Supporting Information

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SI Materials and Methods

Western Blotting. Whole-cell lysates were prepared using a modified radioimmunoprecipitation assay (RIPA) buffer [10× RIPA Lysis Buffer (Millipore; 20-188), 0.1% SDS, protease minitablet (Roche; 1836153)]. Cells were pelleted, washed with ice-cold PBS, resuspended in ice-cold RIPA buffer, and incubated on ice for 5 min. Lysates were sonicated three times for 10 s at 50% power, and then incubated on ice for 10 min. Lysates were centrifuged at maximum speed for 15 min at 4 °C in a table-top centrifuge. Protein concentrations for were determined by BCA assay (Pierce). Ten micrograms of each lysate were fractionated on 10–20% Tris·glycine gel (Bio-Rad), transferred using iBlot (7 min on program 3, using nitrocellulose transfer stacks), and probed with the following antibodies in Odyssey blocking buffer: SMARCB1 (CST; 8745), EZH2 (CST; 5246), and β-actin (CST; 3700).

ELISA. Histones were prepared in equivalent concentrations [0.5 ng/μL for H3 and 4 ng/μL for the trimethylated state of lysine 27 on H3 (H3K27Me3)] in coating buffer (PBS with 0.05% BSA). Sample or standard (100 μL) was added in duplicate to two 96-well ELISA plates (Thermo Labsystems; Immulon 4HBX 3885). Histones isolated from G401 cells that were treated with DMSO or 10 μmol/L EPZ-6438 (N-((4,6-dimethyl-2-oxo-1,2-dihydropyridin-3-yl)methyl)-5-(ethyl(tetrahydro-2H-pyran-4-yl)amino)-4-methyl-4′-(morpholinomethyl)-[1,1′-biphenyl]-3-carboxamide) for 4 d were added to control wells at the same histone concentration as the tumor histone samples. The plates were sealed and incubated overnight at 4 °C. The following day, plates were washed three times with 300 μL per well PBST (PBS with 0.05% Tween 20; 10× PBST; KPL; 51-14-02) on a Bio-Tek plate washer. Plates were blocked with 300 μL per well of diluent [PBS plus 2% (wt/vol) BSA plus 0.05% Tween 20], incubated at room temperature for 2 h, and washed three times with PBST. All antibodies were diluted in diluent. One hundred microliters per well anti-H3K27Me3 (CST; 9733; 50% glycerol stock, 1:1,000) or anti-total H3 (Abcam; ab1791; 50% glycerol stock, 1:10,000) were added to each plate. Plates were incubated for 90 min at room temperature and washed three times with PBST. One hundred microliters per well anti-Rb-IgG-HRP (Cell Signaling Technology; 7074) was added at 1:2,000 to the H3K27Me3 plate and at 1:6,000 to the H3 plate and incubated for 90 min at room temperature. Plates were washed four times with PBST. For detection, 100 μL per well 3,3′,5,5′-tetramethylbenzidine substrate (BioFx Laboratories; TMBS) were added, and plates were incubated in the dark at room temperature for 5 min. Reaction was stopped with 100 μL per well of 0.5 M H_2SO_4. Absorbance at 450 nm was read on SpectraMax M5 Microplate reader.

Pharmacokinetic Analyses. Dexamethasone was used as internal standard. An aliquot of 30-μL plasma sample was added with 30 μL of internal standard (dexamethasone, 1,000 ng/mL) and 150 μL of acetonitrile. The mixture was vortexed for 5 min and centrifuged at 18,000 × *g* for 5 min. An aliquot of 2-μL supernatant was injected for liquid chromatography with tandem mass spectrometry (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 of IS (dexamethasone, 1,000 ng/mL) and 150 μL of acetonitrile. The mixture was vortexed for 5 min and centrifuged at 18,000 × *g* for 5 min. An aliquot of 2-μL supernatant was injected for LC-MS/MS analysis. Tumor samples were homogenized on Beadbeater for 30 s with 3× PBS (wt/vol) to obtain a tumor homogenate. An aliquot of 30-μL tumor homogenate sample was added with 30 μL of internal standard (dexamethasone, 1,000 ng/mL) and 150 μL of acetonitrile. The mixture was vortexed for 5 min and centrifuged at 18,000 × *g* for 5 min. An aliquot of 2-μL supernatant was injected for LC-MS/MS analysis.

