared a non-targeting control analog (compound S10), which lacks a key hydrogen bonding moiety while being otherwise structurally identical to dBRD9 (Fig. S3.5 A). As expected, this molecule lost biochemical BRD9 bromdomain affinity, the ability to degrade BRD9, and accordingly, lost antiproliferative activity in MOLM-13 (MOLM-13 IC50 > 10 mm) (Fig. S5 B–D). Unexpectedly however, S10 retained considerable activity in the multiple myeloma derived MM.1S line, which we had identified as dBRD9 sensitive in studies of activity outside AML (Fig. S5 E). Contemplating the known activity of IMiDs in MM.1S,[168] we were lead to discover that S10 and dBRD9 retain activity against the IKZF family of lineage specific transcription factors; an activity not previously observed with dBET-1 (Fig. S3.5 F).[112] We therefore caution that in select cell lines of lymphoid origin that express and depend on IKZF family proteins, researchers should control for this feature. We suspect that published molecules featuring similar CRBN targeting chemistry may also retain this activity.

In summary, the present work describes the design and characterization of first-in-class chemical degraders of BRD9. These studies demonstrate the utility of the targeted degradation strategy to bromodomain-containing proteins beyond the BET family. This is particularly relevant, as competitive bromodomain inhibition has previously failed to phenocopy the effects of protein knock-down (shRNA) or knock-out (CRISPR-Cas9) for non-BET bromodomain proteins.[100] Thus, using comparative biochemical and biological assays, we have qualified a lead BRD9 chemical degrader, dBRD9, as a selective probe useful for the study of BAF complex biology. The rapid and potent activity of this compound render it ideally suited to the study of fast biological responses such as transcriptional effects and nucleosome positioning. Finally, potent activity of dBRD9 in cellular models of human AML is confirmed to be on target through expression proteomics, alongside chemical and genetic controls within the exemplar MOLM-13 line.
Supplimental Data

Table S3.1: Thienopyridinone degrader data.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>BRD9 IC50 (nM)</th>
<th>BRD4 IC50 (nM)</th>
<th>CRBN-DD1 IC50 (nM)</th>
<th>Dimerization</th>
<th>BRD9/CRBN-DD1 (HUC / Cmax) (nM)</th>
<th>BRD9 Degradation</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1" alt="structure1" /></td>
<td>13.5</td>
<td>3.78</td>
<td>49.9</td>
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<td><img src="image5" alt="image5" /></td>
<td>74577</td>
<td>-35.1</td>
</tr>
<tr>
<td>S1</td>
<td><img src="image7" alt="structure3" /></td>
<td>36.5</td>
<td>3.14</td>
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<td><img src="image8" alt="image8" /></td>
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<td>S3</td>
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<td>5S</td>
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<td>10.8</td>
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<tr>
<td>8</td>
<td>12.3</td>
<td>1.71</td>
<td>11.2</td>
<td>86050 ± 20.2</td>
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Table S3.2: Naphridinone degrader data

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>BRD9 IC50 (nM)</th>
<th>BRD4 IC50 (nM)</th>
<th>CRBN-DO81 IC50 (nM)</th>
<th>Dimerization</th>
<th>BRD9-DO81-DO81 (AUC- Mean) (nM)</th>
<th>BRD9 Degradation</th>
</tr>
</thead>
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<tr>
<td>S6</td>
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<td>&gt;50 µM</td>
<td>8.90</td>
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<td>62596: 6.39</td>
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<tr>
<td>S7</td>
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<td>&gt;50 µM</td>
<td>8.35</td>
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<td>134396: 8.22</td>
<td>BRD9</td>
</tr>
<tr>
<td>S8</td>
<td><img src="structure_s8.png" alt="Structure" /></td>
<td>43.2</td>
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<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>S9</td>
<td><img src="structure_s9.png" alt="Structure" /></td>
<td>48.0</td>
<td>&gt;50 µM</td>
<td>14.8</td>
<td><img src="dimerization_s9.png" alt="Dimerization" /></td>
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<td>BRD9</td>
</tr>
<tr>
<td>6-(BRD9)</td>
<td><img src="structure_6brd9.png" alt="Structure" /></td>
<td>104</td>
<td>&gt;50 µM</td>
<td>31.3</td>
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<td>186320: 92.9</td>
<td>BRD9</td>
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**Figure S3.1.** Overlay of compound 3 cocrystal and I-BRD9 (4UIV) co-crystal structures. Hydrogen bonding to key Asn residue is highlighted.

**Figure S3.2.** Napthiridinone degrader 6 (dBRD9) offers improved biochemical selectivity against BRD4. Vehicle-normalized BRD4(1) displacement (AlphaScreen quadruplicate means +/- SEM).
Figure S3.3. Viability of MOML-13 and MV411 cell lines treated for 3 days with the indicated compounds (ATP-Lite quadruplicate means +/- SEM).
Figure S3.4. A) Immunoblot detection of BRD9 and actin after 24 hr treatment of stably transduced MOLM-13 cells with indicated concentrations of dBRD9; recombinant proteins are equivalent in molecular weight to endogenous and retain endogenous detection epitope. B) Viability of MOLM-13 AML stably transduced with the indicated recombinant BRD9 alleles following 7 day treatment with compound 5 (left) or JQ1 (right) (ATP-Lite quadruplicate means +/- SEM).
Figure S3.5. A) Chemical Structure of S10. B) Vehicle-normalized BRD9 displacement (AlphaScreen quadruplicate means +/- SEM). C) Immunoblot detection of BRD9 and actin after 24hr treatment of MOLM-13 cells with indicated concentrations of S10. D) Viability of MOLM-13 cell line treated for 7 days with S10 (ATP-Lite quadruplicate means +/- SEM). E) Viability of MM1S cell line treated for 3 days with indicated compounds (ATP-Lite quadruplicate means +/- SEM). F) Immunoblot detection of IKZF3 or IKZF1 and actin after 24hr treatment of MOLM-13 cells with indicated concentrations of S10, Lenolidomide, or dBRD9.
Materials and Methods

BRD9(bd) protein purification

A construct of human BRD9 covering residues 134-245 in the pET28PP vector was overexpressed in E. coli BL21 (DE3) in LB medium in the presence of 50 µg/ml of kanamycin. Cells were grown at 37°C to an OD of 0.8, cooled to 17°C, induced with 500 µM isopropyl-1-thio-D-galactopyranoside, incubated overnight at 17°C, collected by centrifugation, and stored at -80°C. Cell pellets were sonicated in buffer A (50 mM hepes 7.5, 300 mM NaCl, 10% glycerol, 10 mM Imidazole, and 3 mM BME) and the resulting lysate was centrifuged at 30,000xg for 40 min. Ni-NTA beads (Qiagen) were mixed with lysate supernatant for 30 min and washed with buffer A. Beads were transferred to an FPLC-compatible column and the bound protein was washed with 15% buffer B (50 mM hepes 7.5, 300 mM NaCl, 10% glycerol, 300 mM Imidazole, and 3 mM BME) and eluted with 100% buffer B. HRV-3C was added to the eluted protein and incubated at 4°C overnight (step skipped for ALPHAScreen protein stock). The eluant was concentrated and passed through a Superdex-75 10/300GL column (GE helathcare) in the following buffer: 20mM HEPES-7.5, 200mM NaCl, 5% glycerol, 2mM DTT, and 1mM TCEP. Fractions were pooled, concentrated to 15 mg/ml, and frozen at -80°C.

BRD9 crystallization

A solution containing 600 µM compound 3 (from a 100 mM DMSO stock) and 500 µM protein was crystallized by sitting-drop vapor diffusion at 20°C in the following crystallization buffer: 20% PEG3350 and 0.2 NH4F. Crystals were transferred briefly into crystallization buffer containing 25% glycerol prior to flash-freezing in liquid nitrogen. Diffraction data from complex crystals were collected at beamline 24ID-E of the NE-CAT at the Advanced Photon Source (Argonne National Laboratory). Data sets were integrated and scaled using XDS.$^{[145]}$ Structures were solved by molecular replacement using the program Phaser$^{[146]}$ and the search model PDB
entry 4YYD. The ligand was positioned and preliminarily refined using Buster and Rhofit.[147]

Iterative manual model building and refinement using Phenix and Coot led to a model with excellent statistics. Docking into the ligand binding site of the published CRBN-pomalidomide cocrystal structure (4CI3) was performed using Glide Docking software (Schrodinger). [148, 170]

**CRBN-DDB1 expression and purification**

Expression and purification of CRBN-DDB1 were performed as described previously using Sf9 cells (Invitrogen). [165] pFastBac vectors encoding human CRBN and DDB1 were a kind gift from Nicolas Thomä and Eric Fischer.

**BRD9(bd) & BRD4(1) ligand-displacement AlphaScreen**

BRD9 assays were performed with minimal modifications from the manufacturer’s protocol (PerkinElmer, USA). All reagents were diluted in 50 mM HEPES, 150 mM NaCl, 0.1% w/v BSA, 0.01% w/v Tween20, pH 7.5. A 2x solution of BRD9 (final assay concentration 25 nM) (see protein expression section), and biotin-probe (10 nM final) (see synthetic procedures) was added at 10ul/well to 384-well plates (Proxiplate-384 White, PerkinElmer, USA). Plates were spun down at 150x g, and 100 nL of DMSO compound stock was added from pre-diluted plates by pin transfer using a Janus Workstation (PerkinElmer, USA). Under low light, a 2x suspension of Ni-coated Acceptor Beads and Streptavidin Donor Beads, was then added at 10ul/well to a final concentration of 5 µg/ml by EL406 automated liquid handler (Biotek, USA). The plates were spun down again at 150g, at room temperature for 1 hour and then read on an Envision 2104 (PerkinElmer, USA) using the manufacturer’s protocol. (BRD4 assays were performed exactly as for BRD9 alphascreen, substituting protein and biotin-probe components for BRD4(1) protein at 20nM final, and Biotin-JQ1 (previously described) [107] at 20nM final. Data were analyzed using GraphPad PRISM v7.
**CRBN-DDB1 ligand-displacement AlphaScreen**

In 384-well AlphaPlates (Perkin Elmer), 50 nM CRBN-DDB1 and 125 nM biotin-thalidomide were plated at 20 uL per well in assay buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 1 mM TCEP, and 0.1% BSA). 100 nL of DMSO compound stock was added from pre-diluted plates by pin transfer using a Janus Workstation (PerkinElmer, USA). Under low light, 20 uL detection solution containing Streptavidin Donor Beads and Nickel Chelate AlphaLISA® Acceptor Beads diluted to 20 ng/uL in assay buffer was added to each well. After 1 hr incubation at RT, luminescence was measured on the Envision 2104 plate reader. Data were analyzed using GraphPad PRISM v7.

**CRBN-DDB1/BRD4 dimerization assay**

GST-BRD4 (2-170) was expressed and purified as previously described.\(^{[109]}\) GST-BRD4 and CRBN-DDB1 were diluted to 125 nM, in assay buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 0.01% Tween 20, and 0.1% BSA) and added at 20 µL per well to 384-well AlphaPlates (PerkinElmer). 100 nL of DMSO compound stock was added from pre-diluted plates by pin transfer using a Janus Workstation (PerkinElmer, USA). Under low light, Nickel Chelate AlphaLISA Acceptor and Glutathione AlphaLISA Donor beads (PerkinElmer) were diluted in assay buffer to 20 µg/mL and added at 20 µl per well. Plates were incubated for 1 hr at room temperature prior to luminescence detection on an Envision 2104 plate reader (PerkinElmer). Data were analyzed using GraphPad PRISM v7.

**Cell lines**

MM1S and MV4;11 cells were purchased from ATCC. MOLM13 cells were a kind gift from Prof. Scott Armstrong, and MM1S-CRBN\(^{-}\) cells were kindly provided by Prof. William Kaelin.\(^{[168]}\) All lines were cultured in RPMI supplemented with 10% FCS and 1% Penicillin/Streptomycin.
**Immunobloting**

Cells were washed with PBS, before being lysed in RIPA buffer supplemented with 1x HALT™ protease inhibitor cocktail (Thermo Scientific) for 10 min on ice, followed by low-amplitude sonication for 10 seconds at 4°C (Q125, QSonica, USA). Insoluble material was removed by centrifugation at 20,000xg for 20 min before protein content was quantified by BCA assay (Pierce). Electrophoretic separation was performed using the Novex Bolt or Nupage systems (Thermo Fisher Scientific) and transferred to Novex 0.45 μM Nitrocellulose membranes. The following primary antibodies were used in this study: BRD9 (Bethyl A303), Actin (Santa Cruz C-2), BRD4 (Bethyl A301), BRD7 (Cell Signaling D9K2T), α-Tubulin (abcam 7291), IKZF1 (Bethyl, A303, 516) IZKF3 (SC 101982). Blots were imaged using fluorescence-labeled secondary antibodies (LI-COR) on the OdysseyCLxImager (LI-COR).

**ATPlite viability assay**

250 to 1000 cells/well were plated at a volume of 40μL/well in white tissue culture treated 384 well assay plates (Thermo Scientific) before DMSO compound stock was added from pre-diluted plates by pin transfer using a Janus Workstation (PerkinElmer, USA). Plates were mixed for two minutes via plate shaker before a 3 or 7 day incubation. Assay plates were allowed to equilibrate to RT for 30 min before reconstituted ATPlite 1Step reagent (PerkinElmer) was dispensed at 1:1 volume /well by EL406 automated liquid handler (Biotek, USA). Plates were mixed for two minutes via plate shaker and incubated at room temperature an additional 10 minutes before being read on an Envision 2104 (PerkinElmer, USA) using the manufacturer's protocol. Values were normalized to DMSO wells and data were analyzed using GraphPad PRISM v7.
Domain-swap vector cloning

The human BRD9 cDNA was purchased from Open Biosystems (clone ID: 5428011) and PCR cloned into the Gateway entry vector pCR8 in accordance with the manufacturer's instructions. BRD9 bromodomain swap cDNAs were generated by ligation mediated PCR and inserted into pCR8 as above. Following sequence verification, wildtype and bromodomain swap BRD9 expression vectors were generated via LR clonase mediated recombination with the Gateway expression vector pLEX305 which was a gift from David Root (Addgene plasmid # 41390).

Virus generation and infection

Viral supernatants were prepared by Lipofectamine 2000 (Thermofisher) transfection of 293T cells with VSV-G/pMD2.G, psPAX2 (addgene), and pLEX305 expression vectors in 10cm dishes at 90% confluence, according to manufacturer's instructions. Supernatants were collected 60 hours post transfection and debris removed using 0.45 µm pore size Steriflip filtration (Millipore); supernatants were used directly without concentration. For each vector, 5x10^6 cells were pelleted and resuspended in 5mL viral supernatant before 750 RCF spinfection for one hour at RT. After outgrowth for 2 days, transductants were selected using 1 ug/mL puromycin (Gibco, Thermofisher).

Quantitative multiplexed proteomics

Samples were prepared as previously described with the following modifications. After 2 hour treatment of MOLM-13 with DMSO or dBRD9, Cells were collected and washed 3x with ice cold PBS. Samples were lysed with 2% SDS in 50mM HEPES pH 8.0 by low-amplitude sonication for 10 seconds at 4°C (Q125, QSonica, USA). The samples were then reduced and denatured with 5 mM DTT for 20 min at 60°C. Final protein concentration of each sample was determined using a micro-BCA assay (Pierce). Sample cysteines were alkylated with 15 mM N-
ethyl maleimide (Sigma) for 20 minutes at RT and then quenched with 5 mM DTT for 10 minutes at RT. Each sample was precipitated using methanol/chloroform. The protein pellet was re-suspended (~5 mg/mL) in 6 M Guanidine HCl in 10 mM EPPS (pH 8.5) and heated at 60°C for 30 min with vortexing every 10 minutes. ~100 µg of protein per condition was diluted with 10 mM EPPS (pH 8.5) to 2 M Guanidine HCl and digested with LysC (Wako Chemicals) at 10 ng/µL at RT for 14 hours. Each sample was further diluted to 0.5 M Guanidine HCl with 10 mM EPPS (pH 8.5) and digested by addition of 10 ng/µL LysC and 10 ng/µL sequencing grade Trypsin (Roche) at 37°C for 14 hours. The solvent from each sample was removed using a SpeedVac, and each sample was re-suspended with 100 µL of 300 mM EPPS (pH 8.0). Each sample (see table) was labeled with 20 µL of the appropriate NHS Tandem Mass Tag (20 µg/µL in dry acetonitrile) (TMT 10 plex, Thermo Scientific) at RT for 2 hours. Each reaction was quenched with 10 mM hydroxylamine for 15 minutes at RT. All 10 samples were combined and acidified by addition of ~3% TFA to a pH of <1. Samples were clarified by spinning at 20,000 rcf at 4°C for 20 min and then subjected to C18 solid-phase extraction (50 mg, SPE) (SepPak, Waters) to desalt and isolate peptides. To reduce sample complexity, peptides were re-suspended in 10 mM sodium bicarbonate (pH 8.0) and fractionated by medium pH reverse-phase HPLC (Zorbax 300Extend-C18, 4.6 x 250 mm column, Agilent) using an acetonitrile gradient from 5% - 39% with 10 mM sodium bicarbonate pH 8.0. With a flow rate of 0.8 mL/min, fractions were collected into a 96 well plate every 38 seconds. Alternative wells were combined to create 24 fractions. The solvent from each fraction was removed by SpeedVac. Each fraction was re-suspended in 1% TFA, and again de-salted with reverse-phase purification. The resulting fractions were resuspended in 8 µL of 1% formic acid. Approximately 2 µL of each fraction were analyzed by LC-MS.
<table>
<thead>
<tr>
<th>Sample</th>
<th>TMT-Tag</th>
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<td>dBRD9, 2 hr, replicate #1</td>
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</tr>
<tr>
<td>dBRD9, 2 hr, replicate #2</td>
<td>129C</td>
</tr>
<tr>
<td>dBRD9, 2 hr, replicate #3</td>
<td>130N</td>
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<tr>
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<td>130C</td>
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<tr>
<td>dBRD9, 2 hr, replicate #5</td>
<td>131</td>
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</table>

**LC-MS**

LC-MS were performed on an Orbitrap Lumos (Thermo Fisher) as previously described with the following modifications.\(^{[171]}\) An EASY-nLC 1200 UHPLC was used (Thermo Fisher). The instrument was operated in data dependent mode using a MultiNotch-MS3 method that simultaneously selects the 5 most abundant fragment ions from the MS2 spectrum.\(^{[169]}\) For the MS3 spectrum we used an MS2 isolation window of 1.2 for \(z = 2\), 1.0 for \(z = 3\) and 0.8 for \(z = 4-6\). The MS3 spectrum was acquired using an Orbitrap Resolution of 50,000.

**MS data analysis**

MS Data analysis was done as previously described with the following modifications.\(^{[171]}\) Peptides were considered quantified if >75% of total intensity in the idealized MS1 isolation window (+/- 0.25Th) was from the precursor and the total number of charges in the MS3 TMT cluster was >1000. The ten conditions were normalized to correct for pipetting errors such that the total TMT signal of each condition (summed signal for all ~78,000 peptides) was the same. Peptides that had Coefficients of Variation for the 5 DMSO controls that were larger than 0.50 (916 / 78207 peptides) were removed from the dataset. The peptide TMT signals were summed and mapped to proteins using the uniprot reference. Fold changes in protein levels were calculated by dividing the median \textbf{dBRD9} 2 hr protein abundance by the median DMSO 2 hr...
protein abundance. Q-values were calculated in Prism 7 by first calculating P-values via performing multiple two-sided t-tests assuming different proteins do not have a consistent standard deviation. P-values were corrected for multiple hypothesis testing using a False Discovery Rate approach with the two-stage step-up method of Benjamini, Krieger and Yekutieli with a desired FDR (Q) of 1%.[175]

Acknowledgements

We thank Dr. Jennifer Perry for critical reading of the manuscript, Eben McCue for assistance with images, N. Gray for technical discussions, Mette Isohey for her work on conditions for linker synthesis, and Stephen DeAngelo for technical contributions to protein production. D.L.B. is a Merck Fellow of the Damon Runyon Cancer Research Foundation (DRG-2196-14). G.L.B. is supported by an EMBO Long-Term Fellowship (ALTF 1235–2015) with additional support from Marie Curie Actions. M.S. was supported by NIH grant F31 GM095450. This work was supported by the Claudia Adams Barr Program for Innovative Cancer Research (to D.L.B.), and by NIH/NCI grant P01-CA066996, to J.E.B. and S.A.A. J.E.B. is now an executive and shareholder in Novartis AG.

Author Contributions

D.R. performed compound synthesis, AlphaScreen, western blotting, and cell viability and domain swap experiments. D.L.B. and S.D. contributed to compound synthesis. J.P. produced recombinant CRBN:DDB1 and assisted with dimerization AlphaScreen. G.L.B. cloned domain swap constructs and contributed to biological studies. M.S. performed mass spectrometry sample prep, data collection, and analysis. H.S.S. and S.D.P. produced protein and performed crystallography. M.W., S.A.A., and J.E.B. contributed direction and supported the study. D.R. wrote the manuscript with contributions from all authors.
Chapter IV

Targeted degradation of BRD9 reverses oncogenic gene expression in synovial sarcoma

Synovial sarcoma tumors contain a pathognomonic chromosomal translocation t(X;18) which fuses the SS18 gene (also known as SYT) on chromosome 18, to one of three related genes, SSX1, SSX2 or SSX4 on the X chromosome.\textsuperscript{[176-178]} This leads to the generation of an oncogenic fusion protein SS18-SSX. Genomic studies have demonstrated that synovial sarcoma tumors have few, if any, significantly co-occurring genetic aberrations.\textsuperscript{[179-180]} This is consistent with most fusion protein associated pediatric cancers; where fusion protein generating translocations are often the sole genetic event and primary driver of disease pathogenesis.\textsuperscript{[181-184]} In support of a central role of the fusion protein, conditional expression of an SS18-SSX fusion leads to the development of a highly penetrant synovial sarcoma-like disease in mice.\textsuperscript{[47]} SS18-SSX is thought to function primarily as an aberrant chromatin regulator, driving oncogenesis by deregulating epigenetic pathways.\textsuperscript{[156, 185]} This suggests that epigenetic mechanisms, which support SS18-SSX dysfunction, exist in synovial sarcoma cells. However, currently our understanding of any such mechanisms is limited; and rational therapeutic options to target SS18-SSX function are lacking.

To identify epigenetic dependencies amenable to therapeutic targeting in synovial sarcoma we used a CRISPR/Cas9 based domain focused screening approach.\textsuperscript{[99]} We generated a custom lentiviral sgRNA library targeting functional regions in 193 chromatin regulators. This library was introduced into Cas9 expressing cell lines and the relative abundance of individual sgRNAs within the population compared between early and late time points by high-throughput sequencing (Fig 4.1 A). This identified 3 independent sgRNAs targeting the bromodomain of BRD9 that were depleted from synovial sarcoma cells (2.2 to 8.2-fold) (Fig 4.1 A,C). Parallel experiments in Ewing sarcoma found that while positive control sgRNAs were depleted from synovial and Ewing sarcoma cultures, BRD9 targeting sgRNAs were only depleted in synovial sarcoma (Fig 4.1 B). Remarkably, of the 52 bromodomains (within 38 proteins) targeted in this screen, only BRD9 had multiple sgRNAs specifically depleted in synovial sarcoma cells (Fig. S4.1 A).
Figure 4.1: The BRD9 bromodomain is a specific functional dependency in synovial sarcoma. 

A) Schematic representation of CRISPR/Cas9 based genomic screening approach. B) Scatter plot representation of biological duplicate sgRNA screening data in synovial and Ewing’s sarcoma cell lines. Each dot denotes and individual sgRNA and axes represent log₂ fold-change in sgRNA abundance between day-3 and day-15. BRD9 bromodomain and control sgRNAs are highlighted. C) Schematic representation of the BRD9 protein structure with sgRNA target sites indicated. D) Negative selection based CRISPR-Cas9 mutagenesis assays. The relative GFP⁺ (sgRNA⁺) subpopulation percentage is depicted at the indicated time-points after lentiviral infection. Mean +/- s.d., n = 3. E) Waterfall plot representing “BRD9 sensitivity” score in a panel of cancer cell lines taken from the Project DRIVE database (ref. 10) (https://oncolognir.shinyapps.io/drive/). F) Negative selection based CRISPR-Cas9 mutagenesis assays in bromodomain functional rescue experiments. The relative GFP⁺ (sgRNA⁺) subpopulation percentage is depicted at the indicated time-points after lentiviral infection. Mean +/- s.d., n = 3.
This is in striking contrast to the bromodomains of BRD4 which are a dependency in both synovial and Ewing sarcoma cells,[186] in addition to several other contexts.[114] To further examine the specificity of this dependency we performed individual sgRNA depletion assays in 2 independent synovial, Ewing and rhabdomyosarcoma cell lines. These experiments demonstrated that BRD9 bromodomain targeting sgRNAs were only depleted in synovial sarcoma (Fig. 4.1 D). Importantly, the sgRNAs used here have comparable/higher genome editing efficiencies in Ewing and rhabdomyosarcoma cell lines, compared to synovial sarcoma cells (Fig. S4.1 B and data not shown); demonstrating that differences in sgRNA depletion cannot be attributed to discrepancies in sgRNA editing. Moreover, using an independent shRNA based approach we observed synovial sarcoma specific effects following knockdown of BRD9 protein levels (Fig S4.1 C-F). Consistent with this, within the recently published Project DRIVE[187] database, we observe, that among the almost 400 cancer cell lines assayed, synovial sarcoma lines are among the most sensitive to BRD9 targeting (Fig. 4.1 E). To confirm the importance of the BRD9 bromodomain we performed functional rescue experiments. To do this we generated a full-length human BRD9 cDNA containing silent point mutations within the sgRNA recognition sequence, conferring resistance to Cas9 targeting (Fig. S4.1 G). Next, we expressed a wildtype (WT), bromodomain deleted (Dbromo) or bromodomain inactivated (N216A) version of this cDNA in synovial sarcoma cells and found that only BRD9-WT can rescue sgRNA depletion (Fig. 4.1 F and Fig. S4.1 H). These data demonstrate that BRD9, and specifically the BRD9 bromodomain, is a selective functional dependency in synovial sarcoma and highlight a novel therapeutic target in this disease.

BRD9 is a component of the SWI/SNF complex in several normal tissues.[155] However, it is unknown whether BRD9 is part of the oncogenic SS18-SSX containing complex in synovial sarcoma. To test this, we first generated HEK293T cell lines stably expressing an SS18-SSX1/2 fusion with an N-terminal 3xHA epitope tag.
Figure 4.2: BRD9 functions as part of SS18-SSX containing SWI/SNF complexes.
A) Volcano plots representing fold enrichment (LFQ intensity) of proteins identified by mass spec in SS18-SSX1 or SS18-SSX2 purifications relative to IgG control purifications. Known SWI/SNF members are indicated in red. B) Western blots analyses of the indicated proteins performed on endogenous BRD9 or IgG purifications in 2 independent synovial sarcoma cell lines (Input = 10% total IP material). Scatter plot representing the normalized protein abundance (IBAQ score) of proteins identified in SS18-SSX1 and SS18-SSX2 purifications. Known SWI/SNF members are indicated in red. C) Tornado plot representing BRD9 and SS18-SSX1 ChIP-seq signal +/- 10kb of all identified BRD9 peaks in HSSYII cells. D) High density sgRNA tiling of BRD9 in 2 independent synovial sarcoma cell lines. Each bar represents the fold-change of an individual sgRNA and its target site along the BRD9 protein. E) Negative selection based CRISPR-Cas9 mutagenesis assays in amino acid 311-345 region functional rescue experiments. The relative GFP+ (sgRNA+) subpopulation percentage is depicted at the indicated time-points after lentiviral infection. Mean +/- s.d., n = 3. F) Western blot analyses of the indicated proteins in anti-V5 purifications performed in control HSSYII cells, or HSSYII cells expressing a full-length, bromodomain deleted or amino acid 311-345 deleted BRD9.
We performed large-scale anti-HA immunoprecipitations (IPs) from nuclear protein lysates prepared from these cells; analyzing these purifications by liquid chromatography-mass spectrometry (LC-MS). This identified ~20 established SWI/SNF complex members, including BRD9, that co-purify with SS18-SSX in this setting (Fig. S4.2 A-C). Next, to test whether SS18-SSX fusions also interact with BRD9 in synovial sarcoma cells, we immunoprecipitated the endogenous fusion proteins in 2 independent synovial sarcoma cell lines (Extended Data Fig. 2d). Significantly, this demonstrates that BRD9 co-purifies with endogenous SS18-SSX containing SWI/SNF complexes in synovial sarcoma cells (Fig. 4.2 A and Fig. S4.2 E). Moreover, SS18-SSX fusion proteins co-purify with BRD9 in reciprocal endogenous IP experiments (Fig. 4.2 B). This demonstrates that by combining genomics and proteomics approaches we have identified BRD9 as a functional dependency, directly linked to SS18-SSX containing complexes in synovial sarcoma.

To ascertain the relative proportion of individual SWI/SNF complex members in SS18-SSX purifications we used the intensity-based absolute quantification (iBAQ) algorithm. This showed that core complex members such as SMARCC1/2 and SMARCA4 have relative abundances approximately equal to, or greater than, SS18-SSX (Fig. 4.2 C); suggesting that these proteins co-exist with the fusion protein in most (if not all) complexes. However, the relative abundance of BRD9 is 10-20% that of SS18-SSX; indicating that BRD9 is a sub-stoichiometric member of SS18-SSX containing complexes. Interestingly, several of the SWI/SNF complex members (PBRM, SMARCA2 and SMARCA4) identified in these proteomics studies were included in our functional genomics screen (Fig. 4.1 A). However, no robust dependencies were evident among these proteins (Fig. S4.2 F). Intriguingly, this suggests that the minor subset of BRD9 containing complexes are particularly important in synovial sarcoma cells.

To understand the extent to which BRD9 and SS18-SSX containing complexes overlap on chromatin we performed chromatin immunoprecipitation with next-generation sequencing
(ChIP-seq). Owing to a lack of high-quality ChIP-grade antibodies for BRD9 and SS18-SSX, we adapted a previously reported CRISPR/Cas9 based approach to knock-in a 3xHA epitope tag at the C-termini of the endogenous BRD9 and SS18-SSX1 loci in HSYII cells[169] (Fig. S4.3 A). BRD9 and SS18-SSX1 bind broadly throughout the genome with ~35% of binding sites occurring at gene promoters and the remaining ~65% at distal inter- and intragenic regions (Fig. S4.3 B). Comparing the binding profiles of BRD9 and SS18-SSX1 demonstrates that these proteins co-localize extensively on the synovial sarcoma genome (Fig. 4.2 D and Fig. S4.3 C). Indeed, a clear majority of all identified BRD9 and SS18-SSX1 binding sites overlap (Fig. S4.3 D), and there is a tight correlation in BRD9 and SS18-SSX1 occupancy genome-wide (Fig. S4.3 E). Considering that BRD9 may be present in only ~15% of SS18-SSX containing complexes, such broad co-localization is remarkable; and suggests BRD9 containing complexes play a particularly important role in supporting SS18-SSX function genome-wide.

Since a key aspect of bromodomain function is to mediate interactions with chromatin[190] we wanted to understand the contribution of the BRD9 bromodomain to chromatin binding. We performed cell-count normalized ChIP-seq (ChIP-Rx) of exogenously expressed 3xHA tagged BRD9; comparing the chromatin occupancy of BRD9-WT and BRD9-N216A (Fig. S4.3 G,H). Intriguingly, despite a reduction in chromatin binding following bromodomain inactivation a significant amount of BRD9 remains associated with chromatin; indicating that BRD9 can bind chromatin independently of bromodomain function presumably via interactions with other SWI/SNF complex members.

Next, we wanted to identify additional regions within BRD9 which contribute to the chromatin binding activity/function of the protein. To do this we used a high-density CRISPR mutagenesis approach, introducing 92 individual sgRNAs targeting across the BRD9 locus into Cas9 expressing synovial sarcoma cells.
Figure 4.3: BRD9 links SS18-SSX1 to super enhancers to support oncogenic transcription.

A) Metaplots representing genome-wide averages for BRD9, SS18-SSX1 and H3K27Ac occupancy at enhancers and active promoters in HSSYII cells. The x-axis shows the regions +/-10kb at enhancers (left) and +/-5kb at promoters (right). The y-axis shows average ChIP-seq signal in rpm/bp.

B) H3K27Ac ChIP-seq signal (rpm/bp) at all enhancer regions in HSSYII cell. Enhancers are ranked by increasing H3K27Ac signal.

C) Box plot representation of the relative abundance of BRD9 and SS18-SSX1 ChIP-seq signal at promoter, typical enhancer and super enhancer elements. P values are from Welch’s two-tailed t-tests. ***P ≤0.001.

D) Tracks showing BRD9 ChIP-seq occupancy on the 98 Mb right arm of chromosome 8 after DMSO or 100nM dBRD9-A treatment. The chromosome 8 ideogram is displayed above the gene tracks with the relevant region highlighted in red.

E) Box plot representations of changes in BRD9 and SS18-SSX1 occupancy at active promoters, typical enhancers and super enhancers comparing DMSO and dBRD9-A treated cells. P values are from Welch’s two-tailed t-tests. *P ≤0.05, ***P≤0.001.

