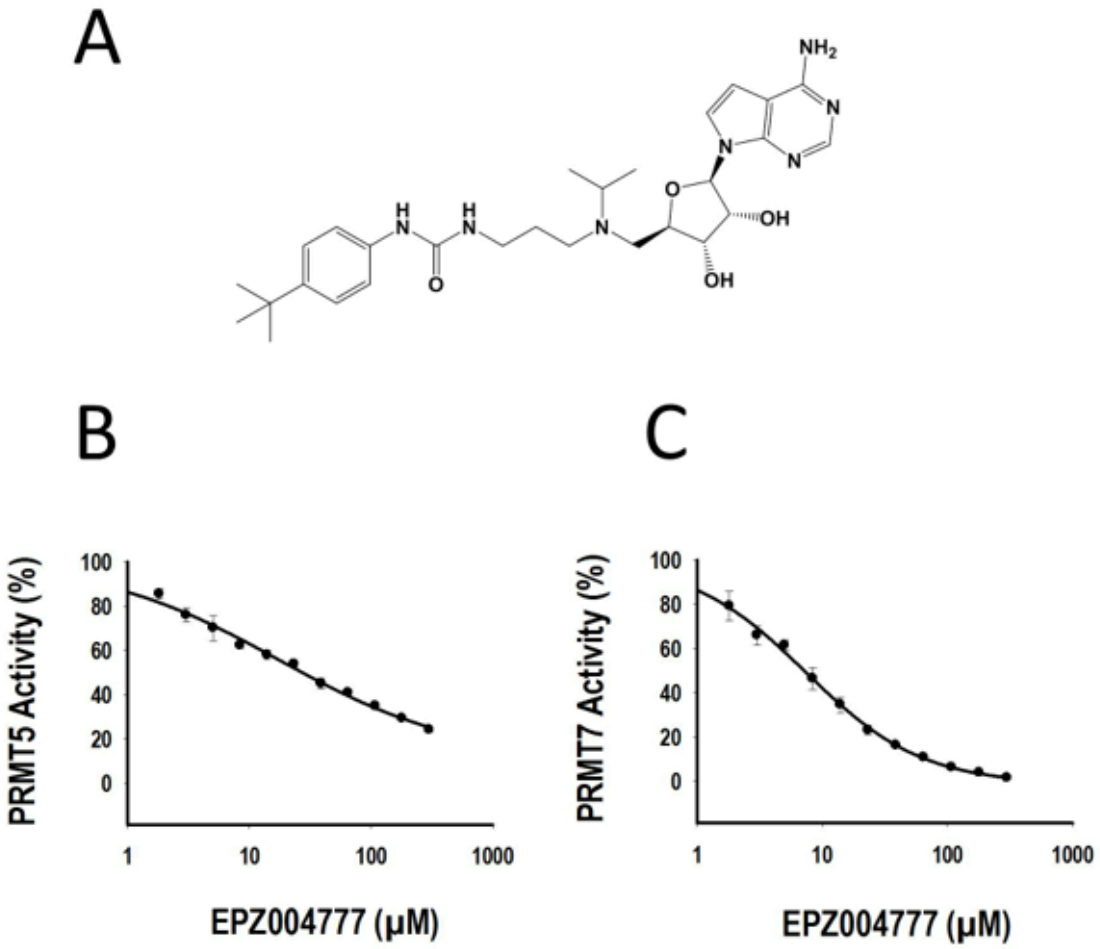
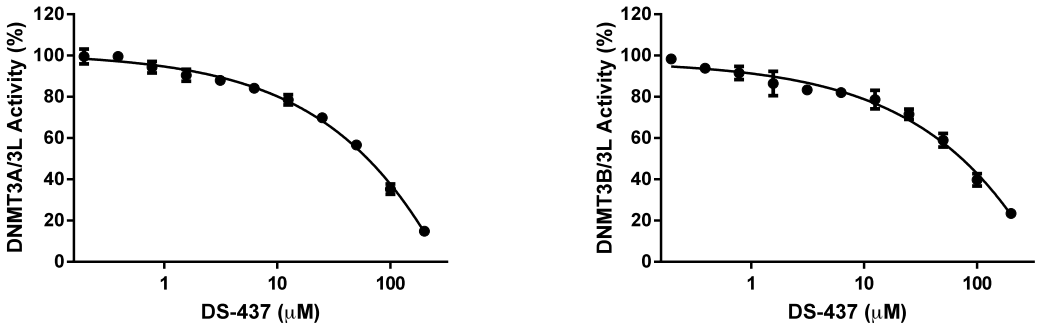
Discovery of a Dual PRMT5-PRMT7 Inhibitor

Smil et al.

