Discovery of First-in-Class, Potent, and Orally Bioavailable Embryonic Ectoderm Development (EED) Inhibitor with Robust Anticancer Efficacy

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Supporting Information

ABSTRACT: Overexpression and somatic heterozygous mutations of EZH2, the catalytic subunit of polycomb repressive complex 2 (PRC2), are associated with several tumor types. EZH2 inhibitor, EPZ-6438 (tazemetostat), demonstrated clinical efficacy in patients with acceptable safety profile as monotherapy. EED, another subunit of PRC2 complex, is essential for its histone methyltransferase activity through direct binding to trimethylated lysine 27 on histone 3 (H3K27Me3). Herein we disclose the discovery of a first-in-class potent, selective, and orally bioavailable EED inhibitor compound 43 (EED226). Guided by X-ray crystallography, compound 43 was discovered by fragmentation and regrowth of compound 7, a PRC2 HTS hit that directly binds EED. The ensuing scaffold hopping followed by multiparameter optimization led to the discovery of 43. Compound 43 induces robust and sustained tumor regression in EZH2MUT preclinical DLBCL model. For the first time we demonstrate that specific and direct inhibition of EED can be effective as an anticancer strategy.

INTRODUCTION

Tumorigenesis is believed to involve multiple epigenetic alterations, in addition to genetic aberrations, that contribute to the progressive transformation of normal cells toward a malignant phenotype. As a result, novel cancer therapies that work by reversing epigenetic effects are being increasingly explored.1 Post-translational modifications of core histone proteins of chromatin are one of the major epigenetic mechanisms regulating gene expression, and paramount among them are methylation events at lysine and arginine residues, catalyzed by histone methyltransferases (HMTs).2

Polycomb repressive complex 2 (PRC2) is a multiprotein complex that catalyzes the methylation of histone H3 at lysine 27 (H3K27). Trimethylated H3K27 (H3K27Me3) is a repressive post-translational modification.3 Overexpression, gain-of-function mutations of EZH2, and hypertrimethylation of H3K27 have been implicated in a myriad of cancers.4 The polycomb group (PcG) proteins SUZ12, EED, EZH2 (or its homolog EZH1), RBBP4, and RBBP7 constitute the "core PRC2"; removal of core subunits by genetic or RNAi-based approaches destabilizes EZH2 and results in the abrogation of all PRC2 functions.5 More importantly, it was known that the recognition of H3K27Me3 by WD40-repeats containing β-propeller protein EED is essential in stimulating basal PRC2 activity and propagating H3K27 methylation in repressive chromatin for gene silencing.6 EED selectively binds the positively charged quaternary amine of the trimethylated lysine via a so-called "aromatic cage" formed by Phe97, Tyr148, and Tyr365, while the neighboring Trp364 interacts with the aliphatic side chain of the lysine residue.6a

EZH2 or EZH1 acts as the catalytic subunit of PRC2.7 Despite high sequence identity, EZH2 and EZH1 are not functionally redundant and have different expression patterns. While EZH2 is found only in actively dividing cells, EZH1 is found in both dividing and nondividing cells.

Although PRC2 containing EZH1 (PRC2–EZH1) has lower catalytic activity compared to that containing EZH2 (PRC2–EZH2), both complexes contribute to the maintenance of cellular H3K27 methylation states.

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Given the association of PRC2/EZH2 with cancer, multiple biotech and pharmaceutical companies have been actively pursuing compounds that can effectively inhibit PRC2 activity (Figure 1). The first such compound was 3-deazaneplanocin A (1, DZNep) which interferes with S-adenosyl-L-homocysteine (SAH). Although the compound has a very short plasma half-life, significant antitumor activity in various cancer types was reported, along with toxicities possibly due to nonspecific inhibition of histone methylation. This result further spurred interest in development of specific PRC2 inhibitors. In 2012, Epizyme and GSK reported the development of S-adenosyl-methionine (SAM) competitive inhibitors 2 (EPZ005687) and 3 (GSK126), respectively, resulting from optimization of hits identified from high-throughput screenings. Compound 3 markedly inhibits the growth of lymphomas carrying activating EZH2 mutations in vivo. Following this, compound 4 (UNC1999) was reported as the first orally bioavailable EZH2 inhibitor that was highly selective for both wild-type and Y641 mutant EZH2, as well as EZH1. Shortly after, compound 5 (EPZ-6438) was reported as Epizyme’s second generation EZH2 inhibitor with better potency and good oral bioavailability. On the basis of the encouraging efficacies in preclinical studies, phase 1/2 clinical trials of compound 5 in advanced solid tumors and B cell lymphomas were launched in June 2013. In the meantime, compound 3 and compound 6 (CPI1205) from Constellation also entered clinical trials in EZH2-mutant B cell NHL and SMARCB1-deficient tumors, respectively. With three independent EZH2 inhibitors in clinical trials, a trove of data are expected on both potential toxicity and efficacy of this approach. However, all three PRC2 inhibitors currently in clinic (compound 5, NCT01897571, NCT02601937, NCT02601950; compound 3, NCT02082977; compound 6, NCT02395601) are pyridone-derived SAM-competitive inhibitors targeting EZH2 with relative weaker activity against EZH1.