F) Box plot representations of changes in gene expression amongst genes associated with typical enhancers and genes associated with super enhancers. P values are from Welch’s two-tailed t-tests. ***P≤0.001.

G) Heat map representing changes in gene expression amongst all super enhancer associated genes in HSSYII cells following 6hrs dBRD9-A treatment at 100nM, or HSSYII cells following infection with 2 independent SS18-SSX1 shRNAs for 96hrs.

H) Tracks showing BRD9 and SS18-SSX1 ChIP-seq occupancy at the indicated genomic loci in DMSO and dBRD9-A treated cells. Also shown is H3K27Ac ChIP-seq signal in untreated cells.
We monitored for changes in sgRNA expressing (GFP-positive) cells over time, and consistent with our pooled screen most sgRNAs targeting the BRD9 bromodomain were robustly out competed in these GFP depletion assays (Fig. 4.2 E). However, we identified an additional hotspot of sgRNA depletion within a previously uncharacterised region of BRD9 (amino acids 311-345). We confirmed the functional importance of this region with rescue experiments showing that a D311-345 BRD9 cDNA was incapable of rescuing the depletion of sgRNAs targeting this region (Fig. 4.2 F). Moreover, ChIP-qPCR analyses demonstrate that deletion of the 311-345 region leads to reduced chromatin binding; comparable to that seen following bromodomain deletion (Fig. S4.3 I). Significantly, co-IP experiments demonstrated that while the BRD9 bromodomain is dispensable for SWI/SNF interaction, the 311-345 amino acid region is essential for association with the complex (Fig. 4.2 G). These data identify a novel SWI/SNF interaction domain within BRD9 and demonstrate that interaction with the complex is essential for BRD9 function. This highlights that targeting bromodomain function in isolation, while at least partially effective at inhibiting synovial sarcoma cell proliferation, is unlikely to completely inactivate BRD9 in synovial sarcoma cells.

The SWI/SNF complex modulates gene transcription through the maintenance of active enhancers.\textsuperscript{191-194} Using H3K27Ac ChIP-seq to define active enhancer elements in HSSYII cells we found that distal BRD9 and SS18-SSX1 binding sites typically overlap H3K27Ac marked enhancers (Fig. 4.3 A). Furthermore, compared to gene promoters these enhancer elements showed higher occupancy of BRD9 and SS18-SSX1 (Fig. 4.3 A and Fig. S 4.4 A). Super enhancers (SEs) represent a critical subclass of enhancers known to drive expression of genes required for maintaining tumour cell identity.\textsuperscript{40,98} Consistent with this, several genes associated with SEs in HSSYII cells including TWIST\textsuperscript{195} and TLE1\textsuperscript{48} are known to play key functional roles in synovial sarcoma (Fig. 4.3 B). Moreover, expression of many of these SE associated genes has previously been linked to primary synovial sarcoma tumour phenotypes, defining both clinical and biological characteristics.\textsuperscript{196-200} SEs have higher levels of BRD9 and SS18-
SSX1 occupancy compared to typical enhancers (Fig. 4.3 C); suggesting that BRD9 and SS18-SSX1 may execute a critical role in the regulation of genes associated with these elements. Next, we leveraged our recent success developing targeted chemical degraders of BRD9\(^{[101]}\) to examine the effects of BRD9 degradation in synovial sarcoma cells. To do this we applied dBRD9-A, a more lipophilic analog of our previously highlighted probe dBRD9. This compound demonstrated greater potency than our previous molecule, degrading BRD9 in a manner that depends E3 ubiquitin ligase component CRBN and BRD9 bromodomain engagement (Fig. 4.4 B,C). Treatment of HSSYII cells with dBRD9-A leads to a genome-wide loss of BRD9 from chromatin (Fig. 4.3 D). Furthermore, loss of BRD9 is associated with reduced SS18-SSX1 occupancy; with the most significant loss of fusion protein binding occurring at SEs (Fig. 4.3 E). Cell count normalized RNA-seq of HSSYII cells treated with dBRD9-A for 6hrs demonstrates that SE associated genes are preferentially downregulated following dBRD9-A treatment (Fig. 4.3 F). Significantly, these genes depend on the fusion protein to maintain their expression, since shRNA mediated knockdown of SS18-SSX1 leads to a collapse of SE associated gene expression (Fig. 4.3 G). dBRD9-A treatment and consequential downregulation of transcription is associated with a loss of SS18-SSX1 binding at SEs associated with many of these genes (Fig. 4.3 H and Fig. S4.4 D). Consistent with a specific role for BRD9 in synovial sarcoma; we found in Ewing sarcoma cells that degradation of BRD9 has minimal effects on gene expression (Fig. S4.4 E). This is despite the fact BRD9 and (wildtype) SS18 are similarly distributed on gene promoters and enhancer elements in Ewing sarcoma cells (Extended Data Fig. 4.4 F). However, degradation of the BET bromodomain proteins,\(^{[56]}\) which also bind promoter and enhancer regions in both cell types leads to a concordant collapse in gene expression in both settings (Fig. S4.4 G,H). Significantly, these data demonstrate that BRD9 is required to maintain SS18-SSX binding at super enhancer elements; and in doing so supports the underlying oncogenic transcriptional program in synovial sarcoma cells.
Figure 4.4: Targeted degradation of BRD9 represents a therapeutic opportunity in synovial sarcoma. A) Chemical structure of our BRD9 degrader compound dBRD9-A. B) Selectivity of phage-displayed bromodomain displacement by dBRD9-A (Bromoscan). C) Western blot analysis of the indicated proteins, in 2 independent synovial sarcoma cell lines following treatment with dBRD9-A at 100nM for 6-72h. D) Cellular viability dose-response data in HSSYII cells treated with dBRD9-A or the BRD9 bromodomain inhibitors I-BRD9 or BI7273. Mean +/- s.d., n = 3. E) Cellular viability dose-response data for 2 independent synovial sarcoma cells line treated with dBRD9-A. Mean +/- s.d. n = 3. F) Relative changes in cell cycle dynamics in 2 independent synovial sarcoma cell lines treated with dBRD9-A for 3/6/9 days at 100nM. Mean +/- s.d., n = 3. G) Relative changes in Annexin-V positive cells in 2 independent synovial sarcoma cell lines treated with dBRD9-A for 3/6/9 days at 100nM. Mean +/- s.d., n = 3. H) Tumour progression in a subcutaneous xenograft model of synovial sarcoma in control vehicle treated mice, and mice treated at 50mg/kg dBRD9-A once daily for 24 days. Mean +/- SEM, 5 mice per treatment group. P value is from 2way ANOVA. I) Western blot analysis of the indicated proteins in protein lysates derived from tumour tissue from 2 independent mice per treatment group as in panel g.
Given our findings that targeting the bromodomain alone would likely not completely inactivate BRD9, we wanted to test the therapeutic efficacy of BRD9 degradation in synovial sarcoma cells. To do this we used our newly developed dBRD9-A compound, which differs from our previous BRD9 degrader in that it contains a more lipophilic alkyl linker and has improved BRD9 degradation properties (Fig. 4.4 A and data not shown). dBRD9-A is a highly specific binder of the BRD9 bromodomain (Fig. 4.4 B) and elicits near complete BRD9 protein degradation at low nanomolar concentrations (Fig. 4.4 C). Significantly, BRD9 degradation leads to a far more robust therapeutic response compared to bromodomain inhibition (Fig. 4.4 D); consistent with the hypothesis that BRD9 also functions independently of its bromodomain.

Synovial sarcoma cell lines are highly sensitive to BRD9 degradation in vitro; while, consistent with our genetic and gene expression data, other sarcoma cell types are unaffected despite robust BRD9 degradation (Fig. 4.4 E and Fig. S4.5 A). Consistent with the on-target activity of dBRD9-A, swapping the BRD9 bromodomain for the closely related BRD7 bromodomain (63%, sequence identity) renders BRD9 and synovial sarcoma cells insensitive to dBRD9-A treatment (Fig. S4.5 B,C). Synovial sarcoma cells treated with dBRD9-A undergo a progressive cell cycle arrest (Fig. 4.4 F), which is further associated with an increase in the proportion of Annexin–V positive cells (Fig. 4.4 G). Using an in vivo synovial sarcoma xenograft model, we found that treatment of mice with dBRD9-A (50mg/kg, once daily via intraperitoneal injection) over 24 days inhibited tumour progression (Fig. 4.4 H). We confirmed in vivo pharmacodynamic activity of dBRD9-A in this system by immunoblotting BRD9 in tumour tissue derived from vehicle and dBRD9-A treated mice (Fig. 4.4 I). Mice treated with dBRD9-A did not suffer any overt side effects associated with treatment, retaining a normal body weight and blood counts (Fig. S4.5 D,E). This demonstrates that our novel approach of targeted BRD9 degradation may provide a therapeutically efficacious option for synovial sarcoma treatment. Moreover, our work demonstrates that BRD9 degradation directly impedes the function of the SS18-SSX fusion
protein unique to synovial sarcoma cells; suggesting that targeting this selective dependency may provide clinical benefits with limited toxicities in patients.

Most cancer treatments target processes that are important in both normal and cancer cells, therefore systemic toxicity resulting in debilitating side-effects remains problematic. Fusion gene driven cancers present a relatively unique opportunity to target cancer cell specific processes since oncogenic fusion proteins are present only in malignant cells. Understanding mechanisms related to fusion protein function may provide opportunities to develop therapies targeting underlying pathologies with limited effects on normal tissues. Indeed, recent studies have demonstrated the importance of chromatin based mechanisms for the oncogenic potential of MLL-fusion proteins in leukemia.\cite{201-206} Our work demonstrates the importance of BRD9 in supporting SS18-SSX function and oncogenic gene expression in synovial sarcoma. Moreover, we provide evidence that a unique approach to inactivation of BRD9 through targeted protein degradation blocks tumour progression \textit{in vivo}. Currently synovial sarcoma has few effective treatment options, and poor overall survival. This study highlights an effective approach to target SS18-SSX function, providing a rationale for further development of this approach and potentially assessment in patients suffering with this terrible disease.
**Materials and Methods**

**Cell culture and lentiviral production**

All cell lines were maintained at 37°C in a humidified incubator. Lentiviral packaging HEK293T, synovial sarcoma (HSSYII, SYO1 and 1273/99) and Ewing’s sarcoma (A673) cell lines were cultured in DMEM (Gibco) media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% Penicillin-Streptomycin and 12.5µg/ml Plasmocin. Synovial sarcoma (CME1), Ewing’s sarcoma (SKNMC) and rhabdomyosarcoma (RH30 and RH41) cells were cultured in RPMI (Gibco) media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% Penicillin-Streptomycin and 12.5µg/ml Plasmocin. Lentiviral supernatants were generated by co-transfection of HEK293T cells of a lentiviral expression vector (cDNA, sgRNA or shRNA) with viral packaging (PAX2) and envelope (VSV-G) vectors using the X-tremegene transfection reagent (Roche) in accordance with the manufacturer’s instructions. Viral supernatants were collected between 24-48hrs post-transfection and used directly for infection of target cells after filtering through a 0.45µm syringe filter and addition of 8.5µg/ml Polybrene.

**Pooled CRISPR screening and data analysis**

The human epigenetic domain U6-sgRNA-EFS-GFP targeting library was pooled at equimolar ratio and used to generate lentiviral supernatant as described above. A dilution series of this virus correlated with GFP positivity in infected cells, and was used to derive an accurate viral multiplicity of infection (MOI). The total number of synovial and Ewing’s sarcoma target cells for infection was chosen to achieve at least 500-fold representation of each sgRNA in the initially infected cell population. To ensure that a single sgRNA was transduced per cell, the viral volume for infection was chosen to achieve an MOI of 0.3–0.4. Genomic DNA was extracted at the indicated time points using QiAamp DNA mini kit (Qiagen #51304), following the manufacturer’s instructions. To maintain >500× sgRNA library representation, 16–20 independent PCR reactions were used to amplify the sgRNA cassette, which were amplified for
20 cycles with 100-200ng of starting gDNA using the 2× Phusion Master Mix (Thermo Scientific #F-548). The PCR products were pooled and end repaired with T4 DNA polymerase (NEB), DNA polymerase I (NEB), and T4 polynucleotide kinase (NEB). An A overhang was added to the end-repaired DNA using Klenow DNA Pol Exo- (NEB). The DNA fragment was then ligated with diversity-increased barcoded Illumina adaptors followed by 5 pre-capture PCR cycles. Bar-coded libraries were pooled at equal molar ratio and subjected to massively parallel sequencing using a Mi-Seq instrument (illumina) using paired-end 150 bp sequencing (MiSeq Reagent Kit v2; Illumina MS-102-2002). The sequence data were trimmed to contain only the sgRNA sequence then were mapped to the reference sgRNA library without allowing any mismatches. The read counts were then calculated for each individual sgRNA. To compare the differential representation of individual sgRNAs between day 3 and day 15 time points, the read counts for each sgRNA were normalized to the counts of the negative control ROSA26 sgRNA.

Cloning and mutagenesis

The human full-length BRD9 cDNA was PCR amplified from MGC clone 5428011 and inserted in the Gateway cloning compatible entry vector pCR8/GW/TOPO (Invitrogen, K250020) in accordance with the manufacturer’s instructions. Clone integrity was confirmed by sanger sequence. Mutagenesis of the wildtype BRD9 sequence was performed using pCR8-BRD9 as template and the Q5 Site-Directed Mutagenesis Kit (NEB, E0554S) in accordance with the manufacturer’s instructions. Sequence verified BRD9 ORF sequences were subsequently cloned into the Gateway expression vector pLEX305 (Addgene vector, 41390) which had been engineered to contain an N-terminal 3xHA epitope tag using LR clonase (Invitrogen, 12538120).

Immunoprecipitation

Immunoprecipitations were performed as previously described.\textsuperscript{[207]} Briefly, nuclear pellets were lysed in buffer C containing protease inhibitors (20 mM HEPES at pH 7.6, 20% [v/v] glycerol,
0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, aprotinin 1 µg mL⁻¹, leupeptin 10 µg mL⁻¹, PMSF 1 mM) and subsequently dialyzed against buffer C-100 (20 mM HEPES at pH 7.6, 20% [v/v] glycerol, 0.2 mM EDTA, 100 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA). Antibody-coupled beads were incubated with dialyzed nuclear extracts containing 250 U of benzonase (Sigma) for 3h at 4°C. Beads were then washed, and elutions were performed with 1xLDS buffer, 1 mg/mL HA peptide or 1mg/mL V5 peptide (Sigma).

Mass spectrometry

In-solution tryptic digestions were performed as described previously. Peptides were analysed with a Q-Exactive mass spectrometer coupled with an EASY-nLC HPLC system (Thermo Fisher) and an in-house packed C18 column (New Objective). Parent ion spectra (MS1) were measured at resolution 70,000, AGC target 3e6. Tandem mass spectra (MS2, up to 10 scans per duty cycle) were obtained at resolution 17,500, AGC target 5e4, collision energy of 25. All mass spectrometry data were processed using the MaxQuant software, version 1.3.0.5. The following search parameters were used; Fixed Mod: carbamidomethylation, Variable Mods: methionine oxidation, Trypsin/P digest enzyme, Precursor mass tolerances 6 ppm, Fragment ion mass tolerances 20 ppm, Peptide FDR 1%, Protein FDR 1%.

Chromatin immunoprecipitation

Cells for H3K27Ac and RNAPII ChIPs were fixed using 1% formaldehyde at room temperature for 10 mins. Formaldehyde crosslinking was quenched by adding Glycine to a final concentration of 0.125M directly to the fixation solution, followed by an additional 5 min incubation at room temperature. Cells for anti-HA (BRD9/SS18-SSX1) ChIPs were subjected to a 2-stage fixation; cells were initially fixed for 30 mins at room temperature using 0.5mM DSG, followed by an additional 10 mins at room temperature using 1% formaldehyde. Formaldehyde crosslinking was quenched as outlined above. Fixed cells were washed 2X with ice-cold PBS.
and pelleted by centrifugation. Nuclei were extracted by resuspending fixed cell pellets in LB1 buffer (50mm HEPES, 140mm NaCl, 1mm EDTA, 10% Glycerol, 0.5% NP40, 0.25% Triton X100) containing 1X protease inhibitor cocktail (Biotools, B14002), followed by 10 mins incubation. Cells were pelleted by centrifugation and resuspended in LB2 buffer (10mM Tris ph8.0, 200mM NaCl, 1mM EDTA, 0.5mM EGTA) containing 1X protease inhibitor cocktail. Extracted nuclei were lysed using Covaris shearing buffer (0.1% SDS, 1mM EDTA and 10mM Tris pH 8.0) containing 1X protease inhibitor cocktail. Nuclei were lysed at a concentration of 10-30 million cells/ml in shearing buffer and sonicated in a Covaris E220, 1ml AFA milltubes (with fiber), Water level = 5, Duty Cycle = 5%, Peak Incidence Power = 140W, Cycle per burst = 200 for 16mins. Sonicated samples were pre-cleared by centrifugation at 14000rpm for 15mins at 4ºC. A 0.25X volume of 5X ChIP buffer (250 mM HEPES, 1.5 M NaCl, 5 mM EDTA pH 8.0, 5% Triton X-100, 0.5% DOC, and 0.5% SDS) was added to pre-cleared lysates, and these samples used directly for immunoprecipitations. For spike-in normalized ChIP experiments (ChIP-Rx) a 1:10 volume of fixed/sonicated chromatin derived for a mouse NIH3T3 cell line expressing a 3x HA epitope tagged BRD9 was added to each sample prior to the immunoprecipitation step.

ChIP-seq library preparation and sequencing

ChIP purified DNA was quantified using a Qubit fluorimeter (Invitrogen), and 2-50ng of DNA/ChIP was used to generate ChIP-seq libraries with the ThruPLEX DNA-seq kit (Rubicon Genomics, R400427). Library DNA was quantified using the Qubit, and size distributions were ascertained on a Tapestation (Agilent) using the D1000 ScreenTape assay reagents (Agilent, 5067-5583). This information was used to calculate pooling ratios for multiplex library sequencing. Pooled libraries were diluted and processed for 75bp single-end sequencing on an Illumina NextSeq instrument using the NextSeq 500 High Output v2 kit (75 cycle) (Illumina, FC-404-2005) in accordance with the manufacturer's instructions.
Cell count RNA-seq library prep and sequencing

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, 74106) in accordance with the manufacturer’s instructions. ERCC spike-in controls were added to isolated RNA to facilitate cell count normalization of RNA-sequencing data. The quality of extracted RNA was confirmed using a Bioanalyzer (Agilent) and 1µg of total RNA was used/sample as library prep input. Libraries were generated using the NEBNext Ultra RNA Library Prep kit for Illumina (NEB, E7530L) in accordance with the manufacturer’s instructions. Library DNA was quantified using the Qubit, and size distributions were ascertained on a Tapestation (Agilent) using the D1000 ScreenTape assay reagents (Agilent, 5067-5583). This information was used to calculate pooling ratios for multiplex library sequencing. Pooled libraries were diluted and processed for 75bp single-end sequencing on an Illumina NextSeq instrument using the NextSeq 500 High Output v2 kit (75 cycle) (Illumina, FC-404-2005) in accordance with the manufacturer’s instructions.

ChIP and RNA-seq data analysis

ChIP-seq analysis was performed using pipelines on the omics analysis platform Basepair (http://www.basepair.io). ChIP fastq files were trimmed to remove adapter and low quality sequences using trim_galore and aligned to the UCSC genome assembly hg19 using Bowtie (version 2.1.0). For spike-in normalized ChIP-seq experiments reads were separately aligned to hg19 and mm9 using Bowtie. Duplicate reads were removed using Picard Mark Duplicates. Peaks were detected using MACS (version 1.4) using a p value cutoff was set to $10^{-5}$. Peaks were annotated to genomic features (Promoter, Gene body, Intergenic) using custom scripts on the Basepair platform, based on the UCSC database for hg19.
RNA-seq fastq files were aligned to NCBI37/hg19 and normalized using STAR. Differential expression data were obtained using the DEseq algorithm. These analyses were all done through the Basepair analysis platform (http://www.basepair.io).

**Western blotting**
Whole cell protein samples were prepared in RIPA buffer (25mM Tris-HCl, pH7.6, 150mM NaCl, 1% NP-40, 1% Sodium Deoxycholate, 0.1% SDS) containing 1X protease inhibitor cocktails. Protein lysates were separated on pre-cast Bolt 4-12% Bis-Tris Plus Gels (Invitrogen, NW04127BOX) and transferred to nitrocellulose membranes. Membranes were subsequently probed using the relevant primary and secondary antibodies and relative protein levels were determined using the Odyssey CLx Imager (LI-COR).

**Cellular viability, cell cycle and apoptosis analysis**
For dose response viability assays, cells were plated in 96-well tissue culture plates (1000 cells/well) in media containing DMSO or the desired concentration or each compound. Media was changed every 3-days up to a total of 9-days, at which point the ATPlite 1-Step luminescence assay system (PerkinElmer, 6016731) was used to determine ATP-dependent luminescence as an approximation of cellular viability. For cell cycle and apoptosis analysis cells were initially seeded on 10cm dishes in media containing DMSO or 100nM dBRD9-A and cultured/passaged in this media for a total of 9 days. For cell cycle analysis control and treated cells were harvested at 3/6/9 days and processed for FACs analysis using the BD Pharmingen BrdU Flow kit (BD, 559619) in accordance with the manufacturer’s instructions. For apoptosis analysis cells were harvested at 3/6/9 days (using Accutase to maintain cell membrane integrity) and processed for FACs analysis using the BD Annexin V Apoptosis Detection kit (BD, 556547) in accordance with the manufacturer’s instructions. Stained cells were analysed on a BD LSRSFortessa Cell Analyzer and data processed using FlowJo software.
**Antibodies:**

**Antibodies used for Western blotting**

**Antibodies used for IP**
mouse anti-HA monoclonal magnetic beads, Pierce (catalogue number: 88837), mouse anti-V5 monoclonal agarose beads, Sigma (catalogue number: A7345-1ML), rabbit anti-BRD9 polyclonal, Bethyl Labs (catalogue number: A303-781A), rabbit anti-SSX1 polyclonal, MyBiosource (catalogue number: MBS9408371), rabbit anti-SSX2 polyclonal, MyBiosource (catalogue number: MBS9127222).

**Antibodies used for ChIP**
rabbit anti-HA monoclonal, Cell Signaling Technology (catalogue number: 3724S), rabbit anti-H3K27Ac polyclonal, Abcam (catalogue number: ab4729), mouse anti-RNAPII monoclonal, Diagenode (catalogue number: C15100055-100).
Figure S4.1: BRD9 is a specific functional dependency in synovial sarcoma. A) Scatter plot representation of sgRNA screening data in synovial and Ewing’s sarcoma cell lines. Each dot denotes and individual bromodomain targeting sgRNA and axes represent log₂ fold-change in sgRNA abundance between day-3 and day-15. BRD4 and BRD9 bromodomain targeted sgRNAs are indicated. Highlighted region denotes sgRNAs depleted >2-fold in synovial sarcoma cells and unchanged in Ewing sarcoma cells. B) Indel quantification by TIDE (Tracking of Indels by sequence trace Decomposition) analysis 4 days after transduction with the indicated sgRNA in the indicated cell lines. Also presented are GFP FACs plots collected at time of harvesting, demonstrating the proportion of GFP+ (sgRNA+) cells in each instance. C) Western blot analysis of the indicated proteins in 4 independent synovial sarcoma cell lines expressing a control shRNA (Renilla) or one of two independent BRD9 targeting shRNAs. D) Western blots (as in Panel b) in Ewing sarcoma (A673) and rhabdomyosarcoma (RH30) cells lines. E) Negative selection based shRNA functional assays in 4 independent synovial sarcoma cell lines. The relative GFP+ (sgRNA+) subpopulation percentage is depicted at the indicated time points after lentiviral infection. Mean +/- s.d., n = 3. F) Negative selection based shRNA assays (as in Panel d) in Ewing sarcoma (A673) and rhabdomyosarcoma (RH30) cell lines. G) Schematic representation of the BRD9 coding region targeted by sgRNA-15. The sgRNA target sequence is highlighted in red and PAM sequence is indicated (top). The silent mutations added to render the allele insensitive to CRISPR/Cas9 targeting are indicated, and sequence of the CRISPR-resistant allele shown (bottom). H) Western blot analysis of the indicated proteins in HSSYII cells used in function rescue experiments (left). Cells were infected with lentiviral constructs expressing either a full-length WT or bromodomain mutant BRD9 or a bromodomain deleted BRD9 cDNA (right).
Figure S4.2: BRD9 is a component of SS18-SSX containing SWI/SNF complexes. A) Silver stains of anti-HA immunoprecipitations performed on nuclear protein lysates prepared from HEK293T cell lines expression GFP (control) or an SS18-SSX1 or SS18-SSX2 fusion protein. B) Volcano plots representing fold enrichment (LFQ intensity) of proteins identified by mass spec in anti-HA-SS18-SSX1/2 purifications relative to GFP control purifications. Known SWI/SNF members are indicated in red. C) Bar chart representing total peptide numbers identified for each of the indicated SWI/SNF complex members in mass spec analysis of anti-HA-SS18-SSX1/2 purifications. D) Silver stains of endogenous immunoprecipitations performed on nuclear protein lysates prepared 2 independent synovial sarcoma cell lines. E) Bar chart representing total peptide numbers identified for each of the indicated SWI/SNF complex members in mass spec analysis of endogenous SS18-SSX1/2 purifications. F) Bar chart representing the fold-change in abundance of individual sgRNAs targeting the bromodomains of the indicated SWI/SNF members between day-15 and day-3 of our functional genomics screening experiments.
Figure S4.3: BRD9 and SS18-SSX1 co-localise genome-wide. 
A) Schematic representation of the CRISPR/Cas9 mediated targeting of a 3xHA epitope tag to the C-termini of the BRD9 and SS18-SSX1 loci. B) Pie charts representing the distribution of BRD9 and SS18-SSX1 binding sites on the synovial sarcoma genome. C) Genomic tracks showing BRD9 and SS18-SSX1 ChIP-seq signal on the 98 Mb right arm of chromosome 8 in HSSYII cells. The chromosome 8 ideogram is displayed above the gene tracks with the relevant region highlighted in red. D) Venn diagram overlaps of all identified BRD9 and SS18-SSX1 ChIP-seq peaks in HSSYII cells. E) Scatter plot representing the correlation of BRD9 and SS18-SSX1 ChIP-seq signal within all identified BRD9 binding peaks. F) Genomic tracks showing BRD9 and SS18-SSX1 ChIP-seq signal at the indicated locus in HSSYII cells. G) Tornado plots showing BRD9-WT and BRD9-N216A ChIP-signal signal within all BRD9 peaks regions. Regions are ranked by change in ChIP-signal (BRD9-N216A/BRD9-WT). H) Genomic tracks showing BRD9-WT and BRD9-N216A ChIP-seq signal at the indicated locus in HSSYII cells. I) Quantitative-ChIP-PCR analysis at the indicated gene promoters of anti-HA ChIPs performed in vector (control) HSSYII cells, or cells expressing BRD9 full-length, D311-345 or Dbromo.
Figure S4.4: Transcriptional regulation by BRD9 in synovial sarcoma cells. 
A) Tornado plots showing BRD9, SS18-SSX1 and H3K27Ac ChIP-seq signal on active enhancers (left) and BRD9, SS18-SSX1 and RNAPII on active promoters (right) in HSSYII cells. B) Western blot analysis of the indicated proteins in wildtype- or CRBN-/- HEK293T cells treated with 100nM DBRD9-A for 0, 24 or 48hrs. C) Western blot analysis of the indicated proteins in HSSYII cells treated with increasing doses of dBRD9-A (100nM, 500nM) for 6h following pre-treatment of cells for 12h with DMSO or BI7273 (5mM). D) Genomic tracks showing BRD9 and SS18-SSX1 ChIP-Rx signal in HSSYII cells treated with DMSO or dBRD9-A at 100nM, and H3K27Ac in untreated cells. E) Volcano plots showing global gene expression changes in synovial sarcoma (HSSYII) (top) and Ewing sarcoma (A673) cells following dBET6 treatment at 100nM for 6d. F) Tornado plots showing BRD9, SS18 (wildtype) and H3K27Ac ChIP-seq signal on active enhancers (left) and BRD9, SS18 (wildtype) and RNAPII on active promoters (right) in A673 cells. G) Tornado plots showing BRD4 ChIP-seq signal on active enhancers and promoters in HSSYII (left) and A673 (right) cells. H) Volcano plots showing global gene expression changes in synovial sarcoma (HSSYII) (top) and Ewing sarcoma (A673) cells following dBET6 treatment at 100nM for 6h.
Figure 4.5: Targeted degradation of BRD9
A) Western blot analysis of the indicated proteins in Ewing (A673) and rhabdomyosarcoma (RH30) cell lines treated with dBRD9-A at 100nM for 6 to 72h. B) Western blot analysis of the indicated proteins in HSSYII cells expressing vector (control), BRD9-WT or BRD9 containing the bromodomain of BRD7 (BRD9-BD7) treated with dBRD9-A at 100nM for 6h. C) Growth assays of HSSYII cells (as in panel b) cultured in the presence of dBRD9-A at 100nM for a total of 9-days. D) Mouse weight measurements of vehicle control and dBRD9-A treated mice. Mean +/- s.d., n = 5. E) Complete blood counts (CBCs) performed on vehicle control and dBRD9-A treated mice. Measurements were taken on day-23 of the 24-day treatment experiment. Mean +/- s.d., n = 5.
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Author Contributions

G.L.B, J.S, C.R.V and S.A.A conceived the study. G.L.B performed most of the experiments, D.R synthesized and contributed to applications of dBRD9-A, J.S generated the custom sgRNA libraries and performed next-generation sequencing related to CRISPR screening experiments, M.L.H assisted with bioinformatics analysis of ChIP and RNA-seq experiments, K.W, E.T.D, and G.C performed mass spec analysis, J.Q, S.F and J.E.B contributed cell lines and reagents. G.L.B and S.A.A interpreted experimental results and wrote the manuscript with contributions from all authors.
Appendix A

Additional materials for Chapter I

Synthetic Procedures


Synthetic scheme 1. Biotin probe synthesis.

Biotin-Probe
4-(((R)-8-cyclopentyl-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)-3-methoxy-N-(13-oxo-17-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-3,6,9-trioxa-12-azaheptadecyl)benzamide

To a solution of (R)-4-(((8-cyclopentyl-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)-3-methoxybenzoic acid (12.7 mg, 0.0298 mmol, 1.0 eq)\(^{[108]}\) in DMF (0.3 mL, 0.1 M) was added HATU (12.5, 0.0328, 1.25 eq), DIPEA (15.6 ul, 0.0894 mmol, 3 eq), and finally BiotinPEG\(_3\)(NH\(_2\)) (15.6 mg, 0.0373, 1.25 eq). After stirring 24 hours at RT the reaction was diluted with MeOH and purified by preparative HPLC to give a yellow oil (21.4mg, 87%).

\(^{1}\)HNMR: (400 MHz, Methanol-d\(_4\)) \(\delta\) 7.85 (d, \(J = 8.3\) Hz, 1H), 7.64 – 7.58 (m, 2H), 7.55 (dd, \(J = 8.3, 1.8\) Hz, 1H), 4.53 – 4.46 (m, 2H), 4.29 (dd, \(J = 7.8, 4.7\) Hz, 2H), 3.98 (s, 3H), 3.72 – 3.57 (m, 12H), 3.54 – 3.47 (m, 2H), 3.41 – 3.32 (m, 2H), 3.19 (dd, \(J = 8.5, 5.1\) Hz, 1H), 3.09 (d, \(J = 22.9\) Hz, 1H), 3.00 (s, 2H), 2.92 (dd, \(J = 12.7, 5.0\) Hz, 1H), 2.69 (d, \(J = 12.6\) Hz, 1H), 2.22 – 2.15 (m, 2H), 2.12 – 2.00 (m, 3H), 1.96 – 1.85 (m, 3H), 1.74 – 1.54 (m, 8H), 1.46 – 1.39 (m, 3H), 0.86 (t, \(J = 7.5\) Hz, 3H).

LCMS: 826.6 (M+H)
Synthetic Procedures (B): dihydropteridinone SAR (Table 1. Compounds).

**Synthetic scheme 2.** dihydropteridinone core and final compound synthesis.

**BI-2536 (Compound 1) (commercially available)**
(R)-4-((8-cyclopentyl-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)-3-methoxy-\(N\)-(1-methylpiperidin-4-yl)benzamide\[^{135}\]

**Compound 2**
(R)-4-((8-cyclopentyl-5,7-diethyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)-3-methoxy-\(N\)-(1-methylpiperidin-4-yl)benzamide\[^{108}\]

\(^1\)H NMR (400 MHz, Methanol-\(d_4\)) \(\delta\) 8.47 (d, \(J = 9.0\) Hz, 1H), 7.78 (s, 1H), 7.52 – 7.44 (m, 2H), 4.48 (q, \(J = 8.7\) Hz, 1H), 4.22 (dd, \(J = 7.8, 3.7\) Hz, 1H), 4.05 (dt, \(J = 14.3, 7.1\) Hz, 1H), 3.99 (s, 3H), 3.89 (tt, \(J = 11.3, 4.1\) Hz, 1H), 3.79 (dt, \(J = 14.2, 7.1\) Hz, 1H), 2.92 (d, \(J = 12.1\) Hz, 2H), 2.30 (s, 3H), 2.20 – 2.08 (m, 3H), 2.02 – 1.62 (m, 13H), 1.22 (t, \(J = 7.1\) Hz, 3H), 0.84 (t, \(J = 7.5\) Hz, 3H).

