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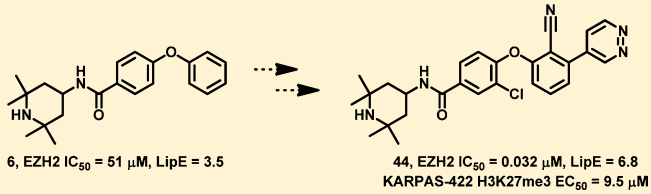
Discovery and Optimization of Tetramethylpiperidinyl Benzamides as Inhibitors of EZH2

Christopher G. Nasveschuk,*[,†] Alexandre Gagnon,[†,‡] Shivani Garapaty-Rao, Srividya Balasubramanian, Robert Campbell, Christina Lee, Feng Zhao, Louise Bergeron, Richard Cummings, Patrick Trojer, James E. Audia, Brian K. Albrecht, and Jean-Christophe P. Harmange

Constellation Pharmaceuticals, 215 1st Street, Suite 200, Cambridge, Massachusetts 02142, United States

Supporting Information

ABSTRACT: The identification and development of a novel series of small molecule Enhancer of Zeste Homologue 2 (EZH2) inhibitors is described. A concise and modular synthesis enabled the rapid development of structure−activity relationships, which led to the identification of **44** as a potent, SAM-competitive inhibitor of EZH2 that dose-dependently decreased global H3K27me3 in KARPAS-422 lymphoma cells.



KEYWORDS: *methyltransferase, PRC2, EZH2, KARPAS-422, tetramethylpiperidine, diphenylether*

Histone lysine methyltransferases (HKMTs; HMTs when referred to both lysine and arginine methyltransferases) contribute to the organization of chromatin structure, and thus play a role in the regulation of gene expression. Enhancer of Zeste Homologue 2 (EZH2) catalyzes methylation of histone H3 lysine 27 (H3K27) and functions as part of a multisubunit complex termed Polycomb Repressive Complex 2 (PRC2) with known application in regulating cell identity.[1] PRC2/EZH2 has been widely implicated in cancer progression, largely due to its prevalent overexpression, which correlates with significant increases in H3K27 methylation, disease stage, and poor prognosis.[2,3] The identification of recurrent gain of function mutations within the EZH2 catalytic domain provides a powerful argument that certain cancers might be dependent on EZH2 catalytic activity. All identified mutated residues, Y641, A677, and A687, alter substrate specificity, facilitating the conversion from H3K27 dimethylated (me2) to trimethylated (me3) states,[4−8] while wild-type (wt) EZH2 preferentially converts H3K27me1 to H3K27me2. To date, EZH2 gain of function mutations have been found exclusively in germinal center B-cell like diffuse large B-cell lymphoma (GCB-DLBCL) and Follicular Lymphoma (FL) but not in activated B-cell DLBCL (ABC-DLBCL) or any other lymphoma subtype, suggesting that EZH2 addiction might be restricted to certain lymphoma subtypes.

Multiple small molecule inhibitors of EZH2 catalytic activity, bearing a common pyridone-amide motif, have recently been disclosed (Figure 1).[9−16] It is of interest to note that the similarities between these compounds is not a result of deliberate differentiation strategies for generating novel inhibitors but rather an indication of the relative paucity of commercial and corporate compound collections for chemical matter with the appropriate structural features to inhibit this enzyme. Several pyridone-amides have been used to unveil new biology linked to the catalytic activity of EZH2. On target *in vitro* and *in vivo* efficacy has been demonstrated with GSK-126, EPZ-6438, and UNC-1999; where EPZ-6438 has recently progressed into phase 1 trials for the treatment of lymphoma.