It has been reported that binding of H3K27Me3 to EED allosterically activates the methyltransferase activity of PRC2. This could partially be explained by the recently published high resolution crystal structures of PRC2 complex, which suggested that this interaction induces conformational change of stimulation-responsive motif (SRM) in EZH2, leading to enhanced catalytic efficiency. We hypothesized that low molecular weight (LMW) compounds interacting with PRC2 via the “Me3 pocket” of EED may inhibit the methyltransferase activities of both PRC2–EZH2 and PRC2–EZH1 and therefore may provide therapeutic(s) similar or complementary to the EZH2 inhibitors in clinic.

RESULTS AND DISCUSSION

In order to find LMW compounds that antagonize PRC2 complex activity, we initiated a high-throughput screening (HTS) campaign, which used the recombinant five-member

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**Figure 1.** Selected PRC2 inhibitors.

**Figure 2.** (a) Chemical structure of HTS hit compound 7. (b) More significant movement of key residues upon binding of compound 7 compared to binding of K27Me3 (green, compound 7; PDB accession code, 5H19; blue, EED residues binding to compound 7; yellow, EED residues binding to K27Me3; K27Me3 peptide is not shown for the purpose of clarity; movement of the residues is indicated by red arrows).
PRC2 complex as enzyme, H3[21-44, K27Me0] as a peptide substrate, and homogeneous time-resolved fluorescence (HTRF) method to detect the dimethylated product. The mechanisms of action of the screening hits were further elucidated by various competition studies using SAM, H3K27Me0 substrate peptide, and/or K27Me3 stimulation peptide. Compound 7 (Figure 2a) was identified as a K27Me3-competitive inhibitor in biochemical assays, and its binding to EED through K27Me3 pocket via the “aromatic cage” was further confirmed by X-ray crystallography (PDB accession code SH19; details of the hits finding are reported in a separate manuscript).15,18

Although the dynamic nature of the Me3 pocket, especially the flexibility of the side chains of Trp364 and Arg367, has been previously reported by comparing the K27Me3 bound structure to apo EED protein structure,6 binding of compound 7 induced more substantial conformational change of side chains of Trp364 and Tyr365, in addition to Arg367 (Figure 2b). As a result, a much deeper pocket was induced with the calculated druggability score improved from previously reported 0.64 to 0.96. Encouraged by this finding, we started our discovery efforts to optimize compound 7 in order to discover potential LMW therapeutic agents targeting the K27Me3 pocket of EED.

During the SAR investigation, biological activities of our compounds were assessed in a cascade of assays (Supporting Information). To determine the binding affinity of the compounds to EED, we developed an EED-H3K27me3 peptide AlphaScreen binding assay and performed competition studies with our compounds. At the same time, the biochemical inhibitory activities of the compounds were evaluated with a liquid chromatography mass spectrometry (LC–MS) based assay which detects SAH formation using either H3K27Me0 peptide or nucleosome as substrate.17 Since results from the two assays correlate reasonably well, in this publication the SAR is discussed only with biochemical activities although AlphaScreen assay was routinely ran as part of SAR support.18 Furthermore, the ability of compounds to reduce global K27Me3 at the cellular level was assessed in G401 cells with an ELISA assay. Antiproliferative activity of selected compounds was determined in KARPAS-422 cells, a diffuse large B-cell lymphoma (DLBCL) cell line harboring a monoallelic Y641N EZH2 mutation. Since we also observed the reported slow kinetics of the antiproliferative activity for SAM-competitive EZH2 inhibitors,10,12 IC50 values at 14 day were required to differentiate in vitro antitumor activity for the compounds. Compound 7, with IC50 of 0.62 μM in AlphaScreen binding assay, IC50 of 4.03 μM in biochemical assay with H3K27Me0 peptide as substrate, and ELISA IC50 of 3.28 μM in G401 cells, represented an excellent starting point for us to start our optimization efforts.

