Journal of Medicinal Chemistry

Drug Annotation

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Identification of *N*-(4-((1*R*,3*S*,5*S*)-3-Amino-5-methylcyclohexyl)pyridin-3-yl)-6-(2,6-difluorophenyl)-5-fluoropicolinamide (PIM447), a Potent and Selective Proviral Insertion Site of Moloney Murine Leukemia (PIM) 1, 2, and 3 Kinase Inhibitor in Clinical Trials for Hematological Malignancies

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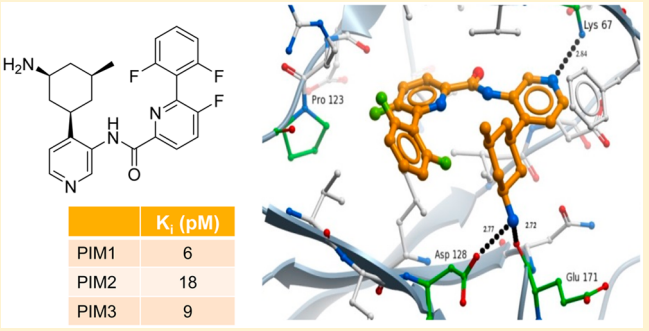
Supporting Information

ABSTRACT: Pan proviral insertion site of Moloney murine leukemia (PIM) 1, 2, and 3 kinase inhibitors have recently begun to be tested in humans to assess whether pan PIM kinase inhibition may provide benefit to cancer patients. Herein, the synthesis, in vitro activity, in vivo activity in an acute myeloid leukemia xenograft model, and preclinical profile of the potent and selective pan PIM kinase inhibitor compound **8** (PIM447) are described. Starting from the reported aminopiperidyl pan PIM kinase inhibitor compound **3**, a strategy to improve the microsomal stability was pursued resulting in the identification of potent aminocyclohexyl pan PIM inhibitors with high metabolic stability. From this aminocyclohexyl series, compound **8** entered the clinic in 2012 in multiple myeloma patients and is currently in several phase 1 trials of cancer patients with hematological malignancies.



	K_i (pM)
PIM1	6
PIM2	18
PIM3	9

INTRODUCTION

Proviral insertion site of Moloney murine leukemia virus kinases, or PIMs 1, 2, and 3 kinases are serine/threonine kinases that normally function in the survival, proliferation, and differentiation of hematopoietic cells in response to growth factors and cytokines.[1] Cytokine signaling through the JAK/STAT pathway leads to increase transcription of the PIM genes with subsequent translation of constitutively active proteins.[1] PIM1 was originally discovered as an oncogene by frequent proximal proviral insertions of murine leukemia virus in lymphomas of infected mice.[2,3] Similar unbiased insertional mutagenesis screens were used to demonstrate the oncogenic potential of PIM2[4] and PIM3[5] in PIM1 and PIM1/2 knockout mice, respectively, and in doing so demonstrated the potential for functional redundancy of the PIM isoforms. In human disease, high expression and/or dysfunction of the three PIMs has been implicated in the progression of hematopoietic and solid tumor cancers.[1,6] High expression of PIM1 has been reported in acute lymphoblastic leukemia, acute myeloid leukemia (AML), and diffuse large B-cell lymphoma, while high expression of PIM2 has been reported in multiple myeloma.[7] The high expression of PIM in cancer, particularly hematopoietic cancers, is thought to play a role in promoting survival and proliferation while suppressing apoptosis. As PIMs display functional redundancy in oncogenesis and are differentially overexpressed in different cancer cell types, a pan-PIM kinase inhibitor rather than an isoform-selective inhibitor would

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be expected to provide superior antitumor efficacy.[8] In addition to cancer, PIM kinases have been reported to play a role in several autoimmune diseases.[9] Not surprisingly, PIM kinases have emerged as attractive therapeutic targets and have elicited several groups to investigate and report novel inhibitors of PIM[10−18] including the clinical compounds SGI-1776[11] and AZD1208,[12] compound **1** and compound **2**, respectively, Figure 1. PIM kinases share a high level of sequence homology

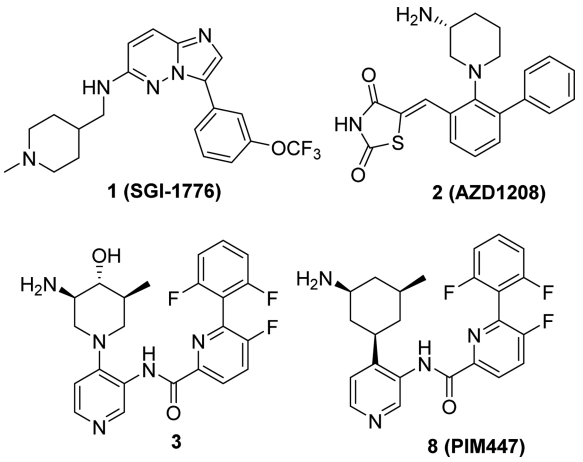

Figure 1. PIM clinical compounds and starting point **3**.

within the family (>61%), and all share the unique feature of being the only kinases with a proline in the hinge,[19] which results in only one hydrogen bond interaction with ATP. As the ATP K_m for PIM2 is 10−100× lower than that for PIM1 and PIM3, cell active pan PIM inhibitors have been more challenging to identify than PIM1/3 inhibitors.

Recently, we described the optimization of a highly kinase selective aminopiperidine, pyridylcarboxamide scaffold,[20] which binds in the PIMs ATP pocket, lacks any hydrogen bonds to the hinge, and exhibits in vitro pan PIM biochemical and cellular potencies greater than compound **1** and compound **2**. From this scaffold, compound **3**, Figure 1, has been characterized in multiple myeloma and AML in vivo hematological mouse xenograft models[7,21] where a PK−PD efficacy relationship has been established. Described herein are our further efforts on the pyridylamide scaffold that have focused on the improvement of metabolic stability. These efforts have led to the identification of *N*-(4-((1*R*,3*S*,5*S*)-3-amino-5-methylcyclohexyl)pyridin-3-yl)-6-(2,6-difluorophenyl)-5-fluoropicolinamide compound **8** (PIM447) which exhibits pan PIM potency, pharmacokinetic properties, and druglike properties suitable for clinical development. Compound **8** entered clinical trials in 2012 and is currently in several phase I trials. Compound **8** was previously known as and is synonymous with LGH447.

While compound **3** has served as a valuable in vitro and in vivo tool for studying PIM biology, the moderate to high in vitro clearance, vide infra, could pose a developability risk, especially since the PK−PD efficacy relationship observed supports maximal efficacy being driven by the maintenance of continuous free plasma levels above cellular antiproliferative EC50's.[7] In vitro met ID studies in human liver microsomes indicate metabolism of compound **3** involves oxidations on carbons of the piperidine ring adjacent to both the internal and external nitrogens. This eventually leads to the removal the piperidine ring altogether, leaving the piperidine nitrogen behind, funneling into a 4-amino 3-aminoacyl pyridyl metabolite, Figure 2. In addition to the high in vitro CL of compound **3**, the formation of an electron rich amino, aminoacyl pyridyl metabolite was not deemed as positive with respect to developability/toxicity. Thus, the challenge for medicinal chemistry was to improve the metabolic stability and reduce or eliminate the formation of a diaminopyridyl metabolite while maintaining the potency and selectivity achieved with compound **3**.

The medicinal chemistry strategy to achieve this goal focused on removing the substituted piperidine portion of the molecule and replacing it with a similarly substituted cyclohexyl ring. With respect to potency, from the SAR developed leading up to compound **3**, it was known that the pyridyl nitrogen in the diaminopyridine ring and the primary amino group on the piperidine were important for potency. The X-ray crystal structure of compound **3** in PIM 1 (PDB code 4N70),[20] Figure 3, indicates these moieties participate in three hydrogen bonds

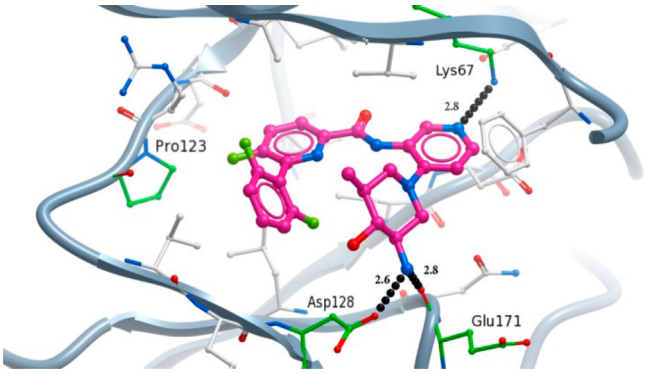

Figure 3. Structure of compound **3** in PIM1.

to the protein (NH_2 to Asp128 side chain and Glu171 backbone carbonyl, and pyridyl to Lys67). Substitution of the cyclohexyl for piperidyl could possibly allow for a similar display of the primary amino group for hydrogen bonding to Asp128 and Glu171 if the piperidine to cyclohexyl change does not change the relative conformation of the saturated six-membered ring relative to the attached *N*-acylpyridine.

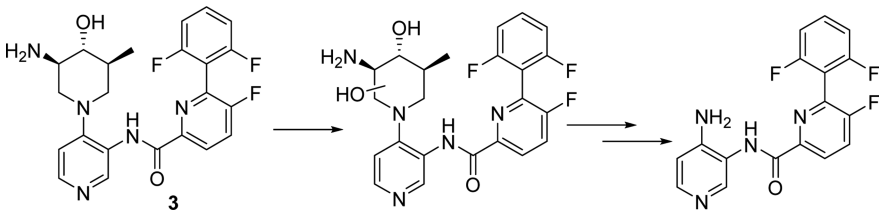

Figure 2. Metabolism of compound **3** in human liver microsomes.

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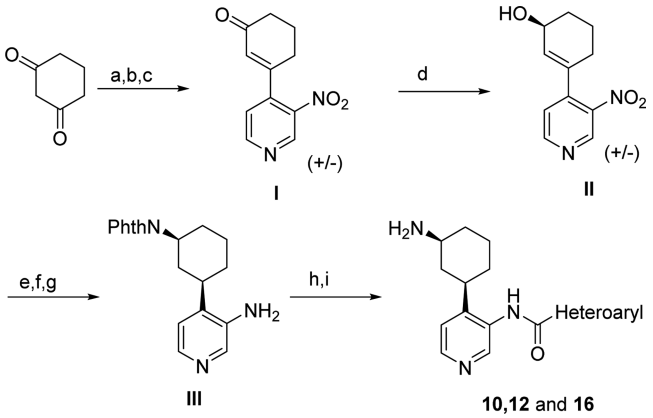
J. Med. Chem. XXXX, XXX, XXX−XXX

Additionally, it was unclear a priori what effect the piperidine to cyclohexyl change might have on potency with respect to the hydrogen bond interaction of the pyridyl nitrogen and Lys67, as the electron density and basicity of the pyridine would be altered. With respect to metabolism, while the piperidine to cyclohexyl change would remove the α or α' N dealkylation metabolic spots adjacent to the piperidine ring nitrogen, it was unclear a priori whether this would improve overall metabolic stability or whether metabolism would just be shifted toward N-dealkylative oxidation α to the primary amino group. This α N-dealkylative oxidation (followed by loss of NH_2 leading to the corresponding ketone) had also been observed in the microsomal studies of compound **3** as one of several piperidine metabolites. Attempts to suppress the metabolism α to the primary amine via installation of an α methyl group led to a loss in PIM potency, unpublished results. Thus, it was unclear beforehand whether the proposed strategy of replacing piperidine with cyclohexyl would yield compounds with improved microsomal stability and with sufficient pan PIM potency. This uncertainty withstanding, cyclohexyl compounds were pursued and synthetic routes were developed.

CHEMISTRY

Initially, aminocyclohexyl targets without additional ring substitution were prepared to establish the viability of the synthetic route, Scheme 1. Cyclohexanedione was converted via

Scheme 1[a]

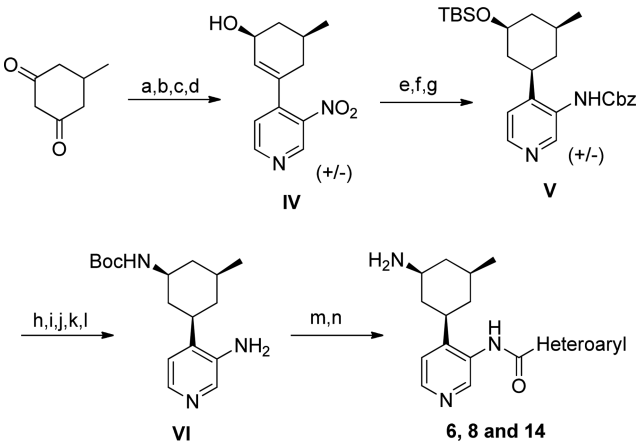


[a]Reagents and conditions: (a) Na_2CO_3, Tf_2O, CH_2Cl_2, 0 °C, 3 h; (b) KOAc, Pd(dppf)Cl_2, pinacolatodiboron, dioxane, 80 °C, 2 h; (c) 4-chloro-3-nitropyridine, Pd(pddf)Cl_2, dioxane/2 M Na_2CO_3, 110 °C, 2 h, 55% (3 steps); (d) $NaBH_4$, $CeCl_3{\cdot}7H_2O$, MeOH, 0 °C, 2 h, 94%; (e) PhthNH, Ph_3P, $(C_4H_9O_2CN)_2$, THF, 0 °C, 2 h, 50%; (f) 400 PSI H_2, Pd/C, MeOH, 50 °C, 48 h, 53%; (g) chiral separation; (h) heteroaryl acid, EDC, HOAT, DMF; 12 h, 91%; (i) H_2NNH_2, MeOH, 50 °C, 2 h, 90%.

the monotriflate to the corresponding cyclohexenoneboronate ester which underwent palladium mediated carbon bond formation with 4-chloro-3-nitropyridine to yield nitropyridyl-cylohexenone **I**. Subsequent 1,2 reduction of the enone yielded the allylic alcohol **II**. The protected amine group was introduced under Mitsunobu conditions with phthalimide. Reduction of the alkene and nitro groups under hydrogenation conditions followed by chiral separation yielded the enantiopure cis substituted cyclohexylpyridylaniline **III**. Amide coupling followed by phthalimide deprotection yielded target compounds **10**, **12**, and **16**.

