Published in final edited form as:
ACS Chem Biol. 2013 June 21; 8(6): 1324–1334. doi:10.1021/cb400133j.

An Orally Bioavailable Chemical Probe of the Lysine Methyltransferases EZH2 and EZH1

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Abstract

EZH2 or EZH1 is the catalytic subunit of the polycomb repressive complex 2 that catalyzes methylation of histone H3 lysine 27 (H3K27). The trimethylation of H3K27 (H3K27me3) is a transcriptionally repressive post-translational modification. Overexpression of EZH2 and hypertrimethylation of H3K27 have been implicated in a number of cancers. Several selective inhibitors of EZH2 have been reported recently. Herein we disclose UNC1999, the first orally bioavailable inhibitor that has high *in vitro* potency for wild-type and mutant EZH2 as well as EZH1, a closely related H3K27 methyltransferase that shares 96% sequence identity with EZH2 in their respective catalytic domains. UNC1999 was highly selective for EZH2 and EZH1 over a broad range of epigenetic and non-epigenetic targets, competitive with the cofactor SAM, and non-competitive with the peptide substrate. This inhibitor potently reduced H3K27me3 levels in cells and selectively killed diffused large B cell lymphoma cell lines harboring the $EZH2^{Y641N}$ mutant. Importantly, UNC1999 was orally bioavailable in mice, making this inhibitor a valuable tool for investigating the role of EZH2 and EZH1 in chronic animal studies. We also designed and synthesized UNC2400, a close analog of UNC1999 with >1,000-fold lower potency than UNC1999 as a negative control for cell-based studies. Finally, we created a biotin-tagged UNC1999 (UNC2399) which enriched EZH2 in pull-down studies, and a UNC1999 – dye conjugate (UNC2239) for co-localization studies with EZH2 in live cells. Taken together, these

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The authors declare no competing financial interests.

Supporting information
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compounds represent a set of useful tools for the biomedical community to investigate the role of EZH2 and EZH1 in health and disease.

Among epigenetic “writers” (the enzymes that produce post-translational modifications (PTMs)), “readers” (the proteins that recognize and bind to PTMs), and “erasers” (the enzymes that remove PTMs), protein lysine methyltransferases (PKMTs, also known as histone methyltransferases (HMTs)), which catalyze mono-, di-, and/or trimethylation of lysine residues of histones and non-histone proteins, have increasingly been recognized as an important target class for modulation to regulate gene expression, cell differentiation and organismal development.[1–12] Small-molecule probes[13] that selectively inhibit the catalytic activity of individual PKMTs are invaluable tools for deciphering the complex regulatory mechanisms enabled by histone and protein lysine methylation. Although the selective PKMT inhibitor discovery field is gaining momentum, only a limited number of selective inhibitors, which target the PKMT substrate binding groove,[14–20] cofactor binding site,[21–30] and a PRMT (protein arginine methyltransferase) allosteric binding site,[31, 32] respectively, have been reported.

Polycomb repressive complex 2 (PRC2) that catalyzes methylation of histone H3 lysine 27 (H3K27) contains either the enzymatic subunit EZH2 (enhancer of zeste homolog 2, also known as KMT6 or KMT6A) or EZH1 (enhancer of zeste homolog 1, also known as KMT6B).[33–36] EZH2 and EZH1 are highly homologous and share 76% sequence identity overall and 96% sequence identity in their respective SET domains,[26] named after *Drosophila* Su(var)3–9 (suppressor of variegation 3–9), E(z) (enhancer of zeste), and Trithorax.[37] Despite their high sequence identity, EZH2 and EZH1 are not functionally redundant and have different expression patterns.[35, 38] While EZH2 is only found in actively dividing cells, EZH1 is found in both dividing and non-dividing cells.[38] Although PRC2 containing EZH1 (PRC2 – EZH1) has lower catalytic activity compared to PRC2 containing EZH2 (PRC2 – EZH2), both complexes contribute to the maintenance of cellular H3K27 methylation states.[35, 36, 38]

The trimethylation of H3K27 (H3K27me3) catalyzed by PRC2 is a transcriptionally repressive epigenetic mark that regulates gene expression, differentiation, and development.[38] Dysregulation of EZH2, other PRC2 components, and H3K27 methylation has been associated with a number of cancers. For example, EZH2 is over-expressed in a broad spectrum of cancers including prostate, breast, myeloma, and lymphoma and high expression correlates with poor prognosis.[38, 39] More recently, hypertrimethylation of H3K27 has been identified in diffused large B cell lymphoma (DLBCL) cells heterozygous for point mutations recurrently targeting tyrosine 641 (Y641).[40, 41] While wild-type (WT) EZH2 is most efficient at catalyzing monomethylation of non-methylated H3K27, the EZH2 Y641 mutant enzymes (Y641F, Y641N, Y641S, and Y641H) have the opposite substrate preference and are most efficient at catalyzing the conversion of di-methylated H3K27 (H3K27me2) into trimethylated H3K27 (H3K27me3).[40, 41] Thus, the cooperation between WT and Y641 mutant EZH2 drives the hypertrimethylation of H3K27 in these heterozygous DLBCL cells.[40, 41] While targeting EZH2 dysfunction has been pursued as a potential therapeutic strategy for the treatment of cancer, it is worth noting that EZH2 and PRC2 play an integral part in regulating stem cell pluripotency and differentiation.[38, 42] Therefore, developing an EZH2 chemical probe that has suitable pharmacokinetic (PK) properties would be extremely useful for assessing therapeutic benefit(s) and potential toxicity of chronically inhibiting EZH2 in animal studies.

The recent discoveries of EPZ005687, GSK126, and El1, the first potent and selective inhibitors of EZH2, were an important advance in the PKMT inhibitor discovery field.[24–26, 28] These inhibitors share a common pyridone indazole/indole scaffold and are competitive with the cofactor S-adenosyl-L-methionine (SAM) and non-competitive with the peptide substrate. In cell-based studies, these inhibitors selectively reduced H3K27me3 and H3K27me2 marks and killed DLBCL cells bearing Y641 point mutations. However, EPZ005687 was reported to not have sufficient PK properties for animal studies and *in vivo* PK properties of El1 were not reported. Although GSK126 was used in animal studies via intraperitoneal (IP) administration, no orally bioavailable EZH2 inhibitors that are more suitable for chronic animal studies have been reported to date. In addition, while EPZ005687, GSK126, and El1 are highly selective for EZH2 over other methyltransferases including EZH1, an inhibitor that has high *in vitro* potency and selectivity for both EZH2 and EZH1 over other methyltransferases has not been reported. Such a tool is expected to inhibit H3K27 methylation mediated by both PRC2 – EZH2 and PRC2 – EZH1, and therefore, could offer potential advantages over EZH2 selective inhibitors in the disease settings where both PRC2 – EZH2 and PRC2 – EZH1 contribute to the methylation of H3K27.

Here we report the design, synthesis, and biological characterization of UNC1999, the first orally bioavailable chemical probe of EZH2 and EZH1. UNC1999 was highly potent and selective for EZH2 wild-type and Y641 mutant enzymes as well as EZH1 over a broad range of epigenetic and non-epigenetic targets. It was competitive with the cofactor and non-competitive with the peptide substrate. In cell-based assays, UNC1999 potently reduced the H3K27me3 mark and selectively killed DB cells, a DLBCL cell line harboring the $EZH2^{Y641N}$ mutant. In mouse PK studies, UNC1999 was orally bioavailable, making it suitable for chronic animal studies. We also report the discovery of UNC2400 which is a close analog of UNC1999 with >1,000-fold less potency as a negative control for cellular studies, a biotinylated UNC1999 (UNC2399) which enriched EZH2 in pull-down studies, and a UNC1999 – dye conjugate (UNC2239) for co-localization studies with EZH2 in live cells.