LCMS 536.55 (M+H).
Compound 3

(R)-4-((8-cyclopentyl-7-ethyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)-3-methoxy-N-(1-methylpiperidin-4-yl)benzamide

^1^H NMR (400 MHz, Methanol-d4) δ 8.46 (d, J = 9.0 Hz, 1H), 7.56 (s, 1H), 7.48 (dd, J = 6.3, 1.9 Hz, 2H), 4.53 – 4.44 (m, 1H), 4.21 (dd, J = 7.5, 3.6 Hz, 1H), 4.03 – 3.88 (m, 4H), 3.02 (d, J = 11.9 Hz, 2H), 2.39 (s, 3H), 2.33 (t, J = 11.3 Hz, 2H), 2.20 – 2.12 (m, 1H), 2.04 – 1.67 (m, 13H), 0.91 (t, J = 7.5 Hz, 3H).

LCMS 508.58 (M+H).

4-amino-N-(1-methylpiperidin-4-yl)benzamide

4-aminobenzoic acid (0.411 g, 3.0 mmol, 1.0 eq), 1-methyl-4-aminopiperidine (0.38 mL, 3.0 mmol, 1.0 eq), and HOBt (0.61 g, 4.5 mmol, 1.5 eq) were dissolved in DMF (12.5 mL, 0.24 M), before DIPEA (1.04 mL, 6.0 mmol, 2 eq) was added and the solution cooled on ice. EDC (1.03 g, 5.4 mmol, 1.8 eq), was added and the solution warmed to RT. After stirring for 16 hours the solution was diluted with sat. Na2CO3, and extracted with 5% MeOH/CHCl3 followed by EtOAC, dried over Na2SO4, filtered, and concentrated. The residue was purified on silica (ISCO 12g, 0-15% DCM/MeOH) to give a white solid (0.468 g, 67%).

^1^H NMR (400 MHz, Methanol-d4) δ 7.63 – 7.56 (m, 2H), 6.70 – 6.61 (m, 2H), 3.85 (ddd, J = 11.3, 7.0, 4.3 Hz, 1H), 2.91 (d, J = 12.2 Hz, 2H), 2.30 (s, 3H), 2.17 (td, J = 12.1, 2.4 Hz, 2H), 1.97 – 1.89 (m, 2H), 1.71 – 1.60 (m, 2H).

LCMS: 234.1 (M+H)

Compound 4

(R)-4-((8-cyclopentyl-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)-N-(1-methylpiperidin-4-yl)benzamide

(R)-2-chloro-8-cyclopentyl-7-ethyl-5-methyl-7,8-dihydropteridin-6(5H)-one (14.7 mg, 0.05 mmol, 1.0 eq), [108] 4-amino-N-(1-methylpiperidin-4-yl)benzamide (14.0 mg, 0.06, 1.2 eq), Pd2(dba)3 (2.3 mg, 5 mol%), Xphos (3.6 mg, 15 mol%), and K2CO3 (27.6 mg, 0.2 mmol, 4 eq), were dissolved in tBuOH(0.5 mL, 0.1 M), in a capped vial and heated to 100°C. After stirring 16 hours the reaction was filtered through celite, concentrated and purified on silica (ISCO 4g, 0-15% DCM/MeOH) to give a yellow solid (7.5 mg, 31%).

^1^H NMR (400 MHz, Methanol-d4) δ 7.87 – 7.65 (m, 5H), 4.52 (q, J = 8.6, 8.1 Hz, 1H), 4.25 (dd, J = 7.8, 3.7 Hz, 1H), 3.96 (ddd, J = 15.2, 11.2, 4.1 Hz, 1H), 3.33 (s, 3H), 3.10 (d, J = 12.2 Hz, 2H), 2.48 (d, J = 3.8 Hz, 5H), 2.19 – 2.10 (m, 1H), 2.07 – 1.96 (m, 3H), 1.94 – 1.66 (m, 10H), 0.86 (t, J = 7.5 Hz, 3H).
LCMS: 492.5 (M+H), 246.6 ((M+H)/2)

![Compound 5](image)

**Compound 5**

(R)-4-((8-cyclopentyl-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)-3-isopropoxy-N-(1-methylpiperidin-4-yl)benzamide

\(^{1}H\) NMR (400 MHz, Methanol-d4) \(\delta\) 8.51 (d, \(J = 8.5\) Hz, 1H), 7.77 (s, 1H), 7.53 – 7.45 (m, 2H), 4.77 (dt, \(J = 12.1\), 6.0 Hz, 1H), 4.51 – 4.43 (m, 1H), 4.28 (dd, \(J = 7.6\), 3.7 Hz, 1H), 4.03 – 3.94 (m, 1H), 3.32 (s, 3H), 3.15 (d, \(J = 12.3\) Hz, 2H), 2.53 (d, \(J = 10.5\) Hz, 5H), 2.16 – 1.70 (m, 14H), 1.42 (d, \(J = 6.0\) Hz, 6H), 0.85 (t, \(J = 7.5\) Hz, 3H).

\(^{13}C\) NMR (100 MHz, cd3od) \(\delta\) 169.37, 165.53, 156.25, 153.67, 146.53, 139.20, 135.46, 127.44, 121.36, 117.59, 117.42, 113.18, 73.07, 61.76, 60.61, 55.38, 47.40, 45.28, 31.53, 30.39, 30.02, 28.59, 28.09, 24.56, 24.30, 22.39, 9.25.

LCMS 550.51 (M+H).

![Compound 6](image)

**Compound 6**

(R)-4-((8-cyclopentyl-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)-3-(cyclopentyloxy)-N-(1-methylpiperidin-4-yl)benzamide

\(^{1}H\) NMR (400 MHz, Methanol-d4) \(\delta\) 8.49 (d, \(J = 8.4\) Hz, 1H), 7.77 (s, 1H), 7.50 – 7.44 (m, 2H), 5.01 (dq, \(J = 5.8\), 2.9 Hz, 1H), 4.43 (p, \(J = 8.3\) Hz, 1H), 4.28 (dd, \(J = 7.5\), 3.6 Hz, 1H), 3.95 (dq, \(J = 8.4\), 5.6, 4.1 Hz, 1H), 3.33 (s, 3H), 3.07 (d, \(J = 12.1\) Hz, 2H), 2.47 – 2.36 (m, 5H), 2.20 – 1.82 (m, 16H), 1.82 – 1.67 (m, 7H), 0.85 (t, \(J = 7.5\) Hz, 3H).

LCMS 575.63 (M+H).

![Methyl 3-cyclobutoxy-4-nitrobenzoate](image)

**Methyl 3-cyclobutoxy-4-nitrobenzoate**

Methyl 3-hydroxy-4-nitrobenzoate (0.50 g, 2.54 mmol, 1.0 eq) and K₂CO₃ (0.525 g, 3.81 mmol, 1.5 eq) were dissolved in DMF (5.1 mL, 0.5 M) before bromocyclobutane (0.29 mL, 3.04 mmol, 1.2 eq) was added and the solution headed to 60°C. After stirring for 16 hours, the reaction was diluted with ½ saturated brine and extracted with EtOAc, dried over Na₂SO₄, filtered, and concentrated. The residue was purified on silica (ISCO 24g, 0-100% Hex/EtOAc) to give a white solid (57.62 mg, 46%).
^1^HNMR (400 MHz, Chloroform-^d^) δ 7.87 – 7.74 (m, 1H), 7.69 – 7.59 (m, 1H), 7.57 (dd, J = 10.5, 1.7 Hz, 1H), 4.82 (p, 1H), 3.94 (s, 4H), 2.66 – 2.40 (m, 2H), 2.35 – 2.11 (m, 2H), 2.05 – 1.83 (m, 1H), 1.83 – 1.59 (m, 1H).

LCMS: Poor ionization

3-cyclobutoxy-4-nitrobenzoic acid
Methyl 3-cyclobutoxy-4-nitrobenzoate (57.6 mg, 0.229 mmol, 1.0 eq) was dissolved in THF/H_2O (1.15 mL, 0.57 mL / 0.2 M, 0.4 M) before LiOH (8.2 mg, 0.344, 1.5 eq) was added. After stirring for 16 hours the reaction was diluted with 1 M HCl and extracted with DCM, dried over Na_2SO_4, filtered, and concentrated to give a cream colored solid (49.5 mg, 91%).

^1^HNMR (400 MHz, Methanol-^d^4) δ 7.78 (d, J = 8.3 Hz, 1H), 7.64 (d, J = 8.3, 1.6 Hz, 1H), 7.59 (s, 1H), 4.82 (p, J = 7.1 Hz, 1H), 2.59 – 2.44 (m, 2H), 2.21 (qdd, J = 10.0, 7.9, 2.8 Hz, 2H), 1.98 – 1.82 (m, 1H), 1.80 – 1.64 (m, 1H).

LCMS: Poor ionization

3-cyclobutoxy-N-(1-methylpiperidin-4-yl)-4-nitrobzamide
To a solution of 3-cyclobutoxy-4-nitrobenzoic acid (49.5 mg, 0.209 mmol, 1.0 eq) in DMF (0.84 mL, 0.25 M) was added 1-methyl-4-aminopiperidine (31.4 uL, 0.250 mmol, 1.2 eq), HOBt (42.3 mg, 0.313 mmol, 1.5 eq), DIPEA (72.6 uL, 0.417 mmol, 2.0 eq), and finally EDC (69.2 mg, 0.313, 1.5 eq). After stirring for 24 hours the solution was diluted with EtOAc, washed with saturated Na_2CO_3, water, and brine, before being dried over Na_2CO_3, filtered and concentrated. The residue was purified on silica (ISCO 4g, 0-15% DCM/MeOH) to give a cream colored solid (41.5 mg, 59.6%).

^1^HNMR (400 MHz, Chloroform-^d^) δ 7.80 (d, J = 8.3 Hz, 1H), 7.41 (s, 1H), 7.22 (d, J = 8.3, 1.7 Hz, 1H), 6.18 (d, J = 7.7 Hz, 1H), 4.83 (p, J = 7.1 Hz, 1H), 4.05 – 3.91 (m, 1H), 2.86 (d, J = 12.0 Hz, 2H), 2.66 (s, 1H), 2.50 (ddd, J = 9.7, 8.1, 6.1, 3.4 Hz, 2H), 2.33 – 2.15 (m, 6H), 2.11 – 1.98 (m, 2H), 1.96 – 1.85 (m, 1H), 1.79 – 1.55 (m, 3H).

LCMS: 333.9 (M+H)

4-amino-3-cyclobutoxy-N-(1-methylpiperidin-4-yl)benzamide
3-cyclobutoxy-N-(1-methylpiperidin-4-yl)-4-nitrobenzamide (41.5 mg, 0.124 mmol, 1.0 eq) was dissolved in EtOH (0.25 mL, 0.5 M), before SnCl2·H_2O (0.140 g, 0.622 mmol, 5 eq) was added and the reaction heated to 70°C. After stirring for 24 hours the solution was diluted with ice
water, and extracted with EtOAc, before combined organics were washed with brine, dried over Na₂CO₃, filtered, and concentrated to give a yellow oil (23.4 mg, 63% yield).

^1^HNMR (400 MHz, Methanol-\textit{d₄}) δ 6.93 (d, \textit{J} = 8.2, 2.0 Hz, 1H), 6.84 (s, 1H), 6.34 (d, \textit{J} = 8.2 Hz, 1H), 4.38 (p, \textit{J} = 7.2 Hz, 1H), 3.51 (tt, \textit{J} = 11.3, 4.1 Hz, 1H), 2.58 (d, \textit{J} = 12.3 Hz, 2H), 2.21 – 2.09 (m, 2H), 1.97 (s, 3H), 1.92 – 1.74 (m, 4H), 1.63 – 1.25 (m, 6H).

**LCMS:** 304.3 (M+H)

**Compound 7**
(R)-3-cyclobutoxy-4-((8-cyclopentyl-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)-N-(1-methylpiperidin-4-yl)benzamide

(R)-2-chloro-8-cyclopentyl-7-ethyl-5-methyl-7,8-dihydropteridin-6(5H)-one (19.1 mg, 0.0649, 1.0 eq), Pd₂(db)(2.3 mg, 5 mol%), Xphos (3.6 mg, 15 mol%), and K₂CO₃ (35.9 mg, 0.260 mmol, 4 eq), were dissolved in tBuOH (0.65 mL, 0.1 M), in a capped vial and heated to 100°C. After stirring for 16 hours the reaction was filtered through celite, concentrated and purified on silica (ISCO 4g, 0-15% DCM/Methanol) to give a yellow solid (8.55 mg, 23%).

^1^HNMR (400 MHz, Methanol-\textit{d₄}) δ 8.49 (d, \textit{J} = 8.5 Hz, 1H), 7.78 (s, 1H), 7.46 (dd, \textit{J} = 8.5, 2.0 Hz, 1H), 7.33 (s, 1H), 4.90 – 4.85 (m, 1H), 4.49 (p, \textit{J} = 8.5 Hz, 1H), 4.28 (dd, \textit{J} = 7.6, 3.7 Hz, 1H), 3.96 (ddt, \textit{J} = 11.3, 8.2, 4.2 Hz, 1H), 3.33 (s, 3H), 3.11 (d, \textit{J} = 12.2 Hz, 2H), 2.61 – 2.52 (m, 2H), 2.48 (s, 5H), 2.33 – 2.09 (m, 4H), 2.08 – 1.69 (m, 15H), 0.86 (t, \textit{J} = 7.5 Hz, 3H).

**LCMS:** 562.6 (M+H)

**Compound 7**
(R)-methyl 2-(((2-chloro-5-nitropyrimidin-4-yl)(cyclopentyl)amino)propanoate

(R)-methyl 2-(cyclopentylamino)propanoate (1.0 g, 5.37 mmol, 1.0 eq) and K₂CO₃ (891 mg, 6.45 mmol, 1.2 eq) were suspended in chilled acetone (21.5 mL, 0.25 M) before 2,4-dichloronitropyrimidine (1.15 g, 5.91 mmol, 1.1 eq) was added and the solution allowed to warm to RT overnight. After stirring for 16 hours the reaction was filtered, taken up in EtOAc, washed with brine, dried over Na₂SO₄, filtered, and concentrated. The residue was purified on silica (ISCO 4g, 0% DCM/EtOAc 0-30%) to give 990 mg solid (54%).

^1^HNMR ^1^H NMR (400 MHz, DMSO-\textit{d₆}) δ 8.79 (s, 1H), 4.42 (q, \textit{J} = 6.6 Hz, 1H), 3.62 (s, 3H), 3.55 – 3.53 (m, 1H), 1.99 (d, \textit{J} = 9.4 Hz, 1H), 1.86 – 1.80 (m, 2H), 1.72 – 1.60 (m, 3H), 1.50 (d, \textit{J} = 6.7 Hz, 3H), 1.42 (d, \textit{J} = 6.4 Hz, 2H).

**LCMS:** Poor ionization
(R)-2-chloro-8-cyclopentyl-7-methyl-7,8-dihydropteridin-6(5H)-one

(R)-methyl 2-((2-chloro-5-nitropyridin-4-yl)(cyclopentyl)amino)propanoate (900 mg, 2.62 mmol, 1.0 eq) was dissolved in AcOH (13 mL, 0.2 M) and heated to 70°C. Fe powder (180 mg, 20% by mass) was added and temperature increased to 100°C and stirred for 2 hours. The reaction was filtered through celite and concentrated to remove AcOH. The residue was purified on silica (ISCO 24g, 0-50% Hex/EtOAc) to recover starting material (95.9 mg, 10%) and give 271 mg brown solid (39%).

1HNMR (400 MHz, DMSO-d6) δ 10.74 (s, 1H), 7.59 (s, 1H), 4.29 – 4.09 (m, 2H), 1.96 – 1.75 (m, 6H), 1.54 (d, J = 6.5 Hz, 2H), 1.28 (d, J = 6.8 Hz, 3H).

LCMS: 328 (M+H)

(R)-2-chloro-8-cyclopentyl-5,7-dimethyl-7,8-dihydropteridin-6(5H)-one

(R)-2-chloro-8-cyclopentyl-7-methyl-7,8-dihydropteridin-6(5H)-one (250 mg, 0.936 mmol, 1.0 eq), was dissolved in DMA (3.74 mL, 0.25 M), before MeI (199 mg, 1.40 mmol, 1.5 eq) was added and the solution cooled to 0°C. NaH (33.6 mg, 1.40 mmol, 1.5 eq) was added, and the solution stirred 30 min at 0°C and 30 min at RT. The reaction was concentrated, taken up in EtOAc, washed with water, brine, and dried over Na2SO4, filtered, and concentrated. The residue was purified over silica (ISCO 12 g, 0-100% Hex/EtOAc), to give a brown solid (226 mg, 86%).

1HNMR (400 MHz, DMSO-d6) δ 7.89 (s, 1H), 4.38 (q, J = 6.8 Hz, 1H), 4.23 (p, J = 7.8 Hz, 1H), 3.21 (s, 3H), 1.92 – 1.75 (m, 6H), 1.58 – 1.47 (m, 2H), 1.26 (d, J = 6.8 Hz, 3H).

LCMS: 280 (M+H)

Compound 8

(R)-4-((8-cyclopentyl-5,7-dimethyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)-N-(1-methylpiperidin-4-yl)benzamide

(R)-2-chloro-8-cyclopentyl-7-methyl-7,8-dihydropteridin-6(5H)-one (28.1 mg, 0.1 mmol, 1.0 eq), 4-amino-N-(1-methylpiperidin-4-yl)benzamide (28.0 mg, 0.12 mmol, 1.2 eq), Xphos (7.15 mg, 0.015 mmol, 0.15 eq), and K2CO3 (55.3 mg, 0.4 mmol, 4.0 eq) was dissolved in t-BuOH (1 mL, 0.1 M) before Pd2(dba)3 was added and the mixture heated to 100°C in a capped vial. After stirring for 16 hours the reaction was cooled, diluted with EtOAc, filtered through celite, and concentrated. The residue was purified over silica (ISCO 4g, 0-15% DCM/MeOH) to give a yellow solid (37.3 mg, 78%).

1HNMR (400 MHz, DMSO-d6) δ 9.31 (s, 1H), 7.95 (d, J = 7.7 Hz, 1H), 7.88 (s, 1H), 7.78 (d, J = 9.0 Hz, 2H), 7.72 (d, J = 9.0 Hz, 2H), 4.51 – 4.40 (m, 1H), 4.33 – 4.23 (m, 1H), 3.75
– 3.60 (m, 1H), 3.22 (s, 3H), 2.76 (d, J = 11.7 Hz, 2H), 2.16 (s, 3H), 2.06 – 1.86 (m, 4H), 1.84 – 1.64 (m, 6H), 1.63 – 1.50 (m, 4H), 1.20 (d, J = 6.7 Hz, 3H).

**LCMS:** 478.4 (M+H)

**Compound 9**

(R)-4-((8-cyclopentyl-5,7-dimethyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)-3-isopropyloxy-N-(1-methylpiperidin-4-yl)benzamide

(R)-2-chloro-8-cyclopentyl-5,7-dimethyl-7,8-dihydropteridin-6(5H)-one (14.7 mg, 0.050 mmol, 1.0 eq), 4-amino-3-isopropyloxy-N-(1-methylpiperidin-4-yl)benzamide (17.5 mg, 0.060 mmol, 1.2 eq), X-phos (3.6 mg, 0.0075 mmol, 0.15 eq), and K$_2$CO$_3$ (27.6 mg, 0.20 mmol, 4.0 eq) were dissolved in t-BuOH (0.50 mL, 0.1 M). Pd$_2$(dba)$_3$ was added, and the reaction heated to 100°C in a capped vial. After stirring for 16 hours the reaction was cooled, filtered through celite, and concentrated. The residue was purified on silica (ISCO 4g, 0-15% DCM/MeOH) to give a yellow oil (7.67 mg, 28%).

$^1$HNMR (400 MHz, Methanol-d$_4$) $\delta$ 8.52 (d, J = 8.5 Hz, 1H), 7.82 (s, 1H), 7.52 – 7.46 (m, 2H), 4.78 (dt, J = 12.2, 6.1 Hz, 1H), 4.55 (dt, J = 16.2, 8.3 Hz, 1H), 4.37 (q, J = 6.8 Hz, 1H), 3.97 (ddd, J = 15.3, 11.3, 4.1 Hz, 1H), 3.32 (s, 3H), 3.10 (d, J = 12.2 Hz, 2H), 2.47 (s, 5H), 2.19 – 2.12 (m, 1H), 2.03 (d, J = 10.0 Hz, 3H), 1.95 – 1.83 (m, 4H), 1.83 – 1.72 (m, 4H), 1.42 (d, J = 6.0 Hz, 6H), 1.33 (d, J = 6.8 Hz, 3H).

**LCMS:** 536.4 (M+H)

(4-amino-3-methoxyphenyl)(4-hydroxypiperidin-1-yl)methanone

4-amino-3-methoxybenzoic acid (0.668 mg, 6.6 mmol, 1.1 eq), 4-hydroxypiperidine (1.0 g, 6 mmol, 1.0 eq), HOBt (1.22 g, 9.0 mmol, 1.5 eq), and DIPEA (2.09 mL, 12 mmol, 2 eq) were dissolved in DMF, and the solution cooled on ice. EDC (2.07 g, 10.8 mmol, 1.8 eq) was added and the solution was allowed to warm to RT overnight. After stirring for 16 hours the reaction was quenched with ice water, extracted with EtOAc, 10% MeOH/DCM, washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated. The residue purified on silica (ISCO 12g, 0-10% DCM/MeOH) to give a white foam (0.095 g, 63%).

$^1$HNMR (400 MHz, Chloroform-d) $\delta$ 6.93 (d, J = 1.7 Hz, 1H), 6.86 (dd, J = 7.9, 1.7 Hz, 1H), 6.65 (d, J = 7.9 Hz, 1H), 4.08 – 3.89 (m, 5H), 3.87 (s, 3H), 3.27 (ddd, J = 13.1, 9.3, 3.4 Hz, 2H), 1.90 (s, 2H), 1.57 – 1.50 (m, 2H).

**LCMS:** 251.1 (M+H)
Synthetic Procedures (C): Kinase inactive SAR (Table 2. Compounds).

**Compound 10**
(R)-4-((8-cyclopentyl-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)(methyl)amino)-3-methoxy-\(N\)-(1-methylpiperidin-4-yl)benzamide\[^{108}\]

\(^1\)H NMR (400 MHz, Methanol-\(d_4\)) \(\delta\) 7.58 (s, 1H), 7.55 (d, \(J = 1.9\) Hz, 1H), 7.51 (dd, \(J = 8.1, 1.9\) Hz, 1H), 7.28 (d, \(J = 8.1\) Hz, 1H), 4.16 (dd, \(J = 7.0, 3.5\) Hz, 1H), 4.06 – 3.98 (m, 1H), 3.82 (s, 3H), 3.79 – 3.70 (m, 1H), 3.33 (s, 3H), 3.28 (s, 3H), 3.23 – 3.12 (m, 2H), 2.56 (d, \(J = 17.6\) Hz, 5H), 2.07 (d, \(J = 10.9\) Hz, 2H), 1.97 – 1.60 (m, 9H), 1.36 (d, \(J = 5.7\) Hz, 4H), 0.80 (t, \(J = 7.5\) Hz, 3H).

LCMS 535.68 (M+H).

**Compound 11**
(R)-4-((8-cyclopentyl-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)oxy)-3-methoxy-\(N\)-(1-methylpiperidin-4-yl)benzamide\[^{108}\]

\(^1\)H NMR (400 MHz, Methanol-\(d_4\)) \(\delta\) 7.64 (s, 1H), 7.49 (d, \(J = 2.0\) Hz, 1H), 7.43 (dd, \(J = 8.2, 2.0\) Hz, 1H), 7.09 (d, \(J = 8.2\) Hz, 1H), 4.15 (dd, \(J = 6.6, 3.3\) Hz, 1H), 3.83 (d, \(J = 4.3\) Hz, 1H), 3.70 (s, 3H), 3.58 – 3.46 (m, 2H), 3.23 (s, 3H), 2.85 (d, \(J = 12.2\) Hz, 2H), 2.23 (s, 3H), 2.11 (t, \(J = 11.1\) Hz, 2H), 1.98 – 1.44 (m, 12H), 1.14 (dd, \(J = 8.3, 5.6\) Hz, 2H), 0.68 (t, \(J = 7.5\) Hz, 3H).

LCMS 522.68 (M+H).
Synthetic scheme 3. dihydropteridinone core & final compound synthesis.

(R)-tert-butyl (1-((2,6-dichloropyridin-3-yl)amino)-1-oxobutan-2-yl)carbamate
3-amino-2,6-dichloropyridine (0.50 g, 3.07 mmol, 1.0 eq) and Boc-D-ethylglycine (0.624 g, 3.07 mmol, 1.0 eq) were dissolved in pyridine (4 mL, 0.75 M) and the solution cooled to 0°C. Propylphosphonic acid (50% EtOAc solution, 9.1 mL, 5.0 eq) was added and the reaction allowed to warm to RT. After stirring for 16 hours the reaction was quenched by pouring into ice water, basificed with Na2CO3, and extracted with EtOAc. Combined organics were dried over Na2SO4, filtered, and concentrated. The residue was purified on silica (ISCO 24 g, 0-25% Hex/EtOAc) to give an off-white solid (0.418 g, 39%).

1H NMR (400 MHz, Chloroform-d) δ 8.71 (d, J = 8.5 Hz, 1H), 7.25 (d, 1H), 5.02 (d, J = 5.9 Hz, 1H), 4.17 (s, 1H), 2.05 – 1.94 (m, 1H), 1.77 – 1.67 (m, 1H), 1.45 (s, 9H), 1.02 (t, J = 7.4 Hz, 3H).

LCMS: (348.2 M+H)

(R)-2-amino-N-(2,6-dichloropyridin-3-yl)butanamide
(R)-tert-butyl (1-((2,6-dichloropyridin-3-yl)amino)-1-oxobutan-2-yl)carbamate (418 g, 1.20 mmol, 1.0 eq) was dissolved in DCM (12 mL, 0.1 M), before HCl (4 M / Dioxane, 4 mL, 25% v/v). After stirring for 10 hours the reaction was concentrated under a stream of N2, taken up in DCM/MeOH and re-concentrated to give an tan solid (0.426 g, mixture of HCl salts). Crude was carried through to next step.

1H NMR (400 MHz, Methanol-d4) δ 8.18 (d, J = 8.4 Hz, 1H), 7.61 (d, J = 8.3 Hz, 1H), 4.19 (t, J = 6.3 Hz, 1H), 2.07 – 1.89 (m, 2H), 1.05 (t, J = 7.5 Hz, 3H).

LCMS: (250.2 M+H)

(R)-2-(cyclopentylamino)-N-(2,6-dichloropyridin-3-yl)butanamide
(R)-2-amino-N-(2,6-dichloropyridin-3-yl)butanamide (0.426 g, 1.33 mmol, 1.0 eq) was dissolved in DCM (1.7 mL, 0.08 M) and a small amount of MeOH added to increase solubility before NaOAc (0.250 g, 3.05 mmol, 2.3 eq) was added. After stirring for 10 minutes NaBH(OAc)3 (0.956 g, 4.51 mmol, 3.4 eq), was added and the reaction allowed to warm to RT overnight. After stirring for 24 hours the reaction was diluted with sat NaHCO3, and extracted with DCM, dried over Na2SO4, filtered, and concentrated. The residue was purified on silica (ISCO 12g, 0-60% Hex/EtOAc to give a viscous oil (0.13 g, 34% over two steps).

1H NMR (400 MHz, Chloroform-d) δ 10.40 (s, 1H), 8.83 (d, J = 8.5 Hz, 1H), 7.25 (d, J = 8.5 Hz, 1H), 3.16 (dd, J = 7.9, 4.5 Hz, 1H), 3.10 (p, J = 6.4 Hz, 1H), 1.92 – 1.30 (m, 10H), 1.00 (t, J = 7.5 Hz, 3H).

LCMS: 316.2 (M+H)
(R)-6-chloro-4-cyclopentyl-3-ethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one

(R)-2-(cyclopentylamino)-N-(2,6-dichloropyridin-3-yl)butanamide (0.134 g, 0.423 mmol, 1.0 eq), was dissolved in DMF (4.2 mL, 0.1 M), before DIPEA (0.589 mL, 3.38 mmol, 8 eq) was added. The solution was equipped with a reflux condenser and heated 160°C. After stirring for 16 hours the reaction was cooled, diluted with water, and extracted with DCM, filtered over Na₂SO₄, and finally MeOH (2.13 mL, 0.13 mmol, 1.12 mmol, 5 eq) was added and the mixture heated to 90°C. After stirring for 16 hours the reaction was diluted with water, and extracted with EtOAc, dried over Na₂SO₄, filtered, and concentrated. The residue was purified on silica (ISCO 4g, 0-100%, Hex/EtOAc) to give a yellow oil (62 mg, 53%).

\[ ^{1}H \text{NMR (400 MHz, Chloroform-d)} \delta \]

for (R)-6-chloro-4-cyclopentyl-3-ethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one (62.4 mg, 0.223, 1.0 eq) was dissolved in dioxane (0.9 mL, 0.25 M), before K₂CO₃ (46.3 mg, 0.335 mmol, 1.5 eq), and finally Me₃PO₄ (0.13 mL, 1.12 mmol, 5 eq) were added and the mixture heated to 90°C. After stirring for 16 hours the reaction was diluted with water, and extracted with EtOAc, dried over Na₂SO₄, filtered, and concentrated. The residue was purified on silica (4g, 0-60% EtOAc/Hex) to give a yellow oil (38.5 mg, 59%).

\[ ^{1}H \text{NMR (400 MHz, Chloroform-d)} \delta \]

for (R)-6-chloro-4-cyclopentyl-3-ethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one (18.3 mg, 0.0624 mmol, 1.0 eq), 4-amino-3-methoxy-N-(1-methylpiperidin-4-yl)benzamide (19.7 mg, 0.0748 mmol, 1.2 eq), Xphos (4.5 mg, 0.00936 mmol, 0.15 eq), K₂CO₃ (34.5 mg, 0.250 mmol, 4 eq), and Pd₂dba₃ (2.9 mg, 0.00312 mmol, 0.05 eq) were dissolved in t-BuOH (0.62 mL, 0.1 M) and the mixture heated to 100°C. After stirring for 16 hours, the reaction was filtered through celite and concentrated. The residue was purified on silica (ISCO 4g, 0-10% DCM/MeOH) to give a yellow oil (13.1 mg, 40%).
\[^{1}\text{H} \text{NMR}\] (400 MHz, Methanol-d\textsubscript{4}) \(\delta\) 8.40 (d, \(J = 8.3\) Hz, 1H), 7.48 – 7.42 (m, 2H), 7.22 (d, \(J = 8.4\) Hz, 1H), 6.42 (d, \(J = 8.4\) Hz, 1H), 4.55 (dt, \(J = 14.2, 7.0\) Hz, 1H), 4.07 (dd, \(J = 8.7, 4.9\) Hz, 1H), 3.98 (s, 3H), 3.96 – 3.90 (m, 1H), 3.05 (d, \(J = 12.2\) Hz, 2H), 2.42 (s, 5H), 2.06 (ddd, \(J = 28.0, 20.0, 9.7\) Hz, 4H), 1.87 – 1.49 (m, 10H), 0.86 (t, \(J = 7.5\) Hz, 3H).

**LCMS:** 521.5 (M+H)

**Synthetic Procedures (D): Dihydropyridopyrazine SAR (Table 3. Compounds).**

**Synthetic scheme 4.** Dihydropyridopyrazine core synthesis.

(R)-tert-butyl (1-((2,6-dichloropyridin-3-yl)amino)-1-oxopropan-2-yl)carbamate

3-amino-2,6-dichloropyridine (4.00 g, 24.5 mmol, 1.0 eq) and Boc-D-Ala (5.57 g, 20.4 mmol, 1.2 eq) were dissolved in pyridine (32.7 mL, 0.75 M) and the solution cooled to 0°C. Propylphosphonic acid (50% EtOAc, 73 mL, 123 mmol, 5.0 eq) was added, and the reaction allowed to slowly warm to RT. After stirring for 24 hours the reaction was quenched by pouring into a mixture of NaOH, Na\_2CO\_3, and ice water, and stirred for 1 hour. The solution was extracted with EtOAc, washed with water, brine, dried over Na\_2SO\_4, filtered, and concentrated to give a reddish solid (carried through next step).

\[^{1}\text{H} \text{NMR}\] (500 MHz, Chloroform-d) \(\delta\) 8.90 (s, 1H), 8.73 (d, \(J = 8.5\) Hz, 1H), 7.27 (d, \(J = 8.5\) Hz, 1H), 5.01 (d, 1H), 4.36 (s, 1H), 1.51 – 1.44 (m, 12H).

**LCMS:** 336 (M+H)

(R)-2-amino-N-(2,6-dichloropyridin-3-yl)propanamide

(R)-tert-butyl (1-((2,6-dichloropyridin-3-yl)amino)-1-oxopropan-2-yl)carbamate (carried from above) was dissolved in 1:1 DCM (98 mL, 0.25 M) and TFA (98 mL, 0.25 M). After stirring for 2 hours at RT the reaction was concentrated, taken up in water, and washed with DCM. The
solution was basified with NaOH to pH 10, and extracted with DCM, dried over Na₂SO₄, filtered, and concentrated to give an off-white solid (4.24 g, 74% over two steps).