Aminocyclohexyl targets containing the 5 position methyl group on the cyclohexyl ring were prepared as described in Scheme 2. (5)-Methylcyclohexanedione was converted via the

Scheme 2[a]



[a]Reagents and conditions: (a) Na_2CO_3, Tf_2O, CH_2Cl_2, 0 °C, 2 h, 78%; (b) KOAc, Pd(dppf)Cl_2, pinacolatodiboron, dioxane, 80 °C, 10 h; (c) 4-chloro-3-nitropyridine, Pd(pddf)Cl_2, dioxane/2 M Na_2CO_3, 110 °C, 1 h, 48% (2 steps); (d) $NaBH_4$, $CeCl_3{\cdot}7H_2O$, EtOH, 0 °C, 1 h, 72%; (e) TBDMSCl, imidazole, DMF, 18 h, 77%; (f) H_2, Pd/C, MeOH, 15 h, 93%; (g) Cbz-OSu, DMAP, CH_2Cl_2, 90 h, 81%; (h) HCl, EtOH, 1 h, 94%; (i) Dess–Martin periodinane, CH_2Cl_2, 1 h, 91%; (j)BnNH_2, $LiBH_4$, MeOH, 3.5 h; (k) H_2, Pd/C, MeOH, Boc_2O, 46 h, 47% (2 steps); (l) chiral separation; (m) heteroaryl acid, EDC, HOAT, DMF; 12 h, 91%; (n) 4 M HCl/dioxane, 17 h, 80% or 25% TFA/CH_2Cl_2, 0.5 h, 90%.

monotriflate to the corresponding cyclohexenoneboronate ester which underwent palladium mediated carbon bond formation with 4-chloro-3-nitropyridine to yield after 1,2 reduction of the enone a cis cyclohexenol **IV**. *tert*-Butyldimethylsilyl protection of the alcohol, hydrogenation of the nitro and alkene groups followed by carbobenzyloxyamine protection yielded the racemic all cis, equatorial, trisubstituted cyclohexane **V**. Deprotection of the silyl ether, Dess–Martin oxidation of the alcohol to the cis disubstituted cyclohexanone, followed by lithium borohydride[22] mediated reductive amination with benzylamine reestablished the all cis equatorial cyclohexyl relative stereochemistry. Removal of the carbobenzyloxy and benzyl groups under hydrogenation conditions followed by in situ primary aliphatic amine Boc protection and chiral resolution yielded the desired all cis trisubstituted Boc protected aminocyclohexylpyridylaniline **VI**. Amide coupling followed by acetate and Boc deprotection yielded target compounds **6**, **8**, and **14**.

Aminocyclohexyl targets with the additional hydroxy substitution present in compound **3** were prepared as described in Scheme 3. Cyclohexenol **IV** was dehydrated to yield a diene which reacted with *N*-bromosuccinimide at the less hindered alkene to yield bromohydrin **VII** with the bromine cis to the methyl group. Upon base treatment intramolecular cyclization yielded the epoxide, trans to the methyl, which was opened with sodium azide yielding azido alcohol **VIII**, therein establishing the relative 1,2 trans, 1,3 cis azido, hydroxy, methyl stereochemical relationship. Subsequent azide reduction, Boc amine protection, hydroxyl acetylation, hydrogenation of the nitro and alkene groups, and chiral resolution yielded the chiral tetrasubstituted, all equatorial, aminohydroxypyridylaniline **IX**.

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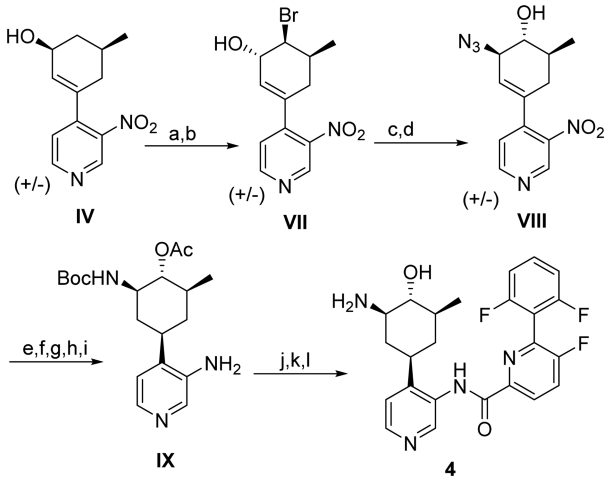
J. Med. Chem. XXXX, XXX, XXX–XXX

Scheme 3[a]



[a]Reagents and conditions: (a) p-TsOH, dioxane, 100 °C, 3 h, 68%; (b) NBS, H_2O, THF, 0.5 h, 80%; (c) KO-*t*-Bu, THF, 0.5 h; (d) NaN_3, NH_4Cl, EtOH, H_2O, 14 h, 55%; (e) PMe_3, pyridine, NH_4OH, 2 h; (f) Boc_2O, dioxane, $NaHCO_{3(sat.)}$, 2 h, 59% (2 steps); (g) Ac_2O, pyridine, 14 h; (h) H_2, Pd/C, MeOH, 72 h, 75% (2 steps); (i) chiral separation; (j) heteroaryl acid, EDC, HOAT, DMF, 14 h; (k) K_2CO_3, EtOH, 14 h; (l) 25% TFA/CH_2Cl_2, 0.5 h, 80% (3 steps).

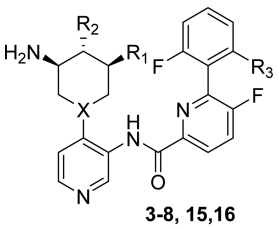
Amide coupling followed by acetate and Boc deprotection yielded the target compound **4**. The piperidyl compound **3** was previously described,[20] and compounds **5**, **7**, **9**, **11**, **13**, and **15** were prepared following a similar route; see experimental part for details.

RESULTS AND DISCUSSION

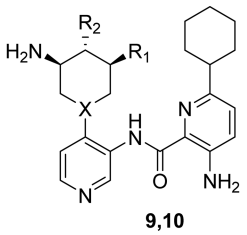
The aminocyclohexanes (**4**, **6**, **8**, **10**, **12**, **14**, **16**) along with matched pair aminopiperidines (**3**, **5**, **7**, **9**, **11**, **13**, **15**) were tested in PIM biochemical and PIM cellular assays to assess the effect of cyclohexyl replacement on target potency. PIM1−3 kinase activity was assessed in a kinase-Glo assay, with [ATP] at or below ATP K_m for each isoform. Potent PIM inhibition, picomolar and nanomolar, for all PIM isoforms was retained for the aminocyclohexyl compounds, Table 1. While many compound matched pairs were at the limits of detection in the low ATP enzymatic assay, for the few (**9**−**14**) that were not there was a ∼3-fold potency difference between piperidine and cyclohexyl, with the piperidine analog being the more potent member of each pair. Cellular activity was assessed in the multiple myeloma derived, PIM2 driven, KMS11-luc cell line where antiproliferative activity was obtained for all compounds except **9** and **10** and target modulation as assessed by phosphorylation of S6 ribosomal protein (pS6RP) was obtained for **3**, **4**, **8**, **14**, and **16**. S6RP, which is not a direct PIM substrate but is downstream from PIM phosphorylation of TSC2, was chosen as a biomarker as it is modulation correlated best with in vivo antiproliferative activity in preclinical models.[7,21] Consistent with prior reports,[7,20,23] there was a large shift upward relative to the low [ATP] enzymatic assays. Additionally, as in the enzymatic assay, the piperidine component of any matched pair was more potent than the corresponding cylcohexyl. The greater potency difference of matched pair **3** and **4** which contain the hydroxyl and amino groups relative to other matched pairs which lack the hydroxyl group is noteworthy, highlighting subtleties of hydrogen bonding, desolvation, and amine basicity for interactions in this region of the protein. Despite the potency decrease in replacing piperidine with cyclohexyl, aminocyclohexyl compounds still exhibit picomolar enzymatic and significant cell potency.

The microsomal stability for the piperidine and cyclohexyl matched pairs **3**−**16** (Table 2) was assessed in human and rat liver microsomes where in addition to NADPH, UDPGA and

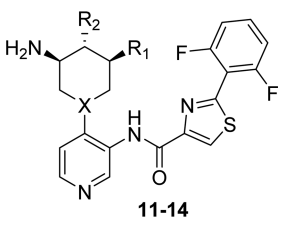
Table 1. PIMs 1, 2, and 3 Enzymatic and Cellular Activity of Compounds 3−16



3-8, 15,16



9,10



11-14

					K_i (μM)			EC_{50} (μM)	
compd	X	R_1	R_2	R_3	PIM1	PIM2	PIM3	KMSIlluc	KMS11luc pS6RP
3	N	CH_3	OH	F	<0.001	<0.003	<0.003	0.02	0.03
4	(*R*)-CH	CH_3	OH	F	<0.001	<0.003	<0.003	0.41	0.91
5	N	CH_3	H	H	<0.001	<0.003	<0.003	1.1	
6	(*R*)-CH	CH_3	H	H	<0.001	0.004	<0.003	1.4	
7	N	CH_3	H	F	<0.001	<0.003	<0.003	0.05	
8	(*R*)-CH	CH_3	H	F	<0.001	<0.003	<0.003	0.17	0.18
9	N	H	H		<0.001	0.004	0.003		
10	(*R*)-CH	H	H		0.005	0.023	0.014		
11	N	H	H		<0.001	0.005	<0.003	1.2	
12	(*R*)-CH	H	H		<0.001	0.029	0.004	5.0	
13	N	CH_3	H		<0.001	<0.003	<0.003	0.39	
14	(*R*)-CH	CH_3	H		<0.001	0.006	0.004	0.91	0.32
15	N	H	H	F	<0.001	<0.003	<0.003	1.4	
16	(*R*)-CH	H	H	F	<0.001	0.005	<0.003	0.54	0.71

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Table 2. Human and Rat Microsomal Stability of Compounds 3−16

	$T_{1/2}$ (min)		CL_H (mL min^{-1} kg^{-1})		ER_H	
compd	rat	human	rat	human	rat	human
3	7	23	49	16	0.88	0.77
4	92	90	20	10	0.36	0.46
5	11	13	45	18	0.82	0.86
6	92	>180	20	<6	0.36	<0.3
7	20	40	40	14	0.72	0.66
8	50	106	28	9	0.51	0.42
9	3	14	52	18	0.94	0.85
10	8	85	47	10	0.86	0.48
11	6	70	49	11	0.89	0.52
12	85	121	21	8	0.38	0.39
13	9	49	47	13	0.85	0.61
14	65	>180	24	<6	0.44	<0.3
15	13	25	44	16	0.79	0.75
16	42	107	30	9	0.55	0.42

alemethicin were added to capture PhII glucoronidation. The compounds were tested at 1 μM for 30 min, and $t_{1/2}$'s were measured from which scaled CL'int, hepatic CL (CL_H), and hepatic extraction ratio (ER_H) were determined using a well-stirred liver model. The measured $t_{1/2}$'s, hepatic CL, and hepatic extraction ratio are listed in Table 2. In human microsomes there is a consistent increase in stability when piperidine is replaced with cyclohexyl with $t_{1/2}$'s increasing and extraction ratio decreasing. For example, the $t_{1/2}$ of the cyclohexyl analog of compound **3**, compound **4**, is 3 times more stable in human microsomes than compound **3**, leading to a ∼2-fold decrease in ER value (∼0.77 versus 0.46). The stability improvement was evident in rat microsomes as well. Improved stability in preclinical species was viewed as a positive with respect to ensuring necessary exposures could be reached in safety studies. The high metabolic stability was also observed in hepatocytes, as compound **8** exhibits clearance values of 12 and 3 mL min^{-1} kg^{-1} in rat and human hepatocytes. From the enzymatic potency, cellular potency, and metabolic stability data depicted in Tables 1 and 2, it is evident replacing the aminopiperidine of aminopiperidine carboxamide inhibitors with an aminocyclohexyl can yield pan PIM potent, cell active compounds with high metabolic stability in human liver microsomes. Of the aminocyclohexanes described in Tables 1 and 2, compound **8** stood out with respect to cellular potency and metabolic stability and was selected for further profiling.

As the biochemical potency for compound **8** reached the limits of detection in the low [ATP] kinase Glo assay format, the potency was further assessed in a high ATP AlphaScreen assay using [ATP]'s of 2800, 500, and 2800 μM for PIMs 1, 2, and 3, respectively. Under these conditions, the K_i's of compound **8** were determined to be 6, 18, and 9 pM for PIMs 1, 2, and 3, respectively. When compared alongside the clinical compounds **1** and **2**, Table 3, compound **8** is more potent in enzymatic and cellular assays.

The kinase selectivity of compound **8** was first determined in biochemical assays for a panel of 68 diverse protein kinases that included PIM2 (Figure 4) as well as 9 lipid kinases. In this panel, only PIM2 was significantly inhibited by compound **8** with an IC_{50} of <0.003 μM, the lowest sensitivity range for the assay. Compound **8** also inhibited GSK3β, PKN1, and PKCτ, but at a significantly lower potency with IC_{50} between 1 and 5

Table 3. Selected SAR of Compound 8 and Comparison to Compounds 1 and 2

compd	Pim1 K_i (nM)	Pim2 K_i (nM)	Pim3 K_i (nM)	KMS11luc EC_{50} (μM)
1	16	610	24	5
2	0.017	0.160	0.230	0.67
8	0.006	0.018	0.009	0.17

Kinase	IC_{50} (μM)
PIM2	<0.00035
GSK3β	1.3
PKCτ	4.1
PKN1	4.9
Protein Kinases with IC_{50} > 10 μM:	
cABL(T315I), cABL, ALK, AURORA-A, BTK, CaMK2,CDK1B, CDK2A, CDK4D1, CK1, COT1, CSK, ERK2, EPHA4, EPHB4, FAK, FGFR1, FGFR2, FGFR3, FGFR4, FGFR3(K650E), FLT3, FYN, HCK, HER1, HER2, HER4, IGF1R, INS1R, IRAK4, JAK1, JAK2, JAK3, JNK2, JNK3, KDR, cKIT, LCK, LYN, cMET, MK2, MK5, MNK1, MNK2, PAK2, PDGFRα, PDK1, PKA, PKBa, PKCα, PKN2, PLK1, RET, ROCK2, RON, S6K, cSRC, SYK, TYK2, WNK1, YES, ZAP70, p38α, p38γ	
Lipid Kinases with IC_{50} > 9.1 μM:	
PI3Kα, PI3Kβ, PI3Kδ, PI3Kγ, PI4Kβ, VPS34, mTOR	

Figure 4. Kinase activity of compound **8** against 68 kinases.

μM (>10^5-fold differential relative to the K_i on PIMs). The biochemical IC_{50} for all other kinases tested in this panel was >9 μM. In follow-up cellular assays of GSK3β inhibition,[7] compound **8** was tested up to 20 μM and was not active.