RESULTS AND DISCUSSIONS

Discovery of UNC1999 and UNC2400

To discover orally bioavailable EZH2 inhibitors, we docked EPZ005687 into an EZH2 homology model, which was built on the basis of the X-ray crystal structure of GLP (PDB: 2RFI), a H3K9 (histone H3 lysine 9) mono- and dimethylase.[43] The docking model of EPZ005687 suggests that the morpholinomethyl moiety is solvent exposed and does not interact with EZH2. Other moieties of EPZ005687 make a number of hydrogen bonds and hydrophobic interactions with the EZH2 SAM binding site, including a hydrogen bond between the central amide and Asn688 (asparagine 688), and two hydrogen bonds between the pyridone and His689 (histidine 689) (Figure 1A – B). Based on this binding hypothesis, we designed multiple compounds which combine several key structural features of EPZ005687 and GSK126 (Figure 1C – D, and Table 1). We focused on modifying the morpholinomethyl region to modulate physicochemical properties of this series without disrupting protein – ligand interactions and kept the pyridone indazole core for maintaining key hydrogen bonds and hydrophobic interactions with the protein. Among these designed inhibitors, UNC1999 was docked into the EZH2 homology model. The docking model of UNC1999 suggests that: (1) the secondary amide and pyridone maintain the respective key hydrogen bonds with Asn688 and His689, (2) the indazole is buried in a hydrophobic pocket, and (3) the *N*-isopropyl piperazine does not interact with the protein (Figure 1C – D). As expected, UNC1999 and EPZ005687 bind to the EZH2 SAM binding site very similarly according to these docking studies (Figure 1).

The compounds in Table 1 were synthesized (Supporting Scheme S1) and evaluated in an EZH2 radioactive biochemical assay which measures the transfer of the tritiated methyl group from the cofactor ^3H – SAM to a peptide substrate. IC_{50} values of these compounds in this assay and their calculated partition coefficient (clogP) values are summarized in Table 1. As expected, the unsubstituted and *N*-alkyl piperazines (compounds **1** – **4**) had high *in vitro* potency for EZH2 (IC_{50} < 10 nM), indicating that modifications to the *N*-capping group are well tolerated. These results support our binding hypothesis: the *N*-alkyl piperazine does not interact with the protein. Among these four inhibitors, UNC1999 (compound **4**) has a more desirable clogP (3.1).[44] This balanced lipophilicity could enhance oral absorption without a significant increase in metabolism. In addition, UNC1999 has a higher clogP than either EPZ005687 (2.2) or GSK126 (2.6), suggesting that UNC1999 may be better absorbed orally than either of the known inhibitors. Interestingly, replacing the 2-piperazinyl pyridin-5-yl moiety (compounds **1** – **4**) with the 3-fluoro pyridin-6-yl group (compound **5**) retained high *in vitro* potency (IC_{50} = 10 ± 1 nM (n = 3)). On the other hand, replacing the 3-fluoro pyridin-6-yl group (compound **5**) with the 4-fluorophenyl group (compound **6**) led to a 6-fold loss of potency. Based on these results, we selected UNC1999 for subsequent mechanism of action (MOA), selectivity, cellular assays, and mouse PK studies.

To provide the research community with a set of useful tools, we aimed to create a structurally similar but significantly less potent EZH2 inhibitor as a negative control for cell-based studies. Based on the UNC1999 docking model which suggests that the secondary amide and pyridone form key hydrogen bonds with Asn688 and His689 (Figure 1C – D), we designed and synthesized UNC2400 (Figure 2A, Supporting Scheme S2), which contains an *N*-methyl group at both the secondary amide and pyridone moieties of UNC1999. We hypothesized that the addition of these two *N*-methyl groups would abolish the hydrogen bond between the secondary amide and Asn688 and impair the hydrogen bonds between the pyridone and His689. Indeed, UNC2400 displayed an IC_{50} = 13,000 ± 3,000 nM (n = 3) in the EZH2 radioactive biochemical assay (Figure 2B), more than 1000-fold less potent than UNC1999, thus supporting our *in silico* binding hypothesis for UNC1999. Importantly, UNC2400 was also inactive in a number of cell-based studies (see below). The high structural similarity and drastic potency difference between UNC1999 and UNC2400 suggest they will be excellent positive and negative control tool compounds.

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

To determine the MOA of EZH2 inhibition by UNC1999, we generated classic Lineweaver-Burk plots[45] (Figure 2C – D), which indicate that UNC1999 was competitive with the cofactor SAM with a K_i of 4.6 ± 0.8 nM (n = 2) and non-competitive with the H3 peptide substrate. We also determined that increasing the H3 peptide concentration had no effect on the IC_{50} values of UNC1999 while increasing SAM concentration dramatically affected the IC_{50} values of UNC1999 (Supporting Figure S1), which further supports that UNC1999 is a SAM-competitive EZH2 inhibitor. We next determined the potency of UNC1999 against EZH2 Y641 mutants. UNC1999 was highly potent for both Y641N and Y641F mutants (Figure 2E and Supporting Figure S2), displaying less than 5-fold higher potency for the WT over the Y641N mutant enzyme and similar potencies for the WT and Y641F mutant enzymes.

To determine the selectivity profile of UNC1999, we first tested UNC1999 against EZH1. Compared with EPZ005687, GSK126 and El1, which are at least 50-fold selective for EZH2 over EZH1,[24, 25, 28] UNC1999 had high *in vitro* potency for EZH1 (IC_{50} = 45 ± 3 nM (n = 3)) (Figure 2F) and was only about 10-fold less potent for EZH1 than EZH2. We also determined the MOA of EZH1 inhibition by UNC1999 (Supporting Figure S3). As indicated by the Lineweaver-Burk plots, UNC1999 was competitive with the cofactor SAM and non-competitive with the H3 peptide substrate. Because EZH1 and EZH2 are highly homologous and share 96% sequence identity in their respective SET domains,[26] it is not surprising that UNC1999 retains high *in vitro* potency for EZH1. However, it is not clear how the structural changes contribute to the switch from GSK126's high EZH2/EZH1 selectivity (> 150-fold) to UNC1999's low EZH2/EZH1 selectivity (approximately 10-fold). The dual inhibition of EZH2 and EZH1 by UNC1999 might result in higher efficacy in cell-based and animal models where the methylation of H3K27 by PRC2 – EZH2 is compensated for by PRC2 – EZH1. This inhibitor may also be useful for assessing potential toxicities resulted from pharmacological inhibition of both EZH2 and EZH1. We next tested UNC1999 against 15 other lysine, arginine or DNA methyltransferases (Figure 2G) and found that UNC1999 was more than 10,000-fold selective for EZH2 (IC_{50} < 10 nM) over these methyltransferases (IC_{50} > 100,000 nM).

We also evaluated the selectivity of UNC1999 over a broad range of non-epigenetic targets. Because UNC1999 was competitive with SAM, an adenine nucleoside, we tested it against a panel of 50 representative kinases (Supporting Table S1). UNC1999 showed no appreciable inhibition (no more than 20% inhibition at 10,000 nM) against these kinases. In addition, we tested UNC1999 in the National Institute of Mental Health (NIMH) Psychoactive Drug Screen Program (PDSP) Selectivity Panel, which consists of a total of 44 GPCRs, transporters, and ion channels (Supporting Table S2). It was found to show no more than 50% inhibition at 10,000 nM against 40 targets and > 50% inhibition at 10,000 nM against 4 targets in the panel. K_i determinations in the radioligand binding assay for each of the 4 interacting targets was subsequently performed (Supporting Table S3). UNC1999 had K_i values of 4,700 nM, 65 nM, 300 nM, and 1,500 nM for sigma1, sigma2, histamine H_3, and NET (norepinephrine transporter), respectively. Although we could not evaluate UNC1999 in sigma2 functional assays because they are unknown, we tested UNC1999 in histamine H_3 functional assays and found that it did not display any agonist or antagonist activities at concentrations up to 1,000 nM. Therefore, with the exception of sigma2, UNC1999 was more than 200-fold selective for EZH2 over a broad range of kinases, GPCRs, transporters, and ion channels.