Gene Expression Analysis. The following TaqMan primer/probe sets were used for quantitative PCR-based gene expression analyses:

Gene	AB no.
MYC	Hs00153408_m1
EZH2	Hs00172783_m1
PTCH1	Hs00181117_m1
PROM1 (CD133)	Hs01009250_m1
GLI1	Hs01110766_m1
DOCK4	Hs00206807_m1
PTPRK	Hs00267788_m1
BIN1	Hs00184913_m1

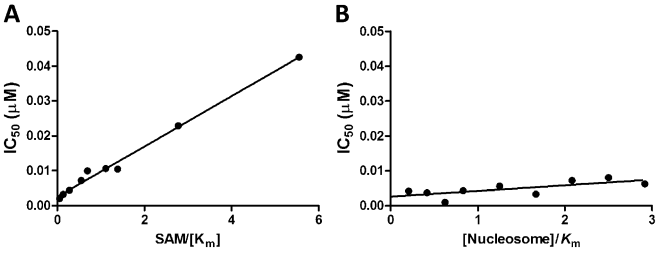

Fig. S1. Biochemical mechanism of action studies. The IC_{50} value of EPZ-6438 increases with increasing SAM concentration (*A*) and is minimally affected by increasing oligonucleosome concentration (*B*), indicating SAM-competitive and nucleosome-noncompetitive mechanism of action.

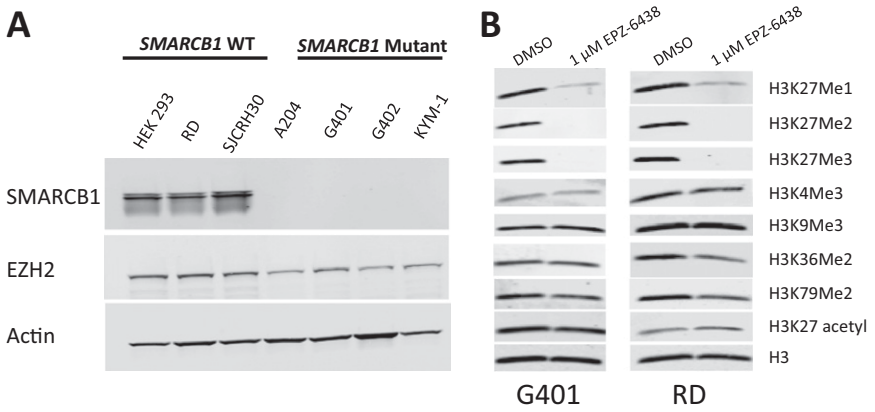

Fig. S2. Verification of SMARCB1 and EZH2 expression in cell lines and specificity of EPZ-6438 for inhibition of cellular histone methylation. (*A*) Cell lysates were analyzed by immunoblot with antibodies specific to SMARCB1, EZH2, and actin (loading control). (*B*) Selective inhibition of cellular H3K27 methylation in G401 and RD cells. Cells were incubated with EPZ-6438 for 4 d, and acid-extracted histones were analyzed by immunoblot.

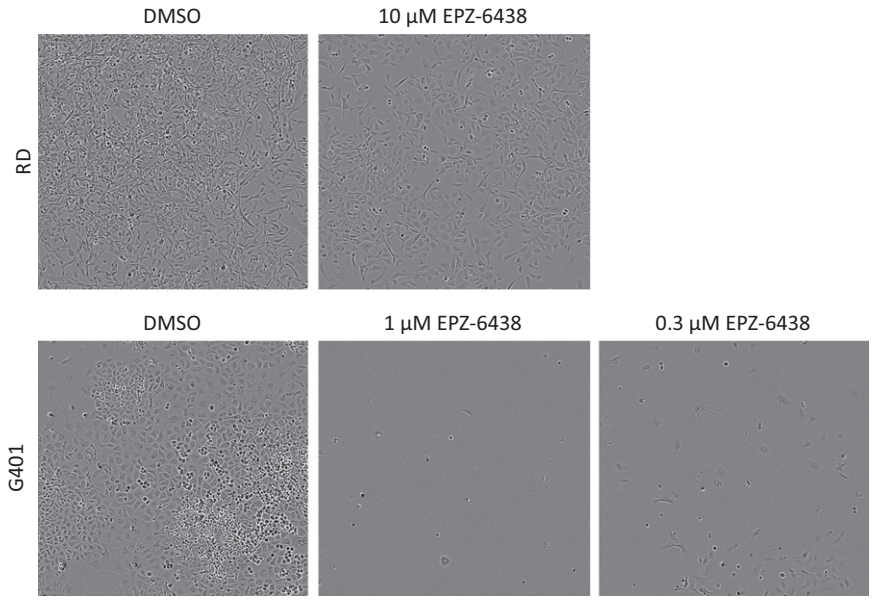

Fig. S3. Morphology of G401 and RD cells incubated with EPZ-6438 in vitro. Cells were incubated in the presence of the indicated concentrations of EPZ-6438 for 14 d. Medium and fresh compound were replenished on days 4, 7, and 11, and cells were replated at the original seeding density on day 7. Note that most of the G401 cells treated at 1 μM were dead by day 14; hence the 0.3 μM concentration condition is included.