Additional kinase profiling of compound **8** at 1 μM against 442 kinases using the KINOMEscan binding displacement assay, Figure 5, indicated high kinase selectivity [$S(35)$ = 0.021

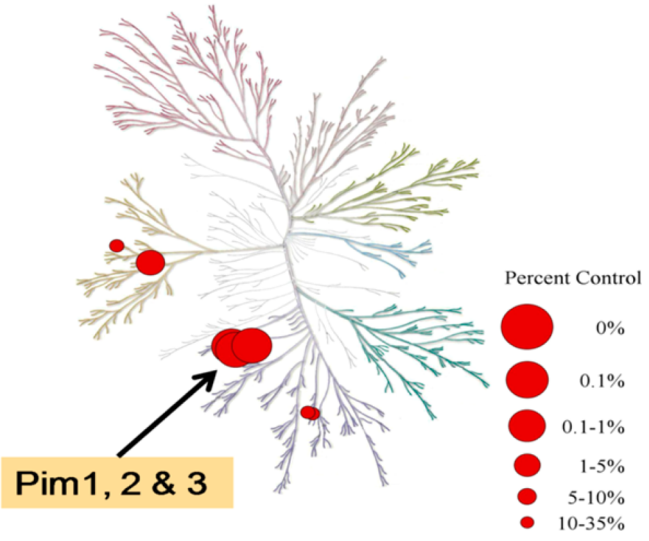

Figure 5. KINOMEscan binding of **8** at 1 μM against 442 kinases.

at 1 μM, 386 nonmutant kinases including PIMs 1, 2, and 3; $S(35)$ = 0.013 at 1 μM, 383 nonmutant kinases not including PIMs 1, 2, and 3], Figure 5. The unlabeled smaller red dots in the kinome tree, Figure 5, correspond to ERK8, GSK3β, and PRKD. The high selectivity of compound **8** is consistent with the scaffold class as reported earlier.[7]

A crystal structure of compound **8** in PIM1 was obtained,[24] Figure 6. The overall binding mode is the same as for **1** with the central amide making no direct hydrogen bonds to the protein

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J. Med. Chem. XXXX, XXX, XXX−XXX

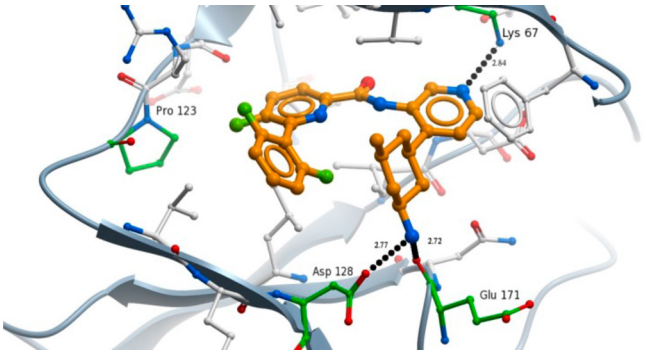

Figure 6. Structure of **8** in PIM1.

and displaying the two sides of the molecule toward the hinge/lower hinge and catalytic Lys region/ribose patch. The amino group on the cyclohexyl ring makes two hydrogen bonds to the Asp128 side chain carboxylate and the Glu171 backbone carbonyl in the ribose patch. Additionally, the methyl group fills a hydrophobic dimple in the glycine rich loop above. The acylaminopyridine ring nitrogen makes a hydrogen bond with the catalytic Lys67. The C ring fluoropyridine fills the Pro123 hinge region with no hydrogen bond interactions, and the difluorophenyl ring extends into the lower hinge region.

With an expanded understanding of the kinase selectivity of compound **8** and the structural features contributing to PIM potency in hand, the cellular activity of compound **8** across an expanded panel of 26 AML cell lines was assessed, Table 4. The

Table 4

cell line	GI_{50} (μM)	cell line	GI_{50} (μM)
Molm16	0.01	OCI-M1	2.57
KG-1	0.01	SKM-1	2.69
EOL-1	0.01	OCI-AML3	2.92
M-07e	0.05	P31/FUJ	5.06
UKE-1	0.09	Mono-MAC-1	5.19
MV-4-11	0.13	THP-1	5.31
CMK	0.28	OCI-AML2	5.53
Set-2	0.48	NB-4	7
CMK-11-5	1.03	PL-21	8.56
Molm-13	1.39	SIG-M5	8.66
Hel92.1.7	1.66	NOMO-1	10
TF-1	1.96	OCI-AML5	10
Mutz8	1.99	F-36P	10

antiproliferative activity spanned a range from low nM to insensitivity at 10 μM, with approximately 1/3 sensitive at ≤500 nM, 1/3 sensitive between 500 nM and 3 μM, and 1/3 sensitive at >3 μM. The activity of compound **8** across the expanded AML panel was consistent with what has been reported for selective PIM inhibitors such as compound **2**[23] and compound **3**[7] wherein a range of sensitivities is evident, with highly sensitive cell lines correlating with the highest expressions of PIM1.

Within the most sensitive AML cell lines, the activity of compound **8** in KG-1 cells was further evaluated both in vitro and in vivo, as a KG-1 in vivo xenograft model was available and synergy with the AML standard of care cytarabine could be assessed given the minimal activity observed with cytarabine at the maximum tolerated dose in the model. Upon treatment of KG-1 cells in vitro at concentrations of 0.05, 0.5, and 5 μM compound **8**, a dose dependent modulation of pS6RP was evident (Figure 7) .

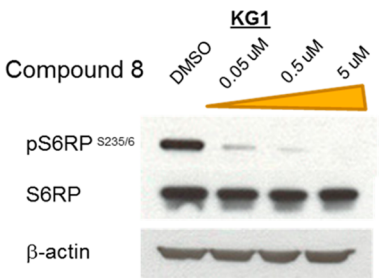

Figure 7. In vitro pS6RP target modulation by compound **8** in KG-1 acute myeloid leukemia cell line.

The activity of compound **8** in an in vivo KG-1 AML xenograft mouse model was assessed next. Following a single oral dose of compound **8** (30 or 100 mg/kg) plasma and tumor samples were collected for pharmacokinetic and pharmacodynamic analysis, respectively. PIM kinase inhibition was determined by assessing the modulation of pS6RP in tumor lysate using the quantitative Meso Scale assay, and the results were expressed as a ratio of their phosphorylated to unphosphorylated forms (Figure 8). The plasma exposure

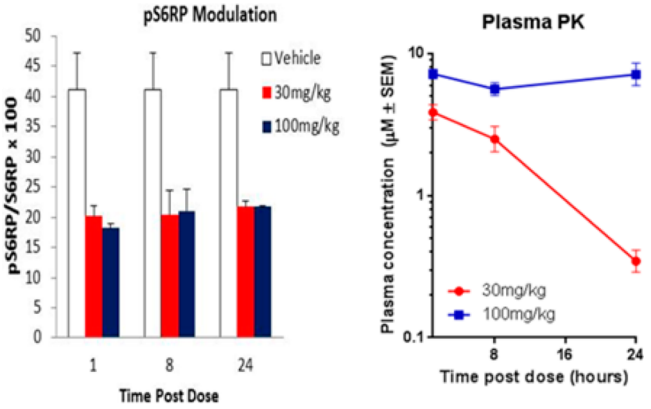

Figure 8. Single dose pS6RP target modulation and plasma exposure of compound **8** in KG-1 acute myeloid leukemia subcutaneous mouse tumor model.

(Figure 8) was roughly dose proportional with a significant difference in exposures at the 24 h time point in particular. With mouse plasma protein binding of 95%, the 30 and 100 mg/kg doses maintain free plasma concentrations of ≥15 and 300 nM, respectively, throughout 24 h. At both doses there was a ∼50% reduction in pS6RP at 1, 8, and 24 h. The lack of a dose response in the target modulation may be a reflection of dynamic range of the detection method, as the ∼50% reduction of pS6RP was the maximal signal seen across multiple xenograft PK−PD studies utilizing compounds in this scaffold class even when dosed up to 300 mg/kg, data not shown. Additionally, in the higher bar KMS11.luc multiple myeloma mouse xenograft model, a dose response in pS6RP modulation at 24 h was observed when compound **8** was dosed at 25 and 100 mg/kg.[25]

The in vivo target modulation translated to an in vivo antiproliferative effect in the KG-1 AML xenograft model when compound **8** was assessed in an 11-day study at 30 and 100 mg/kg QD doses. At the 30 mg/kg daily regimen of compound

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J. Med. Chem. XXXX, XXX, XXX−XXX

8, near stasis was observed with only minor increase in tumor volume relative to the vehicle control (Figure 9). In contrast,

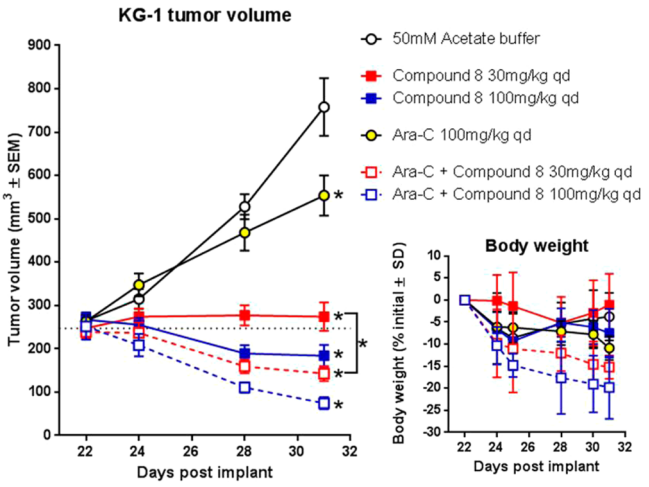

Figure 9. Antitumor activity and tolerability of single agent compound 8 and compound 8 in combination with Ara-C in KG-1 acute myeloid leukemia subcutaneous mouse tumor model.

tumor regression of 31% was observed with the 100 mg/kg QD regimen. Both doses of compound 8 were well tolerated, and the activity observed was similar to that of compound 3 in the same model.[7] In addition to single agent activity, a combination of compound 8 with the nucleoside analog cytarabine (Ara-C), a standard-of-care in the clinical treatment of AML, was assessed. KG-1 tumors exhibited a slight but statistically significant response to Ara-C alone when delivered at 100 mg/kg daily. While this dose results in clinically relevant exposures, it is near the maximum tolerated dose in mice and required a 2-day dosing holiday. When the 30 mg/kg QD dose of compound 8 which led to tumor stasis was combined with the 100 mg/kg QD dose of Ara-C, a synergistic effect resulting in significant regression was achieved (Figure 9). When the higher 100 mg/kg QD dose was similarly combined with Ara-C, the effect did not reach statistical significance compared to the 100 mg/kg dose of compound 8 alone, as this dose achieved regression as a single agent. Similar to the single-agent Ara-C cohort, the combination of Ara-C with compound 8 at both doses induced significant body weight loss following the fifth day of administration which required dosing to be halted only in these arms for 2 days before resuming treatment. Taken together, these results demonstrate the efficacy of compound 8 both as a single agent and in combination with Ara-C, even in a model that is refractory to this standard-of-care.

With these encouraging mouse pharmacology activities, compound 8 was profiled further with respect to developability and druglike properties. The pharmacokinetic properties of compound 8 were evaluated across multiple species (Table 5). Low to moderate in vivo CL was observed for compound 8 across species, as CL values of 20, 28, and 8 mL min^{-1} kg^{-1} were observed in mouse, rat, and dog, respectively. The volume of distribution was consistently large across species, with V_{ss} of 5.3, 6.4, and 3.6 L/kg observed in mouse, rat, and dog, respectively. Additionally, compound 8 exhibited high oral bioavailability across species, as 84%, 70%, and 71% was observed in mouse, rat, and dog, respectively.

Table 5. PK Properties of Compound 8 across Species

species	iv $t_{1/2}$ (h)	CL (mL min^{-1} kg^{-1})	V_{ss} (L/kg)	po F (%)
mouse	3.3	20	5.3	84
rat	2.8	28	6.4	70
dog	6.7	8	3.6	71

The melting point of compound 8 as a free base is 139 °C. Its log D (pH 6.8) is 1.1, and its pK_a values are 9.8 and 4.2. The solubility of crystalline free base at pH 1, 3, and 6.8 is >45, 4, and 0.5 mM. The stability of compound 8 in human plasma is high, >90% after a 3 h incubation, and the human plasma protein binding of compound 8 is 95%. In the manual patch clamp hERG assay compound 8 exhibited a 12 μM IC_{50} and in dog telemetry studies demonstrated no cardiosafety signals wherein a 2× mouse efficacious free C_{max} plasma concentration, 1 μM, was achieved. With a mouse efficacious unbound C_{max} of ~0.5 μM, the window between the free C_{max} in mouse models and hERG IC_{50} is ~20-fold.

On the basis of favorable cellular potency, kinase selectivity, manageable preclinical pharmacology and pharmacokinetic, physical properties, and preclinical safety profiles, compound 8 was advanced into clinical trials in 2012, where a wide therapeutic window demonstrating single agent activity and a tolerable safety profile has been observed in multiple myeloma patients.[26] As opposed to that seen with halted clinical compounds 1 and 2, the observation of early clinical activity and favorable safety profile with compound 8 may be due in part to its increased potency and/or selectivity.

CONCLUSION

In summary, the metabolic stability of a selective pan PIM kinase active aminopiperidine pyridyl carboxamide series exemplified by compound 3 was improved by replacing the chiral trisubstituted aminopiperidine with di-, tri-, and tetrasubstituted aminocyclohexanes. Synthetic routes were developed for the preparation of the substituted cylcohexanes with control of relative stereochemistry. The improved in vitro metabolic stability translated in vivo as cyclohexyl compound 8 exhibits low to moderate CL in mouse, rat, and dog. The metabolically stable aminocyclohexyl pyridyl carboxamides described exhibit picomolar pan PIM enzymatic activity, high kinase selectivity, and nanomolar cellular activity. With the combination of potent in vitro activity and low to moderate CL, compound 8 demonstrates in vivo target modulation (pS6RP), single agent antitumor activity in a KG-1 AML mouse xenograft model, and druglike properties suitable for development. Compound 8 advanced into humans in 2012 and is currently being assessed in several phase I trials.[27]

EXPERIMENTAL SECTION

The compounds and/or intermediates were characterized by high performance liquid chromatography (HPLC) using a Waters Millenium chromatography system with a 2695 separation module (Milford, MA). The analytical columns were Alltima C-18 reversed phase, 4.6 mm × 50 mm, flow 2.5 mL/min, from Alltech (Deerfield, IL). A gradient elution was used, typically starting with 5% acetonitrile/95% water and progressing to 100% acetonitrile over a period of 10 min. All solvents contained 0.1% trifluoroacetic acid (TFA). Compounds were detected by ultraviolet light (UV) absorption at either 220 or 254 nm. HPLC solvents were from Burdick and Jackson (Muskegan, MI) or Fisher Scientific (Pittsburgh, PA). In some instances, purity was assessed by thin layer chromatography (TLC) using glass or plastic backed silica gel plates, such as Baker-Flex silica gel 1B2-F flexible sheets. TLC results were

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J. Med. Chem. XXXX, XXX, XXX−XXX

readily detected visually under ultraviolet light or by employing well-known iodine vapor and other various staining techniques.

Mass spectrometric analysis was performed on one of two LCMS instruments: a Waters system (Alliance HT HPLC and a Micromass ZQ mass spectrometer; column, Eclipse XDB-C18, 2.1 mm × 50 mm; solvent system, 5−95% (or 35−95% or 65−95% or 95−95%) acetonitrile in water with 0.05% TFA; flow rate 0.8 mL/min; molecular weight range 200−1500; cone voltage 20 V; column temperature 40 °C) or a Hewlett-Packard system (series 1100 HPLC; column, Eclipse XDB-C18, 2.1 mm × 50 mm; solvent system, 1−95% acetonitrile in water with 0.05% TFA; flow rate 0.8 mL/min; molecular weight range 150−850; cone voltage 50 V; column temperature 30 °C). All masses were reported as those of the protonated parent ions.

HRMS ESI-MS data were recorded using a Synapt G2 HDMS (TOF mass spectrometer, Waters) with electrospray ionization source. The resolution of the MS system was approximately 15 000. Leucine enkephalin was used as lock mass (internal standards) infused from lockspray probe. The compound was infused into the mass spectrometer by UPLC (Acquity, Waters) from sample probe. The separation was performed on Acquity UPLC BEH C18 1 mm × 50 mm column at 0.2 mL/min flow rate with the gradient from 5% to 95% in 3 min. Solvent A was water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid. The mass accuracy of the system has been found to be <5 ppm with lock mass.

^{1}H nuclear magnetic resonance (NMR) analyses described herein were performed on some of the compounds with a Varian 400 MHz NMR (Palo Alto, CA). The spectral reference was either TMS or the known chemical shift of the solvent. ^{13}C NMR spectra were recorded using a Bruker AVANCE-500 NMR spectrometer operating at a frequency of 125.77 MHz for ^{13}C equipped with a 5 mm BBO probe.