In addition, we determined selectivity of UNC2400 versus other methyltransferases. As expected, UNC2400 displayed poor potencies for EZH1 (IC_{50} = 62,000 ± 7,000 nM (n = 3)) and EZH2 Y641F (IC_{50} > 200,000 nM) (Supporting Figure S4A). It had negligible activities against 15 other methyltransferases (Supporting Figure S4B)

UNC1999 Potently Reduces H3K27me3 in Cells

To assess the cellular potency of UNC1999, we employed an H3K27me3 antibody cell immunofluorescence In-Cell Western (ICW) assay. This assay allows rapid processing of multiple samples for H3K27me3 immunofluorescence signal and normalization to cell number via the use of the nucleic acid dye, DRAQ5. We characterized UNC1999 and UNC2400 in MCF10A cells, which bear the WT EZH2 enzyme. UNC1999 (72 h exposure) exhibited concentration-dependent reductions in H3K27me3 with an IC_{50} of 124 ± 11 nM (n = 3) (Figure 3A). On the other hand, UNC2400 (negative control) showed little or no activity in this ICW assay (Figure 3B), which is consistent with its poor *in vitro* potency. In addition, the treatment of MCF7 cells with UNC1999 at 5,000 nM for 72 h almost completely removed the H3K27me3 mark but did not have significant effects on cellular levels of EZH2 (Figure 3C).

One of the desirable characteristics of a high quality chemical probe is low toxicity due to off-target effects. Both UNC1999 (EC_{50} = 19,200 ± 1,200 nM (n = 3)) and UNC2400 (EC_{50} = 27,500 ± 1,300 (n = 3)) had low cellular toxicity in a standard resazurin (Alamar Blue) reduction assay (Figure 3A – 3B). Interestingly, UNC2400 showed similar cellular toxicity as UNC1999, suggesting that the observed low cellular toxicity is unlikely due to inhibition of EZH2 and EZH1 in this cell type. Taken together, UNC1999 had an excellent separation of cellular potency and toxicity with a function/toxicity ratio of more than 150 (Figure 3A).

UNC1999 Selectively Kills EZH2-mutant DLBCL Cells

We next investigated whether DB cells, a DLBCL cell line harboring the $EZH2^{Y641N}$ mutant,[41, 46] are more sensitive to UNC1999 treatment. UNC1999 (8 day exposure) displayed robust, concentration-dependent inhibition of cell proliferation with an EC_{50} of 633 ± 101 nM (n = 3) (Figure 4A), which is slightly more potent than GSK126.[25] At 5,000 nM, UNC1999 (8 day exposure) completely killed DB cells. Interestingly, we observed a delayed onset of activity for UNC1999: the 3 day treatment with this inhibitor did not have significant effects on cell proliferation at all tested concentrations (Figure 4A), as was also seen with EPZ005687 and GSK126.[24, 25] We also compared the effects of the negative control UNC2400 on cell proliferation with UNC1999. While UNC1999 (3,000 nM, 8 day treatment) significantly inhibited DB cell proliferation, UNC2400 (3,000 nM, 8 day treatment) had negligible effects (Figure 4B). Furthermore, UNC1999 (3,000 nM, 3 day treatment) significantly reduced the H3K27me3 mark but did not significantly change EZH2 levels in DB cells (Figure 4C). On the other hand, treatment with UNC2400 at 3,000 nM for 3 days did not result in a significant reduction in the H3K27me3 mark nor EZH2 levels in DB cells (Figure 4C). Combining the observed low toxicity in MCF10A cells and high sensitivity in DB cells, we provide evidence that UNC1999 selectively kills DLBCL cells heterozygous for Y641 point mutations. In addition, we showed that UNC1999 has robust on-target activities in cells and UNC2400 is an excellent negative control for cell-based studies.

UNC1999 Is Orally Bioavailable in Mice

We next evaluated the *in vivo* PK properties of UNC1999. A single intraperitoneal (IP) injection of UNC1999 at 15, 50, or 150 mg/kg achieved high C_{max} (9,700 – 11,800 nM) and exhibited dose linearity in male Swiss albino mice (Figure 5A). Both the 150 and 50 mg/kg IP doses resulted in the plasma concentrations of UNC1999 above its cellular IC_{50} over the entire 24 h period while the 15 mg/kg IP dose led to the plasma concentrations of UNC1999 above its cellular IC_{50} for approximately 12 h. We next examined whether UNC1999 is orally bioavailable and were pleased to find that a single 50 mg/kg oral dose of UNC1999 achieved high C_{max} (4,700 nM) and good exposure levels in male Swiss albino mice (Figure 5B). The plasma concentrations of UNC1999 were maintained above its cellular IC_{50} for approximately 20 h following this single oral dose. It is worth noting that all doses including the 150 mg/kg IP dose were well tolerated by all test mice, and no adverse effects were observed.

Compared with existing EZH2 inhibitors such as EPZ005687 which was reported to not have sufficient *in vivo* PK properties[24, 28] and GSK126 which was only used via IP injection,[25] UNC1999 is the first orally bioavailable inhibitor of EZH2 and EZH1. An orally bioavailable inhibitor makes chronic animal studies more practical and convenient as such a compound could be simply administered in the food or water of test mice. On the other hand, chronic daily IP injections to test mice could lead to infections, which might complicate long-term animal studies. Therefore, UNC1999 is a valuable tool for the biomedical research community to assess long-term therapeutic benefit(s) and potential toxicity resulting from pharmacological inhibition of EZH2 and EZH1 in mouse models.

Biotinylated UNC1999 Can Be Used to Pull Down EZH2 from Cell Lysates

Because the *N*-alkyl piperazine of UNC1999 is solvent exposed according to our docking model (Figure 1C – 1D) and modifications to the *N*-capping group were well tolerated (Table 1), we hypothesized that biotinylation at this site with a long PEG (polyethylene glycol) linker would not disrupt key protein – ligand interactions. Thus, the resulting compound would retain high affinity to EZH2 and be useful for EZH2 pull-down studies. We therefore designed and synthesized UNC2399 (Figure 6A and Supporting Scheme S3). Indeed, UNC2399 displayed high *in vitro* potency (IC_{50} = 17 ± 2 nM (n = 3)) in the EZH2 radioactive biochemical assay (Figure 6B). This result provides further evidence for our *in silico* binding hypothesis of UNC1999.

To conduct pull-down studies we first conjugated UNC2399 to streptavidin-coated beads. The compound-conjugated beads were used to capture EZH2 protein from HEK293T (human embryonic kidney 293T) cell lysates. We were pleased to find that UNC2399 – streptavidin beads (Figure 6C, well 3) enriched EZH2 from cell lysates as compared to unconjugated streptavidin beads (DMSO control, Figure 6C, well 2). To control for non-specific interactions, we pretreated the cell lysates with a soluble competitor, UNC1999 or UNC2400, before pull-down. As expected, the pretreatment with UNC1999 $^{(100}$ μM) completely blocked the enrichment of EZH2 by UNC2399 – streptavidin beads (Figure 6C, well 4 versus well 3). On the other hand, the pretreatment with UNC2400 (100 μM) resulted in EZH2 levels nearly identical to the samples that were not pretreated (Figure 6C, well 5 versus well 3). Taken together, these results provide evidence that UNC2399, a biotinylated UNC1999, is a useful tool for enriching EZH2 from cell lysates, and lay the groundwork for future use of UNC2399 in chemoproteomics studies.

UNC1999 – Dye Conjugate Co-localizes with EZH2 in Live Cells

We next sought to investigate the potential of UNC1999 to serve as a chemical probe for EZH2 localization in live cells. Based on the same principles used to design UNC2399, we designed UNC2239, which is a UNC1999 – dye conjugate prepared by a click reaction between a UNC1999 analog containing a terminal alkyne and a membrane permeant merocyanine dye with an azide side chain (Figure 7A and Supporting Scheme S4). As expected, UNC2239 displayed high *in vitro* potency (IC_{50} = 21 ± 1 nM (n = 3)) in the EZH2 radioactive biochemical assay despite the addition of the fluorophore (Figure 7B).