**Fragmentation and Regrowth: Bicyclic Triazolopyridine as Scaffold I.** The key interactions in the binding of compound 7 include two hydrogen bonds and a set of π−π interactions. The two hydrogen bonds, one between hydrogenn on 5′-amino and the side chain carbonyl of Asn194 (red dash, Figure 3a) and another one between nitrogen at 2-postion with side chain of Lys211, appear to offer key polar interactions. The π−π interactions, taking full advantage of the aromatic cage
residues, also play indispensable roles. First, the electron-deficient bicyclic [1,2,4]triazolo[4,3-a]pyridine core is held in place by π−π stacking interactions with the electron-rich Tyr148 and Tyr365. Second, the electron-rich furan forms a π−π stacking interaction with guanidinium group of Arg367 and an edge-to-face interaction with Tyr365, which we referred to as "deep pocket" interactions, since the furan group is located in the inner part of the induced pocket. Additionally, the CN group at C6 seems interaction with multiple residues through a water network. (Figure 3a; see Supporting Information (Figure S1) for details of the interactions). Upon closer inspection of the interactions between compound 7 and EED, it appeared to us that the entire piperidine ring connecting C7 to C8 and the benzyl group attached to nitrogen at 9-position did not contribute much to the interaction and likely reduced efficiency of binding due to the nonessential lipophilicity. Compound 8, a fragment of compound 7 (Table 1), was confirmed to retain most of the key interactions with EED (Figure 3b), is equipotent as compound 7. This transformation led to dramatic improvements of both ligand efficiency (LE) and lipophilic efficiency (LipE) (Table 1).

With what appeared to be the minimal pharmacophore required for binding, we set out to improve potency for 8. The cocrystal structure of compound 8 with EED suggested C8 to be a more attractive vector for additional substitution as compared to C7 (red arrow, Figure 3b). Meanwhile, the surprising activities of compound 11 (Figure 3c), another HTS hit, caught our attention. The two key features critical to the binding to EED, the electron-rich "deep pocket" aryl group and hydrogen bond donating NH at 5′-position, are missing in compound 11; and the importance of "the deep pocket" was attested by total loss of activities in compound 12 (Figure 3c). We postulate that an edge-to-face interaction between the phenyl ring of compound 11 and Phe97 (Figure 3c) possibly contributed most to its activity in the absence of "the deep pocket" group. Furthermore, when the cocrystal structure of compound 11 was overlaid with that of compound 7, we hypothesized that the aryl substitution at 8-position of compound 8 would mimic that of compound 11 and help us pick up this edge-to-face interaction. Compound 9

Table 1. Discovery of Bicyclic 6-CN Triazolopyridine Lead Compound 9: Fragmentation of Hit Compound 7 and Regrowth

<table>
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<tr>
<th>compound</th>
<th>Structure</th>
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<th>clogPb</th>
<th>LE (BioChem)b</th>
<th>LipE (BioChem)b</th>
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"IC₅₀ values reported as an average of ≥2 determinations; see Supporting Information for further details. "clogP values generated via ChemBioDraw, version 14.0.0.117. "LE = ligand efficiency = pIC₅₀ − HAC. "LipE = lipophilic efficiency = pIC₅₀ − clogP.

Scheme 1. Synthesis of 5-((Furan-2-ylmethyl)amino)-8-phenyl[1,2,4]triazolo[4,3-a]pyridine-6-carbonitrilea

![Scheme 1](image)

"Reagents and conditions: (a) NaOMe, ethyl propiolate, 90 °C, 2 h, 79%; (b) Br₂, acetic acid, 63%; (c) POCl₃, 160 °C, 2 h, 27%; (d) NH₂NH₂, 0 °C, 93%; (e) CH(OH)₂, TFA, 0 °C, 67%; (f) furan-2-ylmethanamine, EtOH, rt, 73%; (g) phenylboronic acid, PdCl₂(dpff), NaHCO₃, dioxane−H₂O, 90 °C overnight, 10%.
was therefore designed and synthesized, and a close to 20-fold improvement in biochemical activity was achieved, with slight decrease in LE and LipE. Substitutions at C8 position are mostly solvent exposed and achieving additional interactions with the protein was challenging; however some interesting SARs were observed. Addition of a (dimethylamino)methyl group at 4\'-position of C8 aryl group (compound 10) improved biochemical potency 7-fold, and this effect was also observed with similar amino tails and at both 4\'- and 3\'-position of the C8 aryl group (unpublished results).

Compounds in Table 1 were synthesized according to the reaction sequence depicted in Scheme 1. 2-Cyanoacetamide was condensed with ethyl propiolate under basic condition to afford dihydroxylated pyridine compound 14, which was brominated to afford compound 15. Compound 15 was treated with POCl3 followed by aqueous hydrazine solution and then cyclized to give the desired bicyclic 8-bromo[1,2,4]triazolo[4,3-c]pyridine derivative 16. Displacement with furan-2-ylmethanamine at room temperature in ethanol afforded compound 17, which was converted to desired target compound 9 after Suzuki coupling with phenylboronic acid.

