Discovery of Potent, Selective, and Structurally Novel Dot1L Inhibitors by a Fragment Linking Approach

Henrik Möbitz,[‡] Rainer Machauer,[‡] Philipp Holzer,[‡] Andrea Vaupel,[‡] Frédéric Stauffer,[‡] Christian Ragot,[‡] Giorgio Caravatti,[‡] Clemens Scheufler,[‡] Cesar Fernandez,[‡] Ulrich Hommel,[‡] Ralph Tiedt,[‡] Kim S. Beyer,[‡] Chao Chen,[†] Hugh Zhu,[†] and Christoph Gaul*[‡]

[‡]Novartis Institutes for Biomedical Research, Basel, Switzerland

[†]Novartis Institutes for Biomedical Research, Shanghai, China

Supporting Information

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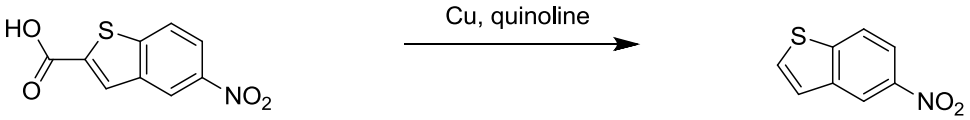
Experimental procedure for the synthesis of **7**

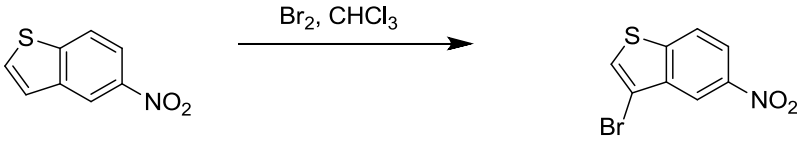
General Methods. All reagents and solvents were of commercial quality and used without further purification. Normal phase column chromatography purifications were carried out in the indicated solvent

system (in the percentage of volume) using pre-packed Silica RediSepRf® cartridges for use on the ISCO CombiFlash®. Nuclear Magnetic Resonance spectra were recorded on a Varian spectrometer. Spectra were taken in the indicated solvent at ambient temperature and the chemical shifts are reported in parts per million (ppm (δ)) relative to the lock of the solvent used. Resonance patterns are recorded with the following notations: br (broad), s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). The LC-MS analyses were performed with a Waters Acquity UPLC BSM, coupled to a Waters Acquity SQD (Single Quadrupole Detector) mass spectrometer with a range of ESI +/- : 100-1200 m/z, utilizing a Waters Acquity HSS T3 1.8 µm 2.1 x 50 mm column with 60 °C column temperature, a flow of 1 mL/min and a solvent gradient of [method 1] 5 to 98% B over 1.4 min, followed by 0.4 min at 98% B; [method 2] 1 to 98% B over 1.4 min, followed by 0.4 min at 98% B; solvent A: water + 0.05% formic acid + 3.75 mM ammonium acetate, solvent B: acetonitrile + 0.04% formic acid. The injection volume was 1 µl and the solvent was typically MeOH. Preparative HPLC was done on a Gilson system with DAD-detector utilizing either a 100x30 mm Waters Sunfire Prep C18 OBD^{TM} 5 µm column with a flow of 30 mL/min, or a 250x30 mm Dr. Maisch GmbH Reprosil 100 Prep C18 5 µm column with a flow of 35 mL/min, and a specified solvent gradient using solvent A: water with 0.1% trifluoroacetic acid, solvent B: acetonitrile.



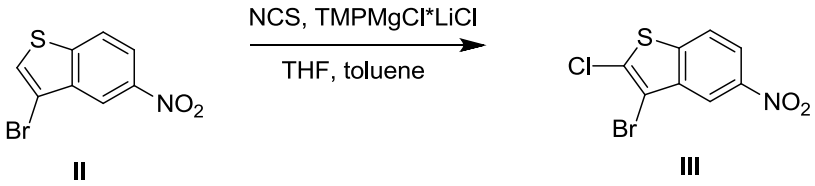
I

5-Nitrobenzo[b]thiophene (I). 5-Nitrobenzo[b]thiophene-2-carboxylic acid (3.0 g, 13.31 mmol) was added to a mixture of copper powder (0.846 g, 13.31 mmol) and quinoline (50 mL) under N_2-atmosphere. After stirring for 2 h at 150 °C, the reaction mixture was diluted with EtOAc (50 mL) and filtered. The filtrate was poured onto ice and acidified with 37% aqueous HCl. EtOAc was added and the mixture was transferred into a separatory funnel. The organic layer was washed with water and brine, dried (Na_2SO_4) and concentrated. The crude product was purified by silica gel chromatography using hexane:TBME as eluent (gradient of 0 to 50% TBME) to give 5-nitrobenzo[b]thiophene (**I)** in 81% yield. ^{1}H NMR (600 MHz, DMSO-d6) δ 8.87 (d, J = 2.3 Hz, 1H), 8.32 (d, J = 8.8 Hz, 1H), 8.19 (dd, J = 8.9, 2.3 Hz, 1H), 8.07 (d, J = 5.5 Hz, 1H), 7.75 (d, J = 5.4 Hz, 1H). LC-MS [method 1] t_{ret} = 1.05 min, no mass hit.