Mouse embryonic fibroblast (MEF) cells stably expressing yellow fluorescent protein (YFP) were treated with the fluorescent EZH2 probe UNC2239 or with the dye component of the probe only as a control (Figure 7D). The dye is known to distribute uniformly in MEF cells without evidence of any compartmental or organelle specific interactions. YFP serves as a uniformly distributed volume control fluorophore. When examining the localization of the probe, the ratio of dye intensity to YFP intensity was normalized for effects arising from varying cell thickness and uneven illumination intensity. Because the concentration of the probe and YFP varied from cell to cell, ratio images were normalized by setting the lowest 5% of pixels within each cell equal to 1. Cells treated with UNC2239 showed significantly higher fluorescence intensity in the nucleus relative to the cytoplasm than cells treated with the dye alone ($p < 0.05$, $n = 6$, Supporting Figure S5). Perinuclear localization of the probe was also observed, potentially due to autophagocytic uptake of the probe. The observed localization of UNC2239 is consistent with the nuclear localization of GFP – tagged EZH2 as seen in transfected HEK 293 cells (Figure 7C), suggesting that UNC2239 co-localizes with EZH2.

Conclusions

We report the discovery of UNC1999, the first orally bioavailable chemical probe of EZH2 and EZH1. UNC1999 was highly potent and selective for EZH2 and EZH1 over a broad range of epigenetic and non-epigenetic targets, and was competitive with the cofactor SAM and non-competitive with the peptide substrate. This inhibitor potently reduced the H3K27me3 mark in cells and selectively killed DB cells, a DLBCL cell line heterozygous with an $EZH2^{Y641N}$ mutant. Importantly, UNC1999 was orally bioavailable in mouse PK studies, thus making this inhibitor more suitable for assessing long-term therapeutic benefit(s) and potential toxicity of pharmacologically inhibiting EZH2 and EZH1 in animal studies than recently reported EZH2 inhibitors including EPZ005687 and GSK126. In addition, UNC1999 had high *in vitro* potency for both EZH2 and EZH1 while EPZ005687, GSK126, and El1 are more selective for EZH2 over EZH1. The dual inhibition of EZH2 and EZH1 by UNC1999 may offer potential advantages over EPZ005687, GSK126, and El1 in the disease settings where both PRC2 – EZH2 and PRC2 – EZH1 contribute to the methylation of H3K27. We also created UNC2400 which is a close analog of UNC1999 with >1,000-fold lower potency than UNC1999 as a negative control for cellular studies, a biotinylated UNC1999 (UNC2399) which enriched EZH2 from cell lysates in pull-down studies, and a UNC1999 – dye conjugate (UNC2239) for co-localization studies with EZH2 in live cells. Taken together, these valuable tools will aid in deciphering the role of EZH2 and EZH1 in health and disease.

METHODS

Synthesis of UNC1999 and its analogs

Synthetic schemes, experimental procedures, and full characterization data of all new compounds are described in Supporting Information.

In-Cell-Western, immunofluorescence microscopy, and cell viability assays

MCF10A cells were grown in DMEM/F12 media 5% horse serum, EGF (20 ng mL^{-1}), hydrocortisone (0.5 μg mL^{-1}), cholera toxin (100 ng mL^{-1}), and insulin (10 μg ml^{-1}) in the presence of inhibitors as stated in Figures. MCF7 cells were cultured in DMEM 10% FBS.

For in-cell-western fixation in 96 well black wall clear bottom plates was performed by 2% formaldehyde in PBS for 10 min. After five washes with 0.1% Triton X100 in PBS, cells were blocked for 1 h RT or overnight at 4°C with 3% BSA, 5% goat serum in PBS. Three replicate wells form each experimental group were incubated in primary H3K27me3 antibody, Diagenode MAb-181-050 at 1/4000 dilution in 3% BSA, 5% goat serum PBS for 18 h at 4°C. The wells were washed five times with 0.1% Tween 20 in PBS, then secondary IR800 conjugated antibody (Li-Cor) in Li-Cor blocking buffer (1:1000) and nucleic acid-intercalating dye, DRAQ5 (Cell Signaling Technologies) added for 1 h RT. After 5 washes with 0.1% Tween 20 PBS, the plates were read on an Odyssey (LiCor) scanner at 800 nm (H3K27me3 signal) and 700 nm (DRAQ5 signal). Fluorescence intensity was quantified, normalized to the background, then to the DRAQ5 signal, and expressed as a percentage of control.

Immunofluorescence microscopy was performed as above except the MCF7 cells were grown on the coverslips, stained for H3K27me3 (Diagenode) and EZH2 (Cell Signaling Technologies) and secondary anti-mouse/rabbit antibodies used were conjugated to Alexa 488 and 555 (Cell Signaling Technologies). After washes with 0.1% Tween 20 PBS, the coverslips were mounted with DAPI Shield (Sigma) and analyzed on Zeiss spinning disc confocal microscope.

Cell viability assays were performed using 0.1 mg mL^{-1} of resazurin (Sigma) in the media. Resazurin reduction was monitored with 544 nm excitation, measuring fluorescence at 590 nm.

DB cell proliferation assay

DB cells, a diffuse-large B-cell lymphoma cell line harboring the EZH2 Y641N mutation,[41, 46] were obtained from ATCC and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, antibiotics, and various concentration of compounds (DMSO control, UNC1999, or UNC2400). The medium containing the test compound or control was refreshed every three days. The numbers of viable cells from at least three independent experiments were measured using TC20 automated cell counter system (Biorad). Total histones were prepared from cell nuclei using an acidic extraction protocol as previously described.[47] About 1 microgram of total histones was separated using 15% of SDS-PAGE, transferred to PVDF membranes, and probed with histone antibodies. Antibodies used in this study are those against EZH2 (BD bioscience 612666), general H3 (Abcam ab1791), and H3K27me3 (Abcam ab6002).

UNC2399 pull-down studies

HEK293T cells were grown in DMEM (Sigma) supplemented with 10% FBS. Approximately 1×10^6 cells were lysed using 500 μL of Cytobuster® supplemented with 1X protease inhibitor cocktail (Roche #05056489001) and 125 U Benzonase® Nuclease (Novagen #D00128165). Protein concentration was quantified using a Bradford assay (BioRad #500-0006) and then 1 mg of lysate was used for each pull-down. Bead conjugates were prepared by incubating 0.1 mM UNC2399 with 200 mg Dynabeads® Streptavidin-coated magnetic beads (Invitrogen #653.06) at room temperature with for 1 h. The beads were then washed 3 times with TBST (20 mM Tris-HCl, pH 8.0/150 mM NaCl/0.1% Tween-20) to remove excess unbound UNC2399. Unconjugated control and compound-conjugated beads were incubated with cell lysate on and end-over-end rotator at 4°C overnight. Beads were washed 3 times with Wash Buffer (10 mM HEPES, pH7.9/0.2% Triton-X-100/0.3 M NaCl/10 mM KCl/1.5 mM $MgCl_2$), resuspended in 1X sample buffer (Invitrogen #LC2676), and incubated at 95°C for 5 min. Each sample was then run on a Mini-PROTEAN TGX gradient gel (BioRad #456-9035) followed by western blotting. Blots were blocked in 5% milk for 1 h, incubated with anti-EZH2 antibody (Abcam #ab110646) overnight at 4°C, washed 3 × 5 min with PBS/0.1 % Tween-20, incubated 1 h with HRP-conjugated secondary (Jackson ImmunResearch Laboratories #211-032-171), washed as before, then developed using ECL2 western blotting substrate (Pierce #80196). Imaging was performed on the Typhoon Trio+ (GE) imaging system. In the case of competitive experiments with free soluble UNC1999 or UNC2400, the lysate was incubated with compound for 1 h at room temperature before being introduced to the beads.

EZH2 homology modeling, radioactive biochemical assays, mechanism of action studies, selectivity assays, mouse PK studies, and UNC2239 imaging studies

Full experimental protocols are described in Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Wenhwa Lee for graphical design and illustration, the University Cancer Research Fund and Carolina Partnership from University of North Carolina at Chapel Hill and the V Foundation for Cancer Research for financial support. The Structural Genomics Consortium is a registered charity (number 1097737) that receives funds from the Canada Foundation for Innovation, Eli Lilly Canada, GlaxoSmithKline, the Ontario Ministry of Economic Development and Innovation, the Novartis Research Foundation, Pfizer, AbbVie, Takeda, Janssen, Boehringer Ingelheim and the Wellcome Trust. This Project was also funded by Genome Canada through the Ontario Genomics Institute per research agreement OGI-055. G.G.W. is supported by NIH/NCI "Pathway to

Independence" Award in Cancer Research (R00CA151683) and a Junior Scholar award of the V Foundation for Cancer Research. This research was also supported by funding from the American Cancer Society (C.J.M., 119169-PF-10-183-01-TBE) and the National Institutes of Health (K.M.H., GM057464 and GM094663).