Core Permutation: [1,2,4]Triazolo[4,3-c]pyrimidine Core as Scaffold II. To continue our SAR exploration, we carried out systematic scaffold hopping efforts of the [6, 5] bicyclic core (Table 2). Removal of the cyano group at the 6-position of compound 9 and replacing it with nitrogen led to compound 18 with similar potency and slightly improved LE and LipE, which is possibly due to a newly formed water-mediated hydrogen bond network (Supporting Information, Figure S1). Having the nitrogen atom at 6-position seems to be critical to the binding. Shifting the nitrogen to 7-position as in compound 22 decreased activity over 60-fold vs compound 18, and removing this nitrogen resulted in a potency decrease over 50-fold (compound 21 vs compound 20). The accessibility of nitrogen at the 2-position as a hydrogen bond acceptor to Lys211 also plays a pivotal role in binding; removing this nitrogen resulted in a 400-fold drop in potency (compound 19 vs compound 18). Although the nitrogen at 1-position does not seem to contribute to meaningful polar interaction judging from X-ray structures, removing the nitrogen led to a 5-fold decrease in LE and LipE.

Table 2. Discovery of Bicyclic 6-CN Triazolopyridine Lead Compound 9: Fragmentation of Hit Compound 7 and Regrowth

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>BioChem (IC50, μM)</th>
<th>clogP</th>
<th>LE (BioChem)</th>
<th>LipE (BioChem)</th>
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*IC50 values reported as an average of ≥2 determinations; see Supporting Information for further details. clogP values generated via ChemBioDraw, version 14.0.0.117. LE = ligand efficiency = pIC50 − HAC. LipE = lipophilic efficiency = pIC50 − clogP.
drop in potency from compound 18 to 21, which is possibly due to less effective $\pi-\pi$ interactions with a less electron-deficient bicyclic core. In addition to pyrimidine-based bicyclic core, we also investigated their pyrazine-based counterparts, such as compounds 22 and 23. In all the cases we examined, alteration of the heterocyclic core of 18 was detrimental to potency. Nonetheless, we had two very promising scaffolds in our hand, namely, 8-aryl[1,2,4]triazolo[4,3-$c$]pyrimidine (scaffold I) and 8-aryl[1,2,4]triazolo[4,3-$c$]pyridine (scaffold II) for further optimization, and we were intrigued to determine if the

Table 3. Investigation of SAR in “The Deep Pocket” (C-Ring)

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$^a$IC$_{50}$ values reported as an average of $\geq$2 determinations; see Supporting Information for further details.
Synthesis of the analogs for 8-aryl[1,2,4]triazolo[4,3-c]-pyrimidine (scaffold II) is detailed in Scheme 2. Commercially available 5-bromo-4-chloro-2-(methylthio)pyrimidine (compound 24) was treated with hydrazine, followed by trimethyl orthoformate to afford bicyclic compound 26, which was transformed to compound 27 after selective displacement with furan-2-ylmethanamine at the 5-position. Compound 27 was further converted to the 8-phenyl analog 28 after Suzuki reaction. In some cases when stronger bases were used, Dimroth rearrangement product 29 was also observed. By using NaHCO₃, we were able to prepare compound 28 in multigram scales with minimum formation of side product 29. Compound 29 can be separated, and its identity was distinctive from singlet at δ 8.50 in ¹H NMR accounting for the proton at C2. When profiled in our biochemical assay, compound 29 lost most of the activity of its isomer 28, which is consistent with the notion that the H-bond acceptor to Lys211 at the 1-position of the bicyclic system is important to maintain the interaction with EED.

Table 4. Selection of in Vivo Candidate Based on Compound Potency and Pharmacokinetic Profile

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"IC₅₀ values reported as an average of more than two determinations; see Supporting Information for further details. LE = ligand efficiency = pIC₅₀ – HAC. "LipE = lipophilic efficiency = pIC₅₀ – clogP. "Global H3K27me3 levels measured in G401 cells and IC₅₀ values reported as an average of more than two determinations. "IC₅₀ value reported for compounds 41 and 42 as single determination, while that of compound 43 reported as an average of 10 measurements with SD = 0.019 and that of 5 as an average of 2 measurement with SD = 0.006. "Hepatic extraction ratio of compounds in mouse liver microsomes. "Fraction unbound to plasma protein (expressed in % unbound). "Dose-normalized total exposure following intravenous (iv) dosing of 2 mg/kg in male CD-1 mice, formulated in PEG300/Soltuol HS15/pH4.65 acetate buffer (20:10:70, v/v/v). "Dose-normalized total exposure following per os (po) dosing of 2 mg/kg in male CD-1 mice, formulated in PEG300/Soltuol HS15/pH4.65 acetate buffer (20:10:70, v/v/v). "Dose-normalized free AUC following po dosing. "Cl = plasma clearance. "Vdss = volume of distribution at steady state. "Terminal half-life. "Oral bioavailability.

SAR at the C-ring and D-ring were transferable between the two scaffolds.