Preparative separations were carried out using a Teledyne ISCO chromatography system, by flash column chromatography using silica gel (230−400 mesh) packing material, or by HPLC using a Waters 2767 sample manager, C-18 reversed phase column, 30 mm × 50 mm, flow 75 mL/min. Typical solvents employed for the Teledyne ISCO chromatography system and flash column chromatography were dichloromethane, methanol, ethyl acetate, heptane, acetone, aqueous ammonia (or ammonium hydroxide), and triethylamine. Typical solvents employed for the reverse phase HPLC were varying concentrations of acetonitrile and water with 0.1% trifluoroacetic acid.

The purity of all compounds screened in the biological assays was examined by LC−MS analysis and was found to be ≥95%.

(*S*)-*tert*-Butyl 1-(3-aminopyridin-4-yl)piperidin-3-ylcarbamate, *tert*-butyl (3*S*,5*R*)-1-(3-aminopyridin-4-yl)-5-methylpiperidin-3-ylcarbamate, *tert*-butyl (3*R*,4*R*,5*S*)-1-(3-aminopyridin-4-yl)-4-(*tert*-butyldimethylsilyloxy)-5-methylpiperidin-3-ylcarbamate, *N*-(4-((3*R*,4*R*,5*S*)-3-amino-4-hydroxy-5-methylpiperidin-1-yl)pyridin-3-yl)-6-(2,6-difluorophenyl)-5-fluoropicolinamide (**1**), and 6-(2,6-difluorophenyl)-5-fluoropicolinic acid were prepared as previously described.[19]

5-Fluoro-6-(2-fluorophenyl)picolinic Acid. To a solution of 6-bromo-5-fluoropicolinic acid (699 mg, 3.2 mmol) in DME and 2 M Na_2CO_3 (3:1, 16 mL) were added 2-fluorophenylboronic acid (672 mg, 4.8 mmol) and Pd(dppf)Cl_2-DCM (130 mg, 0.16 mmol) in a microwave vial. The vial was heated in the microwave at 120 °C for 30 min. The mixture was diluted with ethyl acetate, and 1 N NaOH was added. The organic phase was separated and extracted three more times with 1 N NaOH and once with 6 N NaOH. The combined aqueous phases were filtered and acidified to pH 1 by the addition of concentrated HCl and extracted with ethyl acetate. The organic layer was dried over magnesium sulfate, filtered, and concentrated to yield 5-fluoro-6-(2-fluorophenyl)picolinic acid (301 mg, 40%). LC/MS = 236.1 (M + H). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.19 (dd, J = 8.61, 3.91 Hz, 1 H), 8.02 (dd, J = 9.39, 8.61 Hz, 1 H), 7.54−7.69 (m, 2 H), 7.35−7.44 (m, 2 H).

3-Amino-6-cyclohexylpicolinic Acid. A solution of methyl 3-amino-6-bromopicolinate (1.2 g, 5.2 mmol), cyclohexylzinc bromide 0.5 M solution in THF (13 mL, 6.5 mmol), and tetrakis(triphenylphosphine)palladium (0.25 g, 0.2 mmol) was stirred at 50 °C for 15 min. The reaction was filtered and washed with EtOAc. The organic was washed with H_2O (100 mL), $NaCl_{(sat.)}$ (50 mL), dried over $MgSO_4$, and the volatiles were removed in vacuo. The product was purified on silica utilizing the ISCO 0−65% gradient of hexane/EtOAc to yield methyl 3-amino-6-cyclohexylpicolinate. LCMS (m/z): 235.2 (MH^+). To a solution of methyl 3-amino-6-cyclohexylpicolinate (1.2 g, 5.1 mmol) in THF (10 mL) was added 1 M LiOH (20.4 mL, 20.4 mmol). After stirring for 4 h at room temperature, 1 N HCl (20.4 mL, 20.4 mmol) was added and the THF was removed in vacuo. The resulting solid was filtered, rinsed with cold H_2O (3 × 20 mL), yielding 3-amino-6-cyclohexylpicolinic acid (203 mg, 18%). LCMS (m/z): 221.0 (MH^+).

2-(2,6-Difluorophenyl)thiazole-4-carboxylic Acid. A solution of 2,6-difluorobenzamide (3.14 g, 0.2 mol) and Lawesson's reagent (4.04 g, 0.1 mol) in of toluene (100 mL) was heated at 90 °C for 14 h. Upon cooling, the volatiles were removed in vacuo and purified by SiO_2 chromatography (25% EtOAc/hexanes) yielding 2,6-difluorobenzothioamide (3.97 g) as a light yellow solid LCMS (m/z): 174.1 (MH^+). The material was used as is assuming 3.46 g. A solution of 2,6-difluorobenzothioamide (3.46 g, 0.2 mol) and ethyl bromopyruvate (2.5 mL, 0.2 mol) in ethanol (20 mL) was heated in the microwave at 130 °C for 30 min. Upon removal of volatiles in vacuo, ethyl acetate was added and the solution was washed with $Na_2CO_{3(sat.)}$, with $NaCl_{(sat.)}$, was dried over $MgSO_4$, filtered, and concentrated yielding ethyl 2-(2,6-difluorophenyl)thiazole-4-carboxylate (4.5 g, 84%). LCMS (m/z): 270.1 (MH^+). To a solution of ethyl 2-(2,6-difluorophenyl)-thiazole-4-carboxylate (4.5 g, 0.167 mol) in 2:1 THF/MeOH (100 mL) was added 1.0 M LiOH (33.5 mL, 0.335 mol). After standing for 16 h, 1.0 M HCl (33.5 mL, 0.335 mol) was added and the THF/MeOH was removed in vacuo. The resulting solid was filtered, rinsed with H_2O, and dried, yielding 2-(2,6-difluorophenyl)thiazole-4-carboxylic acid (3.54 g, 88%). LCMS (m/z): 251.1 (MH^+). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.72 (s, 1 H), 7.65 (tt, J = 8.51, 6.36 Hz, 1 H), 7.28−7.39 (m, 2 H).

3-(3-Nitropyridin-4-yl)cyclohex-2-enone (I). To a solution of cyclohexane-1,3-dione (40 g, 357 mmol) in DCM (1 L) was added Na_2CO_3 (41.6 g, 392 mmol), and the heterogeneous solution was cooled to 0 °C. A solution of Tf_2O (66.3 mL, 392 mmol) in DCM (100 mL) was added dropwise over 1 h at room temperature under a nitrogen atmosphere with an internal temperature of <2 °C. Upon addition, the reaction was stirred for 2 h (dark red solution). The solution was filtered, and to the filtrate was added saturated $NaHCO_3$ (carefully), then extracted the organics, dried with brine, then Na_2SO_4, and concentrated. The crude material was used directly assuming 55.5 g of 3-oxocyclohex-1-enyl trifluoromethanesulfonate LC/MS = 244.9/286.0 (M + H and M + CH_3CN). To a solution of 3-oxocyclohex-1-enyl trifluoromethanesulfonate (55.5 g, 227 mmol) in degassed dioxane (1 L) was added bis(pinacolato)diboron (87 g, 341 mmol), KOAc (66.9 g, 682 mmol), and Pd(dppf)Cl_2−DCM (9.28 g, 11.4 mmol). The sealed reaction was heated to 80 °C for 2 h, cooled to rt, and then filtered. The dioxane solution was used for the next step without further purification. LC/MS = 140.9 (M + H of boronic acid). To a solution of 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-cyclohex-2-enone (50.5 g, 227 mmol) in degassed dioxane (1.4 L) and 2 M Na_2CO_3 (335 mL) were added 4-chloro-3-nitropyridine (57.7 g, 364 mmol) and Pd(dppf)Cl_2−DCM (9.28 g, 11.4 mmol) The reaction was placed under a reflux condenser and heated in an oil bath to 110 °C for 2 h. The mixture was cooled to room temperature, filtered through a pad of Celite. The pad was washed with ethyl acetate, and the filtrate was concentrated under vacuo. The residue was partitioned between brine and ethyl acetate, the layers were separated, the aqueous phase was further extracted with ethyl acetate (4×), the organics were combined, dried over sodium sulfate, filtered, and concentrated. The crude was purified via silica gel chromatography to yield 3-(3-nitropyridin-4-yl)cyclohex-2-enone **I** (27.42 g, 55%). LC/MS = 219.0 (M + H). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.32 (s, 1 H), 9.21−9.42 (m, 1 H), 8.88 (d, J = 4.69 Hz, 1 H), 7.28 (s, 1 H), 6.01 (t, J = 1.37 Hz, 1 H), 2.49−2.61 (m, 5 H), 2.17−2.31 (m, 2 H).

3-(3-Nitropyridin-4-yl)cyclohex-2-enol (II). A solution of 3-(3-nitropyridin-4-yl)cyclohex-2-enone (8.0 g, 36.7 mmol) and $CeCl_3 \cdot 7H_2O$ (17.4 g, 46.8 mmol) in MeOH (367 mL) was cooled at 0 °C, and $NaBH_4$ (1.39 g, 36.7 mmol) was added in portions. The reaction

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J. Med. Chem. XXXX, XXX, XXX−XXX

was stirred for 2 h at 0 °C and then quenched by adding water. The volatiles were removed in vacuo, and the residue was dissolved in EtOAc, washed with brine, dried over Na_2SO_4, and concentrated to yield 3-(3-nitropyridin-4-yl)cyclohex-2-enol **II** (7.59 g, 94%). LC/MS = 221.1 (M + H). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.11 (s, 1 H), 8.75 (d, J = 4.99 Hz, 1 H), 7.27 (d, J = 4.74 Hz, 1 H), 5.72−5.85 (m, 1 H), 4.25−4.45 (m, 1 H), 2.13−2.34 (m, 2 H), 1.86−2.06 (m, 2 H), 1.68−1.86 (m, 2 H), 1.64 (d, J = 6.90 Hz, 1 H).

2-(3-(3-Aminopyrin-4-yl)cyclohexyl)isoindoline-1,3-dione (III). To a homogeneous solution of 3-(3-nitropyridin-4-yl)cyclohex-2-enol (7.4 g, 33.6 mmol), triphenylphosphine (13.22 g, 50.4 mmol), and phthalimide (7.42 g, 50.4 mmol) in THF (168 mL) cooled to 0 °C was added di-*tert*-butyl azodicarboxylate (11.61 g, 50.4 mmol) in THF (32 mL). The mixture was stirred at 0 °C for 2 h. The reaction was concentrated in vacuo. The residue was purified by SiO_2 column chromatography (5% methanol in 1:1 ethyl acetate and hexanes) and trituration with DCM and hexanes to yield a solid, which was further triturated with DCM and hexanes to yield 2-(3-(3-nitropyridin-4-yl)cyclohex-2-enyl)isoindoline-1,3-dione (5.83 g, 50%). LC/MS (m/z): MH^+ = 350.2. A solution of 2-(3-(3-nitropyridin-4-yl)cyclohex-2-enyl)isoindoline-1,3-dione (5.63 g, 16.1 mmol) in MeOH (160 mL) was purged with argon for 10 min while in a steel bomb. At this time 10% Pd/C (2.57 g, 2.4 mmol) was added and the steel bomb was sealed and placed under 400 psi of H_2. The reaction mixture was stirred at 50 °C for 2 days. Upon reaction completion, the solids were removed by filtration over Celite, then rinsed with EtOAc and MeOH. The filtrate was concentrated, diluted with EtOAc, and washed 2× with sat. aq 2 M Na_2CO_3, dried with $MgSO_4$, filtered, concentrated, purified by SiO2 chromatography (10% MeOH in EtOAc), and triturated from EtOAc/hexanes to yield 2-(3-(3-aminopyrin-4-yl)-cyclohexyl)isoindoline-1,3-dione (**III**) (2.75 g, 53%). LC/MS (m/z): MH^+ = 322.2. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 8.04 (s, 1 H), 8.01 (d, J = 5.09 Hz, 1 H), 7.80−7.87 (m, 2 H), 7.67−7.75 (m, 2 H), 7.05 (d, J = 5.09 Hz, 1 H), 4.34 (tt, J = 12.28, 3.77 Hz, 1 H), 3.66 (br s, 2 H), 2.60−2.72 (m, 1 H), 2.40−2.50 (m, 1 H), 2.34 (qd, J = 12.52, 3.91 Hz, 1 H), 2.03−2.14 (m, 1 H), 1.82−1.98 (m, 3 H), 1.45−1.65 (m, 2 H). Separation of the enantiomers under the following conditions. Column: Chiralpak OJ (25 × 2 cm). Solvent: hexane/ethanol (70:30). Flow rate: 15 mL/min. Injection volume: 5 mL (10 mg/mL solution in 1:1 MeOH/EtOH). UV: 210 nm. Peak 1: 2-((1*S*,3*R*)-3-(3-aminopyridin-4-yl)cyclohexyl)isoindoline-1,3-dione. Peak 2: 2-((1*R*,3*S*)-3-(3-aminopyridin-4-yl)cyclohexyl)isoindoline-1,3-dione.