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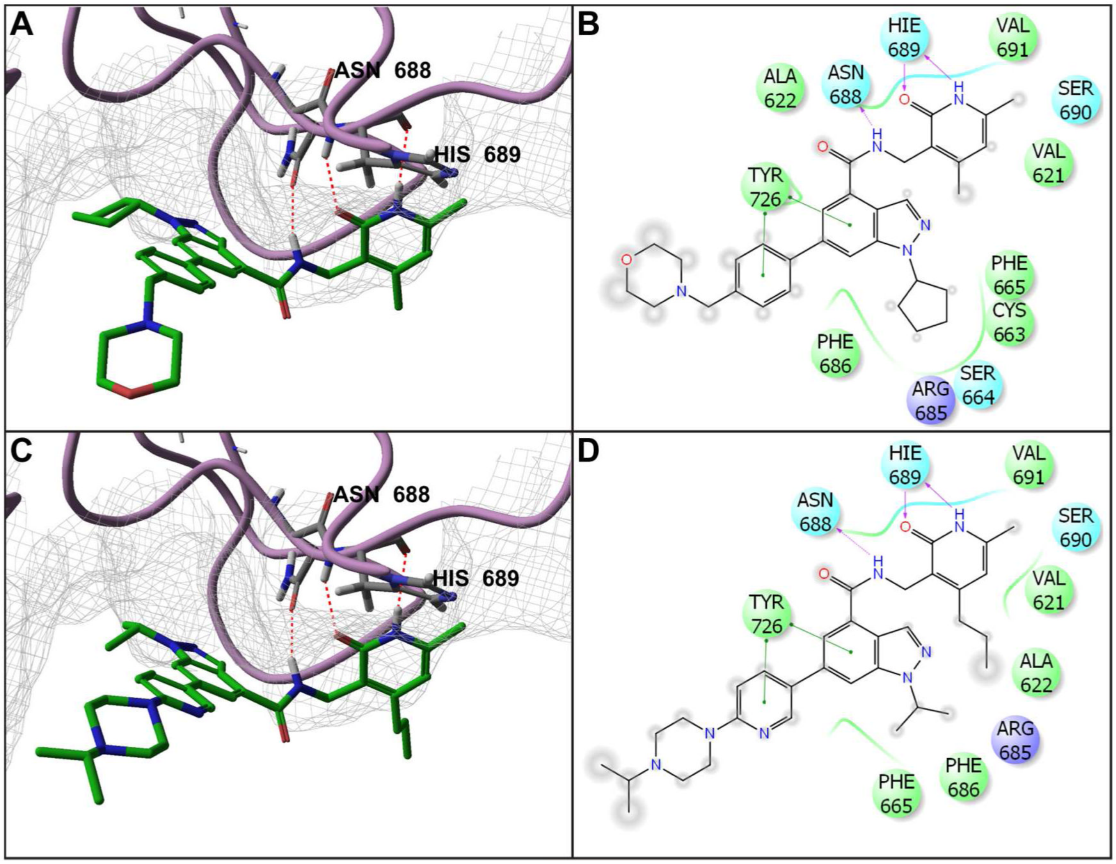

Figure 1. Binding hypothesis: UNC1999 binds to EZH2 similarly as EPZ005687 and its *N*-isopropyl piperazine moiety is solvent exposed

A homology model of EZH2 was constructed using GLP as a template (PDB: 2RFI). (A) EPZ005687 was docked into the SET domain; the ligand (green) orients such that the morpholine group is solvent exposed and therefore does not interact with EZH2 (purple). Additionally, there are three key hydrogen bonds that appear to be requisite for activity (dotted red lines). (B) Proposed ligand interactions of EPZ005687 display a hydrogen bond between the middle amide and Asn688, and two hydrogen bonds between the pyridone and His689 (purple arrows). (C) A docking model of UNC1999 shows it binds to EZH2 similarly as EPZ005687 and its *N*-isopropyl piperazine is solvent exposed. (D) Proposed ligand interactions of UNC1999 display the same key hydrogen bonds and hydrophobic interactions as EPZ005687.

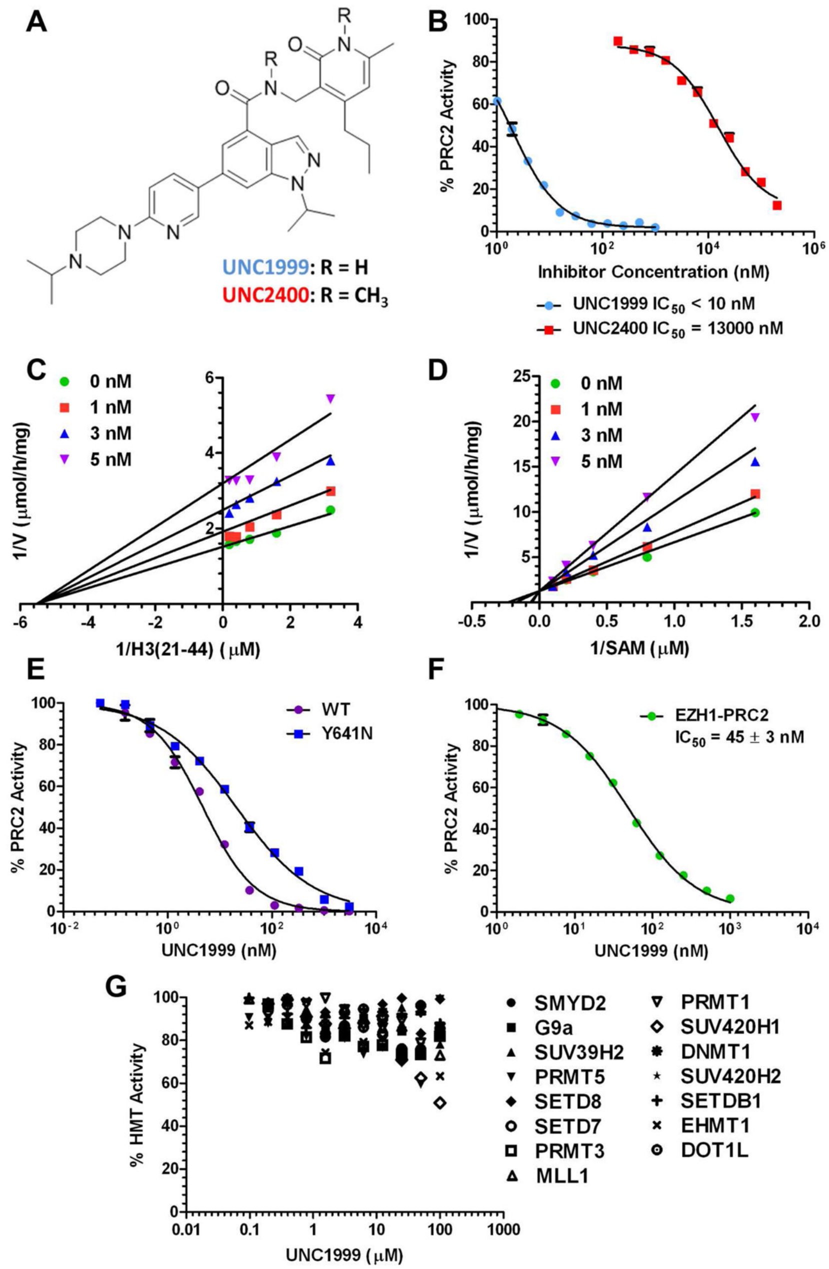

Figure 2. Characterization of UNC1999 and UNC2400 in biochemical assays

(A) Structures of UNC1999, and negative control UNC2400 which differs only by methylation at both amide nitrogens. (B) UNC2400 displayed > 1000-fold decrease in IC_{50} as compared to UNC1999 in the EZH2 radioactive assay. (C) – (D) Lineweaver-Burk plots demonstrated that UNC1999 is non-competitive with the histone H3 substrate (C), and competitive with the co-factor SAM (D). (E) UNC1999 was less than 5-fold more potent for WT EZH2 versus Y641N EZH2. (F) UNC1999 displayed high potency (IC_{50} = 45 ± 3 nM) for EZH1. (G) UNC1999 was selective for EZH2 and EZH1 over 15 other lysine, arginine and DNA methyltransferases.

