Supplementary Materials

Supplementary Materials and Methods

Biochemical Methods
Methods to assay HMT activities have been previously described (*1*).

In vitro cell assays
Proliferation and LCC calculations were performed as previously described (*2*).

ELISA
Histones were isolated from tumors and analyzed as previously described (*3*)

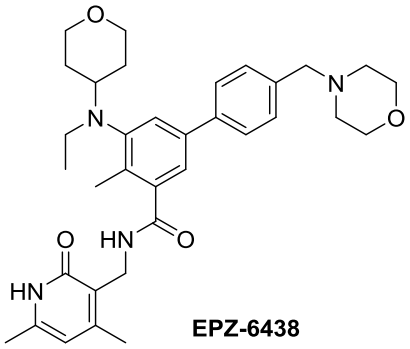
Xenograft study
All the procedures related to animal handling, care and the treatment in this study were performed according to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Chempartner following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). For the *in vivo* study, mice were inoculated subcutaneously at the right flank with KARPAS-422 tumor cells ($1x10^7$ cells/mouse, 50% Matrigel) in 0.2 ml mixture of base media and Matrigel (McCoy's 5A : Matrigel=1:1) for tumor development. The treatments were started when the tumor size reached approximately 107 mm^3 for the anti-tumor activity study (n=10 mice per group) or 410 mm^3 for the PK/PD study (n=12 mice per group). EPZ011989 or vehicle (0.5% NaCMC+0.1% Tween-80 in water) was administered orally BID at a dose volume of 10 µL/g for 7 or 21 days. Animal body weights were measured twice weekly. Tumor size was measured twice weekly in two dimensions using a caliper, and the volume was expressed in mm^3. For the day 7 PK/PD timecourse, tumors were harvested and blood was collected (by mandibular bleeds) from n=2 mice of the 30 minutes, 1, 6, and 12 hour groups, and n=4 mice from the 3 hours post-last dose group. Bone marrow was collected from n=4 mice at the 3 hour post-last dose timepoint.

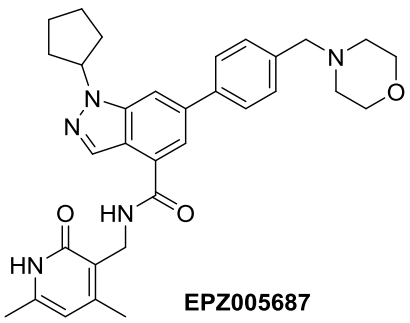
Pharmacokinetic analyses
Dexamethasone was used as internal standard. An aliquot of 30 µL plasma sample was added with 30 µL IS (Dexamethasone, 1000 ng/mL) and 150 µL ACN. The mixture was vortexed for 5 min and centrifuged at 14000 rpm for 5 min. An aliquot of 2 µL supernatant was injected for LC-MS/MS analysis (Q-trap 3200). For 10-fold diluted plasma samples an aliquot of 3 µL plasma sample was added with 27 µL blank plasma, the dilution factor was 10, then added with 30 µL IS (Dexamethasone, 1000 ng/mL) and 150 µL ACN. The mixture was vortexed for 5 min and centrifuged at 14000 rpm for 5 min. An aliquot of 2 µL supernatant was injected for LC-MS/MS analysis.

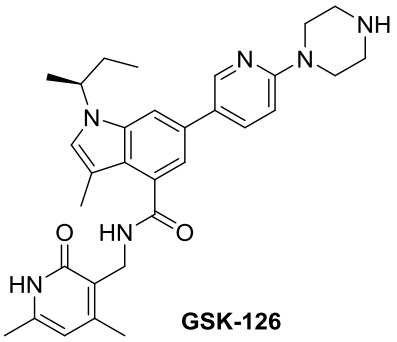
REFERENCES

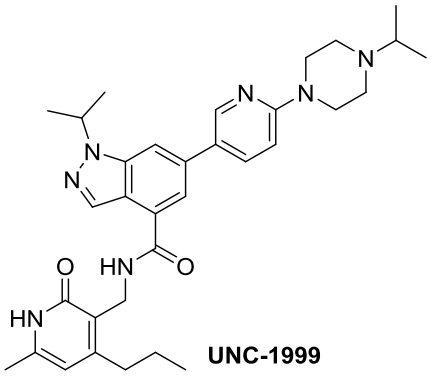
1. S. K. Knutson et al., *Nat. Chem. Biol.* **2012**, *8*, 890-896.
2. S. R. Daigle et al., *Cancer Cell* **2011**, *20*, 53-65.
3. S. K. Knutson et al., *Proc. Natl. Acad. Sci.* **2013**, 7922-7927.