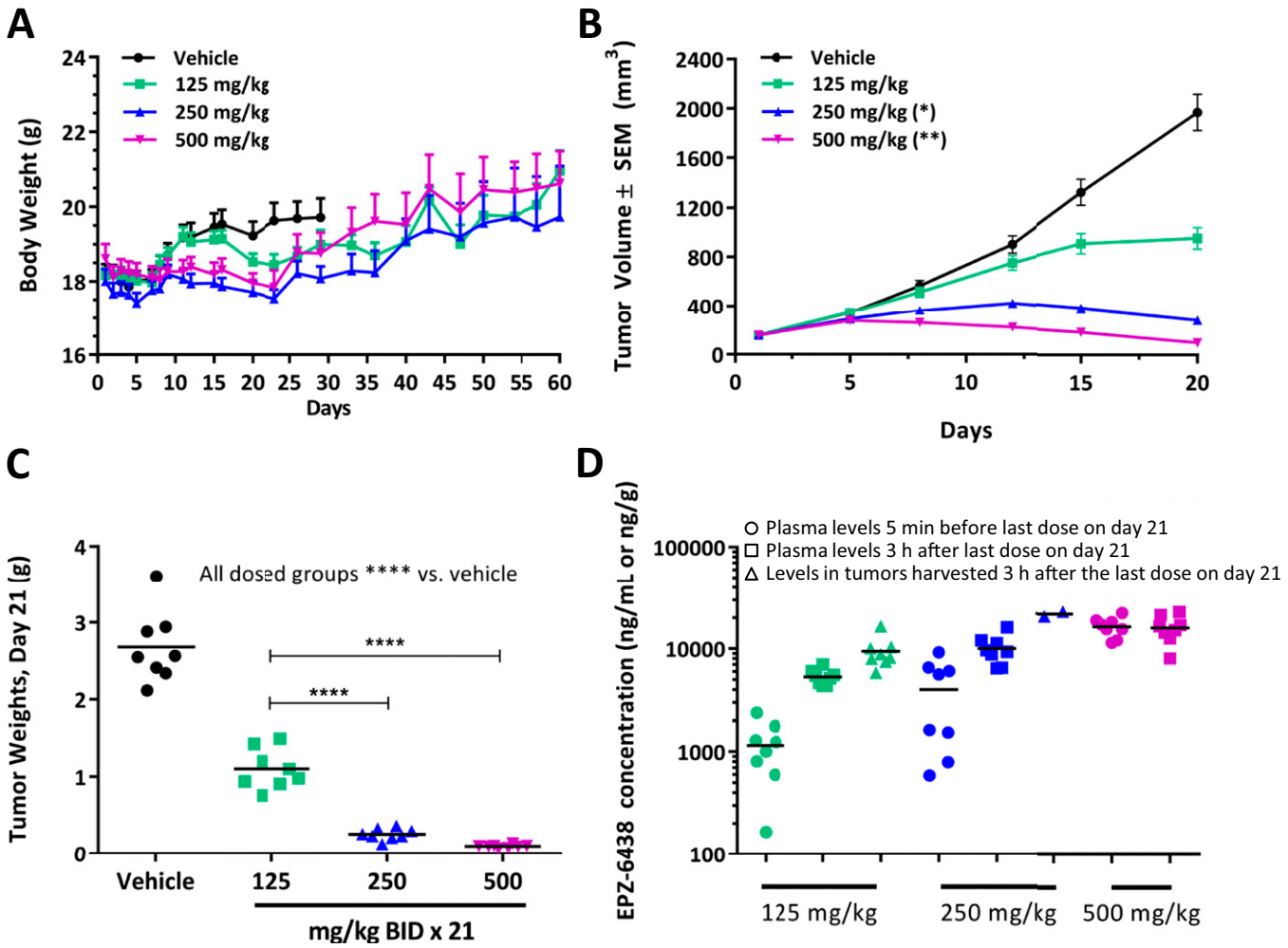

Fig. S4. Body weights, tumor regressions, and plasma levels in G401 xenograft-bearing mice treated with EPZ-6438. (*A*) Body weights were determined twice a week for animals treated with EPZ-6438 on a twice daily (BID) schedule for 28 d. Data are presented as mean values ± SEM ($n = 16$ until day 21, $n = 8$ from day 22–60). (*B*) Tumor regressions induced by BID administration of EPZ-6438 for 21 d at the indicated doses (mean values ± SEM, $n = 16$). *$P < 0.05$, **$P < 0.01$, repeated-measures ANOVA, Dunnett's post test vs. vehicle. (*C*) Tumor weights of eight mice euthanized on day 21. ****$P < 0.0001$, Fisher's exact test. (*D*) Plasma was collected 5 min before and 3 h after dosing of EPZ-6438 on day 21, and compound levels were measured by LC-MS/MS. Animals were euthanized, and tumors were collected 3 h after dosing on day 21. Tumor homogenates were generated and subjected to LC-MS/MS analysis to determine EPZ-6438 concentrations. Note that tumor compound levels could not be determined from all animals especially in the higher dose groups because the xenografts were too small on day 21. The dots represent values for the individual animals; the horizontal lines represent group mean values.

Table S1. Histone methyltransferase inhibition by EPZ-6438

Enzyme assay	IC_{50}, nM	% Inhibition at 1 μM EPZ-6438*
CARM1	>50,000[†]	5 ± 3
DOT1L	>50,000[‡]	2 ± 8
EHMT1	>50,000[‡]	6 ± 6
EHMT2	>50,000[‡]	7 ± 3
EZH1[§,¶]	392 ± 72[‖]	98 ± 1
EZH2 peptide assay[§,¶]	11 ± 5[‖]	ND
EZH2 nucleosome assay[§]	16 ± 12[‖]	100 ± 1
A677G EZH2[§,¶]	2[†]	ND
A687V EZH2[§,¶]	2[†]	ND
Y641F EZH2[§,¶]	14 ± 5[‖]	ND