***cis*-(±)-5-Methyl-3-(3-nitropyridin-4-yl)cyclohex-2-enol (IV).** To a solution of 5-methylcyclohexane-1,3-dione (56.1 g, 445 mmol) in DCM (880 mL, 0.5 M) was added Na_2CO_3 (51.8 g, 863 mmol) and cooled to 0 °C. Tf_2O (132 g, 468 mmol) in DCM (150 mL, 5.0 M) was added dropwise over 1 h at 0 °C under a nitrogen atmosphere. Upon addition, the reaction was stirred for 1 h at room temperature (dark red solution). The solution was filtered and the filtrate was quenched by careful addition of saturated $NaHCO_3$ (∼400 mL) with vigorous stirring until pH = 7. The solution was transferred to a separatory funnel, and the layers were separated. The organic layer was washed with brine, dried with Na_2SO_4, filtered, concentrated under vacuo, and dried under high vacuum to yield 5-methyl-3-oxocyclohex-1-enyl trifluoromethanesulfonate as light yellow oil in 78% yield (89.6 g). The triflate decomposes upon storage and should be used immediately for the next reaction. LC/MS = 259.1/300.1 (M + H and M + CH_3CN). ^{1}H NMR (400 MHz, $CdCl_3$) δ ppm: 6.05 (s, 1H), 2.70 (dd, J = 17.2, 4.3, 1H), 2.53 (dd, J = 16.6, 3.7, 1H), 2.48−2.31 (m, 2H), 2.16 (dd, J = 16.4, 11.7, 1H), 1.16 (d, J = 5.9, 3H). To a solution of 5-methyl-3-oxocyclohex-1-enyl trifluoromethanesulfonate (89.6 g, 347 mmol) in degassed dioxane (478 mL, 0.7 M) was added bis(pinacolato)diboron (176 g, 693 mmol), KOAc (102 g, 1.04 mol), and Pd(dppf)Cl_2−DCM (8.5 g, 10 mmol). The reaction was heated to 80 °C for 10 h (initial heating at large scale results in exothermic formation of an orange foam on top of the solution, and the heating bath should be removed until the foam retracts; reheating to 80 °C at this point appears to be fine), then cooled to room temperature and filtered through a coarse frit glass funnel. The cake was rinsed with more dioxane (∼250 mL), and the filtrate solution was used for the next step without further purification. LC/MS = 155.1 (M + H of boronic acid). To a solution of 5-methyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)cyclohex-2-enone (82 g, 347 mmol) in degassed dioxane (694 mL, 0.5 M) and 2 M Na_2CO_3 (335 mL, 694 mmol) were added 4-chloro-3-nitropyridine (71.5 g, 451 mmol) and Pd(dppf)Cl_2−DCM (14.2 g, 17.4 mmol). The reaction was placed under a reflux condenser and heated in an oil bath to 110 °C for 1 h. The mixture was cooled to room temperature, filtered through a pad of Celite. The pad was washed with ethyl acetate, and the filtrate was concentrated under vacuo. The residue was further pumped at 80 °C on a rotary evaporator for 1 h to remove boronate byproducts (M + H = 101) via sublimation. The residue was partitioned between brine and ethyl acetate, the layers were separated, the aqueous phase was further extracted with ethyl acetate (4×), the organics were combined, dried over sodium sulfate, filtered, and concentrated. The crude was purified via silica gel chromatography loading in DCM and eluting with 2−50% ethyl acetate and hexanes. The pure fractions were concentrated in vacuo to yield an orange oil. The oil was placed under high vacuum (∼500 mTorr) with seed crystals overnight to yield an orange solid. The solid was further purified via trituration in hexanes to yield 5-methyl-3-(3-nitropyridin-4-yl)cyclohex-2-enone (39 g, 48% over 2 steps). LC/MS = 233.2 (M + H). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 9.31 (s, 1H), 8.88 (d, J = 5.1, 1H), 7.30 (d, J = 5.1, 1H), 6.00 (d, J = 2.4, 1H), 2.62 (dd, J = 16.4, 3.5, 1H), 2.53−2.34 (m, 3H), 2.23 (dd, J = 16.1, 11.7, 1H), 1.16 (d, J = 6.3, 3H). To a solution of 5-methyl-3-(3-nitropyridin-4-yl)cyclohex-2-enone (46 g, 198 mmol) in EtOH (990 mL) was added $CeCl_3 \cdot 7H_2O$ (89 g, 238 mmol). The reaction was cooled to 0 °C, then $NaBH_4$ (8.99 g, 238 mmol) was added in portions. The mixture was stirred for 1 h at 0 °C, then quenched by adding water, concentrated to remove the EtOH, added EtOAc, extracted the organics, washed with brine, then dried with Na_2SO_4, filtered, and concentrated and purified by SiO_2 chromatography (0−10% MeOH/CH_2Cl_2) to yield *cis*-(±)-5-methyl-3-(3-nitropyridin-4-yl)cyclohex-2-enol **IV** (33.4 g, 72%). LC/MS = 235.2 (M + H). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.07 (s, 1H), 8.79 (d, J = 4.70 Hz, 1H), 7.50 (d, J = 5.09 Hz, 1H), 5.65 (s, 1H), 4.97 (d, J = 5.48 Hz, 1H), 4.21 (d, J = 4.30 Hz, 1H), 2.04−2.15 (m, 1H), 1.75−1.98 (m, 3H), 1.07−1.19(m, 1H), 0.98 (d, J = 6.26 Hz, 3H).

Synthesis of *cis*-(±)-Benzyl 4-3-(*tert*-Butyldimethylsilyloxy)-5-methylcyclohexyl)pyridin-3-ylcarbamate (V). To a solution of 5-methyl-3-(3-nitropyridin-4-yl)cyclohex-2-enol (**IV**) (21.9 g, 93 mmol) in DMF (93 mL, 1.0 M) were added imidazole (14 g, 206 mmol) and TBDMSCl (18.3 g, 122 mmol). After stirring for 18 h the solution was portioned between EtOAc and H_2O and the layers were separated. After washing further with H_2O (3×) and $NaCl_{(sat.)}$, drying over $MgSO_4$, filtering, and removal of solvents, the crude was purified via silica gel column chromatography, eluting with 0−70% ethyl acetate in hexanes to afford *cis*-(±)-4-(3-(*tert*-butyldimethylsilyloxy)-5-methylcyclohex-1-enyl)-3-nitropyridine (27.3 g, 77%). LC/MS = 349.2 (M + H). To a solution of *cis*-(±)-4-(3-(*tert*-butyldimethylsilyloxy)-5-methylcyclohex-1-enyl)-3-nitropyridine (23.1 g, 66.4 mmol) in methanol (221 mL), at a concentration of 0.3 M, was added 10% palladium on carbon (Degussa type, 101NE/W, 1.4 g). The resultant heterogeneous solution was put under an atmosphere of hydrogen and was stirred for 15 h. At this time the mixture was filtered through a pad of Celite, eluting with methanol. The volatiles were removed in vacuo yielding all *cis*-(±)-4-(3-(*tert*-butyldimethylsilyloxy)-5-methylcyclohexyl)pyridin-3-amine (19.7 g, 93%). LCMS (m/z): 321.3 (MH^+). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.03 (s, 1H), 8.00 (d, J = 5.1, 1H), 7.0 (d, J = 5.1, 1H), 3.80−3.69 (m, 1H), 3.63 (bs, 1H), 3.48 (d, J = 5.1, 1H), 2.55 (tt, J = 12.2, 3.1, 1H), 1.89−2.05 (m, 2H), 1.78 (d, J = 12.9, 1H), 1.52−1.73 (m, 1H), 1.36−1.50 (m, 1H), 1.00−1.16 (m, 1H), 0.98 (d, J = 6.7, 3H), 0.89 (s, 9H), 0.08 (s, 3H), 0.06 (s, 3H). To a solution of *cis*-(±)-4-(3-(*tert*-butyldimethylsilyloxy)-5-methylcyclohexyl)pyridin-3-amine (20.5 g, 64 mmol) in dichloromethane (213 mL) were added benzyl 2,5-dioxopyrrolidin-1-yl carbonate (28.7 g, 115 mmol) and DMAP (0.78 g, 6.4 mmol). After stirring for 90 h the solution was filtered through a glass frit funnel, diluted with 300 mL of DCM, washed with water (2×), brine, dried over sodium sulfate, filtered, and concentrated. The crude material was

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J. Med. Chem. XXXX, XXX, XXX−XXX

purified via silica gel column chromatography, eluting with 10−100% ethyl acetate in hexanes to yield *cis*-(±)-benzyl-4-3-(*tert*-butyldimethylsilyloxy)-5-methylcyclohexyl)pyridin-3-ylcarbamate **V** (23.6 g, 81% yield). LC/MS = 455.3 (M + H). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.77 (bs, 1H), 8.37 (d, J = 5.1, 1H), 7.29−7.49 (m, 5H), 7.17 (d, J = 5.1, 1H), 5.22 (s, 2H), 3.59−3.75 (m, 1H), 2.74−2.67 (m, 1H), 1.94−1.91 (m, 2H), 1.67−1.50 (m, 2H), 1.36−1.45 (m, 1H), 1.09−1.00 (m, 2H), 0.95 (d, J = 6.7, 3H), 0.87 (s, 9H), 0.06 (s, 3H), 0.04 (s, 3H).

***cis*-(±)-*tert*-Butyl (3-(3-Aminopyridin-4-yl)-5-methylcyclohexylcarbamate (VI).** A solution of *cis*-(±)-benzyl 4-3-(*tert*-butyldimethylsilyloxy)-5-methylcyclohexyl)pyridin-3-ylcarbamate (**V**) (18.8 g, 41.3 mmol) in 1:2:1 6 N HCl/THF/MeOH (138 mL) was stirred at rt for 1 h. The pH was then adjusted to pH = 7 by addition of 6 N NaOH, and the volatiles were removed in vacuo. The residue was partitioned between ethyl acetate and water, the aqueous layer was extracted with EtOAc, and the organic phase was washed with $NaCl_{(sat.)}$, dried over $MgSO_4$, filtered, and upon removal of the volatiles in vacuo, silica gel column chromatography eluting with 0−20% (1:1 DCM/MeOH) in DCM afforded 13.2 g of *cis*-(±)benzyl 4-(3-hydroxy-5-methylcyclohexyl)pyridin-3-ylcarbamate in 94% yield. LC/MS = 341.2 (M + H), LC = 2.38 min. ^{1}H NMR (300 MHz, $CDCl_3$) δ J = 5.3, 1H), 6.70 (bs, 1H), 5.20 (s, 2H), 3.72−3.65 (m, 1H), 2.79−2.74 (m, 1H), 2.04−1.97 (m, 2H),1.69−1.50 (m, 2H), 1.37−1.26 (m, 1H), 1.01−0.94 (m, 2H), 0.97 (d, J = 6.5, 3H). To a 0 °C solution of *cis*-(±)-benzyl 4-(3-hydroxy-5-methylcyclohexyl)-pyridin-3-ylcarbamate (13.2 g, 38.8 mmol) in wet CH_2Cl_2 (194 mL) was added Dess-Martin Periodinane (16.5 g, 38.9 mmol) and the solution was stirred for 1 h as it warmed to rt. The solution was partitioned between EtOAc and 1:1 10% $Na_2S_2O_3$/$NaHCO_{3(sat.)}$ and separated. Upon further washing with 1:1 10% $Na_2S_2O_3$/$NaHCO_{3(sat.)}$ (2x) and $NaCl_{(sat.)}$, drying over $MgSO_4$, filtering, removal of solvents, cis-(±)-benzyl-4-(3-methyl-5-oxocyclohexyl)pyridin-3-ylcarbamate was obtained (11.9 g, 91%). LC/MS = 339.2 (M + H), LC: 2.47 min. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.70 (bs, 1H), 8.43 (d, J = 5.3, 1H), 7.30−7.45 (m, 5H), 7.20 (d, J = 5.3, 1H), 6.90 (s, 1H), 5.19 (s, 2H), 3.27−3.04 (m, 1H), 2.52−2.22 (m, 3H), 2.13−1.79 (m, 3H), 1.52 (q, J = 12.4, 1H), 1.06 (d, J = 6.2, 3H). A solution of cis-(±)-benzyl-4-(3-methyl-5-oxocyclohexyl)pyridin-3-ylcarbamate (11.9 g, 35.2 mmol) and benzylamine (11.3 g, 105 mmol) in MeOH (117 mL) was stirred at rt for 2 h. Upon cooling in a −78 °C bath, $LiBH_4$ (19.3 mL, 38.6 mmol, 2.0 M in THF) was added dropwise and the solution was allowed to warm to rt with stirring over 1.5 h. The solution was partitioned between EtOAc and $NaHCO_{3(sat.)}$, separated, washed further with $NaHCO_{3(sat.)}$ and $NaCl_{(sat.)}$, dried over $MgSO_4$, filtered, and after removal of volatiles in vacuo, *cis*-(±)- benzyl 4-(-3-(benzylamino)-5-methylcyclohexyl)pyridin-3-ylcarbamate was obtained as a 4:1 mixture of isomers, with the all cis as predominant (15.3 g, >99% yield). LC/MS = 430.3 (M + H), LC = 2.38 min. To a solution of *cis*-(±)- benzyl 4-(-3-(benzylamino)-5-methylcyclohexyl)-pyridin-3-ylcarbamate (15.1 g, 35.2 mmol) in methanol (176 mL) was added 20% palladium hydroxide on carbon (4.9 g, 0.2 equiv). The resultant heterogeneous solution was put under an atmosphere of hydrogen and was stirred for 14 h. At this time the reaction was purged with Ar, Boc_2O (15.4 g, 70.6 mmol) was added, and the solution was stirred for 8 h. Additional Boc_2O (7.7 g, 35.3 mmol) was added and the solution was stirred for 16 more hours. At this time the mixture was filtered through a pad of Celite eluting with methanol. Upon removal of volatiles in vacuo, purification by silica gel chromatography eluting with 0−20% (1:1 MeOH/DCM) in DCM yielded 7 g of a white foam. Further recrystallization from 10% EtOAc/hexanes yielded *cis*-(±)-*tert*-butyl (3-(3-aminopyridin-4-yl)-5-methylcyclohexylcarbamate (5 g, 47%). LCMS (m/z): 306.3 (MH^+), LC t_R = 2.59 min. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 8.04 (s, 1H), 7.98 (d, J = 5.1, 1H), 6.94 (d, J = 5.1, 1H), 4.3 (m, 1H), 3.77−3.54 (m, 2H), 2.68−2.54 (m, 1H), 2.19 (d, J = 12.5, 1H), 2.06 (d, J = 12.2, 1H), 1.83 (d, J = 12.9, 1H), 1.76−1.62 (m, 1H), 1.44 (s, 9H), 1.20−1.09 (m, 2H), 0.99 (d, J = 6.7, 3H), 0.86 (q, J = 12.0, 1H). Pure enantiomers were by chiral HPLC. Separation of the enantiomers was performed under the following conditions. Column: Chiralpak AD (50 mm × 500 mm). Solvent: heptane/ethanol/methanol (70:25:5). Flow rate: 60 mL/min. Injection volume: 1 g/10 mL 1:1 MeOH/mobile phase. UV: 210 nm. Analytical chiral HPLC on Chiracel OD 10 μM; solvent, heptane/EtOH (80:20); flow rate = 1.2 mL/min where peak 1 comes at 12.12 min and peak 2 comes at 19.07 min. Peak 1 = *tert*-butyl (1*R*,3*S*,5*R*)-3-(3-aminopyridin-4-yl)-5-methylcyclohexylcarbamate. Peak 2 = *tert*-butyl (1*S*,3*R*,5*S*)-3-(3-aminopyridin-4-yl)-5-methylcyclohexylcarbamate

(±)-6-Bromo-5-methyl-3-(3-nitropyridin-4-yl)cyclohex-2-enol (VII). To a solution of 5-methyl-3-(3-nitropyridin-4-yl)cyclohex-2-enol (**IV**) (28.9 g, 123 mmol) in dioxane (1.2 L, 0.1 M) was added p-TSA (25.8 g, 135 mmol), and the reaction was stirred at 100 °C for 3 h. The solution was cooled to room temperature, then passed through a column of neutral alumina eluting with EtOAc to yield (±)-4-(5-methylcyclohexa-1,3-dienyl)-3-nitropyridine as a yellow oil in 68% yield (14.7 g). ^{1}H NMR indicated about 12−15% contamination of another diene isomer. LC/MS = 217.1 (M + H). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.01 (s, 1H), 8.70 (d, J = 5.1, 1H), 7.29 (d, J = 5.3, 1H), 6.14 (dd, J = 5.4, 0.85, 1H), 6.05 (dd, J = 5.4, 2.3, 1H), 5.89 (dd, J = 9.6, 3.7, 1H), 2.55−2.68 (m, 1H), 2.33−2.43 (m, 1H), 2.17−2.27 (m, 1H), 1.1 (d, J = 7.1, 3H). To a solution of 4-(5-methylcyclohexa-1,3-dienyl)-3-nitropyridine (14.7 g, 67 mmol) in THF and water (1:1, 515 mL) was added NBS (18.2 g, 102 mmol), and the reaction was stirred at room temperature for 30 min. Upon completion, ethyl acetate and water were added to the reaction, the organic phase was washed with brine, then dried with sodium sulfate, filtered, and concentrated. The crude material was purified via silica gel column chromatography, eluting with ethyl acetate and hexanes (1:1) to give (±)-6-bromo-5-methyl-3-(3-nitropyridin-4-yl)cyclohex-2-enol **VII** as a yellow oil in 80% yield (17.9 g). LC/MS = 315.0/313.0 (M + H). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.17 (s, 1 H), 8.78 (d, J = 4.70 Hz, 1 H), 7.30 (d, J = 4.70 Hz, 1 H), 5.74−5.79 (m, 1 H), 4.55 (t, J = 3.72 Hz, 1 H), 4.27 (t, J = 2.54 Hz, 1 H), 2.59 (br s, 1 H), 2.28−2.36 (m, 1 H), 2.22−2.28 (m, 2 H), 1.18 (d, J = 6.26 Hz, 3 H).