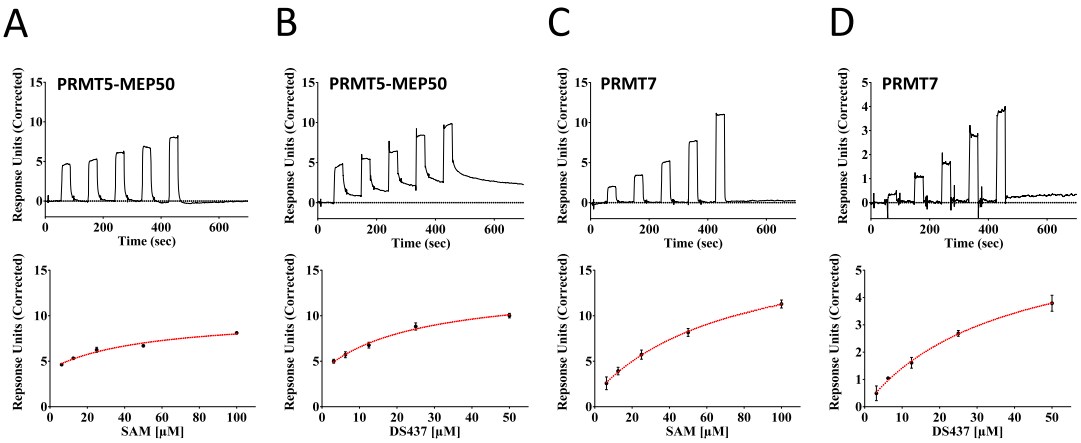
SUPPLEMENTARY FIGURES



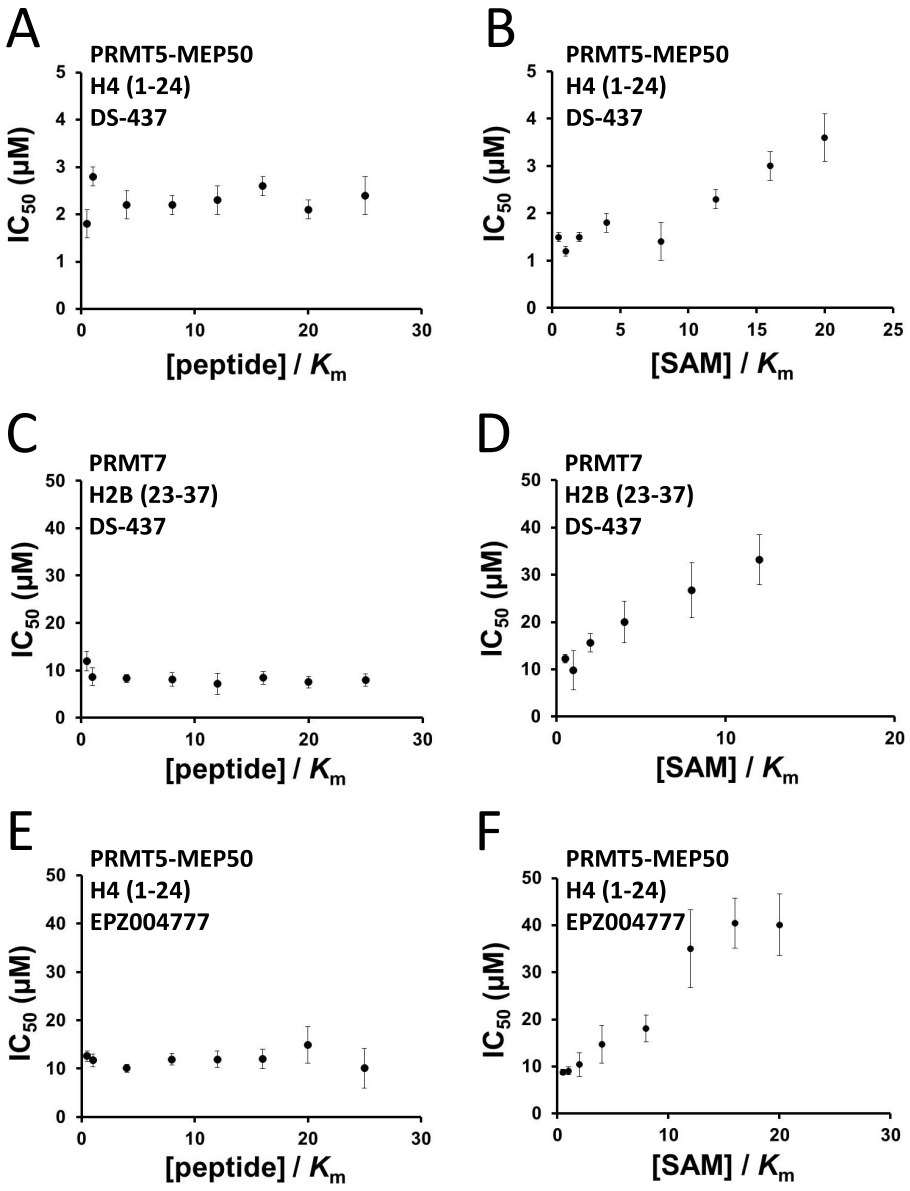
Supplementary Figure 1: Inhibition profile of EPZ004777. (A) The DOT1L inhibitor EPZ004777 is chemically related to DS-437, (B) is weakly active against PRMT5-MEP50 (IC_{50}= 30 µM), (C) and has an IC_{50} value of 7.5 µM with PRMT7.