\(^1\)HNMR (500 MHz, DMSO-d₆) δ 8.69 (d, J = 8.5 Hz, 1H), 7.59 (d, J = 8.5 Hz, 1H), 7.25 – 7.14 (m, 1H), 5.78 (s, 1H), 5.15 (s, 3H), 3.51 (q, J = 7.0 Hz, 1H), 1.28 (d, J = 7.0 Hz, 3H).

**LCMS:** 235.1 (M+H)

(R)-2-(cyclopentylamino)-N-(2,6-dichloropyridin-3-yl)propanamide

(R)-2-amino-N-(2,6-dichloropyridin-3-yl)propanamide (800 mg, 3.42 mmol, 1.0 eq) was dissolved in DCM (0.05 M, 68 mL), before NaOAc (645 mg, 7.86 mmol, 2.3 eq) and cyclopentanone (454 uL, 5.13 mmol, 1.5 eq) were added, and the solution cooled to 0°C. Na(AcO)₃BH (2.46 g, 11.6 mmol, 3.4 eq) was added and the reaction allowed to warm to RT overnight. After stirring for 16 hours the reaction was quenched with water and NaOH, extracted with DCM, dried over Na₂SO₄, filtered, and concentrated. The residue was purified on silica (ISCO 12 g, 0-100% Hex/EtOAc) to give a clear oil (648 mg, 63%).

\(^1\)HNMR (500 MHz, Chloroform-d) δ 10.45 (s, 1H), 8.85 (d, J = 8.5 Hz, 1H), 7.28 (d, J = 8.5 Hz, 1H), 3.34 (q, J = 7.1 Hz, 1H), 3.18 (p, J = 6.3 Hz, 1H), 1.91 (dt, J = 12.9, 5.8 Hz, 1H), 1.83 (dt, J = 12.3, 5.8 Hz, 1H), 1.78 – 1.67 (m, 2H), 1.57 (tt, J = 14.3, 7.1 Hz, 2H), 1.47 – 1.31 (m, 5H).

**LCMS:** 303 (M+H)

(R)-6-chloro-4-cyclopentyl-3-methyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one

(R)-2-(cyclopentylamino)-N-(2,6-dichloropyridin-3-yl)propanamide (648 mg, 2.14 mmol, 1.0 eq) was dissolved in DMF (21.5 mL, 0.1M), DIPEA (3.0 mL, 17 mmol, 8.0 eq) was added, and the solution heated to 160°C under reflux. After stirring for 16 hours the reaction was cooled and diluted with EtOAc, washed with ½ sat Brine, water (3x), brine, dried over Na₂SO₄, filtered, and concentrated to give a yellow solid (501 mg, 88%).

\(^1\)HNMR (500 MHz, DMSO-d₆) δ 10.61 (s, 1H), 7.01 (d, J = 7.8 Hz, 1H), 6.68 (d, J = 7.8 Hz, 1H), 4.20 (p, J = 8.2 Hz, 1H), 4.13 (q, J = 6.8 Hz, 1H), 1.97 – 1.85 (m, 2H), 1.81 – 1.66 (m, 4H), 1.63 – 1.47 (m, 2H), 1.16 (d, J = 6.8 Hz, 3H).

**LCMS:** 268.2 (M+H)

(R)-6-chloro-4-cyclopentyl-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one

(R)-6-chloro-4-cyclopentyl-3-methyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one (501 mg, 1.88 mmol, 1.0 eq) was dissolved in dioxane (7.53 mL, 0.25 M), before K₂CO₃ (389 mg, 2.83 mmol, 1.5 eq), and finally Me₃PO₄ (1.32 g, 9.42 mmol, 5.0 eq) were added and the solution heated to 90°C under reflux. After stirring for 16 hours the reaction was cooled, concentrated, taken up in
EtOAc, washed with water (2X), brine, dried over Na₂SO₄, filtered, and concentrated. The residue was purified on silica (ISCO 12 g, 0-50% Hex/EtOAc) to give a slightly yellow oil which solidifies on standing (353 mg, 67%).

¹H NMR (500 MHz, Chloroform-d) δ 6.97 (d, J = 8.0 Hz, 1H), 6.67 (d, J = 8.0 Hz, 1H), 4.39 (p, J = 8.3 Hz, 1H), 4.29 (q, J = 6.8 Hz, 1H), 3.30 (s, 3H), 2.12 – 2.01 (m, 2H), 1.86 – 1.73 (m, 2H), 1.70 – 1.55 (m, 4H), 1.21 (d, J = 6.8 Hz, 3H).

LCMS: 281 (M+H)

**Synthetic scheme 5:** (General procedure 1) Buchwald-Hartwig coupling with varied arylamines.

**General procedure 1.** (see compound 13 for example)

(R)-6-chloro-4-cyclopentyl-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one (1.0 eq), Aryl amine (1.2 eq), Xphos (0.15 eq), and K₂CO₃ (4eq), were dissolved in t-BuOH (0.1 M) in a 2 mL vial. The headspace flushed with N₂, before Pd₂dba₃ (0.05 eq) was added, the reaction capped and heated to 100°C overnight. The reaction was cooled to RT, filtered through celite, and concentrated. The residue was purified on silica a(ISCO 4g 0-10% DCM/MeOH), b(ISCO 4g 0-15% DCM/MeOH (+2.5 M NH₃)), and/or c Preparative HPLC (acidic).

**Compound 13**

(R)-4-((4-cyclopentyl-1,3-dimethyl-2-oxo-1,2,3,4-tetrahydropyrido[2,3-b]pyrazin-6-yl)amino)-3-methoxy-N-(1-methylpiperidin-4-yl)benzamide

(R)-6-chloro-4-cyclopentyl-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one (21.0 mg, 0.075 mmol, 1.0 eq), 4-amino-3-methoxy-N-(1-methylpiperidin-4-yl)benzamide (39.5 mg, 0.15 mmol, 2.0 eq), (+) BINAP (9.3 mg, 0.015 mmol, 0.2), Cs₂CO₃ (122.2 mg, 0.375 mmol, 5 eq), and Pd(OAc)₂ (3.4 mg, 0.015 mmol, 0.2 eq) were dissolved in toluene (1.9 mL, 0.02 M) and the mixture heated to 110°C in a capped vial. After stirring for 16 hours, the reaction was filtered through celite and concentrated. The residue was purified on silica (ISCO 4g, 0-10% DCM/MeOH) to give a yellow oil (26.8 mg, 70%).

¹H NMR (400 MHz, Methanol-d₄) δ 8.42 (d, J = 8.6 Hz, 1H), 7.50 – 7.40 (m, 2H), 7.24 (d, J = 8.5 Hz, 1H), 6.43 (d, J = 8.4 Hz, 1H), 4.53 (p, J = 7.8 Hz, 1H), 4.26 (q, J = 6.7 Hz, 1H), 3.98 (s, 3H), 3.92 (ddt, J = 11.5, 8.7, 4.4 Hz, 1H), 3.30 (s, 3H), 3.01 (d, J = 12.3 Hz, 2H), 2.38 (s, 3H), 2.34 – 2.25 (m, 2H), 2.08 (ddt, J = 15.8, 7.9, 4.6 Hz, 2H), 1.98 (d, J = 10.2 Hz, 2H), 1.88 – 1.63 (m, 8H), 1.15 (d, J = 6.7 Hz, 3H).
LCMS: 507.6 (M+H)

**Compound 14**

(R)-4-((4-cyclopentyl-1,3-dimethyl-2-oxo-1,2,3,4-tetrahydropyrido[2,3-b]pyrazin-6-yl)amino)-N-(1-methylpiperidin-4-yl)benzamide

4-amino-N-(1-methylpiperidin-4-yl)benzamide was coupled according to General procedure 1a, 0.075 mmol scale.

Brown solid (26.8 mg, 75%)

$^1$HNMR (400 MHz, Methanol-$d_4$) δ 7.80 – 7.63 (m, 4H), 7.24 (d, $J$ = 8.4 Hz, 1H), 6.31 (d, $J$ = 8.4 Hz, 1H), 4.55 (p, $J$ = 7.8 Hz, 1H), 4.26 (q, $J$ = 6.7 Hz, 1H), 3.96 (ddt, $J$ = 11.0, 8.2, 4.1 Hz, 1H), 3.30 (s, 3H), 3.11 (d, $J$ = 12.3 Hz, 2H), 2.49 (s, 5H), 2.15 – 1.99 (m, 4H), 1.75 (dddd, $J$ = 22.2, 18.8, 10.8, 6.6 Hz, 8H), 1.15 (d, $J$ = 6.7 Hz, 3H).

**Compound 15**

(R)-4-((4-cyclopentyl-1,3-dimethyl-2-oxo-1,2,3,4-tetrahydropyrido[2,3-b]pyrazin-6-yl)amino)-3-isopropoxy-N-(1-methylpiperidin-4-yl)benzamide

4-amino-3-isopropoxy-N-(1-methylpiperidin-4-yl)benzamide was coupled according to General procedure 1b, 0.075 mmol scale.

Brown solid (26.7 mg, 67%)

$^1$HNMR (400 MHz, Methanol-$d_4$) δ 8.36 (d, $J$ = 8.5 Hz, 1H), 7.50 – 7.42 (m, 2H), 7.25 (d, $J$ = 8.4 Hz, 1H), 6.43 (d, $J$ = 8.4 Hz, 1H), 4.74 (hept, $J$ = 6.1 Hz, 1H), 4.52 (p, $J$ = 7.8 Hz, 1H), 4.26 (q, $J$ = 6.7 Hz, 1H), 3.94 (tt, $J$ = 11.3, 4.2 Hz, 1H), 3.30 (s, 3H), 3.04 (d, $J$ = 12.2 Hz, 2H), 1.41 (d, $J$ = 6.1 Hz, 6H), 1.16 (d, $J$ = 6.7 Hz, 3H).

**Compound 16**

(R)-4-((4-cyclopentyl-1,3-dimethyl-2-oxo-1,2,3,4-tetrahydropyrido[2,3-b]pyrazin-6-yl)amino)-3-(cyclopentyloxy)-N-(1-methylpiperidin-4-yl)benzamide

4-amino-3-(cyclopentyloxy)-N-(1-methylpiperidin-4-yl)benzamide<sup>[108]</sup> was coupled according to General procedure 1b, 0.086 mmol scale.

Brown solid (38.5, 80%)
HNMR (400 MHz, Methanol-d₄) δ 8.31 (d, J = 8.5 Hz, 1H), 7.51 – 7.40 (m, 2H), 7.24 (d, J = 8.4 Hz, 1H), 6.40 (d, J = 8.4 Hz, 1H), 4.98 (tt, J = 5.8, 2.7 Hz, 1H), 4.49 (p, J = 7.7, 7.2 Hz, 1H), 4.26 (q, J = 6.7 Hz, 1H), 4.01 (tt, J = 11.2, 4.0 Hz, 1H), 3.30 (s, 3H), 3.18 (d, J = 12.5 Hz, 2H), 2.69 – 2.47 (m, 5H), 2.14 – 1.63 (m, 20H), 1.16 (d, J = 6.7 Hz, 3H).

LCMS: 561.4 (M+H)

Synthetic Procedures (E): Tail SAR (Table 4. Compounds).

4-amino-3,5-dimethoxy-N-(1-methylpiperidin-4-yl)benzamide
4-amino-3,5-dimethoxybenzoic acid (98.5 mg, 0.5 mmol, 1.0 eq), 4-amino-1-methylpiperidine (85.7 mg, 0.75 mmol, 1.5 eq), and DIPEA (261 ul, 1.5 mmol, 3.0 eq) were dissolved in DMF (2.0 mL, 0.25 M), before HATU (228 mg, 0.60 mmol, 1.2 eq) was added. After stirring for 16 hours at RT, the reaction was diluted with MeOH and purified by preparative HPLC (acidic) to give a reddish solid (109 mg, 75%).

HNMR (500 MHz, Methanol-d₄) δ 7.19 (s, 2H), 4.13 (tt, J = 11.7, 3.9 Hz, 1H), 3.93 (s, 6H), 3.59 (d, J = 12.7 Hz, 2H), 3.17 (t, J = 12.6 Hz, 2H), 2.89 (s, 3H), 2.24 (d, J = 14.0 Hz, 2H), 1.94 – 1.86 (m, 2H).

LCMS: 295 (M+H)

Compound 17
(R)-4-((4-cyclopentyl-1,3-dimethyl-2-oxo-1,2,3,4-tetrahydropyrido[2,3-b]pyrazin-6-yl)amino)-3,5-dimethoxy-N-(1-methylpiperidin-4-yl)benzamide
4-amino-3,5-dimethoxy-N-(1-methylpiperidin-4-yl)benzamide was coupled according to General procedure 1⁸, 0.05 mmol scale.
Reddish solid (14.3 mg, 53%)

HNMR (500 MHz, Methanol-d₄) δ 7.27 (s, 2H), 7.17 (d, J = 8.4 Hz, 1H), 4.28 – 4.16 (m, 2H), 3.97 (tt, J = 11.3, 4.3 Hz, 1H), 3.89 (s, 6H), 3.02 (d, J = 11.9 Hz, 2H), 2.39 (s, 3H), 2.29 (t, J = 11.6 Hz, 2H), 2.02 (d, J = 11.2 Hz, 2H), 1.96 – 1.88 (m, 2H), 1.81 – 1.54 (m, 8H), 1.15 (d, J = 6.8 Hz, 3H).

LCMS: 537 (M+H)

4-amino-3-methoxy-N-((1-methylpiperidin-4-yl)methyl)benzamide
4-amino-3-methoxybenzoic acid (167 mg, 1.0 mmol, 1.0 eq), (1-methylpiperidinyl)methanamine (192 mg, 1.5 mmol, 1.5 eq), and DIPEA (5.22 mL, 3.0 mmol, 3.0 eq) were dissolved in DMF (5.0 mL, 0.2 M) and cooled to 0°C, before HATU (456 mg, 1.2 mmol, 1.2 eq) was added. After 4 hours the reaction was diluted with water, pH to 4 with 1N HCl, and washed with DCM. The
solution was then basified to pH 12 with NaOH and extracted with DCM/-PrOH 4:1. Combined organics were washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated. The residue was purified on silica (ISCO 12g, 0-15% DCM/MeOH (+2.5 M NH$_3$)) to give a tan solid (234 mg, 84%).

$^1$HNMR (500 MHz, Methanol-d$_4$) δ 7.32 (s, 1H), 7.28 (d, $J = 8.2$ Hz, 1H), 6.70 (d, $J = 8.2$ Hz, 1H), 3.88 (s, 3H), 3.34 (s, 1H), 3.24 (d, $J = 6.9$ Hz, 2H), 2.92 (d, $J = 11.8$ Hz, 2H), 2.29 (s, 3H), 2.08 (t, $J = 11.1$ Hz, 2H), 1.77 (d, $J = 13.1$ Hz, 2H), 1.70 – 1.61 (m, 1H), 1.33 (q, $J = 14.2$, 12.5 Hz, 2H).

LCMS: 278 (M+H)

**Compound 18**

(R)-4-((4-cyclopentyl-1,3-dimethyl-2-oxo-1,2,3,4-tetrahydropyrido[2,3-b]pyrazin-6-yl)amino)-3-methoxy-N-((1-methylpiperidin-4-yl)methyl)benzamide

4-amino-3-methoxy-N-((1-methylpiperidin-4-yl)methyl)benzamide was coupled according to **General procedure 1**b, 0.05 mmol scale.

Yellow solid (22.6 mg, 85%)

$^1$HNMR (500 MHz, Chloroform-d) δ 8.25 (d, $J = 8.4$ Hz, 1H), 7.46 (s, 1H), 7.26 (d, $J = 8.4$ Hz, 1H), 7.04 (d, $J = 8.3$ Hz, 1H), 6.36 (t, $J = 5.8$ Hz, 1H), 6.29 (d, $J = 8.3$ Hz, 1H), 4.55 (p, $J = 7.5$ Hz, 1H), 4.30 (q, $J = 6.7$ Hz, 1H), 3.38 (t, $J = 6.3$ Hz, 2H), 2.94 (d, $J = 11.5$ Hz, 2H), 2.11 – 2.02 (m, 4H), 1.82 – 1.68 (m, 8H), 1.49 – 1.40 (m, 2H), 1.27 (t, $J = 7.1$ Hz, 1H), 1.21 (d, $J = 6.8$ Hz, 3H).

LCMS: 523 (M+H)

**4-methoxy-N-(1-methylpiperidin-4-yl)-3-nitrobenzamide**

4-methoxy-3-nitrobenzoic acid (986 mg, 5 mmol, 1.0 eq), HOBT (1.15 g, 7.5 mmol, 1.5 eq), DIPEA (1.74 mL, 10 mmol, 2.0 eq), and 1-methylpiperidine-4-amine (570 mg, 5 mmol, 1.0 eq) were dissolved in DMF, before EDC (1.16 g, 7.5 mmol, 1.5 eq) was added. After 16 hours the reaction was poured into 1 M K$_2$CO$_3$, extracted and with EtOAc. The combined organics were washed with K$_2$CO$_3$, brine, dried over Na$_2$SO$_4$, filtered, and concentrated. The residue was purified on silica (ISCO 40g, 0-15% DCM/MeOH) to give a yellow solid (1.17 g, 80%).

$^1$HNMR (400 MHz, DMSO-d$_6$) δ 8.36 (d, $J = 8.8$ Hz, 2H), 8.14 (d, $J = 11.1$ Hz, 1H), 7.42 (d, $J = 8.9$ Hz, 1H), 3.96 (s, 3H), 3.71 (d, $J = 7.3$ Hz, 1H), 2.76 (d, $J = 11.9$ Hz, 2H), 2.16 (s, 3H), 1.95 (t, $J = 12.5$ Hz, 2H), 1.75 (d, $J = 15.7$ Hz, 2H), 1.64 – 1.44 (m, 2H).

LCMS: 294 (M+H)

**3-amino-4-methoxy-N-(1-methylpiperidin-4-yl)benzamide**
4-methoxy-N-(1-methylpiperidin-4-yl)-3-nitrobenzamide (117 mg, 4.0 mmol, 1.0 eq) and KF (465 mg, 8.0 mmol, 2.0) were combined in THF (40 mL, 0.1 M), before Pd(OAc)₂ (44.8 mg, 0.2 mmol, 0.05 eq) was added. Et₃SiH (2.55 mL, 15 mmol, 4.0 eq) was added dropwise. After 12 hours the reaction was diluted with water, extracted with EtOAc, and washed with brine, and filtered through celite. The solution was dried over Na₂SO₄, filtered, and concentrated. The residue was purified on silica (ISCO 24g, 0-15% DCM/MeOH) to give a reddish solid (750 mg, 80%).

**¹H NMR** (500 MHz, DMSO-d₆) δ 7.88 (d, J = 7.8 Hz, 1H), 7.13 (s, 1H), 7.07 (d, J = 8.3, 2.2 Hz, 1H), 6.80 (d, J = 8.4 Hz, 1H), 4.80 (s, 2H), 3.80 (s, 3H), 3.68 (d, J = 11.6 Hz, 1H), 2.75 (d, J = 11.7 Hz, 2H), 2.16 (s, 3H), 1.92 (t, J = 11.7 Hz, 2H), 1.71 (s, J = 12.1, 3.8 Hz, 2H).

**LCMS:** 264 (M+H)

**Compound 19**
(R)-3-(((4-cyclopentyl-1,3-dimethyl-2-oxo-1,2,3,4-tetrahydropyrido[2,3-b]pyrazin-6-yl)amino)-4-methoxy-N-(1-methylpiperidin-4-yl)benzamide

3-amino-4-methoxy-N-(1-methylpiperidin-4-yl)benzamide was coupled according to **General procedure** 1⁵, 0.05 mmol scale.

Yellow solid (20.8 mg, 82%)

**¹H NMR** 500 MHz, Chloroform-d) δ 8.38 (s, 1H), 7.17 (d, J = 10.2 Hz, 1H), 6.94 (d, J = 8.3 Hz, 1H), 6.78 (d, J = 8.4 Hz, 1H), 6.68 (s, 1H), 6.24 (d, J = 8.3 Hz, 1H), 5.88 (d, J = 7.8 Hz, 1H), 4.46 (p, J = 7.8 Hz, 1H), 4.20 (q, J = 6.7 Hz, 1H), 3.90 (s, 1H), 3.23 (s, 3H), 2.80 (d, J = 10.8 Hz, 2H), 2.26 (s, 3H), 2.12 (t, J = 11.3 Hz, 2H), 2.02 – 1.93 (m, 4H), 1.66 – 1.51 (m, 8H), 1.13 (d, J = 6.8 Hz, 3H).

**LCMS:** (507 M+H)

**4-methoxy-N-(((1-methylpiperidin-4-yl)methyl)-3-nitrobenzamide**

4-methoxy-3-nitro-benzoic acid (486 mg, 5 mmol, 1.0 eq), (1-methylpiperidin-4-yl)methanamine (128 mg, 1.0 mmol, 1.0 eq), and DIPEA (523 uL, 3.0 mmol, 3.0 eq) were dissolved in DMF (5 mL, 0.2 M), before HATU (456 mg, 1.2 mmol, 1.2 eq) was added. After stirring for 12 hours the reaction was diluted with EtOAc, washed with ½ sat. brine, water (3X), brine, dried over Na₂SO₄, filtered, and concentrated. The residue was purified on silica (ISCO 12 g, 0-15% DCM/MeOH (+2.5 M NH₃)) to give a brown solid (245 mg, 79%).

**¹H NMR** (500 MHz, Methanol-d₄) δ 8.29 (s, 1H), 8.08 (d, J = 8.8 Hz, 1H), 7.36 (d, J = 8.9 Hz, 1H), 4.01 (s, 4H), 3.27 (d, J = 6.8 Hz, 2H), 2.91 (d, J = 11.8 Hz, 2H), 2.28 (s, 4H), 2.05 (t, J = 11.1 Hz, 2H), 1.77 (d, J = 12.9 Hz, 2H), 1.66 (ddd, J = 11.2, 7.4, 4.2 Hz, 1H), 1.34 (tt, J = 12.0, 6.4 Hz, 2H).

**LCMS:** Poor ionization
3-amino-4-methoxy-N-((1-methylpiperidin-4-yl)methyl)benzamide

4-methoxy-N-((1-methylpiperidin-4-yl)methyl)-3-nitrobenzamide (243 mg, 0.791 mmol, 1.0 eq), and KF (91.9 mg, 1.58 mmol, 2.0 eq) were combined in THF (8 mL, 0.1 M), before Pd(OAc)$_2$ (8.9 mg, 0.040 mmol, 0.05 eq) was added. Et$_3$SiH was added dropwise, and the reaction stirred for 16 hours. The reaction was diluted with EtOAc, filtered through celite, dried over Na$_2$SO$_4$, filtered, and concentrated. The residue was purified on silica (ISCO 12 g, 0-15% DCM/MeOH (+ 2.5 M NH$_3$)) to give a reddish solid (175 mg, 63%).

$^{1}$HNMR (500 MHz, Methanol-$d_4$) $\delta$ 7.21 – 7.15 (m, 2H), 6.86 (d, $J$ = 8.0 Hz, 1H), 3.88 (s, 3H), 3.23 (d, $J$ = 6.9 Hz, 2H), 2.88 (d, $J$ = 11.8 Hz, 2H), 2.26 (s, 3H), 2.01 (t, $J$ = 11.0 Hz, 2H), 1.76 (d, $J$ = 13.2 Hz, 2H), 1.68 – 1.57 (m, 1H), 1.38 – 1.19 (m, 2H).

LCMS: 264 (M+H)

Compound 20

(R)-3-((4-cyclopentyl-1,3-dimethyl-2-oxo-1,2,3,4-tetrahydropyrido[2,3-b]pyrazin-6-yl)amino)-4-methoxy-N-((1-methylpiperidin-4-yl)methyl)benzamide

3-amino-4-methoxy-N-((1-methylpiperidin-4-yl)methyl)benzamide was coupled according to General procedure 1b, 0.05 mmol scale.

Yellow solid (16.5 mg, 63%)

$^{1}$HNMR (500 MHz, Chloroform-$d_6$) $\delta$ 8.53 (s, 1H), 7.29 (d, $J$ = 6.7 Hz, 1H), 7.02 (d, $J$ = 8.3 Hz, 1H), 6.86 (d, $J$ = 8.4 Hz, 1H), 6.80 (s, 1H), 4.57 (p, $J$ = 7.9 Hz, 1H), 4.28 (q, $J$ = 6.7 Hz, 1H), 3.94 (s, 2H), 3.30 (s, 3H), 2.92 (d, $J$ = 11.5 Hz, 2H), 2.31 (s, 2H), 2.11 – 2.05 (m, 2H), 1.77 (d, $J$ = 12.1 Hz, 2H), 1.65 (d, $J$ = 11.3 Hz, 4H), 1.41 (q, $J$ = 12.2 Hz, 2H), 1.20 (d, $J$ = 6.8 Hz, 3H).

LCMS: (521 M+H)

1-methyl-4-((4-nitrophenyl)sulfonyl)piperazine

4-nitrobenzene-1-sulfonyl chloride (0.50 g, 2.25 mmol, 1.0 eq) was dissolved in THF (23 mL, 0.1 M) before NEt$_3$ (0.94 mL, 6.78 mmol, 3.0 eq), and finally N-methylpiperazine (0.37 mL, 3.38 mmol, 1.5 eq) were added. After stirring for 12 hours at RT, the reaction was poured into $\frac{1}{2}$ sat NaHCO$_3$, extracted with DCM, dried over Na$_2$SO$_4$, filtered, and concentrated to give a yellow solid (0.58 g, 90%).

$^{1}$HNMR (400 MHz, DMSO-$d_6$) $\delta$ 8.44 (d, 2H), 8.01 (d, 2H), 3.02 – 2.93 (m, 4H), 2.39 – 2.33 (m, 4H), 2.14 (s, 3H).

LCMS: 286.2 (M+H)

4-((4-methylpiperazin-1-yl)sulfonyl)aniline
1-methyl-4-((4-nitrophenyl)sulfonyl)piperazine (0.191 g, 0.669 mmol, 1.0 eq) was dissolved in EtOH (1.3 mL, 0.5 M), before SnCl$_2·$2(H$_2$O) (0.755 mg, 3.34 mmol, 5.0 eq), was added and the reaction heated to 70°C in a capped vial. After stirring for 4 hours the reaction was diluted with water, basified with Na$_2$CO$_3$, and extracted with EtOAc. Combined organics were dried over Na$_2$SO$_4$, filtered, and concentrated to give a brown solid (0.21 g, acceptable impurities).

$^1$HNMR (400 MHz, DMSO-d$_6$) δ 7.34 (d, 2H), 6.65 (d, 2H), 6.06 (s, 2H), 2.78 (s, 4H), 2.34 (s, 4H), 2.13 (s, 3H).

LCMS: 256.2 (M+H)

Compound 21
(R)-4-cyclopentyl-1,3-dimethyl-6-(((4-methylpiperazin-1-yl)sulfonyl)phenyl)amino)-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one

4-((4-methylpiperazin-1-yl)sulfonyl)aniline was coupled according to General procedure 1b, 0.05 mmol scale.

Light yellow solid (9.29 mg, 37%)

$^1$HNMR (500 MHz, Methanol-d$_4$) δ 7.85 – 7.79 (m, 2H), 7.63 – 7.57 (m, 2H), 7.28 (d, J = 8.4 Hz, 1H), 6.35 (d, J = 8.4 Hz, 1H), 4.51 (p, J = 8.6, 8.1 Hz, 1H), 4.29 (q, J = 6.8 Hz, 1H), 3.32 (s, 3H), 3.02 (s, 4H), 2.51 (t, J = 4.6 Hz, 4H), 2.26 (s, 3H), 2.17 – 2.04 (m, 2H), 1.86 – 1.65 (m, 6H), 1.17 (d, J = 6.8 Hz, 3H).

LCMS: 499.2 (M+H)

1-(4-amino-3-methoxyphenyl)piperidin-4-ol

1-(3-methoxy-4-nitrophenyl)piperidin-4-ol (252 mg, 1 mmol, 1.0 eq) (Matrix Sci.), KF (116 mg, 2.0 mmol, 2.0 eq), and Pd(OAc)$_2$ (22.5 mg, 0.1 mmol, 0.1 eq) were dissolved in THF (10 mL, 0.1 M) and H$_2$O (1.25 mL, 0.8). Et$_3$SiH (0.64 mL, 4.0 mmol, 4.0 eq) was added dropwise. After stirring for 12 hours the reaction was filtered through celite, and concentrated. The residue was purified on silica (ISCO 24g, 0-15% DCM/MeOH) to give a black solid (222 mg, 99% yield).

$^1$HNMR (500 MHz, Chloroform-d) δ 6.63 (d, J = 8.3 Hz, 1H), 6.53 (d, J = 2.3 Hz, 1H), 6.43 (dd, J = 8.3, 2.3 Hz, 1H), 3.83 (d, J = 1.4 Hz, 3H), 3.79 (dq, J = 8.7, 4.5 Hz, 1H), 3.39 – 3.29 (m, 2H), 2.78 (td, J = 11.3, 10.1, 2.9 Hz, 2H), 2.06 – 1.95 (m, 2H), 1.73 (ddt, J = 9.5, 6.5, 3.3 Hz, 2H).

LCMS: 223.2 (M+H)

Compound 22
(R)-4-cyclopentyl-6-((4-(4-hydroxypiperidin-1-yl)-2-methoxyphenyl)amino)-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one

1-(4-amino-3-methoxyphenyl)piperidin-4-ol was coupled according to General procedure 1\(^a\), 0.05 mmol scale.

Brown solid (11.7 mg, 50%)

\(^1\)HNMR (500 MHz, Methanol-d\(4\)) \(\delta\) 7.80 (d, \(J = 8.7\) Hz, 1H), 7.16 (d, \(J = 8.5\) Hz, 1H), 6.68 (d, \(J = 2.3\) Hz, 1H), 6.55 (d, \(J = 7.3\) Hz, 1H), 6.21 (d, \(J = 8.3\) Hz, 1H), 4.43 (p, \(J = 8.1\) Hz, 1H), 4.21 (q, \(J = 6.7\) Hz, 1H), 3.87 (s, 3H), 3.74 (tt, \(J = 8.7, 4.0\) Hz, 1H), 3.44 (d, \(J = 19.7\) Hz, 2H), 3.28 (s, 3H), 2.84 (t, \(J = 9.8\) Hz, 2H), 2.08 – 1.95 (m, 4H), 1.83 – 1.60 (m, 8H), 1.14 (d, \(J = 6.7\) Hz, 3H).

LCMS: (466.4 M+H)

Compound 23

(R)-N-(4-cyclopentyl-1,3-dimethyl-2-oxo-1,2,3,4-tetrahydropyrido[2,3-b]pyrazin-6-yl)benzenesulfonamide

(R)-6-chloro-4-cyclopentyl-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one (14.0 mg, 0.05 mmol, 1.0 eq), Benzenesulfonamide (11.8 mg, 0.075 mmol, 1.5 eq), and K\(_2\)CO\(_3\) (13.8 mg, 0.10 mmol, 2.0 eq) were dissolved in MeTHF (500 uL, 0.1 M), before t-BuXphos (0.42 mg, 0.001 mmol, 0.02 eq), and ((allyl)PdCl\(_2\)) (0.37 mg, 0.001 mmol, 0.02 eq) were added in a 2 mL vial. The headspace was flushed with N\(_2\), vial capped, and reaction heated to 90°C. After stirring for 16 hours the reaction was filtered through celite and concentrated. The residue was diluted with acetone and purified on preparative HPLC to give a brown solid (12.6 mg, 63%).

\(^1\)HNMR (500 MHz, Chloroform-d) \(\delta\) 7.81 (d, \(J = 7.7\) Hz, 2H), 7.48 (t, \(J = 7.4\) Hz, 1H), 7.39 (t, \(J = 7.7\) Hz, 2H), 6.91 (d, \(J = 8.3\) Hz, 1H), 6.62 (d, \(J = 8.2\) Hz, 1H), 4.14 (q, \(J = 6.8\) Hz, 1H), 4.05 (p, \(J = 7.7\) Hz, 1H), 3.19 (s, 3H), 1.87 – 1.74 (m, 2H), 1.64 (dd, \(J = 16.8, 10.3\) Hz, 2H), 1.52 – 1.40 (m, 4H), 1.06 (d, \(J = 6.8\) Hz, 3H).

LCMS: 401.5 (M+H)

Compound 24

(R)-6-(benzylamino)-4-cyclopentyl-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one

(R)-6-chloro-4-cyclopentyl-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one (14.1 mg, 0.05 mmol, 1.0 eq) and benzylamine HCl (8.6 mg, 0.060 mmol, 1.2 eq) were slurried in dioxane (500 ul, 0.1 M), before NaO\(_t\)-Bu (14.4 mg, 0.15 mmol, 3.0 eq) was added, followed by Brettphos (1.3 mg, 0.05 mmol, 0.02) and BrettphosPdG3 (2.3 mg, 0.05 mmol, 0.02 eq) in a 2 mL vial. The headspace was flushed with N\(_2\), vial capped, and reaction heated to 100°C. After stirring for 12 hours the reaction was diluted with \(\frac{1}{2}\) sat. NaHCO\(_3\), extracted with EtOAc, dried over Na\(_2\)SO\(_4\), filtered through celite, and concentrated. The residue was purified on silica to give a yellow solid (12.1 mg, 69%).