(±)-2-Azido-6-methyl-4-(3-nitropyridin-4-yl)cyclohex-3-enol (VIII). To a solution of (±)-6-bromo-5-methyl-3-(3-nitropyridin-4-yl)cyclohex-2-enol (**VII**) (16 g, 51.5 mmol) in THF (510 mL) was added potassium *tert*-butoxide (8.6 g, 77 mmol) in portions. The reaction turned from orange to black almost immediately. By TLC, the formation of product is clean in 30 min. The reaction was quenched by adding saturated ammonium chloride and ethyl acetate. The organic phase was washed with brine, then dried with sodium sulfate, filtered, and concentrated. The crude product was dissolved in ethanol and water (3:1, 400 mL, 0.1 M), and ammonium chloride (5.5 g, 103 mmol) and sodium azide (6.6 g, 103 mmol) were added. The dark orange reaction was stirred at room temperature overnight. The conversion to product is clean as indicated by LC/MS. The reaction was concentrated to remove the ethanol, ethyl acetate and water were added, the organic phase was dried with sodium sulfate, filtered, and concentrated. The crude material was purified via silica gel column chromatography, eluting with ethyl acetate and hexanes (1:1) to give **VIII** in 55% yield (12 g). LC/MS = 276.0 (M + H). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.18 (s, 1 H), 8.78 (d, J = 5.09 Hz, 1 H), 7.25 (d, J = 5.09 Hz, 1 H), 5.57 (t, J = 2.15 Hz, 1 H), 4.01−4.09 (m, 1 H), 3.45−3.58 (m, 1 H), 2.26−2.41 (m, 2 H), 2.06−2.22 (m, 2 H),1.12−1.17 (m, 3 H).

(±)-4-(3-Aminopyridin-4-yl)-2-(*tert*-butoxycarbonylamino)-6-methylcyclohexyl Acetate (IX). To a solution of (±)-2-azido-6-methyl-4-(3-nitropyridin-4-yl)cyclohex-3-enol (**VIII**) (12 g, 44 mmol) in pyridine and ammonium hydroxide (8:1, 225 mL) was added trimethylphosphine (9.95 g, 131 mmol), and the brown solution was stirred at room temperature for 2 h (bubbling). Upon completion, EtOH (50 mL) was added and the solution was concentrated in vacuo. More ethanol (50 mL) was added, and the reaction was concentrated again. Dioxane and sat. $NaHCO_3$ (1:1, 200 mL, 0.08 M) were added to the crude, followed by Boc_2O (9.5 g, 44 mmol). The reaction mixture was stirred at room temperature for 2 h, then water and ethyl acetate were added. The organic phase was dried with $MgSO_4$, and concentrated. The crude product was purified via silica gel column chromatography, eluting with ethyl acetate and hexanes (1:1) to afford

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(±)-*tert*-butyl 6-hydroxy-5-methyl-3-(3-nitropyridin-4-yl)cyclohex-2-enylcarbamate (7.9 g, 59%). LC/MS = 350.1 (M + H).^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.12 (s, 1 H), 8.75 (d, J = 5.09 Hz, 1 H), 7.24 (d, J = 4.70 Hz, 1 H), 5.46 (t, J = 2.35 Hz, 1 H), 4.77 (m, 1 H), 4.27 (m, 1 H), 3.48 (m, 1 H), 3.36−3.41 (m, 1 H), 1.95−2.34 (m, 4 H), 1.43−1.50 (m, 9 H), 1.14 (d, J = 6.26 Hz, 3 H). To a solution of (±)-*tert*-butyl 6-hydroxy-5-methyl-3-(3-nitropyridin-4-yl)cyclohex-2-enylcarbamate (7.7 g, 22 mmol) in pyridine (220 mL) was added Ac_2O (6.2 mL, 66 mmol), and the reaction was stirred at room temperature overnight. Upon completion, the reaction was concentrated to dryness, then worked up with ethyl acetate and water. The organic phase was washed with brine, then dried with sodium sulfate, filtered, and concentrated to give (±)-2-(*tert*-butoxycarbonylamino)-6-methyl-4-(3-nitropyridin-4-yl)cyclohex-3-enyl acetate in 99% yield (8.5 g). LC/MS = 392.2 (M + H). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.14 (s, 1 H), 8.75 (d, J = 5.09 Hz, 1 H) 7.23 (d, J = 5.09 Hz, 1 H), 5.50−5.59 (m, 1 H), 4.81 (t, J = 9.78 Hz, 1 H), 4.72 (d, J = 9.00 Hz, 1 H), 4.40−4.53 (m, 1 H), 2.18−2.46 (m, 4 H), 2.13 (s, 3 H), 1.42 (s, 9 H), 1.01 (d, J = 5.87 Hz, 3 H). To a degassed solution of (±)-2-(*tert*-butoxycarbonylamino)-6-methyl-4-(3-nitropyridin-4-yl)cyclohex-3-enyl acetate (8.5 g, 22 mmol) in MeOH and EtOAc (1:1, 200 mL) was added 10% Pd/C (2.3 g, 2.2 mmol), and the reaction was stirred at room temperature under a hydrogen balloon for 3 days. Upon completion, the solution was filtered through a pad of Celite, the pad was washed with ethyl acetate, and the filtrate was concentrated. The crude material contained about 10% of the undesired isomer. The crude was dissolved in ethyl acetate (~20%) and hexanes and heated until all dissolved. The solution was allowed to sit at room temperature for 2 days. The precipitate was then collected to give (±)-4-(3-aminopyridin-4-yl)-2-(*tert*-butoxycarbonylamino)-6-methylcyclohexyl acetate as the pure product in 75% yield (5.9 g). LC/MS = 364.3 (M + H), t_R = 0.63 min. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 8.05 (s, 1 H), 8.00 (d, J = 5.09 Hz, 1 H), 6.93 (d, J = 5.09 Hz, 1 H), 4.64 (d, J = 8.61 Hz, 1 H), 4.52 (t, J = 10.17 Hz, 1 H), 3.77−3.83 (m, 1 H), 3.66 (br s, 2 H), 2.68−2.74 (m, 1 H), 2.26 (ddd, J = 12.91, 6.85, 2.93 Hz, 1 H), 2.12 (s, 3 H), 1.83−1.99 (m, 2 H), 1.42 (s, 9 H), 1.39−1.27 (m, 2 H), 0.99 (d, J = 6.26 Hz, 3 H). Separation of the enantiomers was performed under the following conditions. Column: Chiralpak AD (50 mm × 500 mm). Solvent: heptane/2-propanol (80:20). Flow rate: 80 mL/min. Injection volume: 10 mL. UV: 210 nm. Peak 1, (1*R*,2*R*,4*R*,6*S*)-4-(3-aminopyridin-4-yl)-2-((*tert*-butoxycarbonyl)-amino)-6-methylcyclohexyl acetate (**IX**), at 8.043 min. Peak 2, (1*S*,2*S*,4*S*,6*R*)-4-(3-aminopyridin-4-yl)-2-((*tert*-butoxycarbonyl)-amino)-6-methylcyclohexyl acetate, at 14.581 min.

General Procedure for Amide Coupling and Amine Deprotection for Preparing 4−16. *tert*-Butyl (1*S*,3*R*,5*S*)-3-(3-(6-(2,6-Difluorophenyl)-5-fluoropicolinamido)pyridin-4-yl)-5-methylcyclohexylcarbamate (8). A solution of *tert*-butyl (1*S*,3*R*,5*S*)-3-(3-aminopyridin-4-yl)-5-methylcyclohexylcarbamate (1.46 g, 4.78 mmol), 6-(2,6-difluorophenyl)-5-fluoropicolinic acid (1.57 g, 6.21 mmol), EDC−HCl (1.2 g, 6.21 mmol), and HOAt (0.85 g, 6.21 mmol) in DMF (24 mL, 0.2 M) was stirred at room temperature overnight. The reaction was poured into water and extracted with ethyl acetate (4×). The combined organics were washed with 0.5 N NaOH (3×), brine, dried over sodium sulfate, filtered, and concentrated to afford *tert*-butyl (1*S*,3*R*,5*S*)-3-(3-(6-(2,6-difluorophenyl)-5-fluoropicolinamido)pyridin-4-yl)-5-methylcyclohexylcarbamate in 91% yield (2.34 g). LCMS (m/z): 541.0 (MH^+). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.86 (s, 1H), 9.34 (s, 1H), 8.43 (dd, J = 8.6, 3.9, 1H), 8.39 (d, J = 5.1, 1H), 7.77 (t, J = 8.4, 1H), 7.58−7.43 (m, 1H), 7.17−7.05 (m, 3H), 4.43 (bs, 1H), 3.55 (bs, 1H), 2.94−2.80 (m, 1H), 2.13 (d, J = 12.2, 1H), 2.07−1.94 (m, 1H), 1.83 (d, J = 12.9, 1H), 1.60−1.47 (m, 1H), 1.43 (m, 9H), 1.40−1.30 (m, 1H), 1.05−0.89 (m, 1H), 0.86 (d, J = 6.3, 3H), 0.82−0.71 (m, 1H). A solution of 4 M HCl in dioxane (13.4 mL, 53.6 mmol) was added to *tert*-butyl (1*S*,3*R*,5*S*)-3-(3-(6-(2,6-difluorophenyl)-5-fluoropicolinamido)pyridin-4-yl)-5-methylcyclohexylcarbamate (2.9 g, 5.36 mmol), and the reaction mixture was stirred at room temperature for 17 h. The heterogeneous solution was concentrated in vacuo to yield a white solid which was further dissolved in MeOH and diethyl ether (1:21). The mixture was sonicated, and the precipitate was filtered. The precipitate was dried under high vacuum for several days to yield *N*-(4-((1*R*,3*S*,5*S*)-3-amino-5-methylcyclohexyl)pyridin-3-yl)-6-(2,6-difluorophenyl)-5-fluoropicolinamide as the HCl salt in 80% yield. The free base was obtained by partitioning the salt between EtOAc and Na_2CO_3 (sat.), washing with brine, drying over $MgSO_4$, and removal of volatiles in vacuo. LCMS (m/z): 441.3 (MH^+). HRMS (m/z) calcd for $C_{24}H_{24}F_3N_4O$ (MH^+) 441.1902, found 441.1905. ^{1}H NMR (400 MHz, DMSO-d_6, bis-HCl salt) δ 10.59 (s, 1H), 8.92 (s, 1H), 8.62 (d, J = 6.0, 1H), 8.37 (dd, J = 8.8, 4.0, 1H), 8.23 (t, J = 8.8, 1H), 8.19 (bs, 2H), 7.68−7.71 (m, 2H), 7.36−7.40 (m, 2H),3.01−3.10 (m, 2H), 2.01−2.05 (m, 1H), 1.94−1.97 (m, 1H), 1.72−1.76 (m, 1H), 1.46−1.53 (m, 2H), 1.01−1.13 (m, 2H), 0.89 (d, J = 6.8, 3H). ^{13}C NMR (125.77 MHz, $CDCl_3$, free base) 160.8, 160.5, 159.8, 18, 131.8, 131.4, 125.4, 124.8, 120.7, 111.9, 111.8, 50.3, 44.3, 41.6, 39.8, 36.5, 31.6, 22.1. In variations of this general method, the Boc group could be deprotected by treatment with 25% TFA/CH_2Cl_2 for 30 min, and upon concentration of the volatiles in vacuo, purification via RP-HPLC yielded final compounds as TFA salts after lyophilization. Compounds in free base form were obtained by partitioning HPLC purified fractions between EtOAc and Na_2CO_3(s), washing with brine, drying over $MgSO_4$, and removal of volatiles in vacuo. For compounds **10**, **12**, and **16** the phthalimide group was removed by treating with hydrazine (10 equiv) in MeOH (0.1M) at 60 °C for 2 h or overnight at rt.

***N*-(4-((1*R*,3*R*,4*R*,5*S*)-3-Amino-4-hydroxy-5-methylcyclohexyl)pyridin-3-yl)-6-(2,6-difluorophenyl)-5-fluoropicolinamide (4).** Following the general procedure, (1*R*,2*R*,4*R*,6*S*)-4-(3-aminopyridin-4-yl)-2-(*tert*-butoxycarbonylamino)-6-methylcyclohexyl acetate and 6-(2,6-difluorophenyl)-5-fluoropicolinic acid were coupled and deprotected to yield **2**. The acetate group was removed prior to Boc deprotection by treating with K_2CO_3 (1.2 equiv) in EtOH (0.1 M) overnight. LC/MS = 457.3 (M + H). HRMS (m/z) calcd for $C_{24}H_{24}F_3N_4O_2$ (MH^+) 457.1851, found 457.1855. ^{1}H NMR (DMSO-d_6, HCl salt) δ 10.42 (s, 1H), 8.65 (s, 1H), 8.45 (d, J = 5.1, 1H), 8.33 (dd, J = 8.6, 3.9, 1H), 8.19 (t, J = 8.8, 1H), 8.0 (bs, 2H), 7.69 (m, 1H), 7.39−7.32 (m, 3H), 3.07−2.94 (m, 2H), 2.82 (m, 1H), 2.03−2.00 (m, 1H), 1.74−1.59 (m, 2H), 1.40 (m, 1H), 1.23 (m, 1H), 0.93 (d, J = 6.3, 3H).

***N*-(4-((3*S*,5*R*)-3-Amino-5-methylpiperidin-1-yl)pyridin-3-yl)-5-fluoro-6-(2-fluorophenyl)picolinamide (5).** Following the general procedure *tert*-butyl (3*S*,5*R*)-1-(3-aminopyridin-4-yl)-5-methylpiperidin-3-ylcarbamate and 5-fluoro-6-(2-fluorophenyl)picolinic acid were coupled and deprotected to yield **5**. LC/MS = 424.1 (M + H). HRMS (m/z) calcd for $C_{23}H_{24}F_2N_5O$ (MH^+) 424.1949, found 424.1953. ^{1}H NMR (400 MHz, DMSO-d_6, TFA salt) δ ppm 10.39 (s, 1 H), 9.13 (s, 1 H), 8.39 (d, J = 6.21 Hz, 1 H), 8.34 (dd, J = 8.58, 3.84 Hz, 1 H), 8.18 (t, J = 8.97 Hz, 1 H), 7.99 (br s, 2 H), 7.76−7.84 (m, 1 H), 7.60−7.72 (m, 1 H), 7.41−7.50 (m, 2 H), 7.32 (d, J = 6.26 Hz, 1 H), 3.66−3.79 (m, 1 H), 3.57 (d, J = 10.27 Hz, 1 H), 3.18 (br s, 1 H), 2.85 (t, J = 11.30 Hz, 1 H), 2.28−2.41 (m, 1 H), 1.82 (d, J = 12.32 Hz, 1 H), 1.70 (d, J = 6.46 Hz, 1 H), 1.05 (q, J = 11.88 Hz, 1 H), 0.59 (d, J = 6.55 Hz, 3 H).