Synthesis of the analogs for 8-aryl[1,2,4]triazolo[4,3-c]-pyrimidine (scaffold II) is detailed in Scheme 2. Commercially available 5-bromo-4-chloro-2-(methylthio)pyrimidine (compound 24) was treated with hydrazine, followed by trimethyl orthoformate to afford bicyclic compound 26, which was transformed to compound 27 after selective displacement with furan-2-ylmethanamine at the 5-position. Compound 27 was further converted to the 8-phenyl analog 28 after Suzuki reaction. In some cases when stronger bases were used, Dimroth rearrangement product 29 was also observed. By using NaHCO₃, we were able to prepare compound 28 in multigram scales with minimum formation of side product 29. Compound 29 can be separated, and its identity was distinctive from singlet at δ 8.50 in ¹H NMR accounting for the proton at C2. When profiled in our biochemical assay, compound 29 lost most of the activity of its isomer 28, which is consistent with the notion that the H-bond acceptor to Lys211 at the 1-position of the bicyclic system is important to maintain the interaction with EED.

SAR Investigation of “The Deep Pocket”. Next, we turned our attention to the deep pocket or C-ring SAR for both scaffolds I and II in search for a replacement for the potentially metabolically labile furan ring. Electron-rich five-membered rings, such as furan, are preferred, regardless of the position of the oxygen as seen in compounds 10a and 10b vs 31a and 31b (Table 3). Thiophene isosteres are generally tolerated, albeit slightly less potent than their furan counterparts as in compound 30a and 30b. Compounds bearing less electron-rich C-rings, such as oxazole, isoxazole, and thiazole, are generally less potent. Overall, SAR of the C-ring is quite similar in both scaffolds until substitutions were introduced onto the ring. In both scaffolds, small substitution at the 5′-position of C-ring, such as F (39a and b), is more tolerated than the larger substitutions (40a and 40b). The two scaffolds started to behave quite differently when substitutions were introduced at 3′-position; the substitutions were tolerated in scaffold II, but not tolerated in scaffold I, when comparing 37b vs 37a, and...
38b vs 38a. The same differences were also observed in six-membered ring, as in 39a–40a vs 39a–40b. We believe in that scaffold I there is a steric clash between the CN and the 3′-substituted on the C ring that resulted in the subtle difference in SAR between scaffolds I and II. At this point, we concluded this particular quest without finding a viable replacement for furan in the deep pocket.

Selection of Compound for Pharmacology Studies. We next turned our attention to optimization of the D-ring. For reasons unclear to us, the gain of potency in biochemical assay from varying solvent-exposed D-ring mostly diminished in the reasons unclear to us, the gain of potency in biochemical assay from varying solvent-exposed D-ring mostly diminished in the biochemical assay. We therefore relied on overall pharmacokinetic profile to select our compound that would enable head-to-head comparisons to mouse (M), rat (R), dog (D), and human (H) plasma protein.

Unbound fraction of compound to mouse (M), rat (R), dog (D), and human (H) plasma protein. Dose-normalized free AUC after po dosing, 2 mg/kg in male Sprague Dawley rat, and 2 mg/kg in male Beagle dog, formulated with PEG300/Soltuol HS15/pH 4.65 acetate buffer (10:10:80, v/v/v). ‘Cl = total clearance. ‘Oral bioavailability.

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### Table 5. Characterization of Compound 43

<table>
<thead>
<tr>
<th>MW</th>
<th>mp (°C)</th>
<th>clogP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Caco-2 (A → B, 10&lt;sup&gt;−6&lt;/sup&gt;cm/s per ratio)</th>
<th>HT-Sol&lt;sup&gt;b&lt;/sup&gt;</th>
<th>pH 7</th>
<th>pH 3</th>
<th>SGE&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Fassi&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
<td>369.4</td>
<td>207.6</td>
<td>1.22</td>
<td>3.0/7.6</td>
<td>0.012</td>
<td>0.025</td>
<td>0.026</td>
<td>0.055</td>
<td>0.035</td>
</tr>
</tbody>
</table>

<sup>a</sup>clogP values generated via ChemBioDraw, version 14.0.0.117. <sup>b</sup>Equilibrium solubility. <sup>c</sup>Simulated gastric fluid. <sup>d</sup>Fasted state simulated intestinal fluid.

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tumors were used as our pharmacodynamics markers, which decreased dose-dependently along with the target genes upregulation after 21 days of compound dosing and with all showing good correlation with the plasma exposure of compound 43.\textsuperscript{18}

**Compound 43 Is a Highly Selective EED Inhibitor with Druglike Properties.** To confirm the on-target binding of compound 43 to the H3K27Me3 pocket, we obtained high resolution crystal structures of EED (76-441) in complex with compound 43 and EZH2 (40-68) peptide (EBD) (PDB accession code 5GSA). Compound 43 retained all the interactions of its parent compound 7 (supra vide) with EED, while in addition the phenyl ring of the mostly solvent exposed p-methylsulfonylphenyl group picks up edge-to-face π–π interaction with side chain of Phe97. Compound 43 was subsequently profiled in our in-house histone methyltransferase panel including close to 30 HMT targets. Compound 43 is highly selective against all other targets (>10,000-fold) except EZH1, with almost identical potency as EZH2. In our safety pharmacology panel including 59 targets,\textsuperscript{21} compound 43 showed <50% inhibition at the highest concentration tested (10 μM or 30 μM). IC\textsubscript{50} values in hERG binding assay and QPatch assay are both well above 30 μM.