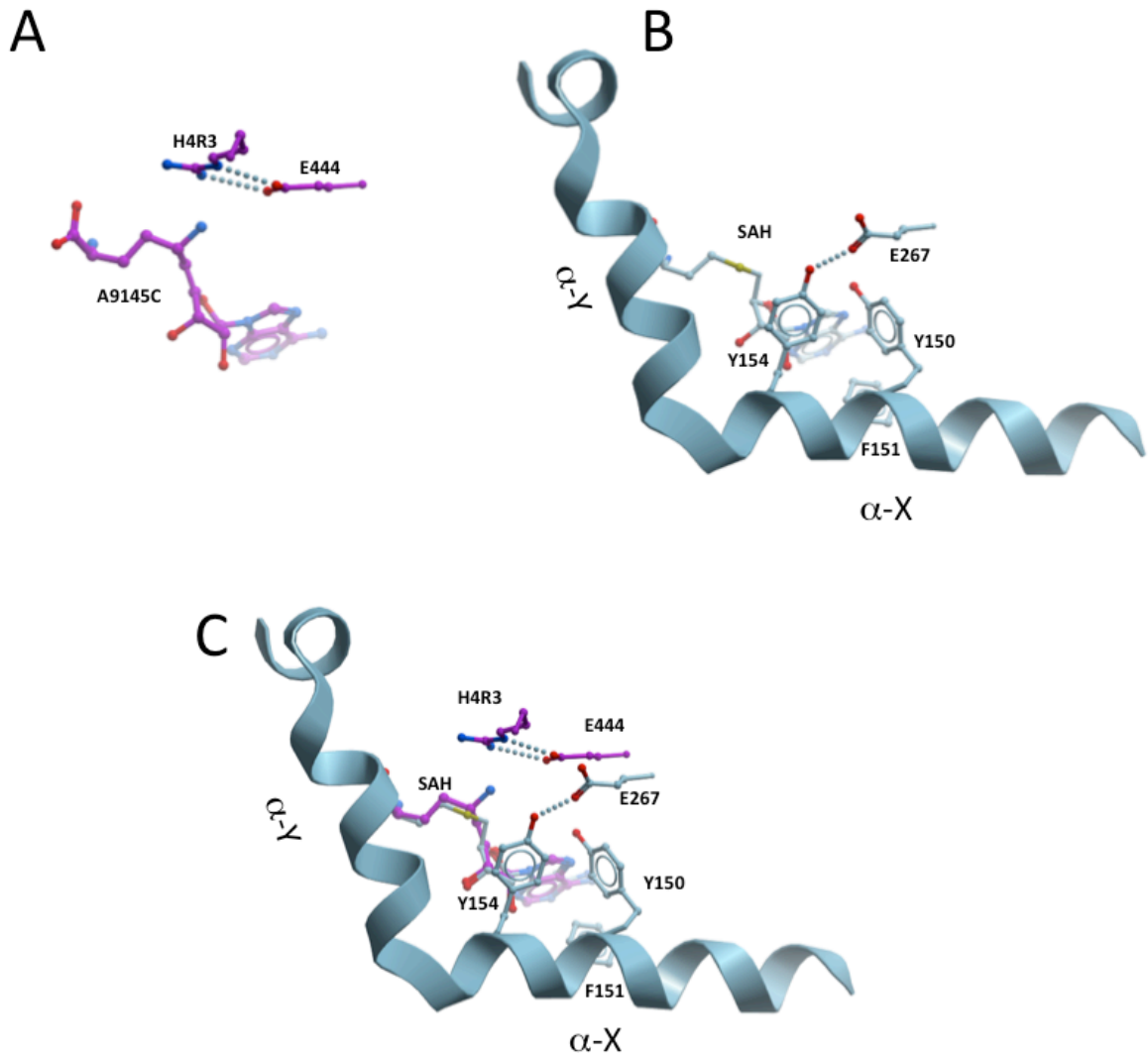
Supplementary Figure 2: Inhibition of DNMT3A and DNMT3B. DS-437 inhibits DNMT3A (left) and DNMT3B (right) with IC_{50} values of 52 ± 2 μM and 62 ± 5 μM respectively.