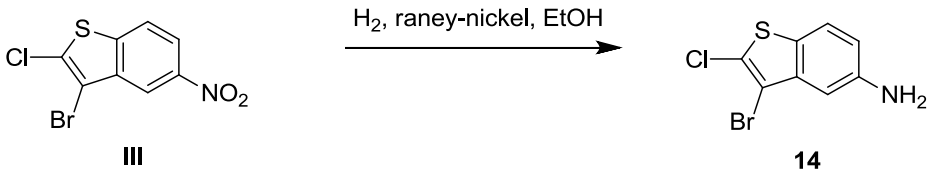
I II

3-Bromo-5-nitrobenzo[b]thiophene (II). 5-Nitrobenzo[b]thiophene (**I**) (1.94 g, 10.72 mmol) in chloroform (45 mL) was treated with bromine (0.773 mL, 15.01 mmol) and the mixture was stirred for 30 h at 60 °C. Then the solvent was evaporated, the residue was suspended in EtOAc (6 mL) and hexane (6 mL) and the crystals were filtered off to give 3-bromo-5-nitrobenzo[b]thiophene (**II**) in 93% yield. ^{1}H NMR (600 MHz, DMSO-d6) δ 8.51 (d, J = 2.1 Hz, 1H), 8.41 (d, J = 8.8 Hz, 1H), 8.33 - 8.27 (m, 2H). LC-MS [method 1] t_{ret} = 1.18 min, no mass hit.



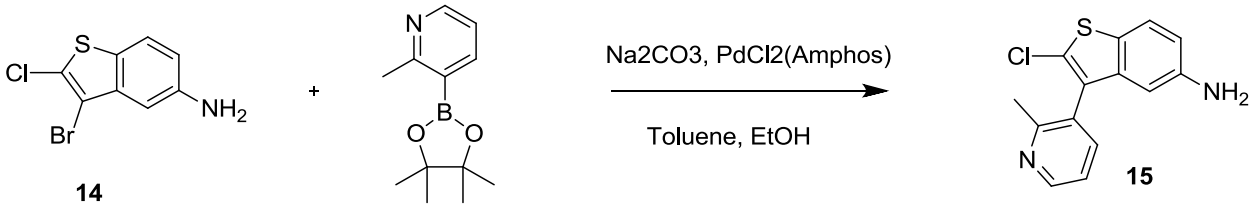
II III

3-Bromo-2-chloro-5-nitrobenzo[b]thiophene (III). 3-Bromo-5-nitrobenzo[b]thiophene (**II**) (1.0 g, 3.80 mmol) was given into a flask with THF (50 mL) and toluene (10 mL). The reaction mixture was cooled down to -75 °C under N_2-atmosphere, then 1 M TMPMgCl*LiCl in toluene/THF (215863-85-7) (7.59 mL, 7.59 mmol) was added dropwise in 10 min. After stirring for 30 min at -75 °C, *N*-chlorosuccinimide (1.04 g, 7.59 mmol) in THF (5 mL) was added dropwise. The reaction mixture was stirred for further 10 min at -75 °C, then was quenched with aqueous NH_4Cl-solution (10 mL) and water (10 mL), transferred into a separatory funnel with water (40 mL) and washed twice with EtOAc (2 x 100 mL). The organic layers were washed with water (50 mL) and brine (50 mL), dried (Na_2SO_4) and evaporated. The crude product was purified with silica chromatography using 100% hexane to 100% TBME as eluent to give 3-bromo-2-chloro-5-nitrobenzo[b]thiophene (**III**) in 80% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.45 (d, *J* = 2.2 Hz, 1H), 8.40 (d, *J* = 8.8 Hz, 1H), 8.34 (dd, *J* = 8.9, 2.2 Hz, 1H). LC-MS [method 1] t_{ret} = 1.32 min, no mass hit.



III 14

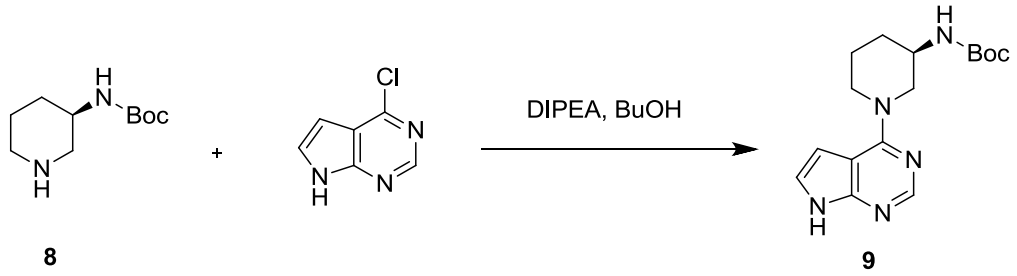
3-Bromo-2-chlorobenzo[b]thiophen-5-amine (14). 3-Bromo-2-chloro-5-nitrobenzo[b]thiophene (**III**) (700 mg, 1.91 mmol) was dissolved in EtOH (20 mL) and transferred into a shaking duck glass under H_2-atmosphere. Raney-nickel (100 mg) was added and the mixture was shaken for 11 h at ambient temperature. The Raney-nickel was filtered off and the filtrate was evaporated to give 3-bromo-2-chlorobenzo[b]thiophen-5-amine (**14**) in 96% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.60 (d, *J* = 8.6 Hz, 1H), 6.86 (d, *J* = 1.9 Hz, 1H), 6.80 (dd, *J* = 8.6, 1.9 Hz, 1H), 5.47 (s, 2H). LC-MS [method 1] t_{ret} = 1.13 min, m/z = 262.0/263.9 $[M+H]^+$.