Y641C EZH2[§,¶]	16[‡]	ND
Y641H EZH2[§,¶]	6[‡]	ND
Y641N EZH2[§,¶]	38[†]	ND
Y641S EZH2[§,¶]	6[‡]	ND
Rat EZH2[§,¶]	4[‡]	ND
PRMT1	>50,000[‡]	5 ± 4
PRMT3	ND	2 ± 2
PRMT5/MEP50	>50,000[‡]	2 ± 6
PRMT6	ND	3 ± 3
PRMT8	>50,000[‡]	7 ± 3
SETD7	ND	4 ± 3
SMYD2	>50,000[‡]	1 ± 2
SMYD3	ND	0 ± 5
WHSC1	>100,000[‡]	8 ± 3
WHSC1L1	>100,000[‡]	9 ± 8

*Values represent the mean and SD of duplicate experiments determined at 10 μmol/L EPZ-6438.
[†]Values represent the mean of duplicate experiments with two replicates per experiment.
[‡]Values represent one experiment with two replicates per experiment.
[§]All EZH1 and EZH2 proteins were assayed in the context of four PRC2 components (EZH1/2, SUZ12, RBAP48, EED).
[¶]Assayed with H3K27 peptides as substrates.
[‖]Values represent mean and SD of replicates (EZH1, $n = 4$; EZH2 peptide assay, $n = 4$; EZH2 nucleosome assay, $n = 6$; Y461F EZH2, $n = 3$).
ND, not determined.

Table S2. EPZ-6438 IC_{50} values for inhibition of H3K27 trimethylation and proliferation in cell lines

Cell line	*SMARCB1* status	Methylation IC_{50}, nM*	Proliferation IC_{50} on day 14, nM[†]
G401	Mutant	2.7	135
A204	Mutant	1.4	1,000
G402	Mutant	1.7	144
KYM-1	Mutant	4.3	32
RD	Wild type	5.6	6,100, >10,000[‡]
293	Wild type	2.4	>10,000
SJCRH30	Wild type	4.9	5,100, >10,000[‡]

*Derived after incubation for 4 d, extraction of histones, immunoblot, and densitometry. Values represent the mean from two experiments.
[†]Compound incubations for each experiment were performed in triplicate, and values represent the mean of two experiments for all cell lines.
[‡]Mean calculation of duplicate experiment not possible.