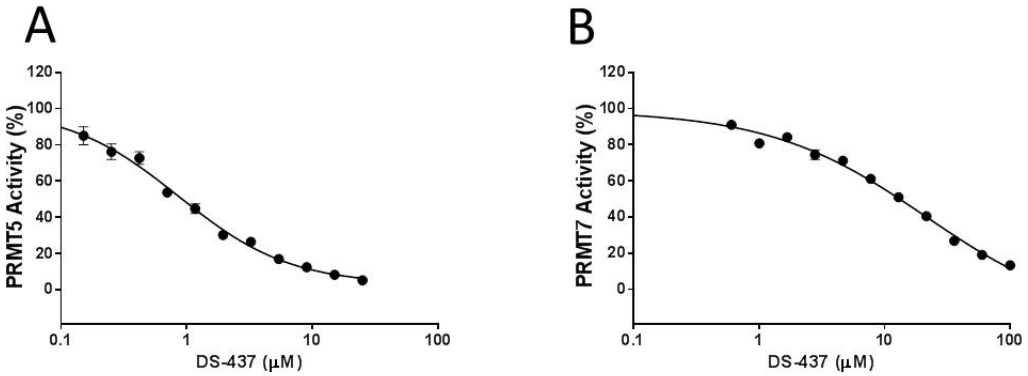
Supplementary Figure 3. Binding confirmation by Surface Plasmon Resonance (SPR). Binding of (A) SAM (K_D: 50 µM; R_{max} of 8) and (B) DS-437 (K_D: 25 µM; R_{max} of 9) to PRMT5-MEP50 complex was assessed in parallel by SPR. Similarly, binding of (C) SAM (K_D: 75 µM; R_{max} of 17) and (D) DS-437 (K_D: 40 µM; R_{max} of 7) to PRMT7 were also confirmed. SPR experiments were performed using a Biacore™ T200 (GE Health Sciences). 3100 RU of biotinylated PRMT5 and 3700 RU of biotinylated PRMT7 were captured onto a streptavidin-conjugated SA chip according to the manufacturer's protocol. The reference cell was left blank and was used for reference subtraction. DS-437 was dissolved in 100% DMSO. The titrated compounds were diluted 1:20 in HBS-EP Buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% Tween-20) with a final DMSO concentration of 5%. HBS-EP buffer with 5% DMSO was utilized as the running buffer. The Biacore was set to a flow rate of 50 µL/min for the experiments with an on time of 30 seconds and off time of 300 seconds. K_D values were determined using steady state curve fitting (curve fitting are shown in red).



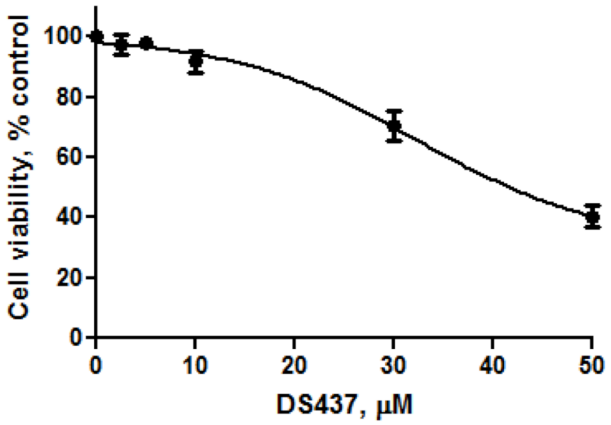
Supplementary Figure 4: Mechanism of action of DS-437 and EPZ0004777. DS-437 doesn't compete with peptide substrates for (A) PRMT5-MEP50 or (C) PRMT7. However, it is clearly a SAM competitive inhibitor of both proteins (B and D respectively). EPZ004777 also shows the same noncompetitive pattern of inhibition as DS-437 toward peptide substrate (E), and a SAM competitive pattern with PRMT5-MEP50 complex (F). Experiments were performed in triplicate as described in material and methods.



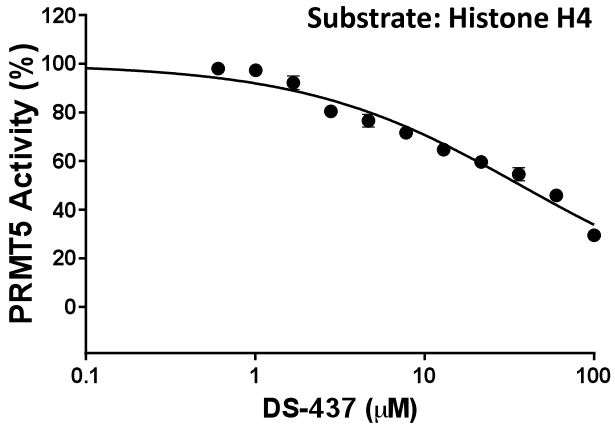
Supplementary Figure 5: (A) E444, a residue predicted to interact with the urea of DS-437, forms a double hydrogen-bond with the substrate arginine in the hPRMT5 structure [PDB code 4GQB]. (B) Similar orientation of the corresponding residue, E267, in CARM1 [PDB code 3B3F] is antagonized by interactions with Y154, a side-chain that is part of the Class I–specific YFxxY motif. (C) Superimposed view of panels A and B.