\(^1\)HNMR (500 MHz, Chloroform-d) \(\delta\) 7.38 – 7.36 (m, 3H), 7.34 – 7.28 (m, 2H), 7.20 (d, \(J = 9.0\) Hz, 1H), 5.86 (d, \(J = 9.0\) Hz, 1H), 4.69 (d, \(J = 8.7\) Hz, 1H), 4.52 – 4.41 (m, 2H), 4.29 (q, \(J = 4.4\) Hz, 2H), 3.19 (s, 3H), 2.84 (t, \(J = 9.8\) Hz, 2H), 2.08 – 1.95 (m, 4H), 1.83 – 1.60 (m, 8H), 1.14 (d, \(J = 6.7\) Hz, 3H).
= 6.8 Hz, 1H), 3.29 (s, 3H), 2.40 – 2.31 (m, 1H), 2.10 – 2.03 (m, 1H), 1.80 – 1.73 (m, 4H), 1.66 – 1.53 (m, 4H), 1.31 (d, J = 6.9 Hz, 3H).

**LCMS:** (352 M+H)

**Compound 25**
(R)-6-(benzyl(methyl)amino)-4-cyclopentyl-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one

1-benzylmethylamine was coupled according to General procedure 1\(^a\), 0.05 mmol scale. Brown solid (9.6 mg, 53%)

\(^1\)HNMR (500 MHz, Chloroform-d) δ 7.26 – 7.23 (m, 3H), 6.92 (d, J = 8.1 Hz, 1H), 6.27 (d, J = 8.2 Hz, 1H), 4.75 (d, J = 14.4 Hz, 1H), 4.61 (d, J = 14.4 Hz, 1H), 4.29 (q, J = 6.8 Hz, 1H), 3.25 (s, 3H), 3.08 (s, 3H), 2.01 – 1.96 (m, 2H), 1.71 – 1.58 (m, 6H), 1.18 (d, J = 6.8 Hz, 3H).

**LCMS:** (366 M+H)

**4-bromo-3-methoxy-N-(1-methylpiperidin-4-yl)benzamide**

4-bromo-3-methoxybenzoic acid (231 mg, 1.0 mmol, 1.0 eq), 4-amino-1-methylpiperidine (137 mg, 1.2 mmol, 1.2 eq), and DIPEA (522 ul, 3.0 mmol, 3.0 eq) were dissolved in DMF (4.0 mL, 0.25 M), before HATU (456 mg, 1.2 mmol, 1.2 eq) was added. After stirring for 12 hours at RT, the reaction was diluted with MeOH, and purified by preparative HPLC to give a white solid (218 mg, 67%).

\(^1\)HNMR (500 MHz, Chloroform-d) δ 7.60 (d, J = 8.1 Hz, 1H), 7.43 (s, 1H), 7.25 (d, J = 8.2 Hz, 1H), 6.93 (d, J = 7.9 Hz, 1H), 4.37 – 4.20 (m, 1H), 3.97 (s, 3H), 3.65 (d, J = 12.0 Hz, 2H), 2.98 – 2.89 (m, 2H), 2.87 (s, 3H), 2.31 – 2.17 (m, 4H).

**LCMS:** 329 (M+H)

**3-methoxy-N-(1-methylpiperidin-4-yl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzamide**

4-bromo-3-methoxy-N-(1-methylpiperidin-4-yl)benzamide (110 mg, 0.25 mmol, 1.0 eq), bis(pinacolato)diboron (76.2 mg, 0.026 mmol, 0.10 eq), and KOAc (123 mg, 1.25 mmol, 5.0 eq) were dissolved in dioxane (2.5 mL 0.1 M), before PdCl\(_2\)(dppf)·CH\(_2\)Cl\(_2\) (20.4 mg, 0.025 mmol, 0.1 eq) was added. The headspace was flushed with N\(_2\), vial capped, and reaction heated to 90°C. After 12 hours the reaction was diluted with EtOAc, washed with mildly basified water (K\(_2\)CO\(_3\)), brine, dried over Na\(_2\)SO\(_4\), filtered, and concentrated to give a brown sludge (carried directly into next step).
LCMS: 376 (M+H)

**Compound 26**

(R)-4-(4-cyclopentyl-1,3-dimethyl-2-oxo-1,2,3,4-tetrahydropyrido[2,3-b]pyrazin-6-yl)-3-methoxy-N-(1-methylpiperidin-4-yl)benzamide

(R)-6-chloro-4-cyclopentyl-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one (28 mg, 0.10 mmol, 1.0 eq), 3-methoxy-N-(1-methylpiperidin-4-yl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzamide (approx. 0.12 mmol), and Na₂CO₃ (250 mL (1 M, H₂O), 0.25 mmol, 2.5 eq) were dissolved in dioxane (1.0 mL, 0.1 M), before PdCl₂(dppf)/gOCH₂Cl₂ (8.2 mg, 0.010 mmol, 0.10 eq) was added. The headspace was flushed with N₂, vial capped, and reaction heated to 100°C. After stirring for 12 hours, the reaction was diluted with EtOAc, filtered through celite, and concentrated. The residue was purified on silica (ISCO 4g 0-15% DCM/MeOH (+2.5 M NH₃)) to give a brown solid (26 mg, 53%).

¹H NMR (500 MHz, Methanol-d₄) δ 7.79 (d, J = 8.0 Hz, 1H), 7.61 – 7.55 (m, 3H), 7.39 – 7.36 (m, 1H), 4.48 – 4.38 (m, 2H), 4.21 – 4.13 (m, 1H), 3.97 (s, 3H), 3.60 (d, 2H), 3.40 (s, 3H), 3.18 (t, 2H), 2.89 (s, 3H), 2.26 (d, J = 13.9 Hz, 2H), 2.15 (s, 4H), 1.96 (q, J = 13.4, 12.3 Hz, 2H), 1.90 – 1.61 (m, 6H), 1.31 (d, J = 6.8 Hz, 3H).

LCMS: 492 (M+H)

¹H NMR (500 MHz, Methanol-d₄) δ 7.18 (s, 1H), 7.02 (s, 1H), 3.83 (s, 3H), 3.79 (s, 3H), 3.64 (s, 2H), 2.36 (s, 6H).

LCMS: 275 M+H

**1-(4-bromo-2,5-dimethoxyphenyl)-N,N-dimethylmethanamine**

4-bromo-2,5-dimethoxybenzaldehyde (245 mg, 1.0 mmol, 1.0 eq), dimethylamine HCl (122 mg, 1.5 mmol, 1.5 eq), and AcOH (57 uL, 1.0 mmol, 1.0 eq), and NaOAc (123 mg, 1.5 mmol, 1.5 eq) were dissolved in DCM. After stirring for 30 minutes, NaBH(OAc)₃ (424 mg, 2.0 mmol, 2.0 eq) was added. After stirring for 12 hours the reaction was diluted with 1 M NaOH, and 1 M K₂CO₃, and stirred 30 min. The solution was extracted with DCM, dried over Na₂SO₄, filtered, and concentrated. The residue was purified on silica (ISCO 12 g, 0-15% DCM/MeOH (+2.5 M NH₃)) to give a white solid (234 mg, 85%).

¹H NMR (500 MHz, Methanol-d₄) δ 7.18 (s, 1H), 7.02 (s, 1H), 3.83 (s, 3H), 3.79 (s, 3H), 3.64 (s, 2H), 2.36 (s, 6H).

LCMS: 275 M+H

**1-(2,5-dimethoxy-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-N,N-dimethylmethanamine**

1-(4-bromo-2,5-dimethoxyphenyl)-N,N-dimethylmethanamine (137 mg, 0.5 mmol, 1.0 eq), bis(pinacolatto)diboron (152 mg, 0.6 mmol, 1.2 eq), and KOAc (256 mg, 2.5 mmol, 5.0 eq) were
slurried in dioxane (5.0 mL, 0.1 M) in a 2 mL vial, before PdCl$_2$(dpdpf)·CH$_2$Cl$_2$ (40.8 mg, 0.05 mmol, 0.1 eq) was added. The headspace was flushed with N$_2$, vial capped, and reaction heated to 90°C. After stirring for 16 hours the reaction was diluted with EtOAc, washed with water/Na$_2$CO$_3$, brine, dried over Na$_2$SO$_4$, filtered, and concentrated to give a brown sludge (carried directly into next step).

**LCMS:** 323 (M+H)

**Compound 27**
(R)-4-cyclopentyl-6-(4-((dimethylamino)methyl)-2,5-dimethoxyphenyl)-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one
(R)-6-chloro-4-cyclopentyl-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one (28 mg, 0.1 mmol, 1.0 eq), 1-(2,5-dimethoxy-4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-N,N-dimethylmethanamine (approx. 0.12 mmol, 1.2 eq), and Na$_2$CO$_3$ (250 ul (1 M), 0.25 mmol, 2.5 eq) were dissolved in dioxane (1.0 mL, 0.1 M) in a 2 mL vial, before PdCl$_2$(dpdpf)·CH$_2$Cl$_2$ (8.17 mg, 0.010 mmol, 0.10 eq) was added. The headspace was flushed with N$_2$, vial capped, and reaction heated to 90°C. After stirring for 16 hours the reaction was diluted with EtOAc, filtered through celite, and concentrated. The residue was purified on silica (ISCO 4g, 0-15% DCM/MeOH (2.5 M NH$_3$)) to give a brown solid (20 mg, 46%).

**1H NMR** (500 MHz, Methanol-d$_4$) δ 7.70 (s, 1H), 7.55 (d, $J = 8.2$ Hz, 1H), 7.43 (d, $J = 8.2$ Hz, 1H), 7.23 (s, 1H), 4.44 – 4.33 (m, 4H), 3.95 (d, $J = 22.6$ Hz, 6H), 3.40 (s, 3H), 2.92 (s, 6H), 2.23 – 2.10 (m, 2H), 1.94 – 1.81 (m, 4H), 1.75 – 1.63 (m, 2H), 1.26 (d, $J = 6.8$ Hz, 3H).

**LCMS:** 439 (M+H)

**Synthetic Procedures (E): Tail SAR (Table 4. Compounds).**

**Synthetic scheme 6.** (General procedure 2) Buchwald-Hartwig coupling with varied aryl halides.

**General procedure 2.**
Acyl chloride (1.0 eq), (4-amino-3-methoxyphenyl)(4-hydroxypiperidin-1-yl)methanone (1.2 eq), X-phos (0.015 eq), and K$_2$CO$_3$ (4.0 eq), were slurried in t-BuOH (0.1 M) in a 2 mL vial, before Pd$_2$dba$_3$ (0.05 eq) was added. The headspace was flushed with N$_2$, vial capped, and reaction heated to 100°C. After stirring overnight, the reaction was diluted with EtOAc, filtered through celite, and concentrated. The residue was purified on silica $^a$(ISCO 4g, 0-10% DCM/MeOH), and/or $^b$preparative HPLC (acidic).
(4-hydroxypiperidin-1-yl)(3-isopropoxy-4-nitrophenyl)methanone
3-isopropoxy-4-nitrobenzoic acid[108] (328 mg, 1.45 mmol, 1.0 eq) and 4-hydroxypiperidine (147 mg, 1.45 mmol, 1.0 eq) were dissolved in DMF (7.3 mL, 0.2 M) before DIPEA (0.76 mL, 4.36 mmol, 3.0 eq), and finally HATU (553 mg, 1.45 mmol, 1.0 eq) were added. After stirring for 16 hours the reaction was diluted with sat. NaHCO₃, extracted with DCM, and washed with water, before being dried over Na₂SO₄, filtered, and concentrated. The residue was separated on silica (ISCO 24 g, 0-10% DCM/MeOH) to give a yellow oil (398 mg, 88%).

¹H NMR (500 MHz, Chloroform-d) δ 7.79 (d, J = 8.2 Hz, 1H), 7.10 (d, J = 1.4 Hz, 1H), 6.97 (dd, J = 8.2, 1.5 Hz, 1H), 4.69 (h, J = 12.2, 6.1 Hz, 1H), 4.03 (dq, J = 7.8, 3.9 Hz, 1H), 3.76 – 3.68 (m, 1H), 3.54 (d, J = 77.1 Hz, 2H), 3.18 (dt, J = 7.4, 3.7 Hz, 2H), 1.52 – 1.39 (m, 10H).

LCMS: 309.3 (M+H)

(4-amino-3-isopropoxyphenyl)(4-hydroxypiperidin-1-yl)methanone
(4-hydroxypiperidin-1-yl)(3-isopropoxy-4-nitrophenyl)methanone (398 mg, containing DMF) was dissolved in THF (13 mL, 0.1 M), before KF (150 mg, 2.58 mmol, 2.0 eq), Pd(OAc)₂(2.29 mg, 0.13 mmol, 0.10 eq), H₂O (1.6 mL, 0.8 M), and finally Et₃SiH (0.82 mL, 5.16 mmol, 4.0 eq) were added. After stirring for 1 hour the reaction was filtered through celite and concentrated. The residue was purified on silica (ISCO 24 g, 0-15% DCM/MeOH) to give a yellow oil (0.837 mmol, 65%).

¹H NMR 500 MHz, Chloroform-d) δ 6.92 (d, J = 1.6 Hz, 1H), 6.85 (dd, J = 7.9, 1.7 Hz, 1H), 6.67 (d, J = 7.9 Hz, 1H), 4.56 (hept, J = 6.0 Hz, 1H), 3.94 (d, J = 33.3 Hz, 5H), 3.26 (ddd, J = 13.2, 9.4, 3.4 Hz, 2H), 1.90 (s, 2H), 1.35 (d, J = 6.1 Hz, 6H).

LCMS: 279.4 (M+H)

Compound 28
(R)-4-cyclopentyl-6-((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one
(R)-6-chloro-4-cyclopentyl-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one was coupled according to General Procedure 2a, 0.05 mmol scale.

Yellow solid (9.68 mg, 39%)

¹H NMR (500 MHz, Methanol-d₄) δ 8.36 (d, J = 8.3 Hz, 1H), 7.25 (d, J = 8.4 Hz, 1H), 7.03 (d, J = 1.8 Hz, 1H), 6.97 (dd, J = 8.3, 1.8 Hz, 1H), 6.42 (d, J = 8.4 Hz, 1H), 4.49 (q, J = 8.0 Hz, 1H), 4.26 (q, J = 6.8 Hz, 1H), 3.95 (s, 3H), 3.90 (tt, J = 8.3, 3.9 Hz, 1H), 3.36 – 3.31 (m, 4H),
3.31 (s, 3H), 2.08 (tdd, J = 13.1, 11.0, 9.2, 5.9 Hz, 2H), 1.98 – 1.62 (m, 8H), 1.53 (s, 2H), 1.16 (d, J = 6.8 Hz, 3H).

**LCMS:** 494.2 (M+H)

Synthetic scheme 7. Dihydropyridopyrazine core cycloalkyl substituent exploration.

**General procedure 3.**

**Intermed 1.**
(R)-2-amino-N-(2,6-dichloropyridin-3-yl)propanamide (1.0 eq) was dissolved in DCM (0.05 M), before NaOAc (2.3 eq) and oxo-alkane (A) (1.5 eq) were added, and the solution cooled to 0°C. Na(AcO)₃BH (3.4 eq) was added and the reaction allowed to warm to RT overnight. After stirring for 16 hours the reaction was quenched with water and NaOH, extracted with DCM, dried over Na₂SO₄, filtered, and concentrated. The residue was purified on silica (ISCO, 0-100% Hex/EtOAc) to give the target compound.

**Intermed 2.**
Intermed 1 (1.0 eq) was dissolved in DMF (0.1M), DIPEA (8.0 eq) was added, and the solution heated to 160°C under reflux. The reaction was stirred to completion by LCMS (12 – 72 hours) before the reaction was cooled, diluted with EtOAc, washed with ½ sat Brine, water (3x), and brine, dried over Na₂SO₄, filtered, and concentrated to give the target compound.

**Intermed 3.**
Intermed 2 (1.0 eq) was dissolved in dioxane (0.25 M), before K₂CO₃ (1.5 eq), and finally Me₃PO₄ (5.0 eq) were added and the solution heated to 90°C under reflux. The reaction was stirred for 16 hours at which point reactions incomplete by LCMS were supplemented with
additional Me₃PO₄ and stirred to completion (12 - 72 hours). The reaction was cooled, concentrated, taken up in EtOAc, washed with water (2X), brine, dried over Na₂SO₄, filtered, and concentrated. The residue was purified on silica (ISCO 12 g, 0-50% Hex/EtOAc) to give the target compound.

\[
\text{(3R)-6-chloro-4-(3,3-dimethylcyclopentyl)-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one (racemic)}
\]
Prepared according to General procedure 3.
Light brown solid
\[
\text{¹H NMR (500 MHz, Chloroform-}d\text{) δ 6.97 (d, } J = 8.0 \text{ Hz, 1H), 6.67 (d, } J = 10.4 \text{ Hz, 1H), 4.54 – 4.38 (m, 1H), 4.28 (p, } J = 6.7 \text{ Hz, 1H), 3.30 (s, 3H), 2.15 – 2.11 (m, 1H), 1.88 – 1.75 (m, 2H), 1.75 – 1.66 (m, 2H), 1.47 (dt, } J = 15.2 \text{, 7.7 Hz, 1H), 1.23 – 1.19 (m, 3H), 1.12 (d, } J = 12.8 \text{ Hz, 3H), 1.06 (d, } J = 3.2 \text{ Hz, 3H).}
\]
LCMS: 310 (M+H)

\[
\text{Compound 29}
\]
(3R)-4-(3,3-dimethylcyclopentyl)-6-((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one (racemic)
(3R)-6-chloro-4-(3,3-dimethylcyclopentyl)-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one was coupled according to General procedure 2, 0.05 mmol scale.
Off-white solid (14.9, 57%)
\[
\text{¹H NMR (500 MHz, Chloroform-}d\text{) δ 8.30 (d, } J = 22.3 \text{ Hz, 1H), 7.11 – 6.92 (m, 4H), 6.25 (d, } J = 8.3 \text{ Hz, 1H), 4.80 (d, } J = 49.7 \text{ Hz, 1H), 4.31 (dq, } J = 13.5 \text{, 6.7 Hz, 1H), 3.99 (dt, } J = 8.2 \text{, 4.4 Hz, 1H), 3.94 (s, 3H), 3.31 (s, 3H), 2.21 – 2.11 (m, 1H), 2.01 – 1.66 (m, 7H), 1.66 – 1.46 (m, 6H), 1.24 – 1.18 (m, 3H), 1.14 (s, 3H), 1.11 (d, } J = 3.3 \text{ Hz, 3H).}
\]
LCMS: 522 (M+H)

\[
\text{(R)-6-chloro-4-cyclohexyl-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one}
\]
Prepared according to General procedure 3.
Light yellow solid
\[
\text{¹H NMR (500 MHz, DMSO-}d_6\text{) δ 7.32 (d, } J = 8.1 \text{ Hz, 1H), 6.77 (d, } J = 8.1 \text{ Hz, 1H), 4.31 (q, } J = 6.7 \text{ Hz, 1H), 4.12 (p, } J = 11.9 \text{, 3.5 Hz, 1H), 3.23 (s, 3H), 1.94 (d, } J = 11.9 \text{ Hz, 1H),}
\]
1.79 (t, J = 12.6 Hz, 2H), 1.74 – 1.66 (m, 1H), 1.66 – 1.49 (m, 3H), 1.41 – 1.26 (m, 2H), 1.22 – 1.15 (m, 1H), 1.11 (d, J = 6.7 Hz, 3H).

**LCMS:** 295 (M+H)

**Compound 30**
(R)-4-cyclohexyl-6-((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one

(R)-6-chloro-4-cyclohexyl-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one was coupled according to **General procedure 2.**

Light brown solid (17.6 mg, 70%)

**1HNMR** (500 MHz, Chloroform-d) δ 8.41 (d, J = 8.1 Hz, 1H), 7.04 – 6.96 (m, 4H), 6.19 (d, J = 8.3 Hz, 1H), 4.35 (dt, J = 12.3, 3.4 Hz, 1H), 4.33 – 4.28 (m, 1H), 4.02 – 3.96 (m, 2H), 3.94 (s, 3H), 3.49 (s, 2H), 3.33 (d, J = 11.8 Hz, 1H), 3.30 (s, 4H), 2.24 (d, J = 11.1 Hz, 1H), 2.05 – 1.82 (m, 4H), 1.75 (t, J = 13.3 Hz, 2H), 1.66 – 1.57 (m, 2H), 1.54 – 1.37 (m, 5H), 1.22 (d, J = 6.7 Hz, 3H).

**LCMS:** (508 M+H)

**Compound 31**
(R)-6-chloro-1,3-dimethyl-4-(tetrahydro-2H-pyran-4-yl)-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one

Prepared according to **General procedure 3.**

Off-white solid

**1HNMR** (500 MHz, DMSO-d6) δ 7.34 (d, J = 8.1 Hz, 1H), 6.81 (d, J = 8.1 Hz, 1H), 4.33 (q, J = 6.7 Hz, 2H), 3.94 (td, J = 13.0, 12.1, 3.3 Hz, 2H), 3.51 – 3.37 (m, 2H), 3.24 (s, 3H), 2.07 – 1.95 (m, 1H), 1.91 – 1.78 (m, 2H), 1.57 (d, J = 12.3 Hz, 1H), 1.13 (d, J = 6.7 Hz, 3H).

**LCMS:** 297 (M+H)

**Compound 32**
(R)-6-((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-1,3-dimethyl-4-(tetrahydro-2H-pyran-4-yl)-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one

(R)-6-chloro-1,3-dimethyl-4-(tetrahydro-2H-pyran-4-yl)-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one was coupled according to **General procedure 2.**

Light brown solid (12.1 mg, 48%)
\[^{1}\text{HNMR}\] (500 MHz, Chloroform-\text{d}) \(\delta 8.27\) (d, \(J = 9.0\) Hz, 1H), \(7.10 - 6.91\) (m, 4H), \(6.26\) (d, 1H), \(5.30\) (s, 1H), \(4.55\) (t, \(J = 11.6\) Hz, 1H), \(4.30\) (q, \(J = 5.7\) Hz, 1H), \(4.11\) (t, \(J = 11.7\) Hz, 4H), \(3.98\) (dd, \(J = 7.9, 4.0\) Hz, 1H), \(3.94\) (s, 3H), \(3.56\) (t, \(J = 12.2\) Hz, 2H), \(3.34\) (s, 1H), \(3.26\) (d, \(J = 12.2\) Hz, 1H), \(2.13\) (d, \(J = 12.2\) Hz, 1H), \(2.05 - 1.79\) (m, 6H), \(1.71\) (d, \(J = 12.4\) Hz, 1H), \(1.58\) (s, 2H), \(1.23\) (d, \(J = 7.8\) Hz, 3H).

**LCMS:** 511 (M+H)

(R)-6-chloro-4-cycloheptyl-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one
Prepared according to General procedure 3.
Yellow solid
\[^{1}\text{HNMR}\] (400 MHz, DMSO-\text{d}_6) \(\delta 7.27\) (d, \(J = 8.1\) Hz, 1H), \(6.73\) (d, \(J = 8.1\) Hz, 1H), \(4.30\) (q, \(J = 6.7\) Hz, 1H), \(3.20\) (s, 3H), \(1.99 - 1.84\) (m, 2H), \(1.82 - 1.40\) (m, 10H), \(1.11\) (d, \(J = 6.7\) Hz, 3H).

**LCMS:** 310 (M+H)

**Compound 32**
(R)-4-cycloheptyl-6-((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one
(R)-6-chloro-4-cycloheptyl-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one was coupled according to procedure General procedure 2., 0.05 mmol scale.
Off-white solid
\[^{1}\text{HNMR}\] (500 MHz, Chloroform-\text{d}) \(\delta 8.39\) (d, \(J = 8.2\) Hz, 1H), \(7.00\) (q, \(J = 9.7, 9.1\) Hz, 4H), \(6.19\) (d, \(J = 8.3\) Hz, 1H), \(4.44\) (q, \(J = 10.2, 8.6\) Hz, 1H), \(4.31\) (q, \(J = 6.7\) Hz, 1H), \(3.98\) (dq, \(J = 8.2, 3.9\) Hz, 2H), \(3.94\) (s, 3H), \(3.48\) (s, 2H), \(3.32\) (d, \(J = 10.2\) Hz, 1H), \(3.29\) (s, 4H), \(2.26 - 2.18\) (m, 1H), \(1.99 - 1.49\) (m, 19H), \(1.22\) (d, \(J = 6.7\) Hz, 3H).

**LCMS:** 528 (M+H)

(R)-4-benzyl-6-chloro-1,3-dimethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one
Prepared according to General procedure 3.
Light brown solid
\[^{1}\text{HNMR}\] (500 MHz, Chloroform-\text{d}) \(\delta 7.38 - 7.27\) (m, 5H), \(7.03\) (d, \(J = 8.0\) Hz, 1H), \(6.74\) (d, \(J = 8.0\) Hz, 1H), \(5.45\) (d, \(J = 15.0\) Hz, 1H), \(4.18 - 4.03\) (m, 2H), \(3.34\) (s, 3H), \(1.25\) (d, \(J = 6.8\) Hz, 3H).

**LCMS:** 302 (M+H)
Compound 33
(R)-4-benzyl-6-((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-1,3-diethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one

(R)-4-benzyl-6-chloro-1,3-diethyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one was coupled according to General procedure 2., 0.05 mmol scale.

Brown solid (10.8 mg, 42%)

$^1$HNR (500 MHz, Methanol-$d_4$) δ 8.09 (d, J = 8.3 Hz, 1H), 7.37 – 7.20 (m, 7H), 6.97 (s, 1H), 6.77 (d, J = 10.0 Hz, 1H), 6.41 (d, J = 8.4 Hz, 1H), 5.48 (s, 1H), 5.25 (d, J = 15.4 Hz, 1H), 4.33 (d, J = 15.4 Hz, 1H), 4.07 (q, J = 6.7 Hz, 1H), 3.91 (s, 3H), 3.87 (p, J = 9.0, 4.9 Hz, 1H), 3.32 (s, 3H), 3.29 – 3.24 (m, 2H), 2.14 (s, 1H), 1.87 (s, 2H), 1.36 – 1.26 (m, 4H), 1.19 (d, J = 6.8 Hz, 3H).

LCMS: 517 (M+H)

Synthetic scheme 8. Compound 34 synthesis.

(R)-tert-butyl 2-((2,6-dichloropyridin-3-yl)carbamoyl)piperidine-1-carboxylate

3-amino-2,6-dichloropyridine (163 mg, 1.0 mmol, 1.0 eq) and N-Boc-D-pipeolic acid (229 mg, 1.2 mmol, 1.2 eq) were dissolved in pyridine (1.33 mL, 0.75 M), and the solution cooled to 0°C. Propylphosphonic anhydride was slowly added, and the reaction allowed to warm to R.T. After 16 hours the reaction was found to be incomplete by LCMS. Additional N-Boc-D-pipeolic acid (114 mg, 0.05 mmol, 0.5 eq) was added and the reaction stirred for 6 hours. The mixture was poured into a solution of NaOH, $K_2CO_3$, and ice, and stirred for 1 hour, before extraction with EtOAc, drying over $Na_2SO_4$, filtering, and concentrating. The residue was purified on silica (ISCO 12g, 0-100% Hex/EtOAc) to give an off-white solid (212 mg, 57%).
HNMR (500 MHz, Chloroform-d) δ 8.74 (d, J = 8.5 Hz, 1H), 7.27 (d, J = 8.5 Hz, 1H), 5.02 – 4.87 (m, 1H), 4.20 – 3.99 (m, 1H), 2.87 – 2.79 (m, 1H), 2.33 (d, J = 10.4 Hz, 1H), 1.75 – 1.53 (m, 4H), 1.50 (s, 9H).

LCMS: 374 (M+H)

(R)-N-(2,6-dichloropyridin-3-yl)-N-methylpiperidine-2-carboxamide
1. (R)-tert-butyl 2-((2,6-dichloropyridin-3-yl)carbamoyl)piperidine-1-carboxylate (117 mg, 0.312 mmol, 1.0 eq) and K$_2$CO$_3$ (216 mg, 1.56 mmol, 5.0 eq) were slurried in dioxane (1.2 mL, 0.25 M), before Me$_3$PO$_4$ was added (182 uL, 1.56 mmol, 5.0 eq), the reaction vial was capped and heated to 90°C. After stirring for 72 hours the reaction was cooled, concentrated, taken up in EtOAc, washed with water, brine, dried over Na$_2$SO$_4$, filtered, and concentrated. The residue was purified on silica (ISCO 12 g, 0-50% Hex/EtOAc) to give a yellow solid (88 mg, 73%).

HNMR (500 MHz, Chloroform-d) δ 8.14 (d, J = 8.2 Hz, 1H), 7.42 (d, J = 8.2 Hz, 1H), 4.03 (dd, J = 8.1, 4.6 Hz, 1H), 3.58 – 3.52 (m, 1H), 3.38 – 3.33 (m, 1H), 3.22 (s, 3H), 2.09 (dt, J = 15.6, 8.7 Hz, 1H), 1.96 – 1.72 (m, 5H), 1.46 (s, 9H).

LCMS: 388 (M+H)

2. The solid was dissolved in DCM (1.1 mL, 0.2 M), before TFA (1.1 mL, 0.2 M) was added and the solution stirred for 4 hours at RT. The reaction was concentrated under a stream of N$_2$, azeotroped with MeOH, and re-concentrated to give an off-white solid (70 mg, quant). The product was carried directly through the next step.

LCMS: 288 (M+H)

(R)-2-chloro-5-methyl-7,8,9,10-tetrahydro-5H-dipyrido[1,2-a:3',2'-e]pyrazin-6(6aH)-one
(R)-N-(2,6-dichloropyridin-3-yl)-N-methylpiperidine-2-carboxamide (crude material, above) was dissolved in DMF, before DIPEA (313 uL, 1.8 mmol, 6.0 eq) was added, and the reaction heated to 160°C in a capped vial. After 16 hours the reaction was cooled, diluted with EtOAc, washed with ½ sat. brine, water (3X), brine, dried over Na$_2$SO$_4$, filtered, and concentrated. The residue was separated on silica (ISCO 12 g, 0-70% Hex/EtOAc) to give a tan solid (66 mg, 87%).

HNMR (500 MHz, Chloroform-d) δ 6.94 (d, J = 8.0 Hz, 1H), 6.66 (d, J = 8.0 Hz, 1H), 4.67 (d, J = 16.3 Hz, 1H), 3.94 (d, J = 11.1 Hz, 1H), 3.32 (s, 3H), 2.69 (d, J = 9.8 Hz, 1H), 2.26 (d, J = 9.7 Hz, 1H), 2.00 (s, 1H), 1.74 (d, J = 9.4 Hz, 1H), 1.59 (d, J = 9.4 Hz, 2H).

LCMS: 253 (M+H)

Compound 34
(R)-2-(((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-5-methyl-7,8,9,10-tetrahydro-5H-dipyrido[1,2-a:3',2'-e]pyrazin-6(6aH)-one
(R)-2-chloro-5-methyl-7,8,9,10-tetrahydro-5H-dipyrido[1,2-a:3',2'-e]pyrazin-6(6aH)-one was coupled according to **General procedure 2.**, 0.045 mmol scale.

Yellow solid (19.8 mg, 94%)

**^1HNMR** (500 MHz, Chloroform-d) δ 7.00 (d, J = 10.1 Hz, 4H), 6.25 (d, J = 8.3 Hz, 1H), 5.31 (s, 1H), 4.68 (s, 1H), 4.12 – 4.05 (m, 1H), 3.98 (td, J = 7.6, 7.0, 3.6 Hz, 2H), 3.93 (s, 3H), 3.34 (d, J = 3.0 Hz, 1H), 3.31 (s, 3H), 2.76 (s, 1H), 2.23 (d, J = 9.0 Hz, 1H), 1.98 – 1.89 (m, 4H), 1.76 (d, J = 11.3 Hz, 1H), 1.59 (s, 2H).

**LCMS:** 467 (M+H)

**Synthetic Procedures (F):** Additional core SAR (**Table 5.** Compounds).  

**Synthetic scheme 9.** (General procedure 3) dihydrobenzopyrazine core synthesis.

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**General procedure 4.** Dihydrobenzopyrazine core synthesis.

**Intermediate 1.**  
1,5-dihalogen-nitrobenzene derivative **A** (1.0 eq), D-alanine (1.2 eq), and K$_2$CO$_3$ (1.5 eq) were dissolved in H$_2$O (0.8M) and EtOH (3:1 ratio). After stirring 12 hours at RT, the product was precipitated with 1 N HCl, allowed to stand at 4°C for 20-40 min, and filtered to give the target compound.

**Intermediate 2.**  
Intermediate **1.** (1.0 eq), and K$_2$CO$_3$ (2.0 eq) were dissolved in water, and cooled to 0°C. Sodium dithionite (2.5 eq) was dissolved in a small amount of basified (K$_2$CO$_3$) water, and slowly added to the cooled reaction mixture over 15 minutes. The reaction was allowed to warm to RT for overnight. Additional aqueous dithionite was added as needed, and reaction stirred to
completion. The reaction was acidified with 1 M HCl and stirred for 10 minutes, before it was basified with Na₂CO₃, and extracted with DCM. The combined organics were washed with water, dried over Na₂SO₄, filtered, and concentrated to give the target compound.