14 15

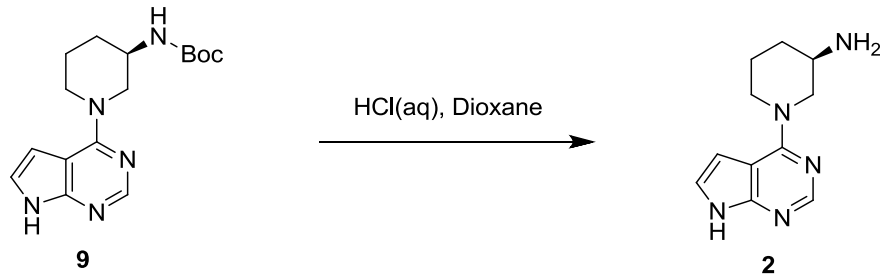
2-Chloro-3-(2-methylpyridin-3-yl)benzo[b]thiophen-5-amine (15). 3-Bromo-2-chlorobenzo[b]thiophen-5-amine (**14**) (147 mg, 0.55 mmol) was given into a vial with EtOH (3 mL) and toluene (3 mL) under Ar-atmosphere. Then 2 M aqueous Na_2CO_3 (0.69 mL, 1.39 mmol), 2-methyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridine (136 mg, 0.622 mmol) and $PdCl_2$(Amphos) (39.2 mg, 0.055 mmol) were added

and the reaction mixture was stirred at 60 °C for 20 min. The reaction mixture was cooled down to rt and filtered through a pad of Hyflo. The cake was washed several times with EtOH and the filtrate was evaporated. The crude product was purified by preparative HPLC (Reprosil column, 10% B to 50% B in 35 min) to give 2-chloro-3-(2-methylpyridin-3-yl)benzo[b]thiophen-5-amine (**15**) in 36% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.55 (dd, 1H), 7.63 (dd, 1H), 7.57 (d, 1H), 7.36 (dd, 1H), 6.73 (dd, 1H), 6.30 (d, 1H), 5.18 (s, 2H), 2.23 (s, 3H). LC-MS [method 1] t_{ret} = 0.94 min, m/z = 275.0/277.1 $[M+H]^+$.



8 **9**

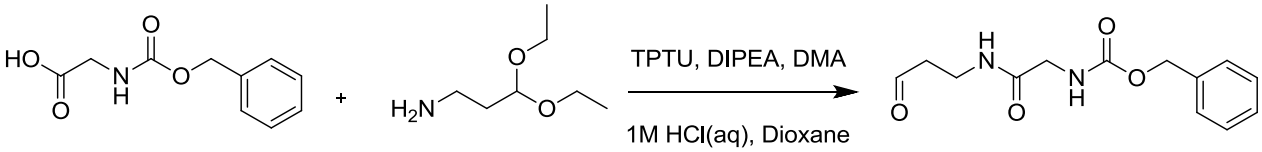
(*R*)-*Tert*-butyl(1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-yl)carbamate (9). 4-chloro-7H-pyrrolo[2,3-d]pyrimidine (730 mg 4.75 mmol) and (*R*)-*tert*-butyl piperidin-3-ylcarbamate (**8**) (1.0 g, 4.99 mmol) were given into a vial with BuOH (8.5 mL) and DIPEA (1.25 mL, 7.13 mmol) under Ar-atmosphere. The vial was sealed and the reaction mixture was stirred for 52 h at 80 °C. Then the reaction mixture was transferred into a separatory funnel with EtOAc (100 mL) and washed three times with brine (3 x 30 mL). The organic layer was then dried (Na_2SO_4) and evaporated to give (*R*)-*tert*-butyl(1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-yl)carbamate (**9**) in a quantitative yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 11.64 (s, 1H), 8.12 (d, J = 0.8 Hz, 1H), 7.17 (m, 1H), 6.95 (d, J = 7.1 Hz, 1H), 6.63 (s, 1H), 4.51 (d, J = 13.6 Hz, 1H), 4.39 (d, J = 12.9 Hz, 1H), 3.49 – 3.33 (m, 1H), 3.18 – 3.07 (m, 1H), 3.04 – 2.94 (m, 1H), 1.94 – 1.84 (m, 1H), 1.83 – 1.74 (m, 1H), 1.54 – 1.44 (m, 2H), 1.40 (s, 9H). LC-MS [method 1] t_{ret} = 0.72 min, m/z = 318.1 $[M+H]^+$.



9 **2**

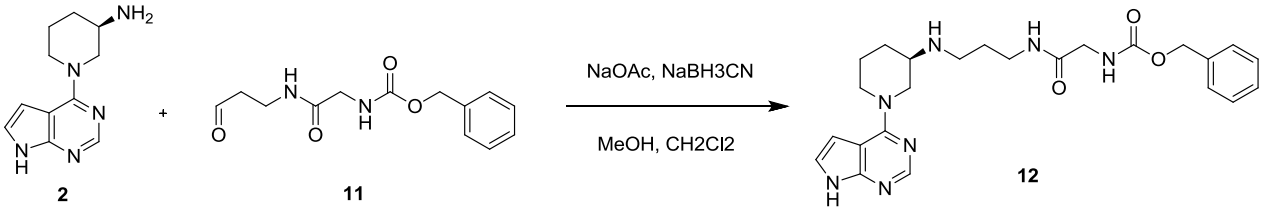
(*R*)-1-(7H-Pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-amine (2). (*R*)-*tert*-butyl(1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-yl)carbamate (**9**) (1.55 g, 4.72 mmol) was introduced in a flask with dioxane (10 mL). Then, 37% aqueous HCl (3 mL, 6.47 mmol) was added and the reaction mixture was stirred at rt for 40 min. The resulting solution was evaporated. The crude product was taken up in MeOH (12 mL) and equally charged onto three H^+ exchange resin cartridges (PoraPak Rxn CX 60 cc 5 g), previously equilibrated with MeOH. Each cartridge was then washed with MeOH (50 mL), before collecting the elution of 7 M ammonia in MeOH (50 mL). The eluates were then evaporated together to give (*R*)-1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-amine (**2**) in 95% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.60 (s,