Supplementary Figure 6. Inhibition of PRMT5-MEP50 and PRMT7 activities by DS-437 in the presence of SmD3 substrate. Inhibitory effect of DS-437 on activity of PRMT5-MEP50 and PRMT7 was assessed using SmD3 peptide (AGRGRGKAAILKAQVAARGRGRGMGRGN) as a substrate for both enzymes. IC_{50} values of 0.9 ± 0.1 μM (Hill slope: 1.0) and 17.4 ± 1.5 μM (Hill slope: 1.0) were determined for PRMT5-MEP50 and PRMT7 respectively at balanced conditions (K_m of both substrates) as described below for each enzyme. PRMT5-MEP50 assay conditions: 20 mM Tris-HCl, pH 8.5, 0.01% Triton X-100, 5 mM TCEP, 25 nM PRMT5-MEP50, 100 nM SmD3 peptide and 0.3 μM SAM. PRMT7 assay conditions: 20 mM Tris-HCl, pH 8.5, 0.01% Tween-20, 5 mM DTT, 50 nM PRMT7, 1.3 μM SmD3 peptide and 3.7 μM SAM.



Supplementary Figure 7: DS437 effect on MDA-MB231 cell viability. The experimental conditions are the same as in Figure 3. Cell viability was measured using Alamar blue.



Supplementary Figure 8: Inhibition of PRMT5-MEP50 activity by DS-437 with histone H4 as substrate. The inhibitory effect of DS-437 on PRMT5-MEP50 activity was determined using the recombinant histone H4 (New England Biolabs ® Inc., Cat. number M2504S) as substrate. Assays were performed in 20 µl of 20 mM Tris-HCl, pH 8.5, 0.01% Tween-20, 10 mM TCEP, 30 nM PRMT5-MEP50, 1.4 µM histone H4, and 5 µM SAM. The IC_{50} value (37 ± 1.2 µM) was determined at concentrations of substrate and cofactor close to their K_m values using a trichloroacetic acid (TCA) precipitation filter plate method as previously described (Hassani et al, J. Biomol Screen. (2014); 19(6):928-935).

Supplementary Table 1: Selectivity profile of DS-437. Raw data (% enzymatic activity upon treatment with 1, 10 and 50 μM of DS-437) used to generate Figure 2B.

Methyltransferase	DS-437: 1μM	DS-437: 10μM	DS-437: 50μM
G9a	102	104	99
EHMT1	91	92	90
SUV39H2	86	108	110
SETDB1	89	91	87
SETD7	111	115	116
SETD8	95	101	96
SUV420H1	88	87	82
SUV420H2	107	100	90
MLL1	106	95	98
EZH1	109	103	108
EZH2	90	87	106
SMYD2	87	78	85
SETD2	89	86	95
PRDM9	94	94	92
PRMT1	89	92	71
PRMT3	91	70	77
PRMT5	86	39	26

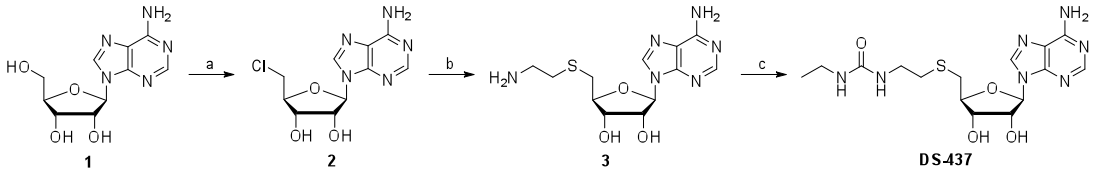
PRMT6	**103**	**97**	**77**
PRMT7	**81**	**45**	**17**
PRMT8	**97**	**91**	**79**
NSD1	**104**	**106**	**84**
NSD2	**110**	**120**	**117**
NSD3	**107**	**108**	**105**
ASH1L	**109**	**94**	**95**
DOT1L	**98**	**104**	**97**
DNMT1	**102**	**104**	**99**
DNMT3A/3L	**89**	**80**	**38**
DNMT3B/3L	**89**	**77**	**45**
BCDIN3D	**101**	**104**	**97**
METTL21A	**99**	**114**	**106**
METTL21D	**100**	**107**	**113**