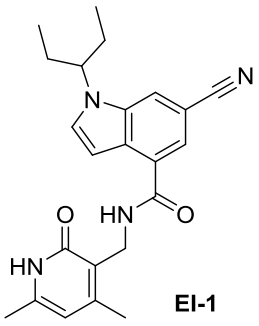
Figure F1: Reported EZH2 inhibitors

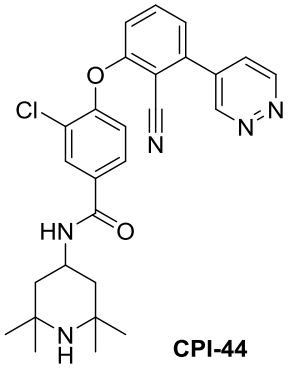


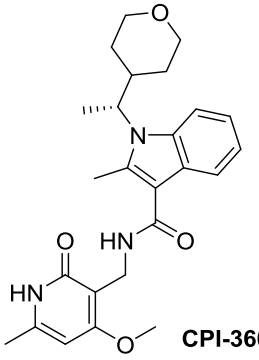












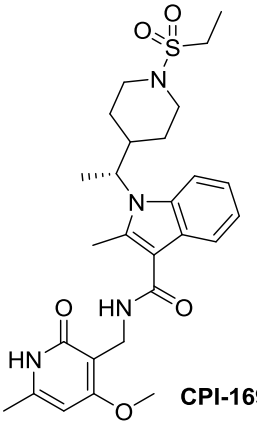


Table S1: Histone Methyltransferase Inhibition by EPZ011989

Enzyme Assay	IC_{50} (nM)	% Inhibition at 1 µM EPZ011989[a]
CARM1	**ND**	**-3 ± 14**
DOT1L	**ND**	**1 ± 4**
EHMT1	**ND**	**9 ± 3**
EHMT2	**ND**	**7 ± 1**
EZH1[b,c]	**103 ± 15**	**98 ± 3**
EZH2 Peptide Assay[b,c]	**6 ± 8**	**100 ± 16**
EZH2 Nucleosome Assay[b]	**ND**	**93 ± 11**
Y641F EZH2[b,c]	**7 ± 5**	**ND**
PRMT1	**ND**	**10 ± 6**
PRMT3	**ND**	**10 ± 12**
PRMT5/MEP50	**ND**	**-4 ± 2**
PRMT6	**ND**	**6 ± 4**
PRMT8	**ND**	**9 ± 5**
SETD2	**ND**	**20 ± 1**
SETD7	**ND**	**6 ± 10**
SUV39H1	**ND**	**11 ± 5**
SMYD2	**ND**	**8 ± 1**
SMYD3	**ND**	**13 ± 6**
WHSC1	**ND**	**26 ± 4**
WHSC1L1	**ND**	**16 ± 10**

a: Values represent the mean and standard deviation of duplicate experiments determined at 10 µmol/L **EPZ011989**.
b: All EZH1 and EZH2 proteins were assayed in the context of 4 PRC2 components (EZH1/2, SUZ12, RBAP48, EED).
c: Assayed with H3K27 peptides as substrates.
ND = Not Determined