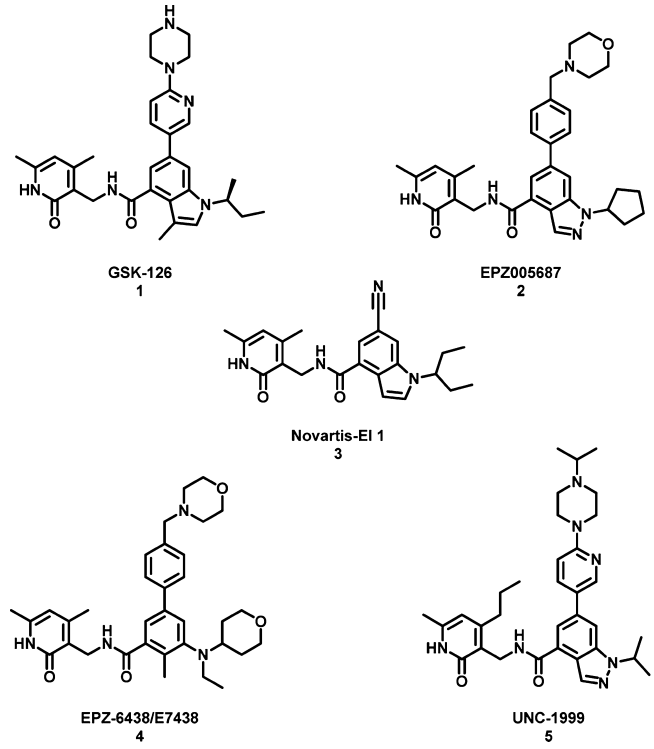


Figure 1. Disclosed EZH2 inhibitors.

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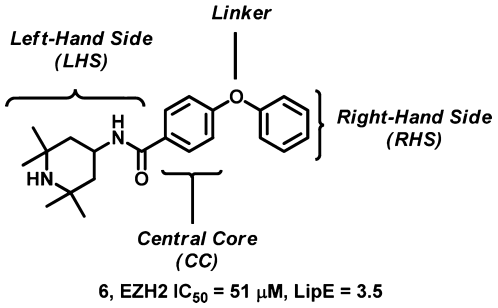
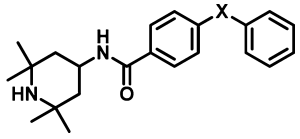


Figure 2. HTS hit compound **6**.

Table 1. Linker SAR



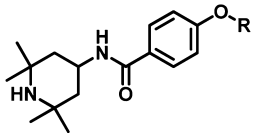
Compound	X	EZH2wt IC_{50} (μM)	Compound	X	EZH2wt IC_{50} (μM)
6		51	13		>80
7		48	14		>80
8		>80	15		>80
9		33	16		>80
10		>80	17		>80
11		>80	18		>80
12		57			

Since all genuine inhibitors of the catalytic activity of EZH2 described to date belong to one chemical series, the pyridone-amides, the identification of structurally unrelated inhibitors enhances the chemical biology toolbox for further exploration of the function of this enzyme. Herein we describe our initial efforts in this area utilizing high throughput screening (HTS), biochemical hit triage, and hit optimization, which led to the identification of structurally distinct EZH2 inhibitors capable of suppressing EZH2 catalytic activity and globally reducing H3K27me3 in a cellular setting.

Our HTS screen utilized a PRC2 enzymatic assay that monitored ^{3}H labeled methyl group transfer from [^{3}H]-*S*-adenosyl methionine (SAM) to oligonucleosome substrate and identified 288 hits.[17] These compounds were initially assessed using a multistage process: hits bearing undesirable functional groups, poor synthetic starting points, and promiscuous binders were removed. The remaining hits were assessed in 10 point concentration−response curves where the Hill coefficient (0.7−1.5) was a prominent filter for further evaluation.[18,19]

Compounds were then assessed in three additional enzymatic assays where titrations were run with (1) 10-fold enzyme, (2) 10-fold K_m SAM, or (3) 10-fold K_m oligonucleosome substrate. These assays allowed another check for nonideal behavior and a preliminary categorization of compounds based on potential mechanism of inhibition (MOI). This MOI assessment was based on whether they