1H), 8.08 (s, 1H), 7.13 (d, 1H), 6.58 (d, 1H), 4.51 (m, 2H), 2.99 (m, 1H), 2.74 (dd, 1H), 2.68 (m, 1H), 1.86 (m, 1H), 1.71 (m, 3H), 1.43 (m, 1H), 1.25 (m, 1H). LC-MS [method 2] t_{ret} = 0.60 min, m/z = 218.2 $[M+H]^+$.



10 **11**

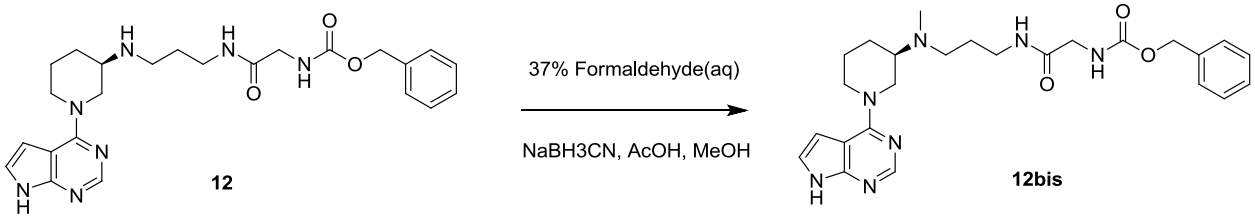
Benzyl(2-oxo-2-((3-oxopropyl)amino)ethyl)carbamate (11). 2-(((Benzyloxy)carbonyl)amino)acetic acid (**10**) (1.0 g, 4.78 mmol) was introduced in a flask with DMA (9 mL) and DIPEA (1.25 mL, 7.17 mmol) under Ar-atmosphere. The reaction mixture was cooled at 0 °C and TPTU (1.7 mg, 5.74 mmol) was added. After 7 min of stirring at 0 °C, 3,3-diethoxypropan-1-amine (774 mg, 5.26 mmol) was introduced and the reaction mixture was stirred at rt for 45 min. The mixture was then taken up in EtOAc (200 mL) and brine (60 mL) and transferred into a separatory funnel. The aqueous layer was separated and extracted with EtOAc (100 mL). The combined organic layers were then washed twice with brine (2 x 50 mL), followed by a mixture of saturated aqueous $NaHCO_3$/brine 1:1 (50 mL) and again with brine (50 mL), dried (Na_2SO_4) and evaporated. The residue was purified by silica gel chromatography using CH_2Cl_2/MeOH as eluent (gradient 0 - 5% MeOH). The resulting oil was taken up in dioxane (35 mL) with 1 M aqueous HCl (4.9 mL, 4.9 mmol) and stirred at rt for 20 min. Then the solution was transferred into a separatory funnel with EtOAc (200 mL) and washed three times with brine (3 x 30 mL), dried (Na_2SO_4) and evaporated. The crude product was purified by preparative HPLC (Sunfire column, 5% B to 50% B in 20 min) to give benzyl(2-oxo-2-((3-oxopropyl)amino)ethyl)carbamate (**11**) in 67% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.62 (s, 1H), 7.92 (t, J = 5.0 Hz, 1H), 7.45 – 7.37 (m, 1H), 7.36 – 7.33 (m, 3H), 7.33 – 7.23 (m, 2H), 5.01 (s, 2H), 3.54 (d, J = 6.1 Hz, 2H), 3.37 – 3.30 (m, 2H), 2.54 (t, J = 6.1 Hz, 2H). LC-MS [method 1] t_{ret} = 0.62 min, m/z = 265.1 $[M+H]^+$; 263.1 $[M-H]^-$.