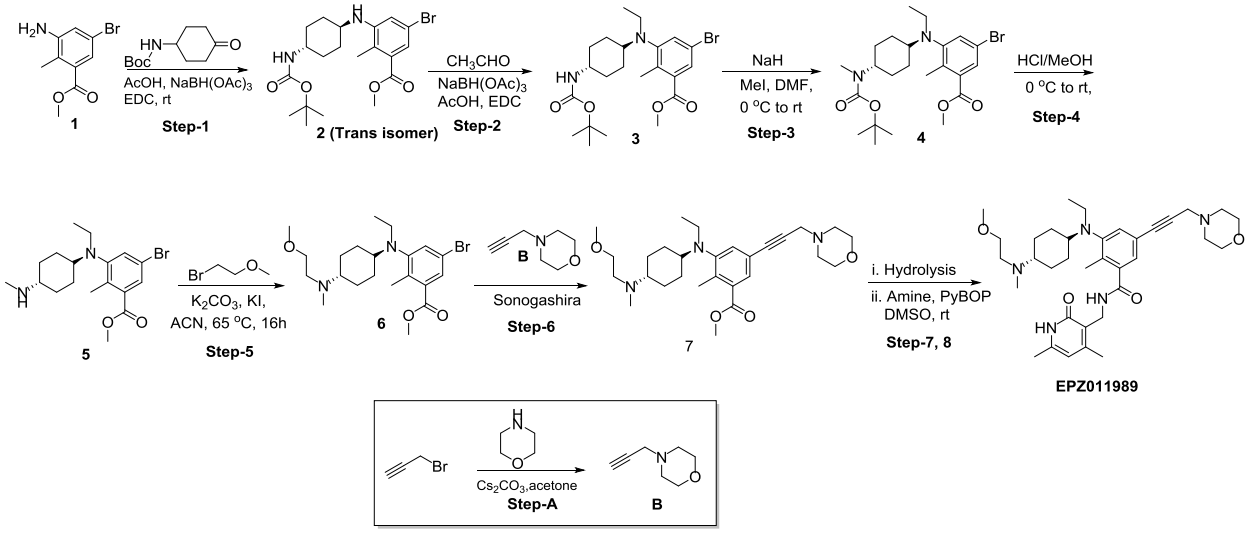
Table S2: Histone Methyltransferase Inhibition by EPZ-6438

(Previously published in the Supporting Info for S. K. Knutson et al., *Proc. Natl. Acad. Sci.* **2013**, 7922-7927)

Enzyme Assay	IC_{50} (nM)	% Inhibition at 1 µM EPZ-6438[a]
CARM1	>50,000[b]	5 ± 3
DOT1L	>50,000[c]	2 ± 8
EHMT1	>50,000[c]	6 ± 6
EHMT2	>50,000[c]	7 ± 3
EZH1[d,e]	392 ± 72[f]	98 ± 1
EZH2 Peptide Assay[d,e]	11 ± 5[f]	ND
EZH2 Nucleosome Assay[d]	16 ± 12[f]	100 ± 1
A677G EZH2[d,e]	2[b]	ND
A687V EZH2[d,e]	2[b]	ND
Y641F EZH2[d,e]	14 ± 5[f]	ND
Y641C EZH2[d,e]	16[c]	ND
Y641H EZH2[d,e]	6[c]	ND
Y641N EZH2[d,e]	38[b]	ND
Y641S EZH2[d,e]	6[c]	ND
rat EZH2[d,e]	4[c]	ND
PRMT1	>50,000[c]	5 ± 4
PRMT3	ND	2 ± 2
PRMT5/MEP50	>50,000[c]	2 ± 6
PRMT6	ND	3 ± 3
PRMT8	>50,000[c]	7 ± 3
SETD7	ND	4 ± 3
SMYD2	>50,000[c]	1 ± 2
SMYD3	ND	0 ± 5
WHSC1	>100,000[c]	8 ± 3
WHSC1L1	>100,000[c]	9 ± 8

a: Values represent the mean and standard deviation of duplicate experiments determined at 10 µmol/L EPZ-6438.
b: Values represent the mean of duplicate experiments with two replicates per experiment.
c: Values represent one experiment with two replicates per experiment.
d: All EZH1 and EZH2 proteins were assayed in the context of 4 PRC2 components (EZH1/2, SUZ12, RBAP48, EED).
e: Assayed with H3K27 peptides as substrates.
f: Values represent mean and standard deviation of replicates (EZH1 n=4, EZH2 Peptide Assay n=4, EZH2 Nucleosome Assay n=6, Y461F EZH2 n=3).
ND = Not Determined