Table 2. Right-Hand Aryl Ring SAR



Compound	Right-Hand Side	EZH2wt IC_{50} (μM)	LipE
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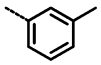
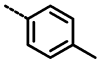
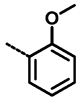
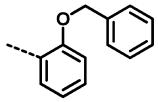
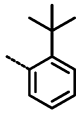
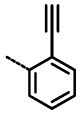
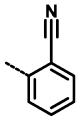
20		61	2.9
21		>80	N/A
22		29	3.2
23		59	3.6
24		3.6	3.1
25		7.9	2.8
26		19	3.8
27		3.4	4.8

did or did not shift in potency at cofactor and substrate concentrations of 10-fold K_m. According to the Cheng−Prusoff relationship, an increase in substrate above K_m should result in predictable decreased inhibitor affinity for a competitive compound, no effect for a noncompetitive compound, and enhanced affinity for an uncompetitive one.[17,20]

After hit triage was complete, several relatively weak (IC_{50} > 20 μM) compounds remained, which met the criteria outlined above. One of these, compound **6** (Figure 2), was of moderate potency ($EZH2_{wt}$ IC_{50} = 51 μM), had a good Hill coefficient (1.1), and LipE of 3.5.[21,22] When funneled through our secondary assays **6** shifted only with an increase in [SAM], which was suggestive of a SAM competitive mechanism of inhibiton.[17] Additionally, **6** was found to be selective when assessed against other HMTs in a biochemical assay.[17] Given these data and the potential for rapid synthesis of analogues, **6** was selected as a starting point for our initial efforts to develop wild-type and disease-relevant mutant EZH2 inhibitors.

The aim of our initial medicinal chemistry campaign was to rapidly navigate the structure−activity landscape of tetramethylpiperidinyl-containing lead compound **6** (Table 1). We first addressed the linker of the bis-phenylether by extending the compound one methylene unit (−CH_2O−, **7**), which

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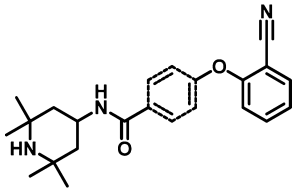
Table 3. Central Core SAR



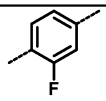
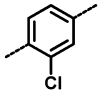
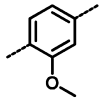
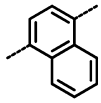
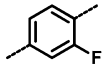
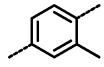
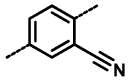
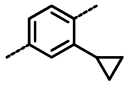
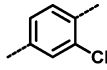
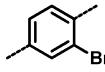
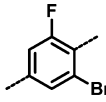
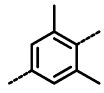
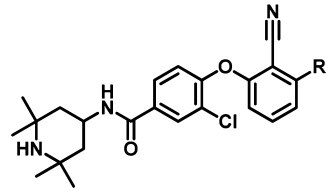
Compound	Central Core	EZH2wt IC_{50} (μM)	LipE
28		15	4.0
29		37	3.2
30		>80	N/A
31		0.84	4.4
32		1.0	5.2
33		0.92	4.9
34		0.93	5.5
35		0.50	4.9
36		0.45	5.1
37		0.32	5.1
38		1.0	4.4
39		51	2.6

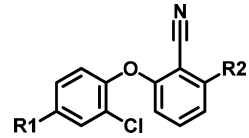
Table 4. Further Modification of Right-Hand Aryl Ring

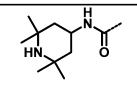
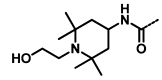
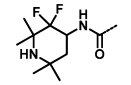
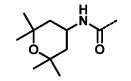
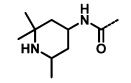
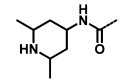
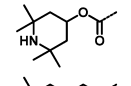
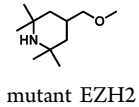


Compound	R	EZH2wt IC_{50} (μM)	EZH2 IC_{50} (μM)[a]	LipE(wt)
40		0.14	1.4	5.1
41		0.14	2.2	5.0
42		0.23	4.0	5.4
43		0.081	0.56	5.4
44		0.032	0.19	6.8
45		0.024	0.13	6.3

[a]Y641N mutant EZH2 used.