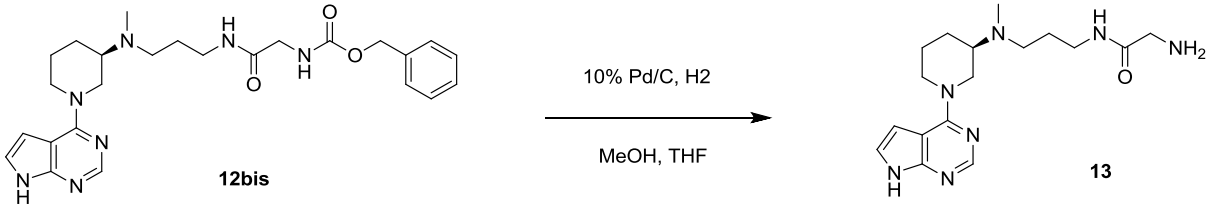
2 **11** **12**

(*R*)-Benzyl(2-((3-((1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-yl)amino)propyl)amino)-2-oxoethyl)carbamate (12). (*R*)-1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-amine (**2**) (363 mg, 1.67 mmol), benzyl(2-oxo-2-((3-oxopropyl)amino)ethyl)carbamate (**11**) (442 mg, 1.67 mmol) and sodium acetate (343 mg, 4.18 mmol) were introduced in a flask with CH_2Cl_2 (6 mL) and MeOH (6 mL) under Ar-atmosphere. The reaction mixture was stirred at rt for 30 min. Then sodium cyanoborohydride (157 mg, 1.12 mmol) was added and the mixture was stirred at rt for 16 h. The reaction mixture was blown off with nitrogen. The residue was taken up in EtOAc (50 mL) and transferred into a separatory funnel with brine (10 mL) and saturated aqueous $NaHCO_3$ (10 mL). The aqueous layer was separated and extracted with EtOAc (50 mL). The combined organic layers were dried (Na_2SO_4) and evaporated. The crude product was purified by preparative HPLC (Reprosil column, 5% B to 60% B in 25 min) to give (*R*)-benzyl (2-((3-

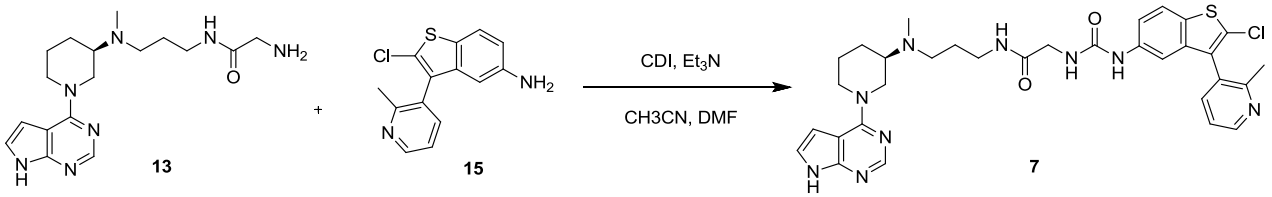
((1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-yl)amino)propyl)amino)-2-oxoethyl)carbamate (**12**) in 58% yield. LC-MS [method 1] t_{ret} = 0.57 min, m/z = 466.2 $[M+H]^+$; 464.2 $[M-H]^-$.



(*R*)-Benzyl(2-((3-((1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-yl)(methyl)amino)propyl)amino)-2-oxoethyl)carbamate (12bis). (R)-Benzyl(2-((3-((1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-yl)amino)propyl)amino)-2-oxoethyl)carbamate (**12**) (454 mg, 0.975 mmol) was introduced in a flask with MeOH (19 mL) and 37% aqueous formaldehyde (0.436 mL, 5.85 mmol) under Ar-atmosphere. Then sodium cyanoborohydride (306 mg, 4.88 mmol) and acetic acid (0.112 mL, 1.95 mmol) were added and the reaction mixture was stirred at rt for 45 min. The mixture was then quenched with saturated aqueous $NaHCO_3$ (20 mL) and transferred into a separatory funnel with EtOAc (120 mL), water (40 mL) and brine (20 mL). The aqueous layer was separated and extracted with EtOAc (120 mL). The combined organic layers were washed with brine (40 mL), dried (Na_2SO_4) and evaporated. The crude product was purified by preparative HPLC (Reprosil column, 5% B to 60% B in 25 min) to give (R)-benzyl(2-((3-((1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-yl)(methyl)amino)propyl)amino)-2-oxoethyl)carbamate (**12bis**) in 80% yield. LC-MS [method 1] t_{ret} = 0.57 min, m/z = 480.3 $[M+H]^+$; 478.2 $[M-H]^-$.



(*R*)-N-(3-((1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-yl)(methyl)amino)propyl)-2-aminoacetamide (13). (*R*)-benzyl(2-((3-((1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-yl)(methyl)amino)propyl)amino)-2-oxoethyl)carbamate (**12bis**) (190 mg, 0.392 mmol) was introduced in a N_2-flushed two-necked flask with MeOH (3 mL) and THF (1.5 mL), followed by 10% Pd/C (100 mg, 0.094 mmol). The reaction mixture was then stirred at rt for 7.5 h under normal pressure H_2-atmosphere. The suspension was then filtered through a pad of Hyflo and the filtrate was evaporated to give (*R*)-N-(3-((1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-yl)(methyl)amino)propyl)-2-aminoacetamide (**13**) in 84% yield. LC-MS [method 1] t_{ret} = 0.24 min, m/z = 346.2 $[M+H]^+$; 344.2 $[M-H]^-$.