Synthetic scheme EPZ011989:



Synthesis of methyl 5-bromo-3-(((*trans*)-4-((tert-butoxycarbonyl)amino)cyclohexyl)-amino)-2-methylbenzoate (2):

To a stirred solution of methyl 3-amino-5-bromo-2-methylbenzoate (140 g, 578 mmol) and tert-butyl (4-oxocyclohexyl)carbamate (147.8 g, 694.2 mmol) in dichloroethane (1L), acetic acid (208.2 g, 3471 mmol) was added and reaction was stirred at room temperature for 20 minutes. Then sodium triacetoxyborohydride (367.9 g, 1735 mmol) was added at 0 ^{o}C and reaction was stirred at room temperature for 16 h. On completion (monitored by TLC), the reaction was quenched with aqueous sodium bicarbonate, the organic layer was separated and the aqueous layer was extracted with dichloromethane (1.5L x 3). The combined organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude compound was purified by silica gel column chromatography (100-200 mesh size) eluting with 2, 4, 6 & 8% ethyl acetate in hexane to remove maximum cis isomer. This afforded 180 g of mixture of cis and trans isomers (40:60 by HPLC). The trans isomer was purified by repetitive recrystallization with ethyl acetate: hexane (1:2) to afford 80 g of pure trans isomer with 99% purity.

^{1}H NMR (DMSO-d_6, 400 MHz) δ 6.94 (s, 1H), 6.84 (s, 1H), 6.79-6.77 (m, 1H), 4.90-4.88 (m,1H), 3.79 (s, 3H), 3.22 (m, 2H), 2.11 (s, 3H), 1.91 (m, 2H), 1.80 (m, 2H), 1.38 (s, 9H), 1.31 (m, 4H).

Synthesis of methyl 5-bromo-3-(((trans)-4-((tert-butoxycarbonyl)amino)cyclohexyl)-(ethyl)amino)-2-methylbenzoate (3):

To a stirred solution of methyl 5-bromo-3-(((*trans*)-4-((tert-butoxycarbonyl)amino)cyclohexyl)-amino)-2-methylbenzoate (70 g, 159 mmol), acetaldehyde (17.49 g, 397.7 mmol) in dichloroethane (700 mL) and acetic acid (57.27 g, 954.5 mmol) was added and reaction was stirred at room temperature for 20 minutes. Then sodium triacetoxyborohydride (101.18 g, 477.2 mmol) was added at 0 ^{o}C and reaction was stirred at room temperature for 16 h. On completion, the reaction was quenched with aqueous sodium bicarbonate, the organic layer was separated and the aqueous layer was extracted with dichloromethane (1L x 3). The combined organic layers were dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude compound was purified by silica gel column chromatography to afford the title compound (58 g, 77.9%).

^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.56 (s, 1H), 7.47 (s, 1H), 6.65-6.64 (m ,1H), 3.82 (s, 3H), 3.14 (m, 1H), 3.05 (m, 2H), 2.61-2.57 (m, 1H), 2.32 (s, 3H), 1.77-1.69 (m, 4H), 1.43-1.40 (m, 2H), 1.36 (s, 9H), 1.15-1.06 (m, 2H). 0.78 (t, J=6.8 Hz, 3H).

Synthesis of methyl 5-bromo-3-(((trans)-4-((tert-butoxycarbonyl)-(methyl)-amino)-cyclohexyl)(ethyl)amino)-2-methylbenzoate (4):

To a stirred solution of methyl 5-bromo-3-(((trans)-4-((tert-butoxycarbonyl)amino)cyclohexyl)-(ethyl)amino)-2-methylbenzoate (30 g, 63.96 mmol) in dry DMF (300 mL), methyl iodide (40 mL, 639.6 mmol) was added at 0 ^{o}C and stirred it at same temperature for 20 min. Then NaH (60% 6.39 g, 159.9 mmol) was added at 0 ^{o}C and reaction was stirred for overnight at room temperature. On completion, the reaction was quenched with ice water and extracted with ethyl acetate (500 mL x 3). The combined organic layers were washed with water, dried, concentrated under reduced pressure. The crude compound was purified by silica gel column chromatography to afford the title compound (30 g, 97.3%).