Table 5. Tetramethylpiperidine SAR



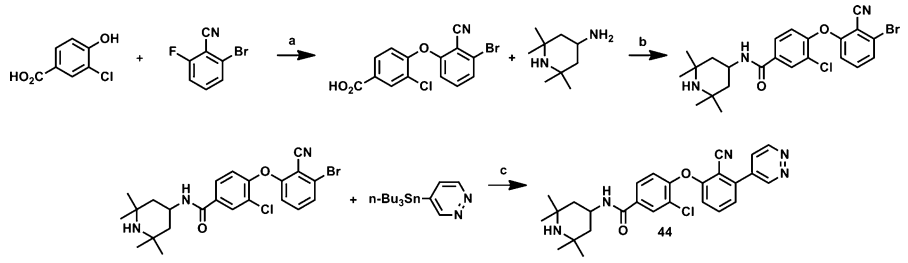
Compound	R1	R2	EZH2wt IC_{50} (μM)	EZH2 IC_{50} (μM)[a]	A to B	B to A	Efflux Ratio[b]
44			0.032	0.19	0.19	3.29	17
46			4.9	62	N/A	N/A	N/A
47			>80	>80	N/A	N/A	N/A
48			1.5	66	21.9	24.8	1.1
49			0.871	10	N/A	N/A	N/A
50			2.1	10	N/A	N/A	N/A
51			0.172	2.5	20.4	47.7	2.3
52			0.189	2.0	0.5	2.1	4.4

[a]Y641N mutant EZH2 used. [b]CACO-2 permeability assay.

maintained activity. The converse methylene insertion (−OCH_2−, **8**) led to a loss of EZH2 affinity. Conversion of ether **6** to thioether **9** maintained potency; however, oxidation of the sulfur led to ablation of activity (Table 1, **10** and **11**). Interestingly, ketone **12** was tolerated, while the corresponding racemic alcohol **13** was not. At this point, we postulated that the ether, thio, and ketone linkages were imparting a conformational bias to the system, which led to enhanced potency over the sulfoxide, sulfone, and benzylic alcohol. Indeed, diphenyl ethers are known to adopt a twisted orientation due to a 1,5-CH-aryl clash and optimized π-overlap with the lone pairs on the ether oxygen.[23,24] Diphenylamine is known to adopt a similar conformation to benzophenone, which is more coplanar than the conformation of diphenyl ethers;[24,25] therefore, absence of activity for compounds **13** and **14** could be attributed to preference of EZH2 for H-bond acceptors and not donors as a linking group in addition to an optimum conformation between the phenyl rings. This hypothesis was further supported by the lack of activity for

dx.doi.org/10.1021/ml400494b | ACS Med. Chem. Lett. XXXX, XXX, XXX−XXX

Scheme 1. Synthesis of Compound 44



(a) K_2CO_3, DMSO, 140 °C, 3 h, 81% yield; (b) EDC, HOBT, TEA, DCM, 23 °C, 3 h, 30% yield; (c) 10 mol% $Pd(PPh_3)_2Cl_2$, LiCl, toluene, 90 °C, 12 h, 60% yield.