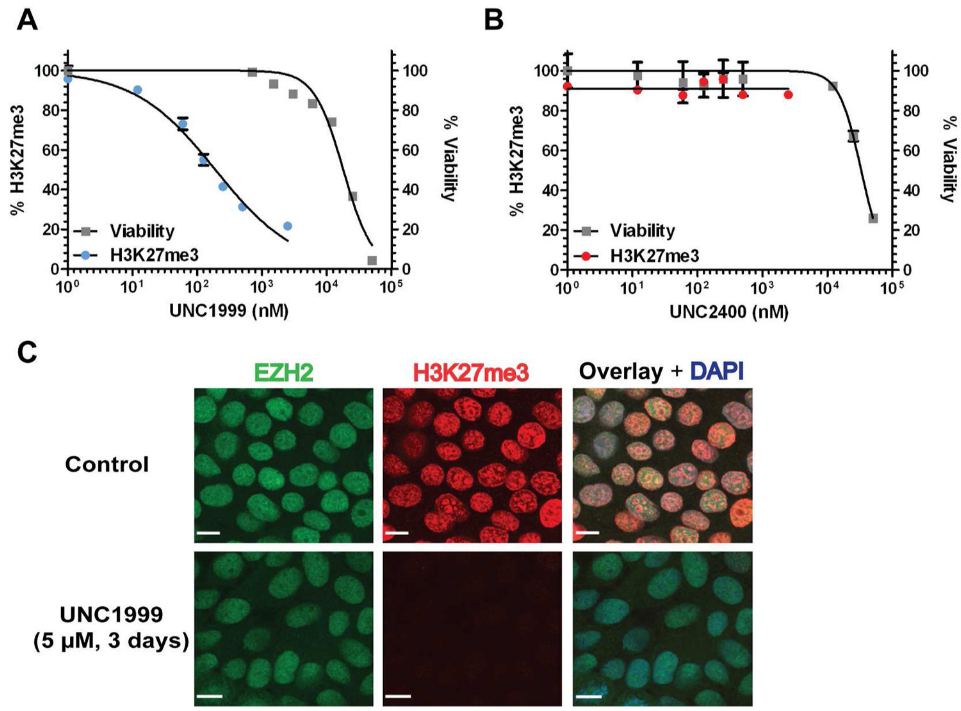

Figure 3. UNC1999 potently reduces H3K27me3 levels in cells and shows low cell toxicity

(A) UNC1999 reduced H3K27me3 levels in MCF10A cells with an IC_{50} = 124 ± 11 nM (n = 3) as determined by the ICW assay (blue), and displayed low cell toxicity (EC_{50} = 19,200 ± 1200 nM (n = 3)) in the resazurin assay (grey). (B) UNC2400 displayed negligible inhibition of H3K27me3 levels (red) in MCF10A cells while displaying similar toxicity (EC_{50} = 27,500 ± 1,300 (n = 3)) in the resazurin assay (grey) as UNC1999. (C) Immunofluorescence staining showed that treatment with UNC1999 (5 μM, 72 h) effectively reduced the H3K27me3 mark but did not affect EZH2 levels in MCF7 cells. Scale bar represents 10 μm.

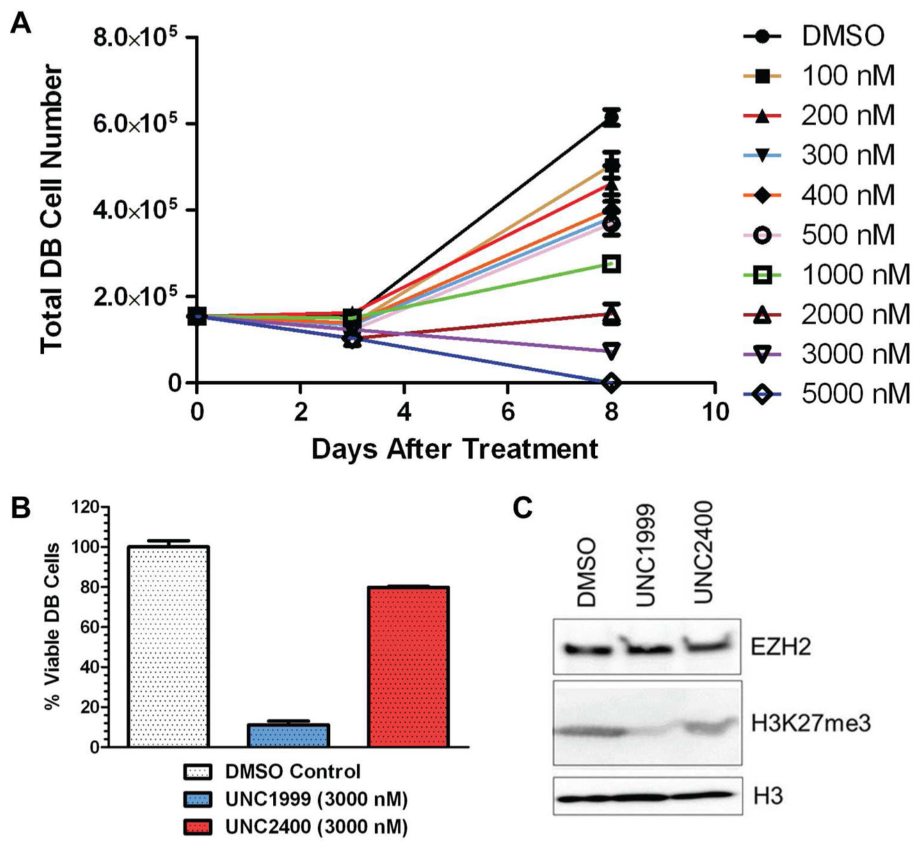

Figure 4. NC1999 selectively kills DB cells, a DLBCL cell line with the EZH2 Y641N mutation

(A) UNC1999 displayed a concentration- and time-dependent inhibition of DB cell proliferation (EC_{50} = 633 ± 101 nM (n = 3)). (B) UNC2400 (3000 nM, 8 days) did not significantly inhibit DB cell proliferation in contrast to UNC1999. (C) Western blotting of EZH2, H3K27me3, and H3 following the treatment of DB cells with UNC1999 or UNC2400 at 3000 nM for 3 days. UNC1999 decreased H3K27me3 but not EZH2 levels while UNC2400 did not significantly reduce H3K27me3 or EZH2 levels in DB cells.