^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.55 (s, 1H), 7.46 (s, 1H), 3.82 (s, 3H), 3.05 (m, 2H), 2.65 (m, 1H), 2.61 (s, 3H), 2.33 (s, 3H), 1.77 (m, 2H), 1.52-1.47 (m, 6H), 1.38 (s, 9H). 0.79 (t, J=6.8 Hz, 3H), 1H merged in solvent peak.

Synthesis of methyl 5-bromo-3-(ethyl((trans)-4-(methylamino)cyclohexyl)amino)-2-methylbenzoate (5):

To a stirred solution of methyl 5-bromo-3-(((trans)-4-((tert-butoxycarbonyl)-(methyl)-amino)-cyclohexyl)(ethyl)amino)-2-methylbenzoate (30 g, 62.24 mmol) in methanol (100 mL) at 0 ^{o}C, methanolic HCl (500 mL) was added and reaction was stirred for 2 h at room temperature. After completion, the reaction was concentrated to dryness. The residue was basified with aqueous sat. bicarbonate solution till pH 8 and aqueous layer was extracted with 10% methanol in DCM (200 mL X 3). The combined organic layers were dried over Na_2SO_4 and solvent removed under reduced pressure to afford the title compound (25g, crude). The isolated compound was used in the next step without further purification.

Synthesis of methyl 5-bromo-3-(ethyl((trans)-4-((2-methoxyethyl)-(methyl)-amino)cyclohexyl)amino)-2-methylbenzoate (6):

To a stirred solution of crude methyl 5-bromo-3-(ethyl((trans)-4-(methylamino)cyclohexyl)amino)-2-methylbenzoate (25 g, 65.44 mmol), 1-bromo-2-methoxyethane (18.19 g, 130.8 mmol) in acetonitrile (250 mL), K_2CO_3 (18.06 g, 130.8 mmol) and KI (6.51 g, 39.21 mmol) were added. The resulting reaction mass was stirred at 65 ^{o}C for 16 h. On completion, the reaction mixture was diluted with water (300 mL) and extracted with DCM (500 mL x 3). The combined organic layers were washed with water, dried over sodium sulfate and concentrated under reduced pressure. The crude compound was purified by silica gel column chromatography to afford the title compound (20 g, 69.3%).

^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.55 (s, 1H), 7.45 (s, 1H), 3.82 (s, 3H), 3.32 (m, 4H), 3.20 (s, 3H), 3.05 (m, 2H), 2.61 (m, 1H), 2.32 (s, 3H), 2.30 (m, 1H), 2.15 (s, 3H), 1.77-1.67 (m, 4H), 1.37-1.31(m, 2H), 1.24-1.18 (m, 2H), 0.78 (t, J=6.8 Hz, 3H).

Synthesis of methyl 3-(ethyl((trans)-4-((2-methoxyethyl)-(methyl)-amino)-cyclohexyl)-amino)-2-methyl-5-(3-morpholinoprop-1-yn-1-yl)benzoate (7):

The solution of methyl 5-bromo-3-(ethyl((trans)-4-((2-methoxyethyl)-(methyl)-amino)cyclohexyl)amino)-2-methylbenzoate (30 g, 68.02 mmol), crude 4-(prop-2-yn-1-yl)morpholine (25.51 g, 204 mmol) and triethylamine (20.61 g, 204 mmol) in DMF (300 mL) was bubbled through Argon for 20 min. Then CuI (3.87 g, 20.36 mmol) and $Pd(PPh_3)_4$ (7.85 g, 6.79 mmol) were added and Argon was bubbled through for further 20 min. The reaction mixture was heated at 105 ^oC for 4 h and then cooled to room temperature. The reaction was quenched with water (100 mL) and the separated aq. phase was extracted with 10 % MeOH/DCM (400 mL x 3). The combined organic extracts were dried over Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography to give the title compound (21 g, 63.7%).