SUPPLEMENTARY MATERIALS AND METHODS

Chemistry General Procedures

All oxygen and/or moisture sensitive reactions were carried out under N_2 atmosphere in glassware purged with N_2 prior to use. All reagents and laboratory grade solvents were purchased from commercial vendors and used as received, without further purification. Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker Avance-III 500 MHz spectrometer (500 MHz ^{1}H, 125 MHz ^{13}C). Proton chemical shifts are reported in ppm (δ) referenced to the NMR solvent; DMSO-d_6 (2.50 ppm for ^{1}H). Data are reported as follows: chemical shifts (δ), multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, quint = quintuplet, m = multiplet); coupling constant(s) (*J*) in Hz; integration. Unless otherwise noted, NMR data were collected at 25 °C. Flash column chromatography was performed using a Biotage SP1 system fitted with a KP-SIL SNAP Silica Gel (60 Å mesh) Flash Cartridge (FSKO-1107). Purity determination was conducted by UV absorbance at 254 nm during Tandem Liquid Chromatography/Mass Spectrometry (LCMS) using a Waters Acquity separations module. Identity was determined via low-resolution mass spectra (LRMS) acquired in positive ion mode using a Waters Acquity SQD mass spectrometer (electrospray ionization source) fitted with a PDA detector. Mobile phase A consisted of 0.01% formic acid in water, while mobile phase B consisted of 0.01% formic acid in acetonitrile. The gradient ran from 5% to 95% mobile phase B over 3 minutes at 0.7 mL/min. An Acquity CSH C18, 1.7 μm, 2.1 x 50 mm column was used with column temperature maintained at 25 °C. The sample solution injection volume was 2 μL.

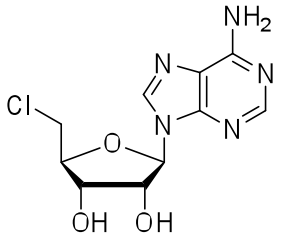
Reaction Scheme for the Preparation of DS-437



Scheme 1. Synthesis of **DS-437**. Reagents and conditions: (a) (i) $SOCl_2$, pyridine, MeCN, 0 °C → rt, overnight, (ii) NH_4OH, $MeOH/H_2O$, rt, 88%; (b) cysteamine, NaH, DMF, 0 °C → rt, overnight, 52%; (c) ethyl isocyanate, MeCN, 80 °C, 3 h, 16%.

Chemistry Procedure for the Preparation of DS-437

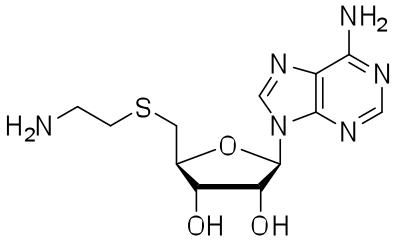
Steps (a) and (b) are adapted from Anglin, J.L.; Deng, L.; Yao, Y.; Cai, G.; Liu, Z.; Jiang, H.; Cheng, G.; Chen, P.; Dong, S.; Song, Y. *J. Med. Chem.* **2012**, *55*, 8066. Additional information relating to the characterization of previously reported compounds (**2**) and (**3**) can be found in the supplementary information and references associated with that publication. ^{1}H and ^{13}C NMR data for (**2**) and (**3**) produced during the synthesis of **DS-437** are included below.



2

Step (a): **(2*R*,3*R*,4*S*,5*S*)-2-(6-Amino-9*H*-purin-9-yl)-5-(chloromethyl)tetrahydrofuran-3,4-diol (2).** To a suspension of adenosine (**1**) (2.36 g, 8.83 mmol) in pyridine (1.44 mL, 17.8 mmol) and MeCN (31 mL) at 0 °C was

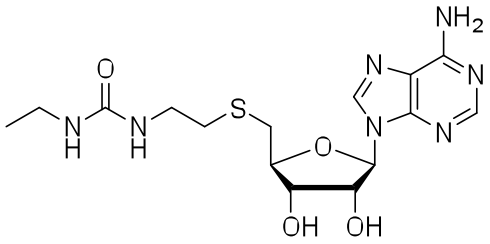
slowly added $SOCl_2$ (3.3 mL, 44.2 mmol). The resulting solution was stirred at 5 °C for 4 h, and subsequently warmed to rt overnight. The precipitate that formed was collected by filtration and dried *in vacuo* to afford a white powder, which was then suspended in MeOH/H_2O (54 mL, v/v = 5:1). Concentrated aqueous NH_4OH solution (5 mL) was added, and after stirring for 30 min at rt, the reaction was concentrated under reduced pressure. The resulting solid was crystallized from water, and dried *in vacuo* to provide (**2**) as a white powder (2.22 g, 88%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.35 (s, 1H), 8.17 (s, 1H), 7.35-7.26 (br s, 2H), 5.94 (d, *J* = 5.5 Hz, 1H), 5.60 (d, *J* = 5.5 Hz, 1H), 5.46 (d, *J* = 5.0 Hz, 1H), 4.79-4.74 (m, 1H), 4.26-4.21 (m, 1H), 4.12-4.08 (m, 1H), 3.95 (dd, *J* = 11.5, 5.0 Hz, 1H), 3.85 (dd, *J* = 11.5, 6.0 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 156.6, 153.2, 149.9, 140.2, 119.6, 87.9, 84.1, 73.1, 71.7, 45.3. LRMS (ESI): 286.2 $[M+H]^+$.