**Intermediate 3.**
Intermediate 2, (1.0 eq), was dissolved in THF (0.15 M), before cyclopentanone (3.0 eq), phenylsilane (3.0 eq), and finally dibutyltin dichloride (1.0 eq) were added. The reaction was stirred at RT for 16 hours, concentrated under N₂, and the residue purified on silica (ISCO 12 g, 0-100% Hex/EtOAc) to give the target compound.

**Intermediate 4.**
Intermediate 3, (1.0 eq), and K₂CO₃ (5.0 eq), were combined in dioxane (0.25 M), before excess Me₃PO₄ was added (approx. 8 eq), and the reaction heated to 90°C in a capped vial. After stirring for 16 hours the reaction was cooled and quenched with water / NaOH, and stirred for 30 minutes. The solution was extracted with DCM, dried over Na₂SO₄, filtered, and concentrated. The residue was purified on silica (ISCO 12g, 0-80% Hex/EtOAc) to give the target compound.

(R)-2-((5-chloro-4-methoxy-2-nitrophenyl)amino)propanoic acid
1-chloro-5-fluoro-2-methoxy-4-nitrobenzene (411 mg, 2.0 mmol, 1.0 eq), D-alanine (214 mg, 2.5 mmol, 1.2 eq), and K₂CO₃ (415 mg, 3.0 mmol, 1.5 eq) were dissolved in H₂O (2.5 mL, 0.8M) and EtOH (7.5 mL (3:1)). After stirring 12 hours at RT, the product was precipitated with 1 N HCl, allowed to stand at 4°C for 20 min, and filtered to give a red solid (523 mg 82%).

¹HNMR (500 MHz, Methanol-d₄) δ 7.75 (s, 1H), 7.02 (s, 1H), 4.37 (q, J = 6.9 Hz, 1H), 3.86 (s, 3H), 1.56 (d, J = 6.9 Hz, 3H).

LCMS: Poor ionization

(R)-6-chloro-7-methoxy-3-methyl-3,4-dihydroquinoxalin-2(1H)-one
(R)-2-((5-chloro-4-methoxy-2-nitrophenyl)amino)propanoic acid (411 mg, 1.5 mmol, 1.0 eq), and K₂CO₃ (415 mg, 3.0 mmol, 2.0 eq) were dissolved in water, and cooled to 0°C. Sodium dithionite (653 mg, 3.75 mmol, 2.5 eq) was dissolved in a small amount of basified (K₂CO₃) water, and slowly added to the cooled reaction mixture over 15 minutes. The reaction was
allowed to warm to RT for 16 hours, and was found to be incomplete by LCMS. Additional aqueous dithionite (261 mg, 1.5 mmol, 1.0 eq) was added, and stirred for 6 hours. The reaction was acidified with 1 M HCl and stirred for 10 minutes, before it was basified with Na$_2$CO$_3$, and extracted with DCM. The combined organics were washed with water, dried over Na$_2$SO$_4$, filtered, and concentrated to give a poorly soluble white solid (115 mg, 34%).

$^1$HNMR (500 MHz, Methanol-d$_4$) δ 6.77 (s, 1H), 6.56 (s, 1H), 3.81 (q, 1H), 3.77 (s, 3H), 1.32 (d, $J = 6.7$ Hz, 3H).

LCMS: 227 (M+H)

(R)-6-chloro-4-cyclopentyl-7-methoxy-3-methyl-3,4-dihydroquinoxalin-2(1H)-one

(R)-6-chloro-7-methoxy-3-methyl-3,4-dihydroquinoxalin-2(1H)-one (105 mg, 0.41 mmol, 1.0 eq), was dissolved in THF (3.3 mL, 0.15 M), before cyclopentanone (107 uL, 1.22 mmol, 3.0 eq), phenylsilane (151 ul, 1.22 mmol, 3.0 eq), and finally dibutyltin dichloride (123 mg, 0.41 mmol, 1.0 eq) was added. The reaction was stirred at RT for 16 hours, concentrated under N$_2$, and the residue purified on silica (ISCO 12 g, 0-100% Hex/EtOAc) to give a white solid (105 mg, 87%).

$^1$HNMR (500 MHz, Methanol-d$_4$) δ 6.86 (s, 1H), 6.75 (s, 1H), 3.98 (q, $J = 6.9$ Hz, 1H), 3.81 (s, 3H), 3.57 (p, $J = 7.4$ Hz, 1H), 3.30 (s, 3H), 1.94 – 1.82 (m, 2H), 1.71 – 1.65 (m, 1H), 1.62 – 1.51 (m, 4H), 1.45 – 1.37 (m, 1H), 0.92 (d, $J = 6.9$ Hz, 4H).

LCMS: 296 (M+H)

(R)-6-chloro-4-cyclopentyl-7-methoxy-1,3-dimethyl-3,4-dihydroquinoxalin-2(1H)-one

(R)-6-chloro-4-cyclopentyl-7-methoxy-3-methyl-3,4-dihydroquinoxalin-2(1H)-one (73.8 mg, 0.25 mmol, 1.0 eq), and K$_2$CO$_3$ (166 mg, 1.2 mmol, 5.0 eq), were combined in dioxane (1.0 mL, 0.25 M), before excess Me$_3$PO$_4$ was added (approx. 8 eq), and the reaction heated to 90°C in a capped vial. After stirring for 16 hours the reaction was cooled and quenched with water / NaOH, and stirred for 30 minutes. The solution was extracted with DCM, dried over Na$_2$SO$_4$, filtered, and concentrated. The residue was purified on silica (ISCO 12 g, 0-80% Hex/EtOAc) to give a clear resin (65 mg, 84%).

$^1$HNMR (500 MHz, Methanol-d$_4$) δ 6.86 (s, 1H), 6.75 (s, 1H), 3.98 (q, $J = 6.9$ Hz, 2H), 3.81 (s, 3H), 3.57 (p, $J = 7.4$ Hz, 1H), 3.30 (s, 3H), 1.95 – 1.82 (m, 2H), 1.69 – 1.40 (m, 6H), 0.92 (d, $J = 6.9$ Hz, 3H).

LCMS: 311 (M+H)

Compound 38
(R)-4-cyclopentyl-6-((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-7-methoxy-1,3-dimethyl-3,4-dihydroquinoxalin-2(1H)-one

(R)-6-chloro-4-cyclopentyl-7-methoxy-1,3-dimethyl-3,4-dihydroquinoxalin-2(1H)-one was coupled according to General procedure 2., 0.05 mmol scale.

Brown solid (8.4 mg, 32%).

\[^1\text{HNMR}\] (500 MHz, Methanol-$d_4$) $\delta$ 7.10 (d, $J = 7.4$ Hz, 1H), 7.02 (s, 1H), 6.94 (d, $J = 6.3$ Hz, 2H), 6.82 (s, 1H), 3.93 (s, 3H), 3.92 – 3.85 (m, 3H), 3.30 (s, 3H), 1.99 – 1.85 (m, 4H), 1.74 – 1.48 (m, 8H), 1.00 (d, $J = 6.8$ Hz, 3H).

LCMS: 523 (M+H)

(R)-6-bromo-4-cyclopentyl-1,3-dimethyl-3,4-dihydroquinoxalin-2(1H)-one

Prepared according to General procedure 4.

Tacky brown resin

\[^1\text{HNMR}\] (500 MHz, Chloroform-$d_4$) $\delta$ 5.99 (d, $J = 8.4$ Hz, 1H), 6.94 (s, 1H), 6.80 (d, $J = 8.4$ Hz, 1H), 4.20 (q, $J = 6.8$ Hz, 1H), 3.77 (p, $J = 7.3$ Hz, 1H), 3.35 (s, 3H), 2.04 – 1.97 (m, 2H), 1.81 – 1.62 (m, 6H), 1.07 (d, $J = 6.8$ Hz, 3H).

LCMS: 324 (M+H)

Compound 35

(R)-4-cyclopentyl-6-((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-1,3-dimethyl-3,4-dihydroquinoxalin-2(1H)-one

(R)-6-bromo-4-cyclopentyl-1,3-dimethyl-3,4-dihydroquinoxalin-2(1H)-one was coupled according to General procedure 2., 0.05 mmol scale.

Yellow solid (9.7 mg, 39%)

\[^1\text{HNMR}\] (500 MHz, Methanol-$d_4$) $\delta$ 7.19 (d, $J = 8.2$ Hz, 1H), 7.05 (d, $J = 8.8$ Hz, 2H), 6.94 (dd, $J = 8.2$, 1.8 Hz, 1H), 6.82 – 6.75 (m, 2H), 4.15 (q, $J = 6.8$ Hz, 1H), 3.95 (s, 3H), 3.91 (tt, $J = 9.8$, 4.7 Hz, 1H), 3.80 (p, $J = 7.3$ Hz, 1H), 3.37 (s, 5H), 2.09 – 1.99 (m, 2H), 1.84 – 1.77 (m, 1H), 1.76 – 1.58 (m, 5H), 1.56 (d, $J = 19.2$ Hz, 2H), 1.05 (d, $J = 6.8$ Hz, 3H).

LCMS: 494 (M+H)

(R)-6-bromo-4-cyclopentyl-1,3,7-trimethyl-3,4-dihydroquinoxalin-2(1H)-one

Prepared according to General procedure 4.

Tacky brown resin
HNMR (500 MHz, Chloroform-\(d\)) \(\delta\) 6.98 (s, 1H), 6.79 (s, 1H), 4.16 (q, \(J = 6.8\) Hz, 1H), 3.72 (p, \(J = 7.3\) Hz, 1H), 3.34 (s, 3H), 2.35 (s, 3H), 2.18 – 2.15 (m, 1H), 2.01 (s, 3H), 1.72 – 1.64 (m, 4H), 1.04 (d, \(J = 6.8\) Hz, 3H).

LCMS: 338 (M+H)

Compound 36
(R)-4-cyclopentyl-6-((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-1,3,7-trimethyl-3,4-dihydroquinoxalin-2(1H)-one
(R)-6-bromo-4-cyclopentyl-1,3,7-trimethyl-3,4-dihydroquinoxalin-2(1H)-one was coupled according to General procedure 2., 0.05 mmol scale.
Light orange solid (11.8 mg, 47%)

HNMR (500 MHz, Chloroform-\(d\)) \(\delta\) 6.95 (s, 1H), 6.87 – 6.66 (m, 4H), 4.11 (s, 1H), 4.00 (s, 1H), 3.87 (s, 3H), 3.59 – 3.51 (m, 1H), 3.32 (d, \(J = 7.6\) Hz, 3H), 3.23 (ddd, \(J = 14.0, 9.7, 3.7\) Hz, 3H), 2.16 (s, 2H), 1.97 – 1.52 (m, 14H), 1.02 (d, 2H).

LCMS: 508 (M+H)

(R)-6-bromo-1,3,4,5-tetramethyl-3,4-dihydroquinoxalin-2(1H)-one
Prepared according to General procedure 4. *with omission of reductive amination step.
Brown solid

HNMR (500 MHz, Chloroform-\(d\)) \(\delta\) 7.38 (d, \(J = 8.7\) Hz, 1H), 6.76 (d, \(J = 8.7\) Hz, 1H), 3.65 (q, \(J = 7.3\) Hz, 1H), 3.38 (s, 3H), 2.61 (s, 3H), 2.44 (s, 3H), 1.16 (d, \(J = 7.3\) Hz, 3H).

LCMS: 284 (M+H)

Compound 37
(R)-6-((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-1,3,4,5-tetramethyl-3,4-dihydroquinoxalin-2(1H)-one
(R)-6-bromo-1,3,4,5-tetramethyl-3,4-dihydroquinoxalin-2(1H)-one was coupled according to General procedure 2., 0.05 mmol scale.
Pale yellow oil (11.7, 51%)

HNMR (500 MHz, Methanol-\(d_4\)) \(\delta\) 7.15 (d, \(J = 8.7\) Hz, 1H), 7.09 – 7.01 (m, 2H), 6.88 (dd, \(J = 8.2, 1.8\) Hz, 1H), 6.57 (d, \(J = 8.2\) Hz, 1H), 3.98 (s, 3H), 3.91 (dt, \(J = 8.1, 3.9\) Hz, 1H), 3.65 (q, \(J = 7.2\) Hz, 1H), 3.41 (s, 3H), 3.36 (d, \(J = 6.7\) Hz, 2H), 3.30 (dd, \(J = 9.7, 3.3\) Hz, 2H), 2.63 (s, 3H), 2.24 (s, 3H), 1.54 (s, 2H), 1.13 (d, \(J = 7.2\) Hz, 3H).

LCMS: 453 (M+H)
(R)-6-bromo-4-cyclopentyl-7-fluoro-1,3-dimethyl-3,4-dihydroquinoxalin-2(1H)-one  
Prepared according to General procedure 4.  
Tan solid  
$^1$HNMR: (500 MHz, Chloroform-$d$) δ 6.85 (d, $J$ = 6.5 Hz, 1H), 6.68 (d, $J$ = 9.6 Hz, 1H), 4.08 (q, $J$ = 6.9 Hz, 1H), 3.63 – 3.57 (m, 1H), 3.25 (s, 3H), 1.99 – 1.87 (m, 2H), 1.74 – 1.43 (m, 6H), 0.97 (d, $J$ = 6.8 Hz, 3H).  
LCMS: 343 (M+H)

Compound 39  
(R)-4-cyclopentyl-7-fluoro-6-((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-1,3-dimethyl-3,4-dihydroquinoxalin-2(1H)-one  
(R)-6-bromo-4-cyclopentyl-7-fluoro-1,3-dimethyl-3,4-dihydroquinoxalin-2(1H)-one was coupled according to General procedure 2., 0.05 mmol scale.  
(13.7 mg, 53% yellow solid)  
$^1$HNMR (500 MHz, Methanol-$d_4$) δ 7.10 (d, $J$ = 7.4 Hz, 1H), 7.02 (s, 1H), 6.94 (d, $J$ = 6.3 Hz, 2H), 6.82 (s, 1H), 3.93 (s, 3H), 3.92 – 3.85 (m, 3H), 3.30 (s, 3H), 1.99 – 1.85 (m, 4H), 1.74 – 1.48 (m, 8H), 1.00 (d, $J$ = 6.8 Hz, 3H).  
LCMS: 523 (M+H)

(R)-1-allyl-6-chloro-4-cyclopentyl-3-methyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one  
(R)-6-chloro-4-cyclopentyl-3-methyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one (50.0 mg, 0.189 mmol, 1.0 eq) was dissolved in DMF (0.76 mL, 0.25 M), and the solution cooled to 0°C. NaH (6.82 mg, 0.284 mmol, 1.5 eq) was added and the reaction stirred on ice for 30 min. Allyl bromide (24.6 uL, 0.284 mmol, 1.5 eq) was added and the reaction allowed to gradually warm to RT overnight. After 24 hours the reaction was quenched with 1/2 sat. NH$_4$Cl and extracted with EtOAc. Combined organics were washed with 1/2 sat brine, water (2X), brine, dried over Na$_2$SO$_4$, filtered, and concentrated. The residue was purified on silica (ISCO 4 g, 0-40% Hex/EtOAc) to give a tan solid (49.0 mg, 85%).  
$^1$HNMR 500 MHz, Chloroform-$d$) δ 6.98 (d, $J$ = 8.1 Hz, 1H), 6.63 (d, $J$ = 8.1 Hz, 1H), 5.83 (ddt, $J$ = 17.1, 9.8, 4.7 Hz, 1H), 5.22 (d, $J$ = 10.4 Hz, 1H), 5.13 (d, $J$ = 17.5 Hz, 1H), 4.77 (dd, $J$ = 16.9, 4.5 Hz, 1H), 4.47 – 4.37 (m, 1H), 4.31 (d, $J$ = 6.8 Hz, 1H), 4.22 – 4.08 (m, 1H), 2.09 – 2.03 (m, 2H), 1.79 (dq, $J$ = 15.7, 5.7, 5.0 Hz, 2H), 1.70 – 1.61 (m, 4H), 1.24 (d, $J$ = 6.8 Hz, 3H).  
LCMS: 308 (M+H)
Compound 40

(R)-1-allyl-4-cyclopentyl-6-((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-3-methyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one

(R)-1-allyl-6-chloro-4-cyclopentyl-3-methyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one was coupled according to General procedure 2.

Brown solid (10.9 mg, 42%).

$\text{HNMR (500 MHz, Chloroform-}d) \delta 8.21 (s, 1H), 7.07 – 6.87 (m, 4H), 6.24 (d, J = 8.3 Hz, 1H), 5.87 (ddd, J = 19.4, 10.2, 4.9 Hz, 1H), 5.22 (d, J = 10.5 Hz, 1H), 5.16 (d, J = 17.2 Hz, 1H), 4.80 – 4.71 (m, 1H), 4.56 (s, 1H), 4.32 (q, J = 6.7 Hz, 1H), 4.19 (d, J = 12.5 Hz, 1H), 3.98 (dq, J = 8.4, 3.9 Hz, 2H), 3.94 (s, 3H), 2.09 (d, J = 17.3 Hz, 2H), 1.93 (s, 4H), 1.86 – 1.54 (m, 10H), 1.25 (d, J = 6.7 Hz, 3H).

$\text{LCMS: 521 (M+H)}$

(R)-1-(but-3-en-1-yl)-6-chloro-4-cyclopentyl-3-methyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one

(R)-6-chloro-4-cyclopentyl-3-methyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one (50.0 mg, 0.189 mmol, 1.0 eq) was dissolved in DMF (0.76 mL, 0.25 M) and the solution cooled to 0°C. NaH (6.82 mg, 0.284 mmol, 1.5 eq) was added and the reaction stirred on ice for 30 min. 4-bromo-1-butene (28.8 uL, 0.28 mmol, 1.5 eq) was added and the reaction allowed to gradually warm to RT overnight. After 16 hours the reaction was quenched with $\frac{1}{2}$ sat NH$_4$Cl and extracted with EtOAc. The combined organics were washed with $\frac{1}{2}$ sat. brine, water (2X), brine, dried over Na$_2$SO$_4$, filtered, and concentrated. The residue was purified on silica (ISCO 4g, 0-100% Hex/EtOAc) to give a tan solid (41 mg, 67%).

$\text{HNMR (500 MHz, Chloroform-}d) \delta 6.98 (d, J = 8.1 Hz, 1H), 6.63 (d, J = 8.1 Hz, 1H), 5.89 – 5.78 (m, 1H), 5.27 – 5.19 (m, 1H), 5.18 – 5.09 (m, 1H), 4.81 – 4.71 (m, 1H), 4.41 (ddd, J = 15.6, 7.7, 4.1 Hz, 1H), 4.31 (q, J = 6.8 Hz, 1H), 4.22 – 4.11 (m, 1H), 2.12 – 2.01 (m, 3H), 1.86 – 1.73 (m, 2H), 1.72 – 1.55 (m, 5H), 1.24 (d, J = 6.8 Hz, 3H).

$\text{LCMS: 320 (M+H)}$
**Compound 41**

(R)-1-(but-3-en-1-yl)-4-cyclopentyl-6-((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-3-methyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one

(R)-1-(but-3-en-1-yl)-6-chloro-4-cyclopentyl-3-methyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one was coupled according to General procedure 2.

Yellow solid (23.2 mg, 87%)

^1H NMR (500 MHz, Chloroform-d) δ 6.99 – 6.84 (m, 4H), 6.20 (d, J = 8.3 Hz, 1H), 5.74 (ddt, J = 17.1, 10.2, 6.9 Hz, 1H), 5.04 – 4.93 (m, 2H), 4.46 (t, J = 8.2 Hz, 1H), 4.17 (q, J = 6.7 Hz, 1H), 3.92 – 3.89 (m, 1H), 3.86 (s, 3H), 3.80 – 3.71 (m, 1H), 3.40 (s, 1H), 2.31 (p, J = 7.0 Hz, 2H), 2.04 – 1.93 (m, 3H), 1.85 (d, J = 11.8 Hz, 2H), 1.78 – 1.65 (m, 3H), 1.63 – 1.46 (m, 6H), 1.11 (d, J = 6.7 Hz, 3H).

**LCMS:** 535 (M+H)

**Synthetic Procedures (G): Supplemental compounds.**

**Compound S1**

(R)-8-cyclopentyl-7-ethyl-2-((4-(4-hydroxypiperidine-1-carbonyl)-2-methoxyphenyl)amino)-5-methyl-7,8-dihydropteridin-6(5H)-one

(R)-6-chloro-4-cyclopentyl-3-ethyl-1-methyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one was coupled according to General procedure 2., 0.075 mmol scale.

Cream colored solid (28.0 mg, 73%)

^1H NMR (400 MHz, Methanol-d4) δ 7.70 (d, J = 8.0 Hz, 1H), 7.55 (s, 1H), 7.07 (dd, J = 1.7 Hz, 1H), 4.48 (dd, J = 6.5, 3.2 Hz, 1H), 4.22 (p, J = 8.8, 8.3 Hz, 2H), 3.98 – 3.87 (m, 4H), 3.74 (s, 1H), 3.44 (d, J = 7.2 Hz, 2H), 3.30 (s, 3H), 2.12 – 2.00 (m, 3H), 2.00 – 1.81 (m, 5H), 1.69 – 1.45 (m, 6H), 0.86 (t, J = 7.5 Hz, 3H).

**LCMS:** 509 (M+H)

**N-(tert-butyl)-4-nitrobenzenesulfonamide**

4-aminobenzene-1-sulfonyl chloride (0.50 g, 2.26 mmol, 1.0 eq) was dissolved in THF (23 mL, 0.1M) before triethylamine (0.98 mL, 6.77 mmol, 3.0 eq), and finally t-butylamine (0.36 mL, 3.38 mmol, 1.5 eq) were added. After stirring for 16 hours at RT, the reaction was diluted with ½ sat. NaHCO₃, extracted with EtOAc, dried over Na₂SO₄, filtered, and concentrated to give a cream colored solid (0.535 g, 92%).
$^1$HNMR (400 MHz, DMSO-d$_6$) δ 8.40 (d, 2H), 8.11 – 8.04 (m, 2H), 7.92 (s, 1H), 1.12 (s, 9H).

LCMS: (poor ionization)

4-amino-$N$-(tert-butyl)benzenesulfonamide
$N$-(tert-butyl)-4-nitrobenzenesulfonamide (0.535 g, 2.07 mmol, 1.0 eq) was dissolved in EtOH (8.3 mL, 0.25 M), before SnCl$_2$·2(H$_2$O) (2.35 g, 10.4 mmol, 5.0 eq) was added and the reaction heated to 70°C. After stirring for 24 hours the reaction was diluted with H$_2$O, basified with sat. Na$_2$CO$_3$, and extracted with EtOAc. Combined organics were washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated to give a yellow solid (0.437 g, 93%).

$^1$HNMR (400 MHz, Methanol-d$_4$) δ 7.55 (d, $J = 8.7$ Hz, 2H), 6.63 (d, $J = 8.6$ Hz, 2H), 3.88 (s, 2H), 2.00 (s, 1H), 1.13 (s, 9H).

LCMS: 229.2 (M+H)

(R)-$N$-(tert-butyl)-4-((8-cyclopentyl-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)benzenesulfonamide
(R)-6-chloro-4-cyclopentyl-3-ethyl-1-methyl-3,4-dihydropyrido[2,3-b]pyrazin-2(1H)-one (29.5 1.1 eq), 4-amino-$N$-(tert-butyl)benzenesulfonamide (25.1 mg, 0.11 mmol, 1.1 eq), XPhos (7.2 mg, 0.015 mmol, 0.15 eq), and K$_2$CO$_3$ (55.3 mg, 0.40 mmol, 4.0 eq) were dissolved in t-BuOH (1.0 mL, 0.1 M), before Pd$_2$dba$_3$ (4.6 mg, 0.005 mmol, 0.05 eq) was added in a 2 mL vial. The headspace was flushed with N$_2$, capped, and the reaction heated to 100°C. After stirring for 16 hours the reaction was cooled, filtered through celite, and concentrated. The residue was purified on silica (ISCO 4g, 0-100% Hex/EtOAc) to give a yellow oil (23.8 mg, 49%).

$^1$HNMR (400 MHz, Chloroform-d) δ 7.81 – 7.65 (m, 5H), 7.16 (s, 1H), 4.49 (s, 1H), 4.44 (q, $J = 7.9$ Hz, 1H), 4.24 (dd, $J = 7.8$, 3.7 Hz, 1H), 3.33 (s, 3H), 2.17 – 2.10 (m, 1H), 2.02 – 1.95 (m, 1H), 1.91 – 1.79 (m, 4H), 1.73 – 1.66 (m, 4H), 1.23 (s, 9H), 0.87 (t, $J = 7.5$ Hz, 3H).

LCMS: 488 (M+H)

Compound S2
(R)-4-((8-cyclopentyl-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)benzenesulfonamide’
(R)-$N$-(tert-butyl)-4-((8-cyclopentyl-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)benzenesulfonamide (17.8 mg, 0.037 mmol, 1.0 eq) was dissolved in TFA (1.0 mL) and anisole was added (10 uL (1:10 by vol)). After stirring for 16 hours at RT the reaction was neutralized with ½ sat Na$_2$CO$_3$, and extracted with DCM. Combined organics were dried over
Na₂SO₄, filtered, and concentrated to give a colorless oil which solidifies on standing (7.07 mg, 45%).

¹H NMR (400 MHz, Chloroform-d) δ 7.87 – 7.73 (m, 4H), 7.67 (s, 1H), 7.08 (s, 1H), 4.73 (s, 2H), 4.50 – 4.38 (m, 1H), 4.24 (dd, J = 7.9, 3.7 Hz, 1H), 3.33 (s, 3H), 2.12 (s, 1H), 1.97 (d, J = 8.6 Hz, 1H), 1.91 – 1.64 (m, 8H), 0.88 (t, J = 7.5 Hz, 3H).

LCMS: 431 (M+H)
Table A1. Data collection and refinement statistics.

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Additional materials for Chapter 2

Synthetic procedures

CRBN warhead and linker synthesis

![Chemical Structure]

### 2-(2,6-dioxopiperidin-3-yl)-4-hydroxyisoindoline-1,3-dione

3-Hydroxyphthalic anhydride (1.641 g, 10 mmol, 1 eq) and 3-aminopiperidine-2,6-dione hydrochloride (1.646 g, 10 mmol, 1 eq) were dissolved in pyridine (40 mL, 0.25 M) and heated to 110 °C. After 14 hours, the mixture was cooled to room temperature and concentrated under reduced pressure. Purification by column chromatography (ISCO, 24 g silica column, 0-10% MeOH/DCM) gave the desired product as a tan solid (2.424 g, 8.84 mmol, 88%).

**^1H NMR** (400 MHz, DMSO-d6) δ 11.08 (s, 2H), 7.65 (dd, \( J = 8.4, 7.2 \) Hz, 1H), 7.36 – 7.28 (m, 1H), 7.25 (dd, \( J = 8.4, 0.6 \) Hz, 1H), 5.07 (dd, \( J = 12.8, 5.4 \) Hz, 1H), 2.88 (ddd, \( J = 17.3, 14.0, 5.4 \) Hz, 1H), 2.63 – 2.50 (m, 2H), 2.08 – 1.95 (m, 1H).

**LCMS:** 275 (M+H)

### tert-butyl 2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)acetate

2-(2,6-dioxopiperidin-3-yl)-4-hydroxyisoindoline-1,3-dione (1.568 g, 5.71 mmol, 1 eq) was dissolved in DMF (57 mL, 0.1 M) at room temperature. Potassium carbonate (1.19 g, 8.58 mmol, 1.5 eq) and tert-butyl bromoacetate (0.843 mL, 5.71 mmol, 1 eq) were then added. After 2 hours, the mixture was diluted with EtoAc and washed once with water then twice with brine. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (ISCO, 24 g silica column, 0-100%EtoAc/hexanes, 21 minute gradient) gave the desired product as a cream colored solid (2.06 g, 5.30 mmol, 93%).

**^1H NMR** (500 MHz, Chloroform-d) δ 7.94 (s, 1H), 7.67 (dd, \( J = 8.4, 7.3 \) Hz, 1H), 7.52 (d, \( J = 6.8 \) Hz, 1H), 7.11 (d, \( J = 8.3 \) Hz, 1H), 4.97 (dd, \( J = 12.3, 5.3 \) Hz, 1H), 4.79 (s, 2H), 2.95 – 2.89 (m, 1H), 2.85 – 2.71 (m, 2H), 2.14 (ddt, \( J = 10.2, 5.0, 2.7 \) Hz, 1H), 1.48 (s, 9H).

**LCMS:** 389.33 (M+H)

### 2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)acetic acid

tert-butyl 2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)acetate (2.06 g, 5.30 mmol, 1 eq) was dissolved in TFA (53 mL, 0.1M) at room temperature. After 4 hours, the solution was diluted with DCM and concentrated under reduced pressure. The resultant cream colored solid...
(1.484 g, 4.47 mmol, 84%) was deemed sufficiently pure and carried onto the next step without further purification.

**1H NMR** (500 MHz, DMSO-d$_6$) δ 11.11 (s, 1H), 7.79 (dd, J = 8.4, 7.4 Hz, 1H), 7.48 (d, J = 7.2 Hz, 1H), 7.39 (d, J = 8.6 Hz, 1H), 5.10 (dd, J = 12.8, 5.4 Hz, 1H), 4.99 (s, 2H), 2.93 – 2.89 (m, 1H), 2.63 – 2.51 (m, 2H), 2.04 (ddd, J = 10.5, 5.4, 3.1 Hz, 1H).

**LCMS:** 333.25 (M+H).

**tert-butyl (8-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)acetamido)octyl)carbamate**

Boc-1,8-diaminoctane (2.10 g, 8.59 mmol, 1.1 eq) was dissolved in DMF (86 mL). In a separate flask, 2-((2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)acetic acid (2.60 g, 7.81 mmol, 1 eq) was dissolved in DMF (78 mL). The solution of Boc-1,8-diaminoctane in DMF was then added, followed by DIPEA (4.08 mL, 23.4 mmol. 3 eq) and HATU (2.97 g, 7.81 mmol, 1 eq). The mixture was stirred for 19 hours at room temperature, then diluted with EtOAc (600 mL). The organic layer was washed sequentially with 200 mL of half saturated sodium chloride, 200 mL 10% citric acid (aq.), 200 mL of half saturated sodium chloride, 200 mL of saturated sodium bicarbonate (aq.), 200 mL water and twice with 200 mL brine. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (ISCO, 40 g column, 0-5% MeOH/DCM, 35 minute gradient) gave the desired product as a white solid (3.53 g, 6.32 mmol, 81%).

**1H NMR** (500 MHz, Chloroform-d) δ 8.49 (s, 1H), 7.74 (dd, J = 8.3, 7.4 Hz, 1H), 7.55 (d, J = 7.2 Hz, 1H), 7.39 (t, J = 5.3 Hz, 1H), 7.19 (d, J = 8.4 Hz, 1H), 4.97 (dd, J = 12.4, 5.3 Hz, 1H), 4.63 (d, J = 2.2 Hz, 2H), 4.59 (d, J = 10.0 Hz, 1H), 3.36 (q, J = 6.9 Hz, 2H), 3.12 – 3.03 (m, 2H), 2.95 – 2.72 (m, 3H), 2.16 (ddt, J = 10.3, 5.2, 2.7 Hz, 1H), 1.59 (p, J = 7.1 Hz, 2H), 1.37 (d, J = 67.6 Hz, 19H).

**LCMS:** 559.47 (M+H).

**N-(8-aminoctyl)-2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)acetamide trifluoroacetate**

tert-butyl (8-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)acetamido)octyl)carbamate (3.53 g, 6.32 mmol, 1 eq) was dissolved in TFA (63 mL, 0.1M) and heated to 50 °C. After 1 hour, the mixture was cooled to room temperature, diluted with MeOH and concentrated under reduced pressure. The crude material was triturated with diethyl ether and dried under vacuum to give a white solid (2.93 g, 5.12 mmol, 81%).

**1H NMR** (500 MHz, Methanol-d$_4$) δ 7.82 (dd, J = 8.4, 7.4 Hz, 1H), 7.55 (d, J = 7.2 Hz, 1H), 7.44 (d, J = 8.4 Hz, 1H), 5.14 (dd, J = 12.5, 5.5 Hz, 1H), 4.76 (s, 2H), 3.33 (dd, J = 6.8, 1.8 Hz, 1H), 3.30 (s, 1H), 2.94 – 2.85 (m, 3H), 2.80 – 2.69 (m, 2H), 2.19 – 2.11 (m, 1H), 1.60 (dq, J = 24.8, 7.0 Hz, 4H), 1.37 (s, 8H).