N-(3-(((*R*)-1-(7H-Pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-yl)(methyl)amino)propyl)-2-(3-(2-chloro-3-(2-methylpyridin-3-yl)benzo[b]thiophen-5-yl)ureido)acetamide (7). 2-Chloro-3-(2-methylpyridin-3-yl)benzo[b]thiophen-5-amine (**15**) (35 mg, 0.121 mmol) and carbonyldiimidazole (22 mg, 0.136 mmol) were introduced in a vial with acetonitrile (1.0 mL) and DMF (0.33 mL) under Ar-atmosphere. The reaction mixture was stirred at rt for 1 h. Then (*R*)-N-(3-((1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-yl)(methyl)amino)propyl)-2-aminoacetamide (**13**) (60 mg, 0.156 mmol) in DMF (0.67 mL) was added, followed by Et_3N (0.034 mL, 0.242 mmol). The vial was sealed and the reaction mixture was stirred at 45 °C for 1 h. The mixture was then concentrated and the residue was purified by preparative HPLC (Sunfire column, 3% B to 60% B in 20 min). The resulting product was purified again by preparative TLC (Merck Silica gel plate 60 F254, 0.5 mm, 20 x 20 cm) using CH_2Cl_2/MeOH/7 M NH_3 in MeOH 36:3:1 as eluent to give N-(3-(((*R*)-1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidin-3-yl)(methyl)amino)propyl)-2-(3-(2-chloro-3-(2-methylpyridin-3-yl)benzo[b]thiophen-5-yl)ureido)acetamide (**7**) in 33% yield. ^{1}H NMR (600 MHz, DMSO-d6) δ 11.67 (s, 1H), 8.97 (s, 1H), 8.58 (d, *J* = 4.7 Hz, 1H), 8.10 (s, 1H), 7.96 – 7.89 (m, 1H), 7.82 (dd, *J* = 8.7, 3.2 Hz, 1H), 7.66 (d, *J* = 7.6 Hz, 1H), 7.41 (s, 1H), 7.40 – 7.37 (m, 1H), 7.37 – 7.33 (m, 1H), 7.17 – 7.14 (m, 1H), 6.49 (s, 1H), 6.27 (t, *J* = 5.4 Hz, 1H), 4.68 (d, *J* = 11.4 Hz, 1H), 4.55 (d, *J* = 11.9 Hz, 1H), 3.62 (d, *J* = 5.4 Hz, 2H), 3.12 – 3.04 (m, 2H), 2.96 – 2.84 (m, 2H), 2.48 – 2.35 (m, 3H), 2.24 (s, 3H), 2.22 (s, 3H), 1.83 (d, *J* = 10.8 Hz, 1H), 1.73 (d, *J* = 12.8 Hz, 1H), 1.57 – 1.44 (m, 3H), 1.43 – 1.33 (m, 1H). LC-MS [method 1] t_{ret} = 0.69 min, m/z = 646.2/648.2 $[M+H]^+$; 644.0/646.0 $[M-H]^-$.

Parameter	**7**
MW	646
PSA	131
logP	3.5
HT-solubility pH 6.8 [mM]	0.187
Caco-2 A-B / B-A [10^{-8} cm/s]	1.0 / 16
LM m/r/h Cl_{int} [µL/min·mg]	very high / very high / 32

Dot1L scintillation proximity assay (SPA)

For IC_{50} determination, compounds were pre-incubated with Dot1L(2-416) for 30 min before the reaction was started by addition of *S*-[methyl-^{3}H-] adenosyl–L-methionine (^{3}H-SAM) (Perkin Elmer, 18-20 Ci/mmol or 55-85 Ci/mmol for higher sensitivity version of the assay) and biotinylated nucleosomes (Wuxi Biortus Biosciences Ltd, China). Final concentrations in an assay volume of 10 µL were 0.5 nM Dot1L (or 0.05 nM Dot1L for higher sensitivity variant of the assay), 200 nM SAM (corresponds to SAM K_M) and 60 nM nucleosomes (in excess of nucleosome K_M). The assay buffer contained 20 mM Tris pH 8, 10 mM $MgCl_2$ and 0.01% Tween-20. Following a reaction time of 90 min (180 min for assay containing 0.05 nM Dot1L), the reaction was stopped/quenched by the addition of 5 µL 4.5 mg/mL Streptavidin SPA Beads (Perkin Elmer, RPNQ0006) diluted in 300 mM MES (pH 6) and 600 µM SAM (A7007, Sigma). Plates were centrifuged and read on a Topcount NXT HTS (Packard) and IC50 data was analysed using XLFit 5.2 (idbs). K_i values were derived applying the Morrison tight binding model for competitive inhibition in Graphpad Prism 5.0.[1] For this, IC_{50} values were determined at a range of Dot1L concentrations (from 3

nM to 0.2 nM). Active enzyme concentration ($[E]_T$) was derived from the slope (m) of IC_{50} vs nominal Dot1L concentration ($[E]_{app}$) using equation $[E]_T = 2m[E]_{app}$. K_i values were then derived using a global fit of the data.

Dot1L surface plasmon resonance assay (SPR)

Ligand association and dissociation rates were determined by surface plasmon resonance (SPR) using a Proteon XPR36 (Biorad). Biotinylated hDot1L (2-416)-Avi was immobilized on SA (GE Healthcare) or GLM (Biorad) sensorchip to 5000-8000 RU. Neutravidin was immobilized to the GLM sensor chips through amine coupling prior to use. A reference channel of neutravidin only was also generated. The running buffer contained 25 mM HEPES, 100 mM NaCl, 0.05% Tween-20, 1 mM TCEP, 1% DMSO, pH 8. Experiments were carried out at 20 °C at a flow rate of 100 µL min^{-1}. SAH (Sigma A9384) was used as internal control and was tested at 5 concentrations in 2.67 fold serial dilutions within range stated in Table S1. For SAH an association time of 60 s and a dissociation time of 120 s was used. High affinity compounds (expected extremely long residence times) were tested at a single concentration of 100 nM with an association time of 245 s and a dissociation time of 14400 s (4 h). Curve fitting was performed using the Proteon Manager software. Data was fit to the 1:1 Langmuir model.