Compound 43 was further evaluated for its physicochemical properties and other DMPK parameters (Table 5). The solubility was relatively low and with little dependency on the pH of the medium. Compound 43 has moderate permeability as the measured in Caco-2 cells at A\textsubscript{π}π→M. Compound 43 has moderate permeability as the measured in Caco-2 cells at A\textsubscript{π}π→M. Compound 43 is a highly potent, effective and selective inhibitor of EZH2 and EZH1 evaluated against a broad range of epigenetic and nonepigenetic targets. This inhibitor potently reduced global H3K27Me3 mark in cells and demonstrated selective cell killing effects in cells carrying a heterozygous Y64N mutation. With favorable pharmacokinetic properties, compound 43 demonstrated very impressive antitumor activities in mouse xenograft model. For the first time we have demonstrated that inhibiting methyltransferase activity of PRC2 through binding to the K27Me3 pocket of EED could constitute a viable strategy for developing anticancer therapeutics. Together with extensive preclinical profiling results of compound 43, especially its favorable pharmacokinetic profile and tolerability in preclinical animal species, compound of this type may be suitable for assessing long-term effects of pharmacologically inhibiting both EZH2 and EZH1 in preclinical models, and potentially in clinic, in terms of therapeutic benefits and potential toxicity.

## EXPERIMENTAL SECTION

**Synthesis and Characterization.** Unless otherwise mentioned, all reagents and solvents were obtained from commercial sources and used without purification. In some cases, intermediates were characterized by LC–MS to confirm that the mass matched the structure and carried on to the next step without further purification. Air sensitive procedures were performed under an atmosphere of nitrogen or argon. Purification of the final compounds to >95% purity was carried out using either prepacked silica gel cartridge (Analogix Biotage or ISCO) or reverse phase C18 column. \textsuperscript{1}H (300 MHz) and \textsuperscript{13}C (100 MHz) NMR spectra were recorded on a Bruker AC300 spectrometer. NMR chemical shifts (δ) are quoted in parts per million (ppm) referenced to the residual solvent peak [DMSO-d\textsubscript{6}] set at 2.49 ppm or [CDCl\textsubscript{3}] set at 7.26 ppm. Purity of all compounds was determined to be >95% by analytical HPLC.

5-((Furan-2-ylmethyl)amino)-8-phenyl[1,2,4]triazolo[4,3-a]pyridine-6-carbonitrile (9). 8-Bromo-5-((furan-2-ylmethyl)amino)-[1,2,4]triazolo[4,3-a]pyridine-6-carbonitrile (compound 17) (40 mg, 0.13 mmol), phenylboronic acid (18.40 mg, 0.15 mmol), PdCl\textsubscript{2}(dpff) (4.60 mg, 6.3 μmol), and NaHCO\textsubscript{3} (31.7 mg, 0.38 mmol) in dioxane (2 mL) and H\textsubscript{2}O (1 mL) were heated to 90 °C under N\textsubscript{2} overnight. The resulting mixture was cooled to room temperature, and H\textsubscript{2}O was added to the mixture and extracted with EtOAc. The combined organic layers were dried (Na\textsubscript{2}SO\textsubscript{4}), filtered, and concentrated. The residue was purified by basic prep-HPLC to afford 3.8 mg (10%) of the title compound. \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}) δ ppm 9.45 (s, 1 H), 8.87 (t, 1 H), 8.12 (d, J = 1.2 Hz, 1 H), 8.11 (s, 1 H), 7.68 (d, J = 0.8 Hz, 1 H), 7.66 (s, 1 H), 7.38–7.48 (m, 3 H), 6.46–6.54 (m, 2 H), 5.03–5.05 (d, J = 6 Hz, 2 H). LC–MS (m/z): 315.9 [M + H]+.

2,6-Dihydroxydicyanocinnitrile (14). To a suspension of 2-cyanacetamide (250 g, 2.55 mol) in methanol (1000 mL) was added NaOMe (137.8 g, 2.55 mol) at 0 °C under N\textsubscript{2}. The reaction mixture was stirred at the same temperature for 30 min. Ethyl propiolate (214.4 g, 2.55 mol) was added dropwise at 0 °C. The reaction mixture was stirred at 0 °C for 2 h, then at 90 °C for 2 h. The reaction mixture was then cooled to rt, and the resulting yellow paste was filtered. The filter cake was suspended with water and neutralized with concentrated HCl until pH reached ~6. A mixed solvent of DCM–MeOH (v/v = 20/1) was added and stirred at 0 °C for 30 min. The solid was filtered and washed with EtO\textsubscript{2}O to give 274 g (79%) of the title compound as light yellow solid. \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}) δ ppm 9.88 (s, 1 H), 7.16 (d, J = 9 Hz, 1 H), 5.06 (d, J = 9 Hz, 1 H). LC–MS (m/z): 137.1 [M + H]+.