**LCMS 459.45 (M+H).**
**tert-butyl 1'-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisocinolin-4-yl)oxy)acetyl)-[4,4'-bipiperidine]-1-carboxylate**

2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisocinolin-4-yl)oxy)acetic acid (166.1 mg, 0.50 mmol, 1 eq) and **tert-butyl [4,4'-bipiperidine]-1-carboxylate** (134.2 mg, 0.50 mmol, 1 eq) were dissolved in DMF (10 mL). DIPEA (261.3 microliters, 1.50 mmol, 3 eq) and HATU (190.1 mg, 0.50 mmol, 1 eq) were added and the mixture was stirred for 14 hours, upon which additional HATU (190.1 mg, 0.50 mmol, 1 eq) was added to ensure complete conversion. After an additional 8 hours, the mixture was diluted with EtOAc and washed with 10% citric acid (aq), saturated sodium bicarbonate, water and twice with brine. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (ISCO, 12 g silica column, 0-10% MeOH/DCM, 25 minute gradient) gave the desired product as a light yellow oil (0.33 g, quant yield), which was carried forward to the next step.

**1H NMR** (500 MHz, Chloroform-d) δ 7.68 (t, J = 7.9 Hz, 1H), 7.51 (d, J = 7.3 Hz, 1H), 7.31 (dd, J = 8.5, 7.3 Hz, 1H), 5.03 – 4.81 (m, 3H), 4.57 (t, J = 13.2 Hz, 1H), 4.19 – 3.92 (m, 3H), 3.02 (q, J = 13.1 Hz, 1H), 2.78 – 2.72 (m, 1H), 2.66 – 2.49 (m, 3H), 2.10 (d, J = 11.0 Hz, 1H), 1.82 – 1.51 (m, 5H), 1.45 (s, 9H), 1.39 – 1.05 (m, 7H).

**LCMS** 583.44 (M+H).

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**4-(2-([4,4'-bipiperidin]-1-yl)-2-oxoethoxy)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione trifluoroacetate**

**tert-butyl 1'-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisocinolin-4-yl)oxy)acetyl)-[4,4'-bipiperidine]-1-carboxylate** (0.33 g, ~0.566 mmol) was dissolved in TFA (5.7 mL) and heated to 50 °C. After 35 minutes, the mixture was cooled to room temperature, diluted with MeOH/DCM and concentrated under reduced pressure. The crude material was triturated with diethyl ether and dried under vacuum to give a cream colored solid (192.8 g, 0.478 mmol, 96% over 2 steps).

**1H NMR** (500 MHz, Methanol-d₄) δ 7.76 (dd, J = 8.5, 7.3 Hz, 1H), 7.50 (d, J = 6.2 Hz, 1H), 7.37 (d, J = 8.5 Hz, 1H), 5.17 (dd, J = 14.5, 5.2 Hz, 1H), 5.11 (dt, J = 12.6, 5.0 Hz, 1H), 5.03 (dd, J = 14.4, 2.4 Hz, 1H), 4.50 (d, J = 13.3 Hz, 1H), 4.07 (d, J = 13.2 Hz, 1H), 3.39 (d, J = 12.8 Hz, 2H), 3.10 (t, J = 12.3 Hz, 1H), 2.94 (t, J = 12.7 Hz, 2H), 2.89 – 2.82 (m, 1H), 2.79 – 2.61 (m, 3H), 2.14 (dq, J = 10.6, 3.1 Hz, 1H), 2.00 – 1.88 (m, 2H), 1.78 (d, J = 11.4 Hz, 2H), 1.53 – 1.27 (m, 5H), 1.09 (d, J = 12.2 Hz, 1H).

**LCMS** 483.35 (M+H).
tert-butyl (8-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)octyl)carbamate 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisindoline-1,3-dione (481.6 mg, 1.74 mmol, 1 eq) and tert-butyl (8-aminooctyl)carbamate (467.7 mg, 1.91 mmol, 1.1 eq) were dissolved in NMP (8.7 mL, 0.2M). DIPEA (606 uL, 3.48 mmol, 2 eq) was added and the mixture was heated to 90 °C. After 15 hours, the mixture was diluted with EtOAc and washed with 10% citric acid (aq), saturated sodium bicarbonate, water and three times with brine. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (ISCO, 12 g column, 0–5% MeOH/DCM, 25 minute gradient) gave the desired product as a yellow oil (0.55 g, 1.099 mmol, 63%).

1H NMR (500 MHz, Chloroform-d) δ 8.00 (s, 1H), 7.53 – 7.46 (m, 1H), 7.09 (d, J = 7.1 Hz, 1H), 6.88 (d, J = 8.5 Hz, 1H), 6.23 (d, J = 5.9 Hz, 1H), 4.92 (dd, J = 12.1, 5.2 Hz, 1H), 4.51 (s, 1H), 3.26 (q, J = 6.6 Hz, 2H), 3.11 (d, J = 5.9 Hz, 2H), 2.90 (d, J = 15.8 Hz, 1H), 2.83 – 2.72 (m, 2H), 2.15 – 2.11 (m, 1H), 1.65 (q, J = 7.1 Hz, 2H), 1.38 (d, J = 59.1 Hz, 19H).

LCMS 501.42 (M+H).

tert-butyl (8-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)octyl)carbamate 2-(2,6-dioxopiperidin-3-yl)-5-fluoroisindoline-1,3-dione (294 mg, 1.06 mmol, 1 eq) and tert-butyl (8-aminooctyl)carbamate (286 mg, 1.17 mmol, 1.1 eq) were dissolved in NMP (5.3 mL, 0.2M). DIPEA (369 uL, 2.12 mmol, 2 eq) was added and the mixture was heated to 90 °C. After 19 hours, the mixture was diluted with EtOAc and washed with water and three times with brine. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (ISCO, 12 g column, 0–10% MeOH/DCM, 30 minute gradient) gave the desired product as a brown solid (0.28 g, 0.668 mmol, 63%).

4-((8-aminooctyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione tert-butyl (8-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)octyl)carbamate (0.55 g, 1.099 mmol, 1 eq) was dissolved in TFA (11 mL) and heated to 50 °C. After 40 minutes, the mixture was cooled to room temperature, diluted with MeOH/DCM and concentrated under reduced pressure. The crude material was triturated with diethyl ether and dried under vacuum to give a cream colored solid (522.97 mg, 1.016 mmol, 93%).

1H NMR (500 MHz, Methanol-d4) δ 7.59 – 7.51 (m, 1H), 7.04 (dd, J = 7.9, 1.7 Hz, 2H), 5.06 (dd, J = 12.4, 5.5 Hz, 1H), 3.34 (d, J = 7.0 Hz, 2H), 2.95 – 2.81 (m, 3H), 2.79 – 2.66 (m, 2H), 2.15 – 2.08 (m, 1H), 1.67 (tt, J = 12.2, 7.2 Hz, 4H), 1.43 (d, J = 22.2 Hz, 8H).

LCMS 401.39 (M+H).
**1H NMR** (500 MHz, Chloroform-\(d\)) \(\delta\) 8.12 (s, 1H), 7.62 (d, \(J = 8.3\) Hz, 1H), 7.02 (s, 1H), 6.81 (d, \(J = 7.2\) Hz, 1H), 4.93 (dd, \(J = 12.3, 5.3\) Hz, 1H), 4.51 (s, 1H), 3.21 (t, \(J = 7.2\) Hz, 2H), 3.09 (d, \(J = 6.4\) Hz, 2H), 2.90 (dd, \(J = 18.3, 15.3\) Hz, 1H), 2.82 – 2.68 (m, 2H), 2.16 – 2.08 (m, 1H), 1.66 (p, \(J = 7.2\) Hz, 2H), 1.37 (d, \(J = 6.2\) Hz, 20H).

**LCMS** 501.41 (M+H).

5-((8-aminooctyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione trifluoroacetate
tert-butyl (8-((2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-5-yl)amino)octyl)carbamate (334.5 g, 0.668 mmol, 1 eq) was dissolved in TFA (6.7 mL) and heated to 50 °C. After 1 hour, the mixture was cooled to room temperature, diluted with DCM and concentrated under reduced pressure. The crude material was triturated with diethyl ether and dried under vacuum to give a dark yellow foam (253.1 mg, 0.492 mmol, 74%).

**1H NMR** (500 MHz, Methanol-\(d_4\)) \(\delta\) 7.56 (d, \(J = 8.4\) Hz, 1H), 6.97 (d, \(J = 2.1\) Hz, 1H), 6.83 (dd, \(J = 8.4, 2.2\) Hz, 1H), 5.04 (dd, \(J = 12.6, 5.5\) Hz, 1H), 3.22 (t, \(J = 7.1\) Hz, 2H), 2.85 – 2.68 (m, 3H), 2.09 (ddd, \(J = 10.4, 5.4, 3.0\) Hz, 1H), 1.70 – 1.61 (m, 4H), 1.43 (d, \(J = 19.0\) Hz, 8H).

**LCMS** 401.36 (M+H).

3-(4-(8-aminooctylamino)-1-oxoisooindolin-2-yl)piperidine-2,6-dione TFA salt

To a solution of lenalidomide (311mg, 1.2 mmol) in MeOH (8 mL) was added tert-butyl (8-o xoctyl)carbamate (291 mg, 1.2 mmol), NaBH₃CN (114 mg, 1.8 mmol) and 1 drop AcOH. The mixture was stirred at 50 °C overnight. The mixture was quenched with H₂O, extracted with EtOAc three times. The combined organic layers was dried over Na₂SO₄, filtered and concentrated. The crude product was used in the next step without further purification. The crude product above was dissolved in CH₂Cl₂/TFA (3 mL/ 3 mL). The mixture was stirred at room temperature for 1 h. The volatile was removed under reduced pressure. The crude product was purified by prep-HPLC to give the desired product (265 mg, 46 % over 2 steps) as a light yellow solid.

**1H NMR** (500 MHz, Methanol-d4) \(\delta\) 7.32 (t, \(J = 7.8\) Hz, 1H), 7.07 (d, \(J = 7.0\) Hz, 1H), 6.82 (d, \(J = 7.9\) Hz, 1H), 5.16 (dd, \(J = 13.3, 5.2\) Hz, 1H), 4.38 – 4.19 (m, 2H), 3.23 (td, \(J = 7.0, 2.4\) Hz, 2H), 2.97 – 2.87 (m, 3H), 2.79 (ddd, \(J = 17.6, 4.5, 2.4\) Hz, 1H), 2.47 (qd, \(J = 13.3, 4.6\) Hz, 1H), 2.18 (dtd, \(J = 12.9, 5.3, 2.4\) Hz, 1H), 1.65 (tq, \(J = 15.0, 7.2\) Hz, 4H), 1.50 – 1.35 (m, 8H).

**tert-butyl (6-((2,6-dioxopiperidin-3-yl)-1,3-dioxoisooindolin-5-yl)amino)hexyl)carbamate**

To a solution of 2-(2,6-dioxopiperidin-3-yl)-5-fluoroisoindoline-1,3-dione (210 mg, 0.76 mmol, 1.0 eq) and tert-butyl (6-aminohexyl)carbamate (Alfa Aesar, 181 mg, 0.84 mmol, 1.1 eq ) and in
NMP (3.8 mL), was added 265 µL DIPEA (1.52 mmol, 2.0 eq). After heating to 90°C for 18 hrs, the mixture was diluted to 60 mL with EtOAc, washed once with a mildly basic (Na₂CO₃) 1:1 solution of deionized water and saturated brine (20 mL), washed three times with deionized water (20 mL), and finally with saturated brine (20 mL) before drying over Na₂SO₄ and concentrating in vacuo. The residue was dissolved in DCM and purified by silica chromatography (DCM/MeOH 0 to 10 % gradient) to give the desired product as a brown oil (335 mg, 93%).

**¹H NMR** (500 MHz, Chloroform-d) δ = 8.31 (s, 1H), 7.49 (dd, J=7.8, 7.0, 1.4, 1H), 7.09 (d, J=7.2, 1.6, 1H), 6.87 (d, J=8.6, 1H), 6.24 (t, J=6.9, 5.6, 1H), 4.56 (s, 1H), 3.29 – 3.20 (m, 2H), 3.12 (q, J=6.8, 2H), 2.92 – 2.86 (m, 1H), 2.82 – 2.70 (m, 2H), 2.16 – 2.09 (m, 1H), 2.04 – 2.00 (m, 1H), 1.70 – 1.63 (m, 2H), 1.50 (p, J=7.2, 2H), 1.44 (s, 9H), 1.40 – 1.35 (m, 2H).

**LCMS**: 473 (M).

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5-((6-aminohexyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione TFA salt
tert-butyl (6-((2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-5-yl)amino)hexyl)carbamate (96.0 mg 0.203 mmol) was dissolved in TFA (2.03 mL and stirred for 4 hr at RT. The mixture was concentrated under a stream of nitrogen, followed by vacuum to give the crude product as a brown oil (75 mg, 76%). This material was used without further purification.

**LCMS**: 373 (M+H).

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tert-butyl (2-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)ethoxy)ethoxy)ethyl)carbamate
To a solution of 2-(2,6-dioxopiperidin-3-yl)-5-fluoroisoindoline-1,3-dione (210 mg, 0.76 mmol, 1.0 eq) and tert-butyl (2-(2-aminoethoxy)ethoxy)ethyl)carbamate (Oakwood Chem., 170.9 mg, 0.84 mmol, 1.1 eq) and in NMP (3.8 mL), was added 265 µL DIPEA (1.52 mmol, 2.0 eq). After heating to 90°C for 18 hrs, the mixture was diluted to 60 mL with EtOAc, washed once with a mildly basic (Na₂CO₃) 1:1 solution of deionized water and saturated brine (20 mL), washed three times with deionized water (20 mL), and finally with saturated brine (20 mL) before drying over Na₂SO₄ and concentrating in vacuo. The resulting brown oil was dissolved in DCM and purified by silica chromatography (DCM/MeOH 0 to 10 % gradient) to give the desired product as a brown oil (330 mg, 86%).

**¹H NMR** (500 MHz, Chloroform-d) δ = 8.57 (s, 1H), 7.53 – 7.46 (m, 1H), 7.11 (d, J=7.1, 1H), 6.92 (d, J=8.5, 1H), 6.52 (s, 1H), 4.96 – 4.88 (m, 1H), 3.73 (d, J=5.2, 2H), 3.66 (td, J=3.3, 1.8, 4H), 3.57 (s, 2H), 3.48 (q, J=5.3, 2H), 3.29 (d, J=32.6, 2H), 2.94 – 2.87 (m, 1H), 2.80 – 2.72 (m, 2H), 2.16 – 2.10 (m, 1H), 2.00 (d, J=7.7, 1H), 1.43 (d, J=3.8, 9H).

**LCMS**: 505 (M).
**Bromodomain Warhead Synthesis**

### ethyl 2-(4-bromo-2-methoxyphenoxy)acetate

To the solution of 4-bromo-2-methoxyphenol (5.0 g, 25 mmol) in acetone (100 mL) was added K₂CO₃ (10.2 g, 74 mmol), ethyl bromacetate (8.2 g, 49 mmol). The resulting solution was stirred at 80 °C overnight, and then cooled to room temperature. The mixture solution was concentrated under reduce pressure, and was diluted by ice water (50 mL) and was extracted with ethyl acetate (3 x 30 mL). The organic layer was washed with brine (300 mL) and dried over anhydrous Na₂SO₄. The organic layer was concentrated under reduce pressure. The residue was purified by column chromatography with PE / EA (2 / 1), to give ethyl 2-(4-bromo-2-methoxyphenoxy)acetate (6.3 g, 95%) as yellow oil.

### ethyl 2-(2-methoxy-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy)acetate

To the solution of ethyl 2-(4-bromo-2-methoxyphenoxy)acetate (2.9 g, 10 mmol) in dioxane (50 mL) was added bis(pinacolato)diboron (3.8 g, 15 mmol), Pd(dppf)Cl₂ (0.82 g, 1 mmol), KOAc (2.5 g, 25 mmol). The resulting solution was stirred at 80 °C overnight and then cooled to room temperature. The resulting solution was concentrated. The residue was purified by column chromatography with PE / EA (2 / 1), to give compound ethyl 2-(2-methoxy-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy)acetate (3.0 g, 90%) as brown oil. Purity is 84%, **LCMS**: 337.2 (M+H)
ethyl 2-{4-(2-cyano-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy}acetate
To the solution of 7-bromo-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridine-2-carbonitrile\textsuperscript{[16]} (2.0 g, 8 mmol) in DMF / water (10 / 1, 44 mL) was added ethyl 2-(2-methoxy-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy)acetate (4.0 g, 12 mmol), Pd(dppf)Cl\textsubscript{2} (0.64 g, 1 mmol), K\textsubscript{3}PO\textsubscript{4} (1.8 g, 8 mmol). The resulting solution was stirred at 70 °C for 5 h, and then cooled to room temperature. The resulting solution was poured into water and extracted with ethyl acetate (3 x 30 mL) and washed with brine (50 mL) and concentrated. The residue was purified by column chromatography (EtOAc) to give compound ethyl 2-(4-(2-cyano-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy)acetate (1.8 g, 60%) as a pale-yellow solid. Purity is 94%.

\textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) δ 8.14 (s, 1H), 7.25 (s, 1H), 6.97 (s, 1H), 6.95 (d, J = 8.0 Hz, 1H), 6.84 (d, J = 8.0 Hz, 1H), 4.68 (s, 2H), 4.22 (q, J = 7.2 Hz, 2H), 3.87 (s, 3H), 3.61 (s, 3H), 1.24 (t, J = 7.2 Hz, 3H).

LCMS: 399.1 (M+H)

ethyl 2-{4-(2-\text{N}(1,1-dioxidotetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy}acetate
To a solution of MeONa (0.54 mg, 0.01 mmol) in (dry) MeOH (80 mL) was added compound ethyl 2-{4-(2-cyano-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy}acetate (0.2 g, 0.5 mmol). The mixture was stirred at room temperature overnight with exclusion of atmospheric moisture. The mixture solution was added compound 4-aminotetrahydro-2H-thiopyran 1,1-dioxide hydrochloride (0.75 g, 5.0 mmol) and stirred at 75 °C for 7 days, and then cooled to room temperature. The resulting solution was concentrated, and the residue was purified by column chromatography (MeOH - DCM) to give compound ethyl 2-{4-(2-\text{N}(1,1-dioxidotetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy}acetate (0.13 g, 52%) as a pale-yellow solid. Purity is 92%.

LCMS: 534.2 (M+H)
2-(4-(2-(N-(1,1-dioxidotetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy)acetic acid

To the solution of ethyl 2-(4-(2-(N-(1,1-dioxidotetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy)acetate (0.2 g, 0.4 mmol) in MeOH (30 mL) was added LiOH (96.0 mg, 4.0 mmol). The result solution was stirred at room temperature for 2 h. Then the mixture solution was concentrated under reduce pressure. The residue was added water (5 mL), and HCl (36%) was carefully added until the pH ~ 3. Then it was filtered, the solid washed with water and dried to give compound ethyl 2-(4-(2-(N-(1,1-dioxidotetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy)acetate (104 mg, 50%) as a solid. Purity is 95%.

\[ ^1H \text{ NMR (400 MHz, DMSO-}d_6) \delta 8.18 (s, 1H), 7.75 (s, 1H), 7.16 (s, 1H), 7.11 (d, J = 8.0 Hz, 1H), 6.94 (d, J = 8.0 Hz, 1H), 4.57 (s, 2H), 3.84 (s, 3H), 3.56 (s, 3H), 3.18-3.08 (m, 4H), 2.03-1.96 (m, 4H). \]

\[ \text{LCMS: 520.2 (M+H)} \]

4-bromo-2-methyl-2,7-naphthyridin-1(2H)-one

To a fine suspension of 4-bromo-2-methyl-2,7-naphthyridin-1(2H)-one (996 mg, 4.43 mmol, 1.0 eq) and Cesium Carbonate (4330 mg, 13.3 mmol, 3.0 eq) in THF (17.7 mL) was added Iodomethane (551 µL, 8.86 mmol, 2.0 eq) and stirred at RT. After 22hrs, the mixture was concentrated in vacuo, and the resulting residue dissolved in DCM. Insoluble material was filtered and washed with both DCM and water before being discarded. Organic filtrate was collected (approx. 150mL), washed three times with deionized water (30 mL), and finally with saturated brine (30 mL), before being dried over Na2SO4 and concentrated in vacuo to give the desired product as an off-white solid (1038 mg, 98%).

\[ ^1H \text{ NMR (500 MHz, DMSO-}d_6) \delta = 9.36 (s, 1H), 8.88 (s, 1H), 8.25 (s, 1H), 7.61 (s, 1H), 3.54 (s, 3H). \]

\[ \text{LCMS: 239 (M)} \]

\[ \text{tert-butyl 2-((4-bromo-2,6-dimethoxybenzyl)(methyl)amino)acetate} \]
Sarcosyl tert-butyl ester hydrochloride (556 mg, 3.06 mmol, 1.5 eq) was dissolved in a solution of NaOAc (251 mg, 3.06 mmol, 1.5 eq), in DCM (8.2 mL), before 167 µL AcOH (2.04 mmol, 1.0 eq) was added, followed by 4-bromo-2,6-dimethoxybenzaldehyde (500 mg, 2.04 mmol, 1.0 eq). The mixture was stirred for 10 min before sodium triacetoxy borohydride was added in one portion (864.8 mg, 4.08 mmol, 2.0 eq), and the mixture stirred for 18 hr. The reaction was basified to approximately pH 11 with 1M K$_2$CO$_3$ and extracted 4 times with DCM (10 mL). The combined organics were washed with deionized water (10 mL), and saturated brine (10 mL), before being dried over Na$_2$SO$_4$ and concentrated in vacuo to give the desired product as an off-white solid (725 mg, 95%).

$^1$H NMR (500 MHz, Chloroform-d) $\delta$ = 6.69 (s, 2H), 3.81 (s, 2H), 3.79 (s, 6H), 3.21 (s, 2H), 2.41 (s, 3H), 1.48 (s, 9H).

LCMS: 376 (M+H).

tert-butyl 2-((2,6-dimethoxy-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)(methyl)amino)acetate

tert-butyl 2-((4-bromo-2,6-dimethoxybenzyl)(methyl)amino)acetate (300 mg, 0.802 mmol, 1.0 eq) and bis(pinacolato)diboron (305 mg, 1.20 mmol, 1.5 eq), were dissolved in DMF, before KOAC (394 mg, 4.01 mmol, 5.0 eq), and PdCl$_2$(dppf) • CH$_2$Cl$_2$ (65.5 mg, 0.080 mmol, 0.1 eq) were added. The mixture was degassed, and headspace flushed with N$_2$ before heating to 90°C for 16 hr. The reaction was diluted to 80 mL with EtOAc, filtered through celite, and washed twice with a 1:1 solution of deionized water and saturated brine (20 mL), three times with deionized water (20 mL), and once with saturated brine (20 mL), before being dried over Na$_2$SO$_4$ and concentrated in vacuo. The residue was dissolved in DCM and purified by silica chromatography (EtOAc/Hexanes 0 to 100% gradient) to give the desired product as a brown solid (158 mg, 47%).

$^1$H NMR: (500 MHz, Chloroform-d) $\delta$ = 6.98 (s, 2H), 3.90 (s, 2H), 3.85 (s, 6H), 3.20 (s, 2H), 2.41 (s, 3H), 1.48 (s, 9H), 1.35 (s, 12H).

LCMS: 423 (M+H).

tert-butyl 2-((2,6-dimethoxy-4-(2-methyl-1-oxo-1,2-dihydro-2,7-naphthyridin-4-yl)benzyl)(methyl)amino)acetate
4-bromo-2-methyl-2,7-naphthyridin-1(2H)-one (45.6 mg, 0.191 mmol, 1.0 eq) and tert-butyl 2-((2,6-dimethoxy-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)(methyl)amino)acetate (96.4 mg, 0.229 mmol, 1.2 eq) were dissolved in DMF (4.6 mL) before a 2N solution of Na$_2$CO$_3$ was added (239 µL, 0.477 mmol, 2.5 eq) followed by Pd(dppf)Cl$_2$ • DCM (15.6 mg, 0.0141 mmol, 0.1 eq). The mixture was degassed and heated to 80°C for 13 hours. Solvent was removed by lyophilization and the residue extracted with DCM and purified by silica chromatography (DCM/MeOH 0 to 15% gradient) to give the desired product as a brown solid (71 mg, 82 %).

$^1$H NMR (500 MHz, Chloroform- $d$) δ = 9.70 (s, 1H), 8.70 (d, J = 5.6, 1H), 7.43 (d, J = 6.2, 1H), 7.28 (s, 1H), 6.54 (s, 2H), 3.93 (s, 2H), 3.84 (s, 6H), 3.68 (s, 3H), 3.30 (s, 2H), 2.50 (s, 3H), 1.52 (s, 9H).

LCMS: 454 (M + H).

2-((2,6-dimethoxy-4-(2-methyl-1-oxo-1,2-dihydro-2,7-naphthyridin-4-yl)benzyl)(methyl)amino)acetic acid
tert-butyl 2-((2,6-dimethoxy-4-(2-methyl-1-oxo-1,2-dihydro-2,7-naphthyridin-4-yl)benzyl)(methyl)amino)acetate (71.0 mg, 0.156 mmol, 1.0 eq) was dissolved in DCM (624 µL) before TFA was slowly added (624 µL). After 1 hr deprotection was incomplete, and the reaction continued for 24 hours. The reaction was concentrated under a stream of N$_2$ followed by high vacuum. The resulting tar-like salt was dissolved in deionized water and lyophilized to give a brown solid, which was triturated with Et$_2$O. The remaining hygroscopic solid was dried under high vacuum overnight to give the desired product as brittle brown solid (90mg, quantitative yield, mixture of TFA salts).

$^1$H NMR (500 MHz, DMSO- $d_6$) δ = 9.76 (s, 1H), 9.48 (s, 1H), 8.75 (d, 1H), 7.94 (s, 1H), 7.64 (d, 1H), 6.87 (s, 2H), 4.42 (s, 2H), 4.02 (s, 2H), 3.87 (s, 6H), 3.63 (s, 3H), 2.76 (s, 3H).

LCMS: 398 (M+H).

tert-butyl 2-((4-bromo-2,6-dimethoxybenzyl)(methyl)amino)acetate
Sarcosyltert-butyl ester hydrochloride (556 mg, 3.06 mmol, 1.5 eq) was dissolved in a solution of NaOAc (251 mg, 3.06 mmol, 1.5 eq), in DCM (8.2 mL), before 167 µL AcOH (2.04 mmol, 1.0 eq) was added, followed by 4-bromo-2,6-dimethoxybenzaldehyde (500 mg, 2.04 mmol, 1.0 eq). The mixture was stirred for 10 min before sodium triacetoxyborohydride was added in one portion (864.8 mg, 4.08 mmol, 2.0 eq), and the mixture stirred for 18hr. The reaction was basified to approximately pH 11 with 1M K$_2$CO$_3$ and extracted 4 times with DCM (10 mL). The combined organics were washed with deionized water (10 mL), and saturated brine (10 mL),
before being dried over Na$_2$SO$_4$ and concentrated in vacuo to give the desired product as an off-white solid (725 mg, 95%).

$^1$H NMR (500 MHz, Chloroform-$d$) $\delta = 6.69$ (s, 2H), 3.81 (s, 2H), 3.79 (s, 6H), 3.21 (s, 2H), 2.41 (s, 3H), 1.48 (s, 9H).

LCMS: 376 (M+H).

**tert-butyl 2-((2,6-dimethoxy-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)(methyl)amino)acetate**

$^1$H NMR: (500 MHz, Chloroform-$d$) $\delta = 6.98$ (s, 2H), 3.90 (s, 2H), 3.85 (s, 6H), 3.20 (s, 2H), 2.41 (s, 3H), 1.48 (s, 9H), 1.35 (s, 12H).

LCMS: 423 (M+H).

**tert-butyl 2-((4-bromo-2,6-dimethoxybenzyl)(methyl)amino)acetate**

tert-butyl 2-((4-bromo-2,6-dimethoxybenzyl)(methyl)amino)acetate (300 mg, 0.802 mmol, 1.0 eq) and bis(pinacolato)diboron (305 mg, 1.20 mmol, 1.5 eq), were dissolved in DMF, before KOAc (394 mg, 4.01 mmol, 5.0 eq), and PdCl$_2$(dppf)•CH$_2$Cl$_2$ (65.5 mg, 0.080 mmol, 0.1 eq) were added. The mixture was degassed, and headspace flushed with N$_2$ before heating to 90°C for 16 hr. The reaction was diluted to 80 mL with EtOAc, filtered through celite, and washed twice with a 1:1 solution of deionized water and saturated brine (20 mL), three times with deionized water (20 mL), and once with saturated brine (20 mL), before being dried over Na$_2$SO$_4$ and concentrated in vacuo. The residue was dissolved in DCM and purified by silica chromatography (EtOAc/Hexanes 0 to 100% gradient) to give the desired product as a brown solid (158 mg, 47%).

$^1$H NMR: (500 MHz, Chloroform-$d$) $\delta = 6.98$ (s, 2H), 3.90 (s, 2H), 3.85 (s, 6H), 3.20 (s, 2H), 2.41 (s, 3H), 1.48 (s, 9H), 1.35 (s, 12H).

LCMS: 423 (M+H).

**tert-butyl 2-((4-(isoquinolin-5-yl)-2,6-dimethoxybenzyl)(methyl)amino)acetate**

5-bromoisoquinoline (40 mg, 0.192 mmol, 1.0 eq) and tert-butyl 2-((2,6-dimethoxy-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)(methyl)amino)acetate (96.4 mg, 0.229 mmol, 1.2 eq) were dissolved in DMF (4.6 mL) before a 2N solution of Na$_2$CO$_3$ was added (239 µL, 0.477 mmol, 2.5 eq) followed by Pd(dppf)Cl$_2$•DCM (15.6 mg, 0.0141 mmol, 0.1 eq). The mixture was degassed and heated to 80°C for 13 hours. The reaction mixture was filtered through Celite, diluted with EtOAc, washed with H$_2$O and brine. The organic layer was dried over Na$_2$SO$_4$, filtered, and concentrated.

LCMS: 423 (M + H).
2-((4-(isoquinolin-5-yl)-2,6-dimethoxybenzyl)(methyl)amino)acetic acid
tert-butylic 2-((2,6-dimethoxy-4-(2-methyl-1-oxo-1,2-dihydro-2,7-naphthridin-4-y1)benzyl)(methyl)amino)acetate (71.0 mg, 0.156 mmol, 1.0 eq) was dissolved in DCM (624 µL) before TFA was slowly added (624 µL). The solution was stirred overnight at rt then concentrated. The crude product was purified by prep-HPLC (0.05 % TFA in H₂O / CH₃CN) to give title compound (49 mg, 70 %).

¹H NMR (500 MHz, MeOH-d₄) δ = 9.80 (s, 1H), 9.48 (s, 1H), 8.55 (m, 2H), 8.34 (m, 1H), 8.19 (m, 1H), 8.07 (m, 1H), 6.94 (s, 2H), 4.65 (s, 2H), 4.08 (s, 2H), 3.98 (s, 6H), 2.99 (s, 3H).

LCMS: 367 (M+H).

Final Compound Synthesis

“Biotin-Probe”
2-(4-((4-(N-(1,1-dioxidotetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy)acetic acid (15.6 mg, 0.030 mmol, 1.0 eq) was dissolved in DMF (300 µL), before N-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide ((+) Biotin-(PEO)₄-Amine, ChemImpex) was added, followed by DIPEA (15.7 µL, 0.090 mmol, 3.0 eq), and lastly HATU (12.5 mg, 0.033 mmol, 1.1). After 16 hr the mixture was diluted with MeOH and purified by preparative HPLC to give the trifluoroacetate salt of Biotin-Probe as a colorless solid (8.2 mg, 30%).

¹H NMR (500 MHz, Methanol-d₄) δ = 8.40 (s, 1H), 8.13 (s, 1H), 7.85 (s, 1H), 7.30 (d, J=2.0, 1H), 7.24 (dd, J=8.3, 2.1, 1H), 7.17 (d, J=8.3, 1H), 4.62 (s, 2H), 4.49 (dd, J=7.8, 5.0, 0.9, 1H), 4.29 (dd, J=7.9, 4.5, 1H), 4.05 (tt, J=10.7, 5.1, 1H), 3.99 (s, 3H), 3.75 (s, 3H), 3.69 – 3.48 (m, 18H), 3.26 – 3.19 (m, 2H), 2.92 (dd, J=12.7, 5.0, 1H), 2.70 (d, J=12.8, 1H), 2.49 – 2.32 (m, 4H), 2.26 – 2.17 (m, 2H), 1.93 – 1.82 (m, 1H), 1.71 – 1.37 (m, 6H).

LCMS: 920 (M+H)
Compound 1

N-(4-aminobutyl)-2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioioisoindolin-4-yl)oxy)acetamide trifluoroacetate salt \(^{[17]}\) (10.3 mg, 0.020 mmol, 1 eq) was added to 2-(4-(2-(N-(1,1-dioioctetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy)acetic acid (10.4 mg, 0.020 mmol, 1 eq) as a 0.1 M solution in DMF (200 microliters). DIPEA (10.5 microliters, 0.060 mmol, 3 eq) was added, followed by HATU (7.6 mg, 0.020 mmol, 1 eq). After 22 hours, the mixture was diluted with DMF/MeOH and purified by preparative HPLC to give the trifluoroacetate salt of 1 as a yellow solid (8.12 mg, 0.00798 mmol, 40%).