Figure S1: Kinetic analysis of binding to immobilized hDot1L. Representative sensorgrams for reference compound SAH (A) and compound **7** (B) are shown. For compound **7** essentially no dissociation was observed over the measurement time resulting in an estimate for τof > 5 h.

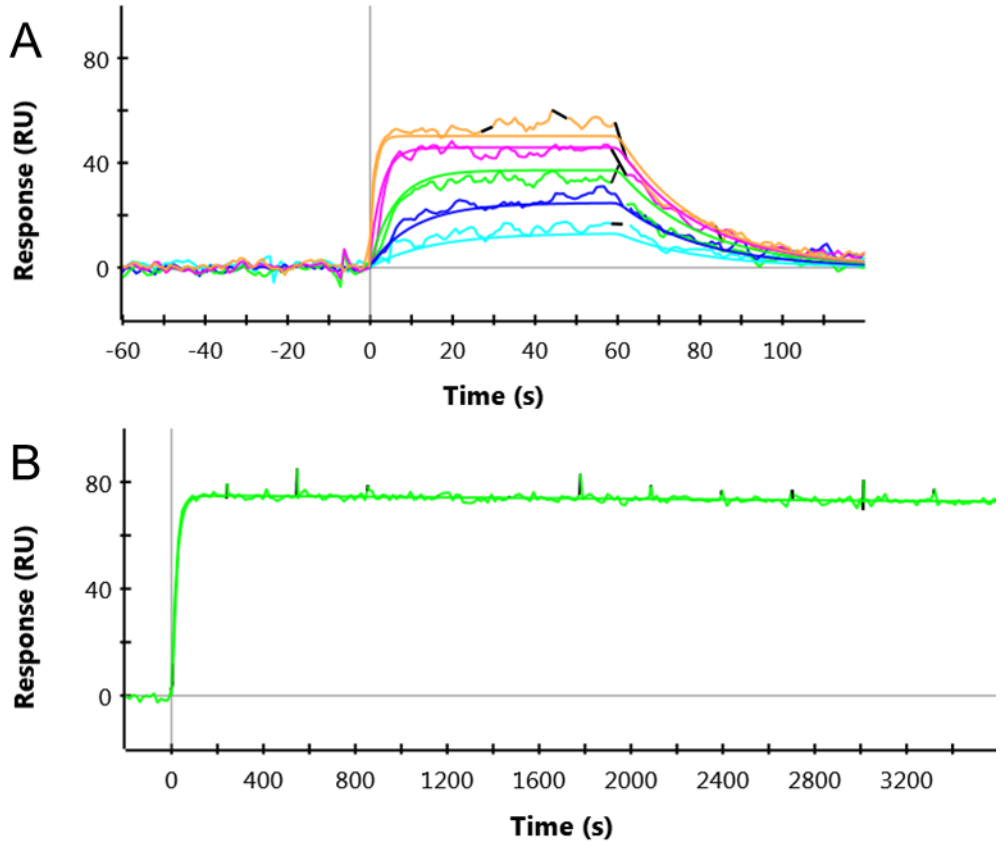


Table S1: Kinetic data representing an average of 3 independent experiments

Compound	k_{on} (1/Ms)	k_{on} SD	k_{off} (1/s)	k_{off} SD	τ (min)	K_d (M)	K_d SD	conc. range
SAH	5.0E+05	2.6E+04	6.9E-02	1.4E-02	0.2	1.4E-7	2.4E-8	36 nM to 1800 nM
7	-	-		-	>300	-	-	100 nM

H3K79me2 ELISA assay in HeLa cells

HeLa cells were seeded (4,000 cells/well) in 96-well cell-culture plates the day before compounds were applied using the HP Digital Dispenser (Tecan, HP D300). 72 hours after compound treatment the media was removed from the plates and wells were rinsed with PBS. PBS was discarded and the cells were lysed with 100 µL/well 0.5 M HCl and incubated on ice for 30 minutes, while shaking. Neutralization buffer (100 µL/well of 0.5 M HNa_2O_4P, 2 mM PMSF, 2.5 mM DTT; pH 11.3) was added to the lysates and mixed. Neutralized lysates (14 µL/well) were transferred to capture plates (NUNC Maxisorp #427111) containing 180 µL/well PBS and incubated overnight at 4 °C. After lysate adsorption, plates were washed with once 300 µL/well TBST (TBS +0.1% Triton X-100) and blocked with 300 µL/well TBST + 3% BSA (Sigma A-7030) for one hour at room temperature. Plates were washed three times with 300 µL/well TBST before incubation with 200 µL/well primary antibody (Anti-H3K79me2, Millipore #04-835; 1:375 dilution in TBST) for 48 h at 4 °C. After incubation plates were washed three times with 300 µL/well TBST and incubated with 200 µL/well secondary antibody (anti-rabbit-HRP from GE Healthcare UK Limited, #NA934V; 1:3000 dilution in TBST) for one hour at room temperature. Before incubation with 200 µL/well ECL reagent (SuperSignal ELISA Pico Chemiluminescent Substrate; Thermo #37069), plates were washed five times with 300 µL/well TBST. Signal detection was performed on a Tecan Synergy HT plate reader. IC_{50}'s were calculated using XLfit. DMSO treated wells were used to define 0% inhibition and 100% inhibition was defined as wells treated with 3 µM EPZ-5676.