5-Bromo-2,6-dihydroxydicyanocinnitrile (15). A suspension of 2,6-dihydroxydicyanocinnitrile (274 g, 2 mol) in acetic acid (1500 mL) was stirred at 70 °C for 30 min. To the mixture at 10 °C was added a solution of Br\textsubscript{2} (100 mL, 2 mol) in acetic acid (100 mL). The resulting mixture was stirred for 20 min. The solid was filtered to give 271 g (63%) of the title compound as light yellow solid. \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}) δ ppm 7.86 (s, 1 H). LC–MS (m/z): 212.9 [M + H]+.

8-Bromo-5-chloro[1,2,4]triazolo[4,3-a]pyridine-6-carbonitrile (16). A solution of compound 15 (130 g, 0.6 mol) in POCI\textsubscript{3} (500 mL) was sealed and heated at 160 °C for 2 h. The mixture was cooled to room temperature and concentrated to remove extra POCI\textsubscript{3}. Ethyl acetate (2000 mL) and ice-water (2000 mL) were added to the residue, and the resulting mixture was neutralized with aqueous NaHCO\textsubscript{3} until pH reached ~7. The aqueous layer was separated and extracted with ethyl acetate. The combined organic layers were washed with brine, dried (Na\textsubscript{2}SO\textsubscript{4}), filtered, and concentrated. The mixture was purified with silica gel column chromatography (eluted with PE–EA: 50/1) to give 41 g (27%) of the 8-bromo-2,6-dichloronitrosonitrile compound as light yellow solid. \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}) δ ppm 9.11 (s, 1 H). LC–MS (m/z): 250.5 [M + H]+.

8-Bromo-5-((furan-2-ylmethyl)amino)[1,2,4]triazolo[4,3-a]-pyridine-6-carbonitrile (17). To a mixture of compound 16 (1.03 g, 4 mmol) in EtOH (7 mL) at room temperature was added furan-2-ylmethanamine (0.855 g, 8.80 mmol) in EtOH (1 mL) dropwise. The resulting mixture was stirred at room temperature for 2 h under N\textsubscript{2}. After the starting material was consumed, DCM (20 mL) was added.
Solvant was removed under vacuum after silica gel (~4 g) was added. The silica gel coated with crude product was loaded on a silica gel column and eluted with DCM–MeOH to give 1.2 g (94%) of the title compound as a light green solid. 1H NMR (400 MHz, DMSO-d6) δ ppm 9.63 (s, 1 H), 8.85 (s, 1 H), 7.73 (s, 1 H), 7.66 (d, J = 8.8 Hz, 1 H), 6.51 (d, J = 2.8 Hz, 1 H), 4.64 (dd, J = 3.2, 2 Hz, 1 H), 4.97 (s, 2 H). LC–MS (m/z): 318.0 [M + H]+.

5-Bromo-4-hydrazinyl-2-(methylthio)pyrimidine (25). To a solution of 5-bromo-4-chloro-2-(methylthio)pyrimidine (24, 49.0 g, 0.205 mol) in ethanol (1000 mL) was added hydrazine (21.5 g, 0.43 mol). The mixture was heated at reflux and stirred for 3 h. The resulting suspension was filtered, washed with hexane, and dried in vacuo to give the title compound (44.1 g, 92%) as a white solid. 1H NMR (400 MHz, CD3OD) δ ppm 8.08 (s, 1 H), 2.42 (s, 3 H). LC–MS (m/z): 234.9, 236.9 [M + H]+.

5-Bromo-(furan-2-ylmethyl)-8-(4-(methylsulfonyl)phenyl)[1,2,4]triazolo[4,3-d]pyrimidine (26). Compound 25 (40.0 g, 0.17 mol) was dissolved in 200 mL of triethylamine. The mixture was heated at reflux and stirred for 3 h. The reaction mixture was concentrated under reduced pressure, and the residue was purified by flash chromatography (eluted with EA/PE = 1:1 to 1:1) to give the title compound (38.3 g, 92%) as a white solid. 1H NMR (400 MHz, CDCl3) δ ppm 9.87 (s, 1 H), 8.03 (s, 1 H), 2.82 (s, 3 H). LC–MS (m/z): 245.0, 247.0 [M + H]+.