\(^1\)H NMR (500 MHz, Methanol-\(d_4\)) \(\delta\) 8.30 (d, \(J = 4.7\) Hz, 1H), 7.75 (dd, \(J = 8.4, 7.4\) Hz, 1H), 7.62 (d, \(J = 4.9\) Hz, 1H), 7.51 (d, \(J = 7.3\) Hz, 1H), 7.28 (d, \(J = 8.4\) Hz, 1H), 7.12 (d, \(J = 8.2\) Hz, 2H), 7.04 (d, \(J = 8.1\) Hz, 1H), 5.02 (dd, \(J = 11.6, 6.2\) Hz, 1H), 4.61 (dd, \(J = 8.9, 3.7\) Hz, 2H), 4.56 (s, 2H), 4.03 (dt, \(J = 10.0, 5.0\) Hz, 1H), 3.92 (s, 3H), 3.70 (s, 3H), 3.41 – 3.32 (m, 4H), 3.26 (d, \(J = 4.6\) Hz, 3H), 3.14 (d, \(J = 15.1\) Hz, 2H), 2.81 – 2.71 (m, 3H), 2.42 – 2.29 (m, 4H), 2.15 – 2.09 (m, 1H), 1.66 – 1.56 (m, 4H).

LCMS: 904.47 (M+H).

Compound 2

N-(8-aminooctyl)-2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioioisoindolin-4-yl)oxy)acetamide trifluoroacetate salt (11.5 mg, 0.020 mmol, 1 eq) was added to 2-(4-(2-(N-(1,1-dioioctetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy)acetic acid (10.4 mg, 0.020 mmol, 1 eq) as a 0.1 M solution in DMF (200 microliters). DIPEA (10.5 microliters, 0.060 mmol, 3 eq) was added, followed by HATU (7.6 mg, 0.020 mmol, 1 eq). After 23 hours, the mixture was diluted with DMF/MeOH and purified by preparative HPLC to give the trifluoroacetate salt of 2 as a light brown oily residue (9.83 mg, 0.00915 mmol, 46%).

\(^1\)H NMR (500 MHz, Methanol-\(d_4\)) \(\delta\) 8.35 (s, 1H), 7.84 – 7.76 (m, 2H), 7.52 (d, \(J = 7.0\) Hz, 1H), 7.40 (d, \(J = 8.6\) Hz, 1H), 7.27 (d, \(J = 2.1\) Hz, 1H), 7.21 (dd, \(J = 8.2, 2.1\) Hz, 1H), 7.12 (d, \(J = 8.3\) Hz, 1H), 5.14 – 5.10 (m, 1H), 4.72 (s, 2H), 4.58 (s, 2H), 3.97 (d, \(J = 10.8\) Hz, 4H), 3.70 (s,
3H), 3.29 – 3.18 (m, 6H), 2.98 (s, 1H), 2.89 – 2.84 (m, 1H), 2.80 – 2.68 (m, 3H), 2.43 – 2.31 (m, 4H), 1.52 (s, 4H), 1.37 – 1.25 (m, 8H).

LCMS: 960.49 (M+H).

Compound S1
N-(3-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)-2-((2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)acetamide trifluoroacetate salt[17] (13.0 mg, 0.020 mmol, 1 eq) was added to 2-(4-((1,1-dioxidotetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy)acetic acid (10.4 mg, 0.020 mmol, 1 eq) as a 0.1 M solution in DMF (200 microliters). DIPEA (10.5 microliters, 0.060 mmol, 3 eq) was added, followed by HATU (7.6 mg, 0.020 mmol, 1 eq). After 24 hours, the mixture was diluted with DMF/MeOH and purified by preparative HPLC to give the trifluoroacetate salt of S1 as a brown oily residue (14.11 mg, 0.01227 mmol, 61%).

^1H NMR (500 MHz, Methanol-d₄) δ 8.35 (s, 1H), 7.81 (s, 1H), 7.76 (dd, J = 8.4, 7.4 Hz, 1H), 7.49 (d, J = 7.1 Hz, 1H), 7.37 (d, J = 8.3 Hz, 1H), 7.25 (d, J = 2.1 Hz, 1H), 7.19 (dd, J = 8.2, 2.1 Hz, 1H), 7.12 (d, J = 6.3 Hz, 1H), 5.11 (dd, J = 12.9, 5.5 Hz, 1H), 4.69 (s, 2H), 4.56 (s, 2H), 4.05 – 3.99 (m, 1H), 3.95 (s, 3H), 3.70 (s, 3H), 3.61 – 3.52 (m, 12H), 3.43 – 3.36 (m, 4H), 3.29 – 3.18 (m, 4H), 2.89 – 2.81 (m, 1H), 2.77 – 2.68 (m, 2H), 2.46 – 2.30 (m, 4H), 2.11 (ddt, J = 10.2, 5.4, 2.5 Hz, 1H), 1.80 (dt, J = 12.8, 6.4 Hz, 4H).

LCMS: 1036.43 (M+H).

Compound 3
4-((6-aminohexyl)oxy)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (0.03 mmol, 1.0 eq) was added to 2-(4-((1,1-dioxidotetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy)acetic acid (15.6 mg, 0.030 mmol, 1 eq) as a 0.1 M solution in DMF (200 microliters). DIPEA (15.7 microliters, 0.090 mmol, 3 eq) was added, followed by HATU (12.5 mg, 0.033 mmol, 1.1 eq). After 16 hours, the mixture was diluted with MeOH and purified by preparative HPLC to give the trifluoroacetate salt of 3 as a pale yellow solid (14.11 mg, 0.01227 mmol, 61%).

^1H NMR (500 MHz, Methanol-d₄) δ 8.32 (s, 1H), 7.79 (s, 1H), 7.69 – 7.65 (m, 1H), 7.35 (d, J=7.1, 1H), 7.31 (d, J=8.5, 1H), 7.24 (d, J=2.1, 1H), 7.20 (dd, J=8.3, 2.1, 1H), 7.11 (d, J=8.3, 1H), 5.07 (dd, J=12.7, 5.5, 1H), 4.58 (s, 2H), 4.11 (d, J=5.4, 2H), 3.95 (s, 3H), 3.68 (s, 3H), 3.28 – 3.19 (m, 5H), 2.97 (s, 2H), 2.86 – 2.80 (m, 1H), 2.74 – 2.66 (m, 2H), 2.41 (d, J=12.2,
2H), 2.33 (d, J=12.6, 2H), 2.09 (d, J=10.1, 1H), 1.80 – 1.75 (m, 2H), 1.62 – 1.56 (m, 3H), 1.55 – 1.48 (m, 3H), 1.37 (q, J=8.0, 3H), 1.28 (s, 1H).

**LCMS:** 875 (M + H).

**Compound S2**
4-2(2-[4,4'-bipiperidin]-1-yl)-2-oxoethoxy)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione trifluoroacetate salt (11.9 mg, 0.020 mmol, 1 eq) was added to 2-(4-(2-(N-(1,1-dioxidotetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy)acetic acid (10.4 mg, 0.020 mmol, 1 eq) as a 0.1 M solution in DMF (200 microliters). DIPEA (10.5 microliters, 0.060 mmol, 3 eq) was added, followed by HATU (7.6 mg, 0.020 mmol, 1 eq). After 24 hours, the mixture was diluted with DMF/MeOH and purified by preparative HPLC to give the trifluoroacetate salt of S2 as yellow solid (11.6 mg, 0.01016 mmol, 51%).

**1H NMR** (500 MHz, Methanol-d4) δ 8.34 (s, 1H), 7.75 – 7.71 (m, 2H), 7.49 (dd, J = 6.7, 3.9 Hz, 1H), 7.35 – 7.32 (m, 1H), 7.21 – 7.11 (m, 2H), 7.04 (d, J = 8.3 Hz, 1H), 5.11 – 4.95 (m, 3H), 4.87 (d, J = 14.1 Hz, 1H), 4.52 (s, 2H), 4.06 – 3.96 (m, 2H), 3.92 (d, J = 2.5 Hz, 3H), 3.72 (s, 3H), 3.18 (d, J = 11.9 Hz, 2H), 3.08 (d, J = 11.0 Hz, 2H), 2.93 (d, J = 17.9 Hz, 2H), 2.81 – 2.72 (m, 2H), 2.62 (s, 1H), 2.43 – 2.30 (m, 3H), 2.13 (s, 1H), 1.74 (d, J = 27.5 Hz, 4H), 1.45 (s, 2H), 1.29 (d, J = 29.7 Hz, 7H).

**LCMS:** 984.57 (M+H).

**Compound S3**
(2S,4R)-1-((S)-14-amino-2-(tert-butyl)-4-oxo-6,9,12-trioxa-3-azatetradecan-1-yl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide hydrochloride salt[16] (13.1 mg, 0.020 mmol, 1 eq) was added to 2-(4-(2-(N-(1,1-dioxidotetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy)acetic acid (10.4 mg, 0.020 mmol, 1 eq) as a 0.1 M solution in DMF (200 microliters). DIPEA (10.5 microliters, 0.060 mmol, 3 eq) was added, followed by HATU (7.6 mg, 0.020 mmol, 1 eq). DMF (200 microliters) was added to improve solubility. After 20 hours, more HATU (7.6 mg) was added to ensure
complete conversion. After an additional 2 hours, the mixture was diluted with DMF/MeOH and purified by preparative HPLC to give the trifluoroacetate salt of \textbf{S3} (5.99 mg, 27%).

$^1$H NMR (500 MHz, Methanol-$d_4$) $\delta$ 8.98 (s, 1H), 8.36 (s, 1H), 7.81 (d, $J = 3.2$ Hz, 1H), 7.46 – 7.40 (m, 4H), 7.26 (dd, $J = 9.5$, 2.1 Hz, 1H), 7.19 (dd, $J = 8.2$, 2.1 Hz, 1H), 7.12 (d, $J = 8.3$ Hz, 1H), 4.72 – 4.67 (m, 1H), 4.56 (d, $J = 7.6$ Hz, 2H), 4.50 (d, $J = 8.3$ Hz, 2H), 4.35 (d, $J = 15.5$ Hz, 1H), 4.03 – 3.93 (m, 5H), 3.86 (d, $J = 11.1$ Hz, 1H), 3.79 (dd, $J = 10.9$, 3.8 Hz, 1H), 3.73 – 3.62 (m, 10H), 3.59 (t, $J = 5.5$ Hz, 2H), 3.29 – 3.17 (m, 4H), 2.51 – 2.45 (m, 4H), 2.36 (dt, $J = 37.2$, 12.8 Hz, 4H), 2.23 (dd, $J = 13.2$, 7.6 Hz, 1H), 2.08 (ddd, $J = 13.3$, 9.3, 4.4 Hz, 1H), 1.03 (d, $J = 12.3$ Hz, 9H).

**LCMS**: 1121.53 (M+H).

\begin{center}
\textbf{Compound S4}
\end{center}

(2S,4R)-1-((S)-2-(2-(2-aminoethoxy)ethoxy)acetamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide hydrochloride salt (12.2 mg, 0.020 mmol, 1 eq) was added to 2-(4-(4-(1,1-dioxidotetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy)acetic acid (10.4 mg, 0.020 mmol, 1 eq) as a 0.1 M solution in DMF (200 microliters). DIPEA (10.5 microliters, 0.060 mmol, 3 eq) was added, followed by HATU (7.6 mg, 0.020 mmol, 1 eq). Additional DMF (200 microliters) was added to improve solubility. After 27 hours, extra HATU (7.6 mg) was added to ensure complete conversion. After 3 more hours, the mixture was diluted with DMF/MeOH and purified by preparative HPLC to give the trifluoroacetate salt of \textbf{S4} as yellow oily residue (12.35 mg, 0.0104 mmol, 52%).

$^1$H NMR (500 MHz, Methanol-$d_4$) $\delta$ 8.95 (s, 1H), 8.36 (d, $J = 5.0$ Hz, 1H), 7.80 (d, $J = 6.6$ Hz, 1H), 7.45 – 7.39 (m, 4H), 7.23 (d, $J = 2.0$ Hz, 1H), 7.17 (dd, $J = 8.3$, 2.0 Hz, 1H), 7.09 (d, $J = 8.3$ Hz, 1H), 4.74 – 4.70 (m, 1H), 4.58 – 4.49 (m, 4H), 4.34 (d, $J = 15.4$ Hz, 1H), 4.04 – 3.97 (m, 3H), 3.93 (s, 2H), 3.87 – 3.78 (m, 2H), 3.73 – 3.62 (m, 10H), 3.51 (dt, $J = 28.6$, 5.1 Hz, 2H), 3.24 (dd, $J = 29.6$, 14.3 Hz, 4H), 2.48 (d, $J = 15.7$ Hz, 4H), 2.36 (dd, $J = 25.4$, 12.2 Hz, 4H), 2.24 – 2.18 (m, 1H), 2.10 (dt, $J = 13.1$, 6.6 Hz, 1H), 1.03 (s, 9H).

**LCMS**: 1077.72 (M+H).

\begin{center}
\textbf{Compound S5}
\end{center}

5-((8-aminooctyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione trifluoroacetate salt (10.3 mg, 0.020 mmol, 1 eq) was added to 2-(4-(4-(1,1-dioxidotetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-
methoxyphenoxy)acetic acid (10.4 mg, 0.020 mmol, 1 eq) as a 0.1 M solution in DMF (200 microliters). DIPEA (10.5 microliters, 0.060 mmol, 3 eq) was added, followed by HATU (7.6 mg, 0.020 mmol, 1 eq). After 20 hours, the mixture was diluted with DMF/MeOH and purified by preparative HPLC to give the trifluoroacetate salt of S5 as yellow solid (6.27 mg, 0.00617 mmol, 31%).

\(^1\)H NMR (500 MHz, Methanol-d\(_4\)) δ 8.34 (s, 1H), 7.80 (s, 1H), 7.49 (d, J = 8.4 Hz, 1H), 7.26 (s, 1H), 7.21 (d, J = 8.4 Hz, 1H), 7.12 (d, J = 8.3 Hz, 1H), 6.90 (s, 1H), 6.75 (d, J = 8.0 Hz, 1H), 5.02 (dd, J = 12.8, 5.4 Hz, 1H), 4.58 (s, 2H), 3.95 (s, 4H), 3.71 (s, 3H), 3.30 – 3.17 (m, 6H), 3.12 (t, J = 7.1 Hz, 2H), 2.87 – 2.79 (m, 1H), 2.76 – 2.64 (m, 2H), 2.44 – 2.29 (m, 4H), 2.11 – 2.05 (m, 1H), 1.64 – 1.55 (m, 4H), 1.37 (d, J = 17.9 Hz, 8H).

LCMS: 902.54 (M+H).

Compound 4
4-((8-aminooctyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione trifluoroacetate salt (20.6 mg, 0.040 mmol, 1 eq) was added to 2-(4-(2-(N-(1,1-dioxidotetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyrind-7-yl)-2-methoxyphenoxy)acetic acid (20.8 mg, 0.040 mmol, 1 eq) as a 0.1 M solution in DMF (400 microliters). DIPEA (21.0 microliters, 0.120 mmol, 3 eq) was added, followed by PyBOP (20.8 mg, 0.040 mmol, 1 eq). After 21 hours, the mixture was diluted with DMF and purified by preparative HPLC to give the trifluoroacetate salt of 4 as yellow solid (22.35 mg, 0.0220 mmol, 55%).

\(^1\)H NMR (500 MHz, Methanol-d\(_4\)) δ 8.34 (s, 1H), 7.81 (s, 1H), 7.49 (d, J = 8.5, 7.1 Hz, 1H), 7.25 (d, J = 2.1 Hz, 1H), 7.20 (dd, J = 8.3, 2.1 Hz, 1H), 7.12 (d, J = 8.3 Hz, 1H), 6.97 (dd, J = 10.7, 7.7 Hz, 2H), 5.04 (dd, J = 12.6, 5.5 Hz, 1H), 4.58 (s, 2H), 4.03 – 3.98 (m, 1H), 3.95 (s, 3H), 3.71 (s, 3H), 3.30 – 3.15 (m, 8H), 2.85 (ddd, J = 17.5, 13.9, 5.2 Hz, 1H), 2.77 – 2.64 (m, 2H), 2.44 – 2.29 (m, 4H), 2.10 (dtd, J = 13.0, 5.8, 2.8 Hz, 1H), 1.60 (ddd, J = 29.2, 13.9, 6.8 Hz, 4H), 1.38 (d, J = 23.7 Hz, 8H).

\(^1\)H NMR (500 MHz, Methanol-d\(_4\)) δ 174.61, 171.77, 170.97, 170.81, 169.28, 160.21, 158.44, 152.92, 151.81, 149.19, 148.22, 137.20, 136.79, 133.86, 132.61, 131.25, 130.53, 128.62, 121.31, 117.90, 117.23, 113.03, 111.71, 70.31, 56.78, 51.20, 50.23, 50.19, 43.34, 40.03, 37.53, 32.20, 30.23, 30.14, 29.87, 27.74, 27.66, 23.81.

LCMS: 902.87 (M+H).

Compound 5
3-(4-((8-aminoctyl)amino)-1-oxoisindolin-2-yl)piperidine-2,6-dione trifluoroacetate salt (10.0 mg, 0.020 mmol, 1 eq) was added to 2-(4-(2-(N-(1,1-dioxidotetrahydro-2H-thiopyran-4-yl)carbamimidoyl)-5-methyl-4-oxo-4,5-dihydrothieno[3,2-c]pyridin-7-yl)-2-methoxyphenoxy)acetic acid (10.4 mg, 0.020 mmol, 1 eq) as a 0.1 M solution in DMF (200 microliters). DIPEA (10.5 microliters, 0.060 mmol, 3 eq) was added, followed by PyBOP (10.4 mg, 0.020 mmol, 1 eq). After 18 hours, the mixture was diluted with DMF and purified by preparative HPLC to give the trifluoroacetate salt of 5 as an oily, yellow solid (13.3 mg, 0.01329 mmol, 66%).

$^1$H NMR (500 MHz, Methanol-$d_4$) $\delta$ 8.33 (s, 1H), 7.80 (s, 1H), 7.28 – 7.24 (m, 2H), 7.19 (dd, $J$ = 8.3, 2.1 Hz, 1H), 7.12 (d, $J$ = 8.3 Hz, 1H), 7.00 (d, $J$ = 7.1 Hz, 1H), 6.74 (d, $J$ = 7.9 Hz, 1H), 5.14 (dd, $J$ = 13.3, 5.2 Hz, 1H), 4.58 (s, 2H), 4.32 – 4.21 (m, 2H), 3.99 (ddt, $J$ = 13.6, 6.6, 3.3 Hz, 1H), 3.94 (s, 3H), 3.72 (s, 3H), 3.30 – 3.17 (m, 6H), 3.15 (t, $J$ = 7.2 Hz, 2H), 2.91 (ddd, $J$ = 18.7, 13.5, 5.4 Hz, 1H), 2.79 (ddd, $J$ = 17.6, 4.5, 2.4 Hz, 1H), 2.53 – 2.27 (m, 5H), 2.19 (tdd, $J$ = 12.9, 5.3, 2.5 Hz, 1H), 1.65 – 1.51 (m, 4H), 1.43 – 1.30 (m, 8H).

$^{13}$C NMR (126 MHz, MeOD) $\delta$ 174.67, 172.44, 170.98, 160.23, 158.42, 152.91, 151.79, 149.17, 144.94, 136.76, 132.95, 132.60, 131.25, 130.56, 128.61, 128.06, 121.32, 117.87, 117.20, 113.83, 113.03, 111.90, 70.29, 56.75, 53.61, 51.18, 50.22, 47.39, 44.45, 40.01, 37.52, 32.39, 30.39, 30.27, 30.25, 30.04, 29.86, 28.01, 27.72, 24.25.

LCMS: 888.88 (M+H).
5-((6-aminohexyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindole-1,3-dione trifluoroacetate salt (0.03 mmol, 1 eq) was added to 2-((2,6-dimethoxy-4-(2-methyl-1-oxo-1,2-dihydro-2,7-naphthyridin-4-yl)benzyl)(methyl)amino)acetic acid trifluoroacetate salt (18.8 mg, 0.037 mmol, 1.2 eq) as a 0.1 M solution in DMF (300 microliters). DIPEA (15.7 microliters, 0.090 mmol, 3.0 eq) was added, followed by HATU (12.5 mg, 0.033 mmol, 1.1 eq). After 20 hours the mixture was diluted to 10 mL with EtOAc, washed once with a mildly basified (Na₂CO₃) 1:1 solution of deionized water and saturated brine (2 mL), washed three times with deionized water (2 mL), and finally with saturated brine (2 mL) before drying over Na₂SO₄ and concentrating in vacuo. The residue was dissolved in 0.75 mL DCM and purified by silica chromatography (DCM/MeOH 0 to 20 % gradient) to give S6 as a yellow solid (12.5 mg, 56%).
\(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 11.05 (s, 1H), 9.44 (s, 1H), 8.72 (d, \(J = 5.7\) Hz, 1H), 7.87 (s, 1H), 7.69 (s, 1H), 7.56 (d, \(J = 6.2\) Hz, 1H), 7.54 (d, \(J = 8.4\) Hz, 1H), 7.08 (t, \(J = 5.3\) Hz, 1H), 6.92 (s, 1H), 6.81 (dd, \(J = 8.4, 1.8\) Hz, 1H), 6.77 (s, 2H), 5.02 (dd, \(J = 12.7, 5.5\) Hz, 1H), 3.84 (s, 6H), 3.60 (s, 4H), 3.14 (dq, \(J = 12.9, 6.6\) Hz, 4H), 2.96 (s, 1H), 2.92 – 2.83 (m, 2H), 2.61 – 2.58 (m, 1H), 2.55 (s, 1H), 2.18 (s, 3H), 1.99 (dd, \(J = 10.7, 5.2\) Hz, 1H), 1.56 (p, \(J = 6.9\) Hz, 2H), 1.46 (p, \(J = 7.1\) Hz, 2H), 1.42 – 1.23 (m, 6H).

LCMS: 752.6 (M+H).

**Compound S7**

5-((6-aminohexyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione trifluoroacetate salt (0.03 mmol, 1 eq) was added to 2-((2,6-dimethoxy-4-(2-methyl-1-oxo-1,2-dihydro-2,7-naphthyridin-4-yl)benzyl)(methyl)amino)acetic acid trifluoroacetate salt (18.8 mg, 0.037 mmol, 1.2 eq) as a 0.1 M solution in DMF (300 microliters). DIPEA (15.7 microliters, 0.090 mmol, 3.0 eq) was added, followed by PyBOP (15.6 mg, 0.030 mmol, 1.1 eq). After 12 hours, the reaction was incomplete; an additional 3 eq DIPEA and 0.5 eq PyBOP was added. After 7.5 hrs, the mixture was purified by preparative HPLC to give S7 as a yellow solid (6.8 mg, 41%).

\(^1\)H NMR (500 MHz, Methanol-\(d_4\)) \(\delta\) 9.54 (s, 1H), 8.67 (d, \(J = 6.1\) Hz, 1H), 7.89 (s, 1H), 7.77 (d, \(J = 6.0\) Hz, 1H), 7.51 (dd, \(J = 8.5, 7.2\) Hz, 1H), 6.99 (dd, \(J = 7.8, 2.2\) Hz, 2H), 6.84 (s, 2H), 5.48 (s, 2H), 5.03 (dd, \(J = 12.6, 5.5\) Hz, 1H), 4.51 (d, \(J = 4.9\) Hz, 2H), 3.95 (s, 6H), 3.70 (s, 3H), 3.34 (s, 1H), 3.27 (t, \(J = 6.9\) Hz, 2H), 2.92 (s, 3H), 2.85 (ddd, \(J = 17.5, 13.9, 5.2\) Hz, 1H), 2.76 – 2.65 (m, 2H), 2.13 – 2.06 (m, 1H), 1.61 (p, \(J = 6.9\) Hz, 2H), 1.52 – 1.46 (m, 2H), 1.43 – 1.25 (m, 11H).

LCMS: 780.9 (M+H).

**Compound S8**

\((2R,4S)-1-((R)-14-amino-2-(tert-butyl)-4-oxo-6,9,12-triaza-tetrade-cane-1-yl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide trifluoroacetate salt (0.02 mmol, 1 eq) was added to 2-((2,6-dimethoxy-4-(2-methyl-1-oxo-1,2-dihydro-2,7-naphthyridin-4-yl)benzyl)(methyl)amino)acetic acid trifluoroacetate salt (10.2 mg, 0.030 mmol, 1 eq) as a 0.1 M solution in DMF (200 microliters). DIPEA (10.4 microliters, 0.060 mmol, 3.0 eq) was added, followed by PyBOP (11.4 mg, 0.022 mmol, 1.1 eq). After 7 hours, the reaction was incomplete;
an additional 3 eq DIPEA and 0.5 eq PyBOP was added. After 14 hrs, the reaction was diluted to 10 mL with EtOAc, washed once with a mildly basified (Na$_2$CO$_3$) 1:1 solution of deionized water and saturated brine (2 mL), washed three times with deionized water (2 mL), and finally with saturated brine (2 mL) before drying over Na$_2$SO$_4$ and concentrating in vacuo. The residue was dissolved in 0.5 mL DCM and purified by silica chromatography (DCM/MeOH 0 to 10% gradient) to give S8 as a colorless solid (6.23, 31%).

$^1$H NMR (500 MHz, Methanol-d$_4$) δ = 9.50 (s, 1H), 8.86 (s, 1H), 8.66 (d, J=5.8, 1H), 7.73 (s, 1H), 7.61 (d, J=5.8, 1H), 7.45 – 7.42 (m, 2H), 7.39 (d, J=8.3, 2H), 6.75 (s, 2H), 4.68 (s, 1H), 4.61 – 4.48 (m, 4H), 4.34 (d, J=15.5, 1H), 4.00 (d, J=5.7, 2H), 3.89 (s, 6H), 3.87 (s, 2H), 3.69 (s, 3H), 3.67 (d, J=4.9, 2H), 3.62 (d, J=2.3, 3H), 3.56 (d, J=1.3, 1H), 3.53 (t, J=5.1, 2H), 3.40 (t, J=5.2, 2H), 2.45 (s, 3H), 2.41 (s, 2H), 2.23 (dd, J=11.5, 7.7, 1H), 2.11 – 2.05 (m, 1H), 1.96 (s, 1H), 1.94 (s, 3H), 1.02 (s, 9H).

LCMS: 1000 (M+H).

Compound S9

$N$-(8-aminooctyl)-2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)acetamide trifluoroacetate salt (0.02 mmol, 1 eq) was added to 2-((2,6-dimethoxy-4-(2-methyl-1-oxo-1,2-dihydro-2,7-naphthyridin-4-yl)benzyl)(methyl)amino)acetic acid trifluoroacetate salt (10.2 mg, 0.02 mmol, 1 eq) as a 0.1 M solution in DMF (200 microliters). DIPEA (10.4 microliters, 0.060 mmol, 3.0 eq) was added, followed by PyBOP (11.4 mg, 0.022 mmol, 1.1 eq). After 16 hours the reaction was diluted to 10 mL with EtOAc, washed once with a mildly basified (Na$_2$CO$_3$) 1:1 solution of deionized water and saturated brine (2 mL), washed three times with deionized water (2 mL), and finally with saturated brine (2 mL) before drying over Na$_2$SO$_4$ and concentrating in vacuo. The residue was dissolved in 0.5 mL DCM and purified by silica chromatography (DCM/MeOH 5 to 10% gradient) to give S9 as a colorless solid (6.85 mg, 41%).

$^1$H NMR (500 MHz, Methanol-d$_4$) δ = 9.46 (s, 1H), 8.65 (d, J=5.8, 1H), 7.78 (dd, J=8.4, 7.4, 1H), 7.73 (s, 1H), 7.61 (d, J=5.7, 1H), 7.50 (d, J=7.2, 1H), 7.38 (d, J=8.4, 1H), 6.78 (s, 2H), 5.11 (dd, J=12.6, 5.5, 1H), 4.70 (s, 2H), 4.09 (s, 2H), 3.91 (s, 6H), 3.66 (s, 3H), 3.46 (s, 2H), 3.27 – 3.17 (m, 5H), 2.91 – 2.82 (m, 1H), 2.76 – 2.65 (m, 2H), 2.60 (s, 3H), 2.16 – 2.08 (m, 1H), 1.53 – 1.43 (m, 5H), 1.28 (s, 10H).

LCMS: 838 (M+H).
Compound 6 (dBRD9)

4-((2-(2-(aminoethoxy)ethoxy)ethyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione trifluoroacetate salt (0.02 mmol, 1 eq) was added to 2-((2,6-dimethoxy-4-(2-methyl-1-oxo-1,2-dihydro-2,7-naphthyridin-4-yl)benzyl)(methyl)amino)acetic acid trifluoroacetate salt (10.2 mg, 0.02 mmol, 1.0 eq) as a 0.1 M solution in DMF (200 microliters). DIPEA (10.4 microliters, 0.060 mmol, 3.0 eq) was added, followed by PyBOP (11.4 mg, 0.022 mmol, 1.1 eq). After 16 hours the reaction was found incomplete; an additional 3.0 eq DIPEA and 0.5 eq PyBOP was added. After 24 hours the reaction was diluted to 10 mL with EtOAc, washed once with a mildly basified (Na₂CO₃) 1:1 solution of deionized water and saturated brine (5 mL), washed three times with deionized water (5 mL), and finally with saturated brine (5 mL) before drying over Na₂SO₄ and concentrating in vacuo. The residue was dissolved in 0.5 mL DCM and purified by silica chromatography (DCM/MeOH 5 to 20% gradient) to give 6 (dBRD9) as a yellow solid (4.9 mg, 31%).

**¹H NMR** (1H NMR (500 MHz, Chloroform-d) δ 9.68 (s, 1H), 8.89 (s, 1H), 8.70 (d, J = 5.6 Hz, 1H), 7.85 (s, 1H), 7.46 (t, 1H), 7.42 (d, J = 5.6 Hz, 1H), 7.30 (s, 1H), 7.08 (d, J = 7.1 Hz, 1H), 6.86 (d, J = 8.5 Hz, 1H), 6.54 (s, 2H), 6.49 (t, J = 5.3 Hz, 1H), 4.93 – 4.85 (m, 1H), 3.84 (s, 6H), 3.73 (s, 2H), 3.70 – 3.64 (m, 9H), 3.60 (t, J = 5.0 Hz, 2H), 3.50 – 3.46 (m, 3H), 3.45 – 3.40 (m, 2H), 3.14 (s, 2H), 2.88 – 2.82 (m, 1H), 2.76 – 2.69 (m, 2H), 2.30 (s, 3H), 2.14 – 2.09 (m, 1H).

**¹³C NMR** (126 MHz, Chloroform-d) δ 171.23, 168.61, 167.57, 159.46, 151.67, 150.92, 146.73, 141.73, 136.05, 135.99, 132.55, 120.49, 117.86, 117.40, 116.65, 111.72, 110.41, 105.30, 70.83, 70.28, 70.21, 69.32, 60.65, 55.97, 48.90, 48.65, 43.07, 42.33, 39.01, 37.12, 31.39, 29.70, 22.94.

**LCMS**: 784 (M+H).
$^1$H NMR (CDCl$_3$)
13C NMR (CDCl3)

S10
4-((2-(2-(2-aminoethoxy)ethoxy)ethyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione trifluoroacetate salt (0.03 mmol, 1 eq) was added 2-((4-(isoquinolin-5-yl)-2,6-dimethoxybenzyl)(methyl)amino)acetic acid (12.1 mg, 0.03 mmol, 1.0 eq) as a 0.1 M solution in DMF (300 microliters). DIPEA (15.7 microliters, 0.090 mmol, 3.0 eq) was added, followed by PyBOP (17.2 mg, 0.033 mmol, 1.1 eq). After 16 hours the reaction was diluted to 10 mL with EtOAc, washed once with a mildly basified (Na₂CO₃) 1:1 solution of deionized water and saturated brine (5 mL), washed three times with deionized water (5 mL), and finally with saturated brine (5 mL) before drying over Na₂SO₄ and concentrating in vacuo. The residue was dissolved in 0.5 mL DCM and purified by silica chromatography (DCM/MeOH 5 to 20% gradient). A second purification by prep HPLC yielded (S10) as a yellow solid (9.63 mg, 41%).
\(^1\)H NMR (500 MHz, Methanol-\(d_4\)) \(\delta\) 9.26 (s, 1H), 8.40 (d, \(J = 6.1\) Hz, 1H), 8.10 (d, \(J = 8.0\) Hz, 1H), 7.84 (d, \(J = 6.1\) Hz, 1H), 7.79 – 7.71 (m, 2H), 7.47 – 7.42 (m, 1H), 6.96 (t, \(J = 7.5\) Hz, 2H), 6.74 (s, 2H), 5.00 (dd, \(J = 12.8, 5.5\) Hz, 1H), 3.86 (s, 6H), 3.76 (s, 2H), 3.64 (d, \(J = 5.3\) Hz, 6H), 3.57 (t, \(J = 5.3\) Hz, 2H), 3.43 (t, \(J = 5.3\) Hz, 2H), 3.38 (t, \(J = 5.3\) Hz, 2H), 3.13 (s, 2H), 2.85 – 2.77 (m, 1H), 2.73 – 2.60 (m, 2H), 2.31 (s, 3H), 2.07 – 2.00 (m, 1H).

\(^1\)H NMR (CD\(_3\)OD)
Table S3. Single-point screening of Compound 5 and dBRD9 at 1 µM using BromoScan.

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Table S3. Data collection and refinement statistics.

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\(^a\) Data collection on APS-24ID \(^b\) Resolution statistics for the highest-resolution shell are shown in parentheses.
References