^1H NMR (DMSO-d_6, 400 MHz) δ 7.46 (s, 1H), 7.32 (s, 1H), 3.82 (s, 3H), 3.62-3.57 (m, 6H), 3.50 (s, 2H), 3.35-3.32 (m, 2H), 3.21 (s, 3H), 3.17 (m, 1H), 3.05 (m, 2H), 2.61-2.58 (m, 2H), 2.38 (s, 3H), 2.33 (m, 1H), 2.18 (m, 2H), 1.77-1.70 (m, 4H), 1.36-1.20 (m, 4H), 0.77 (t, J=6.8 Hz, 3H), 3H merged in solvent peak.

Synthesis of 4-(prop-2-yn-1-yl) morpholine (B):

To a stirred solution of propargyl bromide (50 g, 420 mmol) in acetone (300 mL), CS_2CO_3 (136.5 g, 420 mmol) was added at 0 ^oC. Then morpholine (36.60 g, 420 mmol) in acetone (200 mL) was added dropwise and reaction was stirred at room temperature for 16h. On completion, reaction mass was filtered and the filtrate was concentrated under reduced pressure to afford the title compound (50 g, crude). The isolated compound was taken directly to next step without further purification.

Synthesis of N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-3-(ethyl((trans)-4-((2-methoxyethyl)(methyl)amino)cyclohexyl)amino)-2-methyl-5-(3-morpholinoprop-1-yn-1-yl)benzamide (EPZ011989):

Aqueous NaOH (2.59 g, 64.91 mmol in 10 mL H_2O) was added to the solution of 3-(ethyl((trans)-4-((2-methoxyethyl)-(methyl)-amino)-cyclohexyl)-amino)-2-methyl-5-(3-

morpholinoprop-1-yn-1-yl)benzoate (21 g, 43.29 mmol) in EtOH (100 mL) and stirred at 60 ^{o}C for 1 h. After completion of the reaction, ethanol was removed under reduced pressure and acidified using dilute HCl up to pH 6 and pH 4 was adjusted using citric acid. Extraction was carried out using 10 % MeOH/DCM (200 mL x 3). Combined organic layers were dried and concentrated to afford the desired hydrolyzed acid (15.5 g, 76.0%).

To the solution of acid (15.5 g, 32.90 mmol) in DMSO (50 mL), 3-(amino methyl)-4, 6-dimethylpyridin-2(1H)-one (10 g, 65.80 mmol) and triethylamine (23 mL, 164.5 mmol) were added. The reaction mixture was stirred at room temperature for 15 min before PYBOP (25.66 g, 49.34 mmol) was added to it at 0 ^{o}C and further stirred for overnight at room temperature. After completion, the reaction mass was poured into ice water (100 mL) and extraction was carried out using 10 % MeOH/DCM (200 mL x 3). Combined organic layers were dried over sodium sulphate and concentrated under reduced pressure. The crude compound was purified by column chromatography over basic alumina eluting with MeOH: DCM to afford the desired compound **EPZ011989** as light brown solid (11g, 55.3%).

Analytical data for EPZ011989 free base:

LCMS: 606.50 [M+1]; HPLC: 99.07% (@ 210 nm-400 nm) (Rt; 3.791; Method: Column: YMC ODS-A 150 mm x 4.6 mm x 5 µ; Mobile Phase: A; 0.05% TFA in water/ B; 0.05% TFA in acetonitrile; Inj. Vol: 10 µL, Col. Temp.: 30 ^{o}C; Flow rate: 1.4 mL/min.; Gradient: 5% B to 95% B in 8 min, Hold for 1.5 min, 9.51-12 min 5% B); ^{1}H NMR (MeOD, 400 MHz) δ 7.23 (s, 1H), 7.09 (s, 1H), 6.11 (s, 1H), 4.46 (s, 2H), 3.74-3.72 (m, 4H), 3.51 (s, 2H), 3.47 (t, J=5.6 Hz, 2H), 3.32 (s, 3H), 3.07 (q, J=7.2 Hz, 2H), 2.64-2.63 (m, 7H), 2.38 (m,1H), 2.37 (s, 3H), 2.27 (s, 3H), 2.26 (s, 3H), 2.25 (s, 3H), 1.89-1.86 (m, 4H), 1.50-1.30 (m, 4H), 0.83 (t, J=7.2 Hz, 3H).