8-Bromo-N-(furan-2-ylmethyl)[1,2,4]triazolo[4,3-c]-pyrimidin-5-amine (28). To a solution of 8-bromo-N-(furan-2-ylmethyl)[1,2,4]triazolo[4,3-c]-pyrimidin-5-amine (27) and potassium carbonate (28.2 mg, 0.204 mmol), (4-(methylsulfonyl)phenyl)boronic acid (17.7 mg, 0.088 mmol) in mixed solvent (dioxane/MeCN/water = 3 mL:0.3 mL:0.3 mL) were added potassium carbonate (28.2 mg, 0.204 mmol), (4-(methylsulfonyl)phenyl)boronic acid (17.7 mg, 0.088 mmol) in mixed solvent (dioxane/MeCN/water = 3 mL:0.3 mL:0.3 mL) were added potassium carbonate (28.2 mg, 0.204 mmol), and Pd(PPh3)4 (7.86 mg, 6.80 mmol). The resulting mixture was heated at 80 °C for 20 min. The mixture was then cooled to room temperature, and solvent was removed in vacuo. The residue was purified with silica gel column chromatography (eluted with DCM–MeOH (10:1)) to give 5 mg (22.3%) of the title compound. 1H NMR (400 MHz, CD3OD) δ ppm 7.93 (t, J = 7.4 Hz, 1H), 7.81 (d, J = 7.4 Hz, 1H), 7.49 (d, J = 1.1 Hz, 1H), 6.98 (d, J = 1.1 Hz, 1H), 6.54 (s, 1 H), 4.74 (s, 2 H). LC–MS (m/z): 261.1 [M + H]+.

8-Bromo-N-(furan-2-ylmethyl)[1,2,4]triazolo[4,3-c]-pyrimidin-5-amine (27). To a solution of 8-bromo-N-(furan-2-ylmethyl)[1,2,4]triazolo[4,3-c]-pyrimidin-5-amine (20.0 mg, 0.068 mmol) in mixed solvent (dioxane/MeCN/water = 3 mL:0.3 mL:0.3 mL) were added potassium carbonate (28.2 mg, 0.204 mmol), (4-(methylsulfonyl)phenyl)boronic acid (17.7 mg, 0.088 mmol), and Pd(PPh3)4 (7.86 mg, 6.80 mmol). The resulting mixture was stirred at rt (for 1 h). The solvent was removed and the residue was purified with silica gel column chromatography, eluting with DCM–MeOH (20:1), to give 20 mg (16.7%) of the title compound. 1H NMR (400 MHz, CD3OD) δ ppm 9.28 (s, 1 H), 7.87 (s, 1 H), 7.47 (s, 1 H), 6.38–6.40 (m, 2 H), 4.77 (s, 2 H). LC–MS (m/z): 290.9, 292.0 [M + H]+.

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8-Bromo-N-(furan-2-ylmethyl)-8-phenyl[1,2,4]triazolo[4,3-c]-pyrimidin-5-amine (28). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b01576.

Synthetic methods for compounds 19–23; characterization data for selected compounds; experimental conditions for crystallization and structure determination; key interactions with EED protein; experimental protocols of biological assays, including AlphaScreen binding assay and biochemical and cellular assay; mouse pharmacokinetic study and MTD determination for compound 43 (PDF)

Molecular formula strings (CSV)

Accession Codes

Protein Data Bank codes are the following: compound 7, 5H19; compound 8, 5H24; compound 11, 5H25. Authors will release the atomic coordinates and experimental data upon article publication.
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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS USED
PRC, polycomb repressive complex; EED, embryonic ectoderm development; H3K27, histone H3 lysine 27; SAM, S-adenosylmethionine; HTRF, homogeneous time-resolved fluorescence; SAH, S-adenosyl-L-homocysteine hydrolase; HTS, high-throughput screening; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; HAC, heavy atom count; LE, ligand efficiency; LipE, lipophilic efficiency; SAR, structure–activity relationship; HPLC, high performance liquid chromatography.

REFERENCES


(18) To understand how the binding to EED affects the EZH2 methylation activity, we have studied the mechanism of action of our EED compounds. Using compound 43 as an example, we established evidence that our compounds bind directly to the K27Me3 pocket: Qi, W.; Zhao, K.; Gu, J.; Huang, Y.; Wang, Y.; Zhang, H.; Zhang, M.; Zhang, J.; Yu, Z.; Li, L.; Teng, L.; Chua, Z.; Zhang, C.; Zhao, M.; Chan, H.; Chen, Z.; Fang, D.; Fei, Q.; Lying Feng, L.; Feng, L.; Gao, Y.; Ge, H.; Ge, X.; Li, G.; Lingel, A.; Lin, Y.; Liu, Y.; Luo, F.; Shi, M.; Wang, L.; Wang, Z.; Yu, Y.; Zeng, J.; Zeng, C.; Zhang, L.; Zhang, Q.; Zhou, S.; Oyang, C.; Atadja, P.; Li, E. Unpublished results.