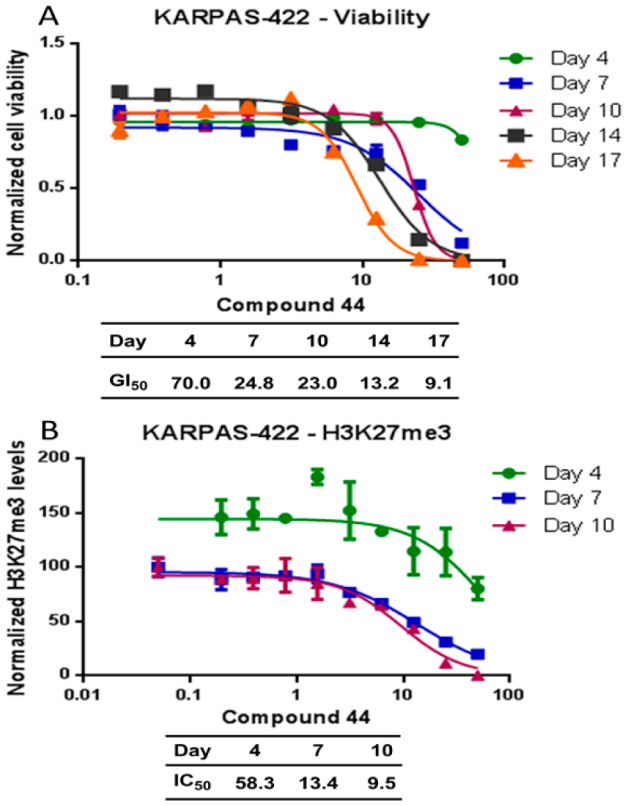


Figure 3. Activity of compound **44** in KARPAS-422 cells. (A) Viability of KARPAS-422 cells with growth inhibition observed with varying [**44**] and days of exposure. (B) Compound **44** treatment results in dose- and time-dependent reduction in global H3K27me3 levels in KARPAS-422 cells. Total H3 levels were determined and used for normalization purposes. Experiments were carried out in triplicate ± SD.

carbon linked compounds **16**–**18**, where the phenyl rings should adopt a near orthogonal orientation.

Holding the diphenylether constant, we next evaluated the right-hand aryl ring. Incorporation of a 2-methyl group (Table 2, **19**) was nearly equipotent with **6**, and the continued scan led to progressive loss of potency at the 3- and 4-positions (Table 2, **20** and **21**). Focusing on the 2-aryl position, 2-chloro (**21**) provided a modest improvement, while 2-methoxy (**23**) was equipotent to **6**. Extension of the 2-methoxy to 2-benzyloxy (**24**) or branching of the 2-methyl to 2-*t*-butyl (**25**) led to a 10-fold boost in potency with no increase in LipE, illustrating that this position was tolerant of diverse functionality and potentially reinforced an optimal conformation. A modest boost in LipE was coupled with smaller rigid groups at the 2-position (**26**, LipE = 3.8). Further exploration revealed a 15-fold boost in potency with the incorporation of a 2-cyano substituent (**27**, LipE = 4.8), suggesting the cyano nitrogen was making a favorable interaction with the binding site in EZH2. The 2-cyano substituent was utilized as the preferred substituent for further SAR development.

We next addressed elaboration of the central aromatic ring. While substitution proximal to the amide linkage did not yield an improvement in biochemical potency relative to **27** (Table 3, **28**–**30**), modification of the central core to a naphthyl system did provide a 4-fold enhancement (Table 3, **31**). We suspected that monosubstitution adjacent to the ether linkage would stabilize the intrinsic twist of the biphenylether and thus enhance potency. We were gratified to find that this was the case as fluoro, methyl, and cyano substitution all led to a minimum of a 3-fold improvement (Table 3, **32**–**34**). Both cyclopropyl (**35**) and chloro (**36**) substitution improved potency 6-fold over **27** with **36** being the more efficient modification (0.45 μM, LipE = 5.1) at this position and provided a compound with meaningful levels of potency against EZH2 Y641N mutant (**36**, 7.9 μM). Switching from chloro (**36**) to bromo (**37**) increased lipophilicity leading to a modest boost in potency with no increase in efficiency as indicated by the static LipE (5.1). Disubstitution of the central core did not provide an advantage (Table 3, **38** and **39**). The chloro group was selected for further optimization.

In an attempt to improve the biochemical potency of the tetramethylpiperidinyl-class of inhibitors, we chose to further evaluate the right-hand aryl ring (Table 4). Methyl and chloro substitution alpha to the 2-cyano group improved binding to EZH2 wild-type and Y641N mutant 3- and 4-fold over **36**, respectively (Table 4, **40** and **41**). Introduction of a pyrazole alpha to the 2-cyano group (**42**) led to a modest improvement; however, inclusion of a 6-membered heterocycle enabled sub-100 nM potency against the wild-type enzyme (Table 4, **43**–**45**). Changing the nitrogen aryl substitution from 4-pyridyl (**43**, LipE = 5.4) to pyridazine (**44**, LipE = 6.8) then pyrimidine (**45**, LipE = 6.3) led to improvement in both affinity and efficiency.