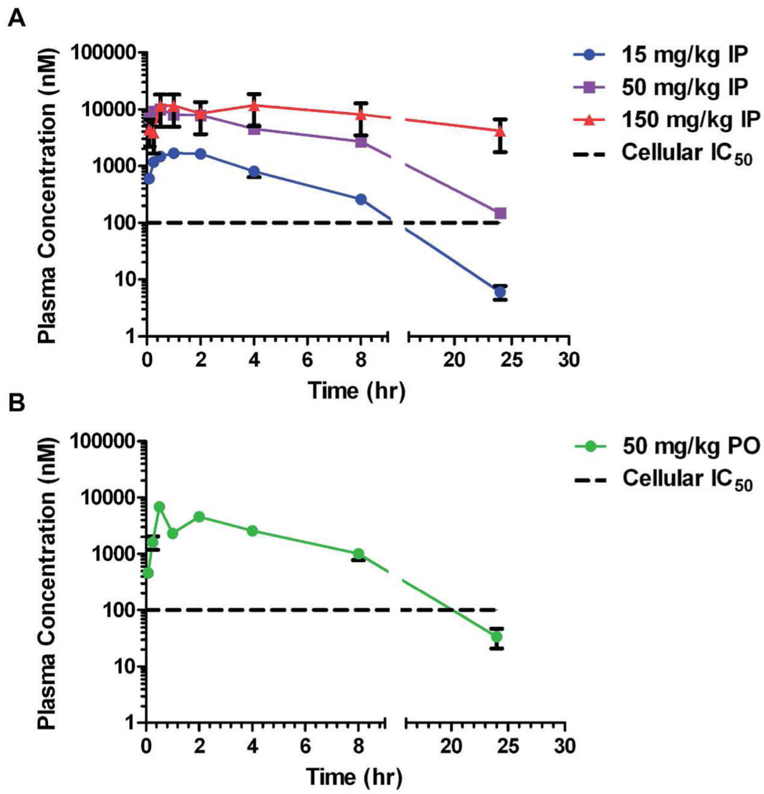

Figure 5. Pharmacokinetic profiles of UNC1999 in male Swiss albino mice

Plasma concentrations of UNC1999 following (A) a single IP injection (15, 50, or 150 mg/kg) or (B) a single oral dose (50 mg/kg) over the 24 h period. The dashed black line indicates the cellular IC_{50} of UNC1999.

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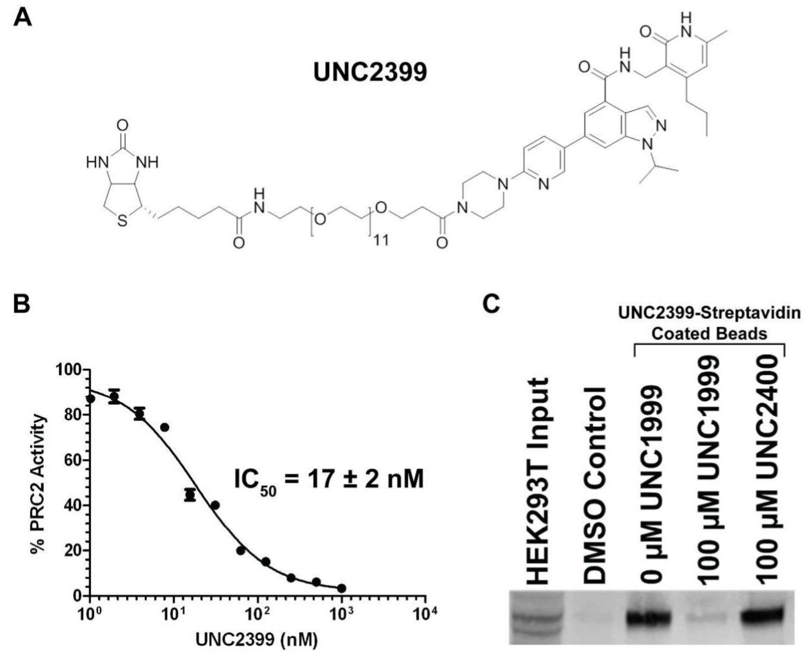


Figure 6. Biotinylated UNC1999 (UNC2399) enriches EZH2 from HEK293T cell lysates

(A) Structure of UNC2399, a biotinylated UNC1999. (B) UNC2399 displayed high *in vitro* potency (IC_{50} = 17 ± 2 nM) for EZH2. (C) In UNC2399 pull-down experiments EZH2 levels were markedly enriched (well 3) as compared to the DMSO control (well 2). The ability to pull-down EZH2 out of cell lysates was abolished by the pretreatment with 100 μM UNC1999 (well 4), but was not affected by the pretreatment with 100 μM UNC2400 (well 5). This blot is representative of three biological replicates.

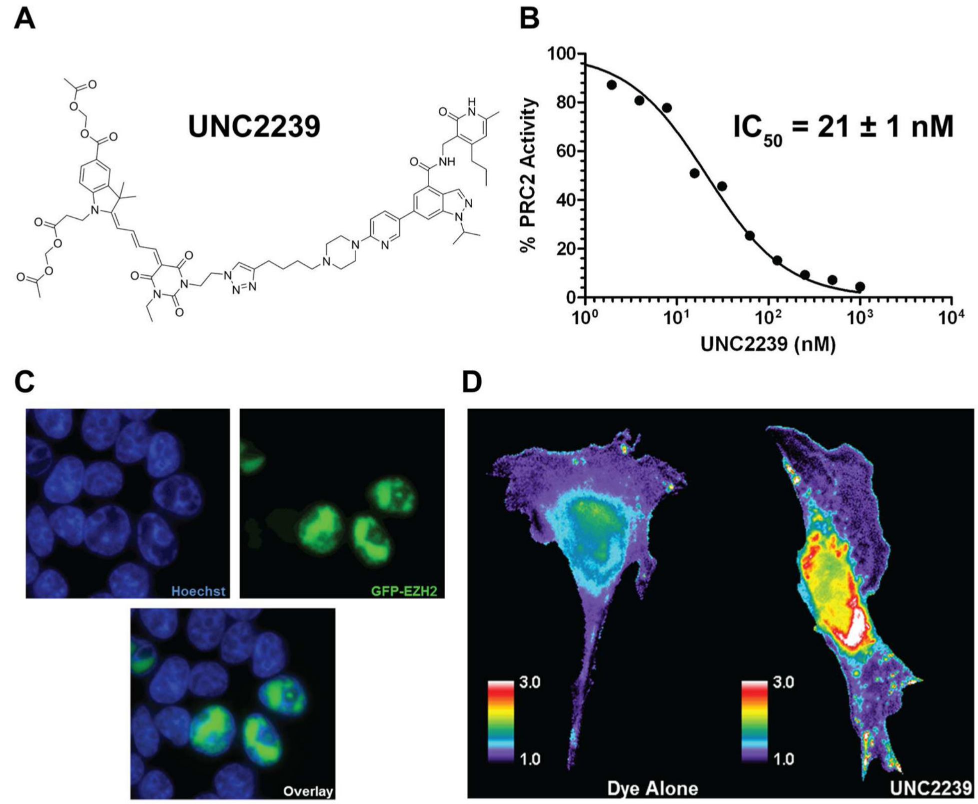
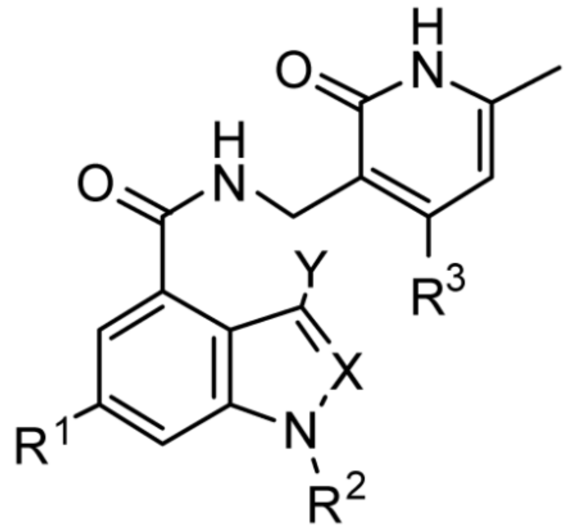


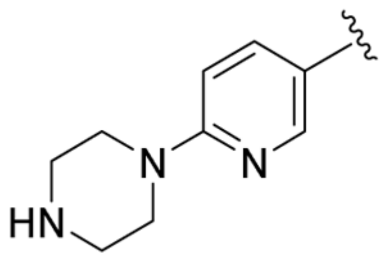
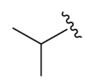
Figure 7. Live Cell Imaging with the Fluorescent Probe for EZH2