***N*-(4-((1*R*,3*S*,5*S*)-3-Amino-5-methylcyclohexyl)pyridin-3-yl)-5-fluoro-6-(2-fluorophenyl)picolinamide (6).** Following the general procedure *tert*-butyl (1*S*,3*R*,5*S*)-3-(3-aminopyridin-4-yl)-5-methylcyclohexylcarbamate and 5-fluoro-6-(2-fluorophenyl)picolinic acid were coupled and deprotected to yield **6**. LC/MS = 423.1 (M + H). HRMS (m/z) calcd for $C_{24}H_{25}F_2N_4O$ (MH^+) 423.1996, found 423.1996. ^{1}H NMR (400 MHz, DMSO-d_6, TFA salt) δ ppm 10.42 (s, 1 H), 8.72 (s, 1 H), 8.47 (d, J = 5.18 Hz, 1 H), 8.27 (dd, J = 8.61, 3.91 Hz, 1 H), 8.13 (t, J = 9.05 Hz, 1 H), 7.89 (td, J = 7.52, 1.79 Hz, 1 H), 7.81 (br s, 2 H), 7.59−7.70 (m, 1 H), 7.35−7.49 (m, 3 H), 3.09 (d, J = 3.62 Hz, 1 H), 2.96 (t, J = 12.13 Hz, 1 H), 2.00 (d, J = 11.88 Hz, 1 H), 1.89 (d, J = 12.91 Hz, 1 H), 1.75 (d, J = 12.52 Hz, 1 H), 1.52 (br s, 1 H), 1.41 (q, J = 11.95 Hz, 1 H), 0.95−1.12 (m, 2 H), 0.89 (d, J = 6.50 Hz, 3 H).

***N*-(4-((3*S*,5*R*)-3-Amino-5-methylpiperidin-1-yl)pyridin-3-yl)-6-(2,6-difluorophenyl)-5-fluoropicolinamide (7).** Following the general procedure *tert*-butyl (3*S*,5*R*)-1-(3-aminopyridin-4-yl)-5-meth-

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ylpiperidin-3-ylcarbamate and 6-(2,6-difluorophenyl)-5-fluoropicolinic acid were coupled and deprotected to yield **7**. LC/MS = 442.3 (M + H). HRMS (m/z) calcd for $C_{23}H_{23}F_3N_5O$ (MH^+) 442.1855, found 442.1856. ^{1}H NMR (400 MHz, methanol-d_4, TFA salt) δ ppm 9.29 (s, 1 H), 8.47 (dd, J = 8.68, 3.94 Hz, 1 H), 8.39 (d, J = 6.46 Hz, 1 H), 8.07 (t, J = 8.68 Hz, 1 H), 7.59−7.76 (m, 1 H), 7.48 (d, J = 6.55 Hz, 1 H), 7.25 (t, J = 8.56 Hz, 2 H), 3.81−3.97 (m, 1 H), 3.72 (d, J = 12.67 Hz, 1 H), 3.32−3.41 (m, 1 H), 3.01 (t, J = 11.32 Hz, 1 H), 2.47 (t, J = 12.25 Hz, 1 H), 2.00 (d, J = 12.28 Hz, 1 H), 1.79−1.93 (m, 1 H), 1.20 (q, J = 12.13 Hz, 1 H), 0.73 (d, J = 6.60 Hz, 3 H).

(*S*)-3-Amino-*N*-(4-(3-aminopiperidin-1-yl)pyridin-3-yl)-6-cyclohexylpicolinamide (9). Following the general procedure (*S*)-*tert*-butyl 1-(3-aminopyridin-4-yl)piperidin-3-ylcarbamate and 3-amino-6-cyclohexylpicolinic acid were coupled and deprotected to yield **9**. LC/MS = 395.2 (M + H). HRMS (m/z) calcd for $C_{22}H_{31}N_6O$ (MH^+) 395.2559, found 395.2560. ^{1}H NMR (400 MHz, methanol-d_4, TFA salt) δ ppm 9.33 (s, 1 H), 8.37 (d, J = 6.50 Hz, 1 H), 7.50 (d, J = 6.50 Hz, 1 H), 7.16−7.34 (m, 2 H), 3.83 (d, J = 12.08 Hz, 1 H), 3.65 (d, J = 12.76 Hz, 1 H), 3.51−3.60 (m, 1 H), 3.04−3.18 (m, 2 H), 2.61−2.73 (m, 1 H), 2.26 (dd, J = 13.30, 3.42 Hz, 1 H), 1.86−2.07 (m, 5 H), 1.81 (d, J = 12.37 Hz, 1 H), 1.64−1.77 (m, 1 H), 1.26−1.62 (m, 6 H).

3-Amino-*N*-(4-((1*R*,3*S*)-3-aminocyclohexyl)pyridin-3-yl)-6-cyclohexylpicolinamide (10). Following the general procedure 2-(3-(3-aminopyrin-4-yl)cyclohexyl)isoindoline-1,3-dione and 3-amino-6-cyclohexylpicolinic acid were coupled and deprotected to yield **10**. LC/MS = 394.2 (M + H). HRMS (m/z) calcd for $C_{23}H_{32}N_5O$ (MH^+) 394.2607, found 394.2610. ^{1}H NMR (400 MHz, cd3od, TFA salt) δ ppm 9.47 (s, 1 H), 8.49 (d, J = 5.67 Hz, 1 H), 7.77 (d, J = 5.72 Hz, 1 H), 7.16−7.33 (m, 2 H), 3.11−3.24 (m, 1 H), 2.63−2.74 (m, 1 H), 2.26 (d, J = 11.98 Hz, 1 H), 2.11−2.22 (m, 2 H), 1.96−2.11 (m, 3 H), 1.91 (d, J = 12.81 Hz, 2 H), 1.77−1.86 (m, 1 H), 1.66−1.77 (m, 2 H), 1.40−1.66 (m, 6 H), 1.23−1.40 (m, 2 H).

(*S*)-*N*-(4-(3-Aminopiperidin-1-yl)pyridin-3-yl)-2-(2,6-difluorophenyl)thiazole-4-carboxamide (11). Following the general procedure (*S*)-*tert*-butyl 1-(3-aminopyridin-4-yl)piperidin-3-ylcarbamate and 2-(2,6-difluorophenyl)thiazole-4-carboxylic acid were coupled and deprotected to yield **11**. LC/MS = 416.1 (M + H). HRMS (m/z) calcd for $C_{20}H_{20}F_2N_5OS$ (MH^+) 416.1357, found 416.1354. ^{1}H NMR (400 MHz, DMSO-d_6, HCl salt) δ ppm 10.25 (s, 1 H), 8.88 (s, 1 H), 8.80 (s, 1 H), 8.41 (d, J = 6.80 Hz, 1 H), 8.33 (br s, 3 H), 7.64−7.76 (m, 1 H), 7.34−7.46 (m, 3 H), 3.86 (d, J = 9.34 Hz, 1 H), 3.56 (d, J = 13.84 Hz, 1 H), 3.37 (br s, 2 H), 3.27 (br s, 1 H), 1.93−2.05 (m, 1 H), 1.79−1.93 (m, 1 H), 1.51−1.77 (m, 2 H).

***N*-(4-((1*R*,3*S*)-3-Aminocyclohexyl)pyridin-3-yl)-2-(2,6-difluorophenyl)thiazole-4-carboxamide (12).** Following the general procedure 2-(3-(3-aminopyrin-4-yl)cyclohexyl)isoindoline-1,3-dione and 2-(2,6-difluorophenyl)thiazole-4-carboxylic acid were coupled and deprotected to yield **12**. LC/MS = 415.0 (M + H). HRMS (m/z) calcd for $C_{21}H_{21}F_2N_4OS$ (MH^+) 415.1404, found 415.1403. ^{1}H NMR (400 MHz, DMSO-d_6, TFA salt) δ ppm 10.21 (s, 1 H), 8.74 (s, 1 H), 8.64 (s, 1 H), 8.49 (d, J = 5.23 Hz, 1 H), 7.82 (br s, 3 H), 7.63−7.77 (m, 1 H), 7.32−7.47 (m, 3 H), 3.10 (br s, 1 H), 2.94 (t, J = 11.79 Hz, 1 H), 1.91−2.05 (m, 2 H), 1.71−1.90 (m, 2 H), 1.46 (q, J = 12.23 Hz, 1 H), 1.26−1.38 (m, 3 H).

***N*-(4-((3*S*,5*R*)-3-Amino-5-methylpiperidin-1-yl)pyridin-3-yl)-2-(2,6-difluorophenyl)thiazole-4-carboxamide (13).** Following the general procedure *tert*-butyl (3*S*,5*R*)-1-(3-aminopyridin-4-yl)-5-methylpiperidin-3-ylcarbamate and 2-(2,6-difluorophenyl)thiazole-4-carboxylic acid were coupled and deprotected to yield **13**. LC/MS = 430.0 (M + H). HRMS (m/z) calcd for $C_{21}H_{22}F_2N_5OS$ (MH^+) 430.1513, found 430.1514. ^{1}H NMR (400 MHz, DMSO-d_6, HCl salt) δ ppm 9.85 (s, 1 H), 9.24 (s, 1 H), 8.81 (s, 1 H), 8.33 (d, J = 5.53 Hz, 1 H), 8.12 (br s, 3 H), 7.65−7.76 (m, 1 H), 7.41 (t, J = 8.83 Hz, 2 H), 7.22 (d, J = 5.53 Hz, 1 H), 3.51 (d, J = 9.19 Hz, 1 H), 3.31−3.32 (m, 2 H), 2.64−2.75 (m, 1 H), 2.34 (t, J = 11.57 Hz, 1 H), 2.06 (d, J = 12.18 Hz, 1 H), 1.95 (d, J = 6.31 Hz, 1 H), 1.13 (q, J = 12.05 Hz, 1 H), 0.80 (d, J = 6.55 Hz, 3 H).

***N*-(4-((1*R*,3*S*,5*S*)-3-Amino-5-methylcyclohexyl)pyridin-3-yl)-2-(2,6-difluorophenyl)thiazole-4-carboxamide (14).** Following the general procedure *tert*-butyl (1*S*,3*R*,5*S*)-3-(3-aminopyridin-4-yl)-5-methylcyclohexylcarbamate and 2-(2,6-difluorophenyl)thiazole-4-carboxylic acid were coupled and deprotected to yield **14**. LC/MS = 429.2 (M + H). HRMS (m/z) calcd for $C_{22}H_{23}F_2N_4OS$ (MH^+) 429.1561, found 429.1561. ^{1}H NMR (400, DMSO-d_6, HCl salt): δ 10.21 (s, 1H), 8.74 (s, 1H), 8.64 (bs, 1H), 8.47 (d, J = 4.8, 1H), 7.99 (bs, 2H), 7.64−7.12 (m, 1H), 7.35−7.42 (m, 3H), 3.11 (bs, 1H), 2.93−3.00 (m, 1H), 2.01 (d, J = 12.4, 1H), 1.92 (d, J = 11.6, 1H), 1.74 (d, J = 12.4, 1H), 1.52−1.60 (m, 1H), 1.42 (q, J = 12.0, 1H), 1.00−1.10 (m, 2H), 0.92 (d, J = 6.4, 3H).

(*S*)-*N*-(4-(3-Aminopiperidin-1-yl)pyridin-3-yl)-6-(2,6-difluorophenyl)-5-fluoropicolinamide (15). Following the general procedure (*S*)-*tert*-butyl 1-(3-aminopyridin-4-yl)piperidin-3-ylcarbamate and 6-(2,6-difluorophenyl)-5-fluoropicolinic acid were coupled and deprotected to yield **3**. LC/MS = 428.1 (M + H). HRMS (m/z) calcd for $C_{22}H_{21}F_3N_5O$ (MH^+) 428.1698, found 428.1699. ^{1}H NMR (400 MHz, methanol-d_4) δ ppm 9.15 (s, 1 H), 8.46 (dd, J = 8.66, 3.91 Hz, 1 H), 8.38 (d, J = 6.70 Hz, 1 H), 8.06 (t, J = 8.68 Hz, 1 H), 7.59−7.71 (m, 1 H), 7.50 (d, J = 6.75 Hz, 1 H), 7.23 (t, J = 8.49 Hz, 2 H), 3.88 (dd, J = 12.10, 3.40 Hz, 1 H), 3.66 (d, J = 13.25 Hz, 1 H), 3.38−3.48 (m, 1 H), 3.11−3.29 (m, 2 H), 1.98−2.09 (m, 1 H), 1.75−1.86 (m, 1 H), 1.58−1.75 (m, 2 H).

***N*-(4-((1*R*,3*S*)-3-Aminocyclohexyl)pyridin-3-yl)-6-(2,6-difluorophenyl)-5-fluoropicolinamide (16).** Following the general procedure 2-(3-(3-aminopyrin-4-yl)cyclohexyl)isoindoline-1,3-dione and 6-(2,6-difluorophenyl)-5-fluoropicolinic acid were coupled and deprotected to yield **4**. LC/MS = 427.2 (M + H). HRMS (m/z) calcd for $C_{23}H_{22}F_3N_4O$ (MH^+) 427.1746, found 427.1749. ^{1}H NMR (CDCl3, free-base): δ 9.93(s, 1H), 9.38(s, 1H), 8.40−8.45(m,1H), 8.40(d, 1H), 7.74−7.80(m, 1H), 7.47−7.55(m, 1H), 7.19(d, 1H), 7.06−7.13(m, 2H), 2.68−2.83(m, 2H), 1.97−2.05(m, 1H), 1.65−1.95(m, 5H), 1.22−1.40(m, 3H), 1.04−1.15(m, 1H).

Microsomal Stability Testing. The test compounds were incubated at a concentration of 1 μM in the presence of a 0.5 mg/mL microsomal protein suspension, 1 mM UDPGA, 3 mM $MgCl_2$, 1 mM NADPH, and 25 μg of alamethicin/mg microsomal of protein. Over the time course of the incubation, duplicate samples were collected at intervals of 0, 5, 15, and 30 min for determination of metabolic rate and parent compound remaining at the end of a 30 min incubation. Phenolphthalein was used as a reference control. Data were obtained utilizing semiquantitative LC−MS/MS methods. Calculation of scaled intrinsic clearance: CL_{int} = 0.693 × [1/$t_{1/2}$ (min)] × (g of liver weight/kg of body weight) × (mL incubation/mg of microsomal protein) × (45 mg of microsomal protein/g of liver weight) with rat = 45 g of liver per kg of body weight and with human = 25.7 g of liver per kg of body weight. Hepatic clearance was extrapolated from microsomal data using well-stirred liver model CL_h = [Q_h × (f_{ub}/f_{ui}) × CL_{int}]/Q_h + (f_{ub}/f_{ui}) × CL_{int}, where assumption is f_{ub}(fraction unbound in blood) = f_{ui}(fraction unbound in incubation) and Q_h values of 55 and 20.7 mL min^{-1} kg^{-1} were used for rat and human. Calculation of hepatic extraction ratio was with E = CL_h/Q_h.

PIM Enzymatic Assays. *Kinase-Glo Pim1, Pim2, Pim3 ATP Depletion Assays.* Pim1, Pim2, and Pim3 kinase-Glo assays using ATP (at or below ATP Km) were used as previously described[20] to determine the biochemical activity of the compounds **1**−**14** as shown in Table 1.

High ATP Pim1, Pim2, Pim3 AlphaScreen Assays. Pim1, Pim2, and Pim3 AlphaScreen assays using high ATP (11−125 × APP K_m) were used as previously described[20] to determine the biochemical activity of compounds **1**, **2**, and **8** as shown in Table 3.