Compound **44** was evaluated in a CACO-2 assay and found to have low passive permeability and a high efflux ratio, indicative of a compound with the potential for poor oral bioavailability.[26,27] We therefore sought to address this liability through modification of the tetramethylpiperidine (Table 5). One approach was to attenuate the basicity the nitrogen via inductive effects; unfortunately, *N*-ethanol **46** lost 10-fold against EZH2 wild-type, while difluoropiperidine **47** was inactive (compare to **36**). Indeed, a strongly basic nitrogen is preferred for activity as pyran **48** was 38-fold less potent than

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44. It is worthy of note that **48** no longer displays efflux (efflux ration = 1.1) and was quite permeable (A to B 21.9 × 10^{-6} cm/s), lending support to the hypothesis that poor permeability and efflux were a direct result of the basic nitrogen of **44**. Further manipulation of the tetramethylpiperidine through selective methyl group deletion also provided compounds with a significant reduction in activity (**49**−**50**). In the face of an apparent SAR cliff around the modification of the tetramethylpiperidine, we attempted to improve the permeability of this series by manipulating the amide linker between the tetramethylpiperidine and central core. Both ester (**51**) and ether (**52**) were tolerated displaying a 4-fold loss in potency, while improving permeability and reducing efflux. The improved permeability achieved with **52** did not translate into an improved shift between biochemical potency and reduction of H3K27me3 in HeLa cells (HeLa cell assay **44** = 7 μM;[17] **52** ≥ 15 μM); therefore, compound **44** was prioritized for further evaluation as a tool compound for the study of EZH2 disease biology.

The synthesis of **44** commenced with a S_NAr reaction to establish the diphenyl ether linkage (Scheme 1). EDC-mediated peptide coupling furnished the tetramethylpiperidinyl-benzamide scaffold primed for exploring diversity on the right-hand side aryl ring. The pyridazine was coupled using standard Stille conditions to provide **44** in three chemical steps and 15% overall yield.

Time and concentration-dependent exposure of KARPAS-422 cells to **44** led to growth arrest (Figure 3A) as well as dose-dependent reduction in global H3K27me3 levels (Figure 3B). These results are consistent with previous reports and validate this scaffold as a useful tool compound to interrogate the inhibition of EZH2.[9−16]

This work described the identification and optimization of a new class of inhibitors of the catalytic activity of EZH2/PRC2, the tetramethylpiperidinyl benzamides. Compound **6** was initially identified as a SAM-competitive compound with acceptable LipE and a modular synthesis well suited for rapid SAR development. Optimization of **6** provided **44**, a SAM competitive compound that was >1000-fold more potent against EZH2. We also showed that upon treatment with **44** KARPAS-422 lymphoma cells displayed dose-dependent reduction in global levels of H3K27me3 with a characteristic delay in onset of phenotype. Compound **44** is an effective tool for the evaluation of EZH2 inhibition *in vitro*.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic procedures, characterization data, the synthesis of compound **44**, and KARPAS-422 assay information. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(C.G.N.) Tel: 617-714-0538. E-mail: chris.nasveschuk@constellationpharma.com.

Present Address

‡Département de Chimie, Université du Québec à Montréal, C.P. 8888, Succ. Centre-Ville, Montréal, QC H3C 3P8, Canada.

Author Contributions

†These authors (C.G.N. and A.G.) contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

K_2CO_3, potassium carbonate; DMSO, dimethylsulfoxide; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; HOBT, 1-hydroxybenzotriazole hydrate; TEA, triethylamine; DCM, dichloromethane; $Pd(PPh_3)_2Cl_2$, bis(triphenylphosphine)palladium(II) dichloride; LiCl, lithium chloride; EZH2, enhancer of zeste homologue 2; PRC2, polycomb repressive complex 2; H3K27me3, histone 3 lysine 27 trimethyl

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