3

Step (b): **(2*R*,3*R*,4*S*,5*S*)-2-(6-Amino-9*H*-purin-9-yl)-5-(((2-aminoethyl)thio)methyl)tetrahydrofuran-3,4-diol (3).** To a solution of cysteamine (178 mg, 2.31 mmol) in DMF (7 mL) at 0 °C was slowly added NaH (92 mg, 60% dispersion in mineral oil, 2.31 mmol). When evolution of H_2 ceased after 15 min, (2*R*,3*R*,4*S*,5*S*)-2-(6-amino-9*H*-purin-9-yl)-5-(chloromethyl)tetrahydrofuran-3,4-diol (**2**) (600 mg, 2.10 mmol) was added, and the reaction mixture warmed to rt with stirring overnight. The reaction was subsequently quenched with water (10 mL), and adjusted to pH = 7 with dilute aqueous HCl solution. All solvents were then removed under reduced pressure, and the residue purified by flash column chromatography (silica gel, 0-100% MeOH/EtOAc gradient) to afford (**3**) as a white powder (356 mg, 52%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.33 (s, 1H), 8.14 (s, 1H),

7.30-7.24 (br s, 2H), 5.92 (br d, *J* = 5.5 Hz, 1H), 5.60 (br d, *J* = 5.5 Hz, 1H), 5.44 (d, *J* = 5.0 Hz, 1H), 5.31-5.26 (br s, 2H), 4.73-4.69 (m, 1H), 4.25-4.19 (m, 1H), 4.10-4.06 (m, 1H), 3.05-2.99 (m, 2H), 2.85-2.79 (m, 2H), 2.75-2.70 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 156.5, 153.2, 149.7, 140.1, 119.5, 88.1, 83.8, 73.0, 72.0, 41.1, 37.8, 36.5. LRMS (ESI): 327.2 $[M+H]^+$.



DS-437

Step (c): **1-(2-((((2*S*,3*S*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)thio)ethyl)-3-ethylurea (DS-437).** To a suspension of (2*R*,3*R*,4*S*,5*S*)-2-(6-amino-9*H*-purin-9-yl)-5-(((2-aminoethyl)thio)methyl)tetrahydrofuran-3,4-diol (**3**) (100 mg, 0.306 mmol) in MeCN (7 mL) was added ethyl isocyanate (24 μL, 0.306 mmol). The resulting solution was then heated to 80 °C for 3 h prior to cooling back down to rt. The evident precipitate was removed by filtration, and the filtrate concentrated under reduced pressure to afford (**DS-437**) as a white powder (20 mg, 16%) requiring no further purification (HPLC purity >95% at 254 nm, retention time = 0.32 min using the conditions and column outlined above). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.46 (s, 1H), 8.26 (s, 1H), 7.95-7.72 (br s, 2H), 5.96 (s, 1H), 5.90 (d, *J* = 5.6 Hz, 1H), 5.81 (s, 1H), 4.80-4.72 (m, 1H), 4.19-4.13 (m, 1H), 4.08-4.01 (m, 1H), 3.60-3.50 (br s, 2H), 3.48-3.45 (m, 2H), 3.31-3.26 (m, 2H), 2.88-2.80 (m, 2H), 2.78-2.74 (m, 2H), 1.16 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 162.8, 155.0, 151.3, 149.7, 141.0, 119.5, 87.9, 84.5, 73.3, 73.0, 45.9, 36.3, 34.6, 33.0, 16.2. HRMS (ESI) *m/z* calcd for $C_{15}H_{24}N_7O_4S$ $[M+H]^+$: 398.1610, found: 398.1612.