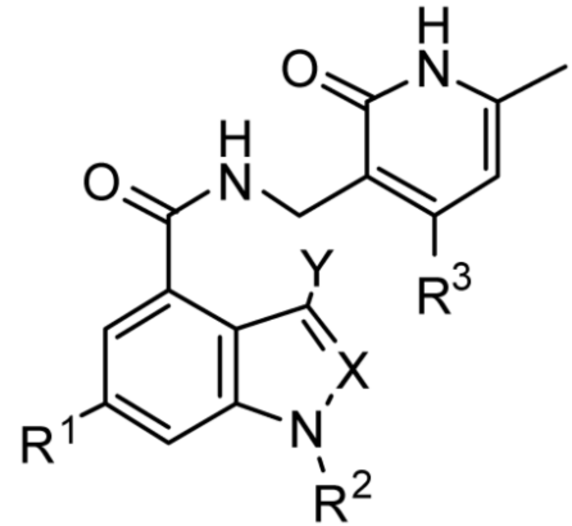
(A) Structure of UNC2239, a fluorescent dye conjugate of UNC1999. (B) UNC2239 displayed high *in vitro* potency (IC_{50} = 21 ± 1 nM) for EZH2. (C) HEK293 cells transfected with EZH2-GFP and stained with nuclear marker Hoechst 33342 showed nuclear localization of EZH2-GFP. (D) Ratio images showing localization of the EZH2 probe in MEF cells. The ratio of dye fluorescence intensity over YFP volume control fluorophore intensity was determined. Cells were normalized by setting the lowest 5% of cytoplasmic ratio values = 1. Cells treated with the EZH2 probe showed greater fluorescence intensity in the nucleus than did cells treated with dye only.

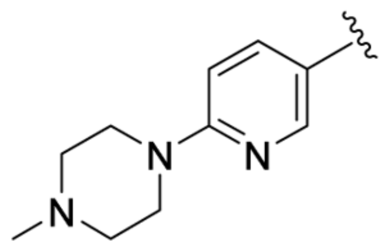
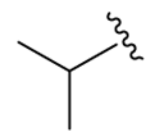
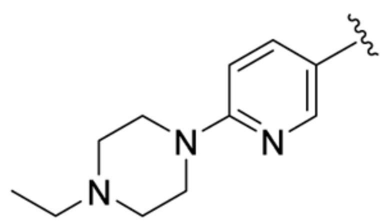
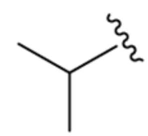
Table 1

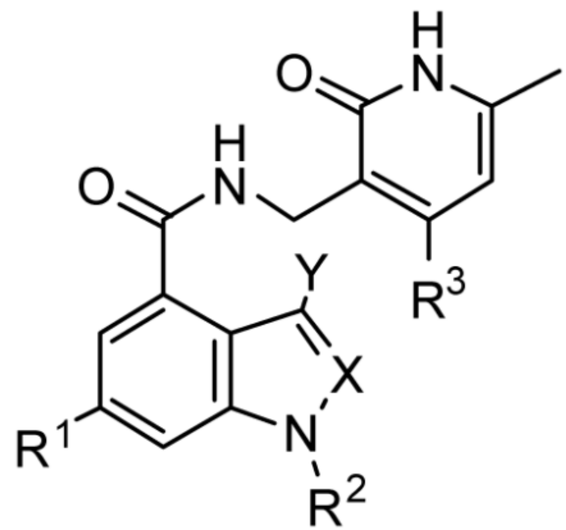
in vitro potencies of newly synthesized inhibitors.

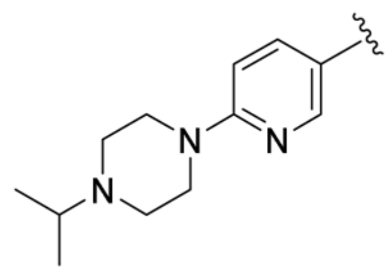
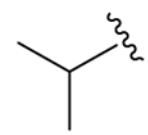
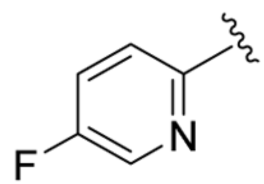
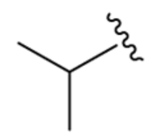


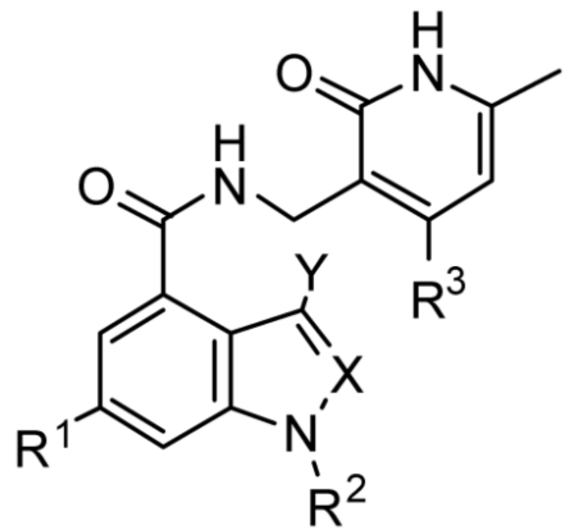
Compound	R^1	R^2	R^3	X	Y	EZH2 IC_{50} (nm)	clogP
1			*n*-Pr	N	H	<10[a,b]	2.1

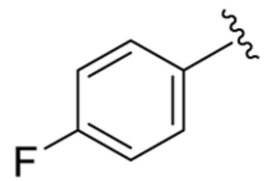
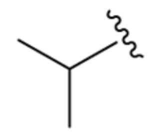
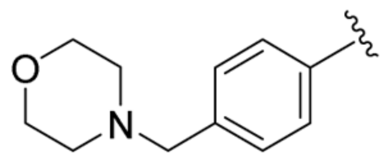
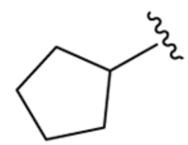


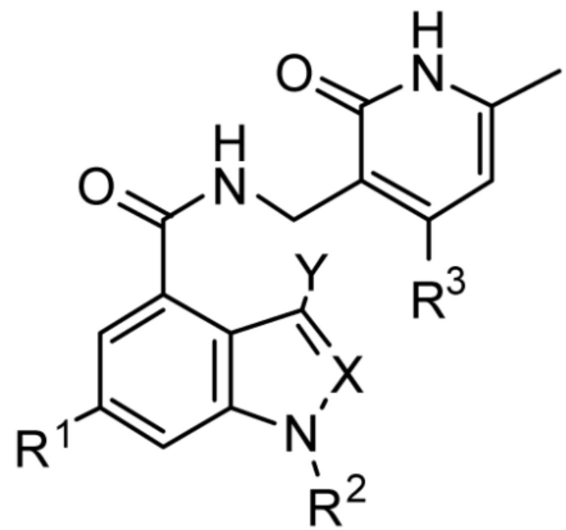
Compound	R^1	R^2	R^3	X	Y	EZH2 IC_{50} (nm)	clogP
2			*n*-Pr	N	H	<10[a,b]	2.5
3			*n*-Pr	N	H	<10[a,b]	2.8

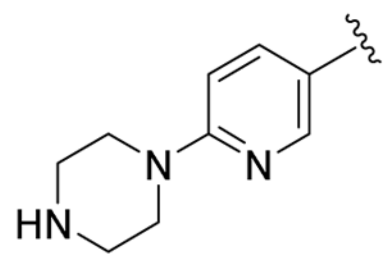
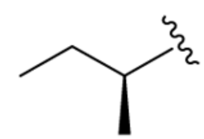


Compound	R^1	R^2	R^3	X	Y	EZH2 IC_{50} (nm)	clogP
UNC1999 (**4**)			*n*-Pr	N	H	<10[a,b]	3.1
5			*n*-Pr	N	H	10 ± 1[a]	2.3



Compound	R^1	R^2	R^3	X	Y	EZH2 IC_{50} (nm)	clogP
6			*n*-Pr	N	H	63 ± 2[a]	3.2
EPZ005687			Me	N	H	54 ± 5[c]	2.2



Compound	R^1	R^2	R^3	X	Y	EZH2 IC_{50} (nm)	clogP
GSK126			Me	CH	Me	10[c]	2.6

[a] IC_{50} determination experiments were performed in triplicate.

[b] The IC_{50} limit of the EZH2 radioactive biochemical assay is 10 nM because the concentration of EZH2 used in this assay is 20 nM.

[c] IC_{50} values obtained from references 24 and 25, respectively.