Biochemical Kinase Specificity Profile of 8. The kinase specificity profile for compound **8** reported in Table 5 was determined as previously described.[28] Compound **8** was assessed in the Ambit KINOMEscan binding assay at 1 μM with activity presented as % of control = [(test compound signal − positive control signal)/(negative control signal − positive control signal)] × 100. Data for the 442 kinase KINOMEscan are in Supporting Information.

Cell Lines and Reagents. The KMS-11.luc human multiple myeloma tumor cell line, a KMS-11 clone expressing firefly luciferase, was obtained from the University Health Network (UHN), Toronto,

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J. Med. Chem. XXXX, XXX, XXX−XXX

Ontario, Canada. KMS-11.luc was cultured in RPMI-1640 (ATCC, Manassas, VA) supplemented with 10% FBS. The AML cell line KG-1 and all others included in Table 5 were obtained from commercial sources (ATCC or DSMZ) and were cultured in RPMI or IMDM plus 10−20% FBS (Invitrogen) as supplier recommended.

Cellular Proliferation Assays. Proliferation assays in KMS-11.luc were conducted as previously described[20] with the concentration at which 50% maximal inhibition is reached reported as EC_{50}. Proliferation assays in KG-1 cells lines as well as for cell lines in the expanded AML panel, Table 5, were conducted as previously described[7] with the concentration at which growth is inhibited by 50% reported as GI_{50}.

Western Blot Analysis. Following treatment of KG-1 cells with **8** for 2 h at the indicated concentrations, cells were lysed in RIPA buffer. Protein concentration was determined using a BCA assay (Thermo Scientific 23227), and 50 μg of lysate was separated by SDS−PAGE using 10% bis-Tris gels. Proteins were transferred onto 0.2 μm nitrocellulose membrane (Invitrogen LC2002), and pS6RP was detected using an antibody from Cell Signaling Technology (CST no. 4857) while total S6RP was detected with CST no. 2217. Following incubation with secondary antibodies, antibody binding was detected using ECL Advance (GE Healthcare RPN2135).

Phosphoprotein Assays. Commercial electrochemiluminescence (ECL) assay kits from Meso Scale Discovery (MSD; Rockville, MD) were used to quantify the effects of compounds on the levels of phosphorylated S6RP in KG-1 cells and KMS11-luc cells. For in vitro assays, KMS11-luc cells were seeded in 96-well tissue culture plates, and then compounds were serially diluted and added to cell plates to achieve a final concentration range of 10 μM to 2 nM in 0.1% DMSO and incubated with cells for 1 h at 37 °C. Cells were pelleted by centrifugation for 7 min at 1500 rpm. Medium was gently aspirated, and MSD lysis buffer was added. Cells were lysed by placing plates on a DELFIA (PerkinElmer, Waltham MA) plate shaker at 4 °C and shaking the plates for 30 min at 600 rpm. For in vivo assays, MDS lysis buffer (MSD, Rockville, MD) was added to frozen pulverized tumor samples on ice and homogenates were prepared using the MagNA Lyser bead instrument (Roche Applied Science, Indianapolis, IN) by disrupting the samples with four cycles of 6000 rpm for 30 s at 4 °C. Supernatants were created following centrifugation at 11 000 rpm for 15 min at 4 °C, and protein concentration was determined using the BCA protein assay kit according to the manufacturer's instructions (Pierce Chemical Company, Rockford, IL). For both assays, samples were transferred to ECL assay plates previously blocked with 3% BSA, sealed, and incubated at 4 °C overnight while undergoing gentle shaking on a DELFIA (PerkinElmer, Waltham MA) plate shaker. After overnight incubation, assay plates were processed according to manufacturer instructions.

In Vivo Studies. The single dose PK/PD and multiday efficacy studies in KG-1 tumored mice were carried out as previously described.[7] Compound **8** was formulated for oral administration in 50 mM acetate buffer, pH 4. The concentration of **8** in plasma was determined following extraction in acetonitrile using liquid chromatography and tandem mass spectroscopy (LC/MS/MS). Cytarabine (Hospira, Lake Forest IL) was diluted with bacteriostatic water and prepared fresh daily.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b01275.

Data for the 442 kinase KINOMEscan (PDF)

Accession Codes

Coordinates and structure factors have been deposited in the PDB with accession code 5DWR for the PIM1:**8** complex.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AML, acute myeloid leukemia; ATP, adenosine triphospate; [ATP], concentration of adenosine triphospate; Ara-C, cytarabine; BAD, proapoptotic protein of the BCl2 family; BOC, *tert*-butoxycarbonyl; CL, clearance; ER, extraction ratio; hERG, human-ether-a-go-go-related gene; JAK/STAT, Janus kinase and signal transducer and activator of transcription; K_m, Michaelis constant; log $D_{7.4}$, log of the octanol−water distribution coefficient at pH 7.4 of ionized and un-ionized compound; PD, pharmacodynamics; PIM, proviral insertion of Moloney virus; PK, pharmacokinetics; PSA, polar surface area; S6RP, ribosomal protein S6; $S(35)$, number of nonmutant kinases tested with ≥65% inhibition/number nonmutant kinases tested

■ REFERENCES

(1) Brault, L.; Gasser, C.; Bracher, F.; Huber, K.; Knapp, S.; Schwaller, J. PIM serine/threonine kinases in the pathogenesis and therapy of hematologic malignancies and solid cancers. *Haematologica* **2010**, *95*, 1004−1015.

(2) Cuypers, H. T.; Selten, G.; Quint, W.; Zijlstra, M.; Mandaag, E. R.; Boelens, W. Murine leukemia virus-induced T-cell lymphomagenesis: integration of proviruses in a distinct chromosomal region. *Cell* **1984**, *37*, 141−150.

(3) Meeker, T. C.; Nagarajan, L.; ar-Rushdi, A.; Croce, C. M. Cloning and characterization of the human PIM-1 gene: a putative oncogene related to the protein kinases. *J. Cell. Biochem.* **1987**, *35*, 105−112.

(4) van der Lugt, N. M.; Domen, J.; Verhoeven, E.; Linders, K.; vad der Gulden, H.; Allen, J.; Berns, A. Proviral tagging in E mu-myc transgenic mice lacking the Pim-1 proto-oncogene leads to compensatory activation of Pim-2. *EMBO J.* **1995**, *14*, 2536−2544.

(5) Mikkers, H.; Allen, J.; Knipscheer, P.; Romeyn, L.; Hart, A.; Vink, E.; Berns, A. High-throughput retroviral tagging to identify components of specific signaling pathways in cancer. *Nat. Genet.* **2002**, *32*, 153−159.

(6) Nawijn, M. C.; Alendar, A.; Berns, A. For better or for worse: the role of PIM oncogenes in tumorigenesis. *Nat. Rev. Cancer* **2011**, *11*, 23−34.

(7) Garcia, P.; Langowski, J.; Wan, Y.; Chen, M.; Castillo, J.; Fanton, C.; Ison, M.; Zavorotinskaya, T.; Dai, Y.; Lu, J.; Niu, X.-H.; Basham, S.; Chan, J.; Yu, H.; Doyle, M.; Feucht, P.; Warne, R.; Narberes, J.; Tsang, T.; Fritsch, C.; Kauffmann, A.; Pfister, E.; Drueckes, P.; Trappe, J.; Wilson, C.; Han, W.; Lan, J.; Nishiguchi, G.; Bellamacina, C.; Aycinena, J. A.; Zang, R.; Holash, J.; Burger, M. Pan-PIM kinase inhibition provides a novel therapy for treating hematological cancers. *Clin. Cancer Res.* **2014**, *20*, 1834−1845.

(8) Blanco-Aparicio, C.; Carnero, A. PIM kinases in cancer: diagnostic, prognostic and treatment opportunities. *Biochem. Pharmacol.* **2013**, *85*, 629−643.

(9) Jackson, L. J.; Pheneger, J. A.; Pheneger, T. J.; Davis, G.; Wright, A. D.; Robinson, J. E.; Allen, S.; Munson, M. C.; Carter, L. L. The role of PIM kinases in human and mouse CD4+ T cell activation and inflammatory bowel disease. *Cell. Immunol.* **2012**, *272*, 200−213.

(10) Morwick, T. PIM kinase inhibitors: a survey of the patent literature. *Expert Opin. Ther. Pat.* **2010**, *20* (2), 193−212 and references therein..

DOI: 10.1021/acs.jmedchem.5b01275
J. Med. Chem. XXXX, XXX, XXX−XXX

(11) Mumenthaler, S. M.; Ng, P. Y.; Hodge, A.; Bearss, D.; Berk, G.; Kanekal, S.; Redkar, S.; Taverna, P.; Agus, D. B.; Jain, A. Pharmacologic inhibition of PIM kinases alters prostate cancer cell growth and resensitizes chemoresistant cells to taxanes. *Mol. Cancer Ther.* **2009**, *8*, 2882−2893.
(12) Dakin, L. A.; Block, M. H.; Chen, H.; Code, E.; Dowling, J. E.; Feng, X.; Ferguson, A. D.; Green, I.; Hird, A. W.; Howard, T. Discovery of novel benzylidene-1,3-thiazolidine-2,4-diones as potent and selective inhibitors of the PIM-1, PIM-2, and PIM-3 protein kinases. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 4599−4604.
(13) Tsuhako, A. L.; Brown, D. S.; Koltun, E. S.; Aay, N.; Arcalas, A.; Chan, V.; Du, H.; Engst, S.; Franzini, M.; Galan, A. The design, synthesis, and biological evaluation of PIM kinase inhibitors. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 3732−3738.
(14) Xia, Z.; Knaak, C.; Ma, J.; Beharry, Z. M.; McInnes, C.; Wang, W.; Kraft, A. S.; Smith, C. D. Synthesis and evaluation of novel inhibitors of PIM-1 and PIM-2 protein kinases. *J. Med. Chem.* **2009**, *52*, 74−86.
(15) Tao, Z.; Hasvold, L. A.; Leverson, J. D.; Han, E. K.; Guan, R.; Johnson, E. F.; Stoll, V. S.; Stewart, K. D.; Stamper, G.; Soni, N. Discovery of 3H-benzo[4,5]thieno[3,2-d]pyrimidin-4-ones as potent, highly selective, and orally bioavailable inhibitors of the human protooncogene proviral insertion site in moloney murine leukemia virus (PIM) kinases. *J. Med. Chem.* **2009**, *52*, 6621−6636.
(16) Haddach, M.; Michaux, J.; Schwaebe, M. K.; Pierre, F.; O'Brien, S. E.; Borsan, C.; Tran, J.; Raffaele, N.; Ravula, S.; Drygin, D. Discovery of CX-6258. a potent, selective, and orally efficacious pan-PIM kinase inhibitor. *ACS Med. Chem. Lett.* **2012**, *3*, 135−139.
(17) Nishiguchi, G. A.; Atallah, G.; Bellamacina, C.; Burger, M. T.; Ding, Y.; Feucht, P. H.; Garcia, P. D.; Han, W.; Klivansky, L.; Lindvall, M. Discovery of novel 3,5-disubstituted indole derivatives as potent inhibitors of Pim-1, Pim-2, and Pim-3 protein kinases. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 6366−6369.
(18) Wang, X.; Magnuson, S.; Pastor, R.; Fan, E.; Hu, H.; Tsui, V.; Deng, W.; Murray, J.; Steffek, M.; Wallwever, H.; Moffat, J.; Drummond, J.; Chan, G.; Harstad, E.; Eben, A. J. Discovery of novel pyrazolo[1,5-a]pyrimidines as potent pan-PIM inhibitors by structure- and property-based drug design. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 3149−3153.
(19) Qian, K. C.; Wang, L.; Hickey, E. R.; Studts, J.; Barringer, K.; Peng, C.; Kronkaitis, A.; Li, J.; White, A.; Mische, S.; Farmer, B. Structural basis of constitutive activity and a unique nucleotide binding mode of human Pim-1 kinase. *J. Biol. Chem.* **2005**, *280*, 6130−6137.
(20) Burger, M.; Han, W.; Lan, J.; Nishiguchi, G.; Bellamacina, C.; Lindvall, M.; Atallah, G.; Ding, Y.; Mathur, M.; McBride, C.; Beans, E. L.; Muller, K.; Tamez, V.; Zhang, Y.; Huh, K.; Feucht, P.; Zavorotinskaya, T.; Dai, Y.; Holash, J.; Castillo, J.; Langowski, J.; Wang, Y.; Chen, M. Y.; Garcia, P. Structure guided optimization, in vitro activity and in vivo activity of pan-PIM kinase inhibitors. *ACS Med. Chem. Lett.* **2013**, *4*, 1193−1197.
(21) Lu, J.; Zavorotinskaya, T.; Dai, Y.; Niu, X.; Castillo, J.; Sim, J.; Yu, J.; Wang, Y.; Langowski, J. L.; Holash, J.; Shannon, K.; Garcia, P. D. Pim2 is required for maintaining multiple myeloma cell growth through modulating TSC2 phosphorylation. *Blood* **2013**, *122*, 1610−1620.
(22) Cabral, S.; Hulin, B.; Kawai, M. Lithium borohydride: a reagent of choice for the selective reductive amination of cyclohexanones. *Tetrahedron Lett.* **2007**, *48*, 7134−7136.
(23) Keeton, E. K.; McEachern, K.; Dillman, K. S.; Palakurthi, S.; Cao, Y.; Grondine, M. R.; Kaur, S.; Wang, S.; Chen, Y.; Wu, A.; Shen, M.; Gibbons, F. D.; Lamb, M. L.; Zheng, X.; Stone, R. M.; DeAngelo, D. J.; Platanias, L. C.; Dakin, L. A.; Chen, H.; Lyne, P. D.; Huszar, D. AZD1208, a potent and selective pan-PIM kinase inhibitor, demonstrates efficacy in preclinical models of acute myeloid leukemia. *Blood* **2014**, *123*, 905−913.
(24) Structure of compound **8** in PIM1 submitted under PDB accession code 5DWR.
(25) Garcia, P. D.; Langowski, J. L.; Holash, J.; Burger, M.; Zang, R.; Zavorotinskaya, T.; Fanton, C.; Saci, A.; Growney, J.; Vanasse, K. G. The Pan-PIM kinase inhibitor LGH447 shows activity in PIM2-dependent multiple myeloma and in AML models. *Blood* **2013**, *122*, 1666.
(26) Raab, M. S.; Ocio, E. M.; Thomas, S. K.; Gunther, A.; Lebovic, D.; Jakubowiak, A. J.; Song, D.; Xiang, F.; Patel, A.; Vanasse, K. G.; Kumar, S. K. Phase 1 study update of the novel pan-PIM kinase inhibitor LGH447 in patients with relapsed/refractory multiple myeloma. *Am. Soc. Hemat. Annual Meet.* **2014**, Abstract 301.
(27) www.clinicaltrials.gov and PIM447, LGH447.
(28) Manley, P. W.; Drueckes, P.; Fendrich, G.; Furet, P.; Liebetanz, J.; Martiny-Baron, G.; Mestan, J.; Trappe, J.; Wartmann, M.; Fabbro, D. Extended kinase profile and properties of the protein kinase inhibitor nilotinib. *Biochim. Biophys. Acta, Proteins Proteomics* **2010**, *1804*, 445−53.

DOI: 10.1021/acs.jmedchem.5b01275
J. Med. Chem. XXXX, XXX, XXX−XXX