Protein expression and purification. Full-length human PRMT5 and MEP50 were expressed in Sf9 cells grown in HyQ® SFX insect serum-free medium (TermoScientific). Cells were harvested by centrifugation at 7000 rpm for 15 min and the pellet was re-suspended in 20 mM Tris-HCl, pH 7.5 containing 500 mM NaCl, 5% glycerol, 5 mM imidazole, 2 mM DTT, 0.6% (v/v) NP-40, 1 mM PMSF, and 50 U/ml Benzonase (Novagene). The cell lysis was completed by sonication at a frequency of 8 Hz with 10 s on and 10 s off for 2 min. The cell lysate was clarified by high-speed centrifugation (60 min at 36,000 g at 4 ˚C). The recombinant protein complex was purified by loading the cleared lysate on a Talon metal affinity resin (Clontech) followed by washing with 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5% glycerol, 15 mM imidazole). The recombinant protein complex was eluted from the cobalt-based affinity column using the elution buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5% glycerol, 250 mM imidazole). The eluate was concentrated and loaded onto a gel filtration Superdex-200 column (GE Healthcare) and eluted with 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM DTT. Pure fractions as judged by SDS–PAGE were pooled, concentrated, flash frozen and stored at -80 ˚C.

Full-length PRMT7 was expressed in Sf9 cells grown in HyQ® SFX Insect serum-free medium (ThermoScientific). Cells were harvested and lysed as described above and the cleared lysate was incubated with 5 ml anti-Flag M2-Agarose (Sigma) in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol and washed with the same buffer with 500 mM NaCl. The pure recombinant protein was eluted from the column using the same buffer with 0.1 mg/ml Flag peptide (Sigma). Pure PRMT7 was flash frozen and stored at -80 ˚C.

Radioactive activity assay. The scintillation proximity assay was used to determine the selectivity of the compounds against a panel of 30 methyltransferase as we previously reported[24]. IC_{50} values were determined in triplicate at K_m concentration of both substrates. In the case of PRMT5-MEP50 complex the reaction mixture (20 µl) contained 20 mM Tris-HCl, pH 8.5 containing 0.01% Tween-20, 10 mM Tris(2-carboxyethyl)phosphine (TCEP), 15 nM PRMT5-MEP50 complex, 0.07 µM H4 1-24 peptide (SGRGKGGKGLGKGGAKRHRKVLRDK-Biotin) and 0.6 µM SAM.

For PRMT7, the assay mixture contained 20 mM Tris-HCl, pH 8.5, 0.01% Tween-20, and 5 mM dithiothreitol (DTT), 25 nM PRMT7, 0.3 μM H2B 23-37 peptide (Ac-KKDGKKRKRSRKESYK-Biotin) and 1.1 μM SAM. Both reactions were incubated at 23 ˚C for 30 min. The reactions were quenched by addition of equal volume of 7.5 M guanidine hydrochloride and the volume finalized to 200 by the addition of 20 mM Tris-HCl, pH 8.0. After the transfer to the SPA plates (FlashPlate® PLUS; PerkinElmer Life Sciences), the plates were incubated for at least 1 h before measuring the signals using a TopCount NXT™ Microplate Scintillation and Luminescence Counter (PerkinElmer Life Sciences). For the IC_{50} determination the compounds were serially diluted in the reaction buffer and tested under the same conditions.

To assess the mechanism of action of the compounds, IC_{50} values were determined using the radioactive assay as described above and in triplicates against the compounds at saturating concentrations of H4 1-24 (0.8 μM) and varying concentrations of SAM (0.3, 0.6, 1.2, 2.4, 4.8, 7.2, 9.6, and 12 μM).To assess the competition of the compounds with the peptide substrate, SAM was kept at saturation (3 μM) and IC_{50} values were determined at various peptide concentrations (0.035, 0.07, 0.28, 0.56, 0.84, 1.12, 1.4, and 1.75 μM). The reactions contained 20 mM Tris-HCl, pH 8.5 containing 0.01% Tween-20, 10 mM TCEP, and 15 nM PRMT5-MEP50 complex.

Cellular Assays. MDA-MB231 (ATTC) cells were grown in RPMI with 10% FBS and treated with the inhibitor, changing media and compound every day for 4 days. After 4 days, cells were harvested and lysed. Lysates were resolved on SDS PAGE, transferred to the PVDF membrane and immunoblotted with Rme2s (Cell Signalling Technology 13222), Y12 (Abcam 3138) SmD1 (Abcam 50940), SmD3 (Abcam 121129), Actin (Abcam 3280), rabbit anti-H3R2me2s (Millipore), rabbit anti-H4R3me2s (Abcam), mouse anti-H3 (Abcam), mouse anti-H4 (Abcam). Signal detection was achieved with IRDye®680RD Goat anti-Mouse IgG (LI-COR Biosciences) and IRDye®800CW Goat anti-Rabbit IgG (LI-COR Biosciences) on the Odyssey scanner (LI‑COR Biosciences). Cell viability was determined using Alamar

blue (resazurin, Sigma) 0.1mg/ml in the media. Resazurin reduction was monitored with 544 nm excitation, measuring fluorescence at 590 nm.