HoxA9 RGA assay in Molm-13 cells

MOLM-13 cells were stably transfected with pGL4.17-HOXA9-(-5K~0)-luc, a luciferase sensor under the control of the HoxA9 promoter, and maintained in the presence of 500 µg/mL G418. For the RGA assay, cells were seeded in 96-well plates (10,000 cells/well; Costar #3610) immediately before compounds were applied using the HP Digital Dispenser (Tecan, HP D300) to duplicate plates and incubated for 72 hours. Following compound treatment, one set of duplicate plates was used to determine cell growth effects using resazurin (10 µL/well of resazurin; 130mg/L in PBS, 4 hours). Luciferase signal was measured on the second set of plates using the Bright-Glo Luciferase Assay System (100 µL/well; Promega #E2650). Fluorescent and luminescence signal detection was performed on a Tecan Synergy HT plate reader. To account for possible growth effects, the luciferase signal was normalized to the corresponding resazurin signal. IC_{50}'s were calculated using XLfit. DMSO treated wells were used to define 0% inhibition and 100% Inhibition was defined as wells treated with 3 µM EPZ-5676.

MV4-11 proliferation assay

In order to investigate the antiproliferative activity of the DotL1 inhibitors, 8-day proliferation assays using MV4-11 cells were performed. Cells were seeded in 96-well black/clear bottom plates (Costar #3904) at a density of 1'000 cells/well and a volume of 150 µL/well. The compound treatment was performed 6 hours post seeding on the same day, using a HP D300 Digital non-contact dispenser (Hewlett-Packard Development Company, L.P.). For each compound a 9-point serial dilution (1:3, vol/vol) in triplicates was performed, using final 0 CellTiter-Glo readout (Promega #G7573) acquired on a Mithras LB940 multimode plate reader (Berthold Technologies). IC_{50}'s were determined by four-parameter curve fitting using the in-house developed analysis software.

Experimental procedure for the generation of the Dot1L cocrystal structures

Dot1L crystals were grown at 20 °C using the hanging drop vapor diffusion method. Purified Dot1L methyltransferase domain (aa2–aa332) at 8 mg/ml in 20 mM Tris pH 8.0, 200 mM NaCl, 1 mM TCEP, 1 mM EDTA was pre-incubated with inhibitor compounds at final concentrations between 1-2 mM resulting in a DMSO concentration of 4-10%. For crystallization the protein inhibitor complex was mixed with an equal volume of a reservoir solution. Seeding was applied prior to sealing the setups. Seed stocks were made out of previously obtained Dot1L crystals. Three different reservoir conditions have been identified yielding Dot1L co-crystals. Condition one is 1.0-1.6 M potassium sodium tartrate tetrahydrate, 0.1 M Hepes pH 6.6-7.2. The second condition is 1.3-1.6 M lithium sulfate monohydrate, 0.1 M Hepes or Tris pH 7.0-8.5. Finally, the third reservoir composition is 0.9–1.4 M lithium sulfate monohydrate, 0.1-0.3 M ammonium sulfate, 0.1 M sodium citrate tribasic dihydrate. Prior to flash cooling the crystals in liquid nitrogen, they were rapidly soaked in reservoir solution containing 20-25% ethylene glycol. In some cases inhibitor compounds were added to the cryo buffer as well. During data acquisition, the crystal temperature was kept at 100 K. Diffraction data were collected at the Swiss Light Source (beamline X10SA) using a Pilatus pixel detector with an incident monochromatic X-ray beam. Raw diffraction data were processed and scaled using XDS/XSCALE[2] software. The complex structures were determined by molecular replacement with PHASER[3] using as search model the coordinates of Dot1L (PDB code 1NW3)[4] or other previously solved in-house structures of Dot1L. The program BUSTER[5] was used for full structure refinement. The refined coordinates of the complex structures have been deposited in the RCSB Protein Data Bank. All figures below were prepared using Pymol[6].

Table S2: Crystallographic data collection and refinement statistics

	ADO + CPD1	CPD1+CPD2	CPD7
Data collection*			
Space group	$P6_3$	$P6_3$	$P6_3$
Cell dimensions			
a, *b*, *c* (Å)	158.2, 158.2, 73.8	158.5, 158.5, 74.1	158.4, 158.4, 74.6
Resolution (Å)	2.18 (2.24-2.18)**	2.09 (2.14-2.09)	2.19 (2.25-2.19)
R_{sym} (%)	10.4 (110.4)	11.6 (109.6)	9.7 (121.1)
I/*sigI*	18.0 (2.5)	15.5 (2.5)	15.4 (2.1)

Completeness (%)	99.8 (97.7)	99.9 (99.4)	99.9 (99.2)
Redundancy	10.1 (8.6)	10.2 (9.6)	10.3 (9.5)
Refinement[***]			
Resolution (Å)	2.18	2.09	2.19
No. reflections	54951	62898	54967
R_{work}/ R_{free} (%)	16.9 / 18.7	17.5 / 19.3	18.4 / 20.2
R.m.s deviations			
Bond lengths (Å)	0.010	0.010	0.010
Bond angles (º)	0.97	0.94	1.01
PDB ID	5MVS	5MW3	5MW4

*Values as defined in XDS/Xscale[2]
**Highest resolution shell is shown in parenthesis
***Values as defined in BUSTER[5]

Figure S2: Unbiased difference electron density contoured at 3.5 sigma for 5MVS.pdb. The final refined models of ADO and CPD1 are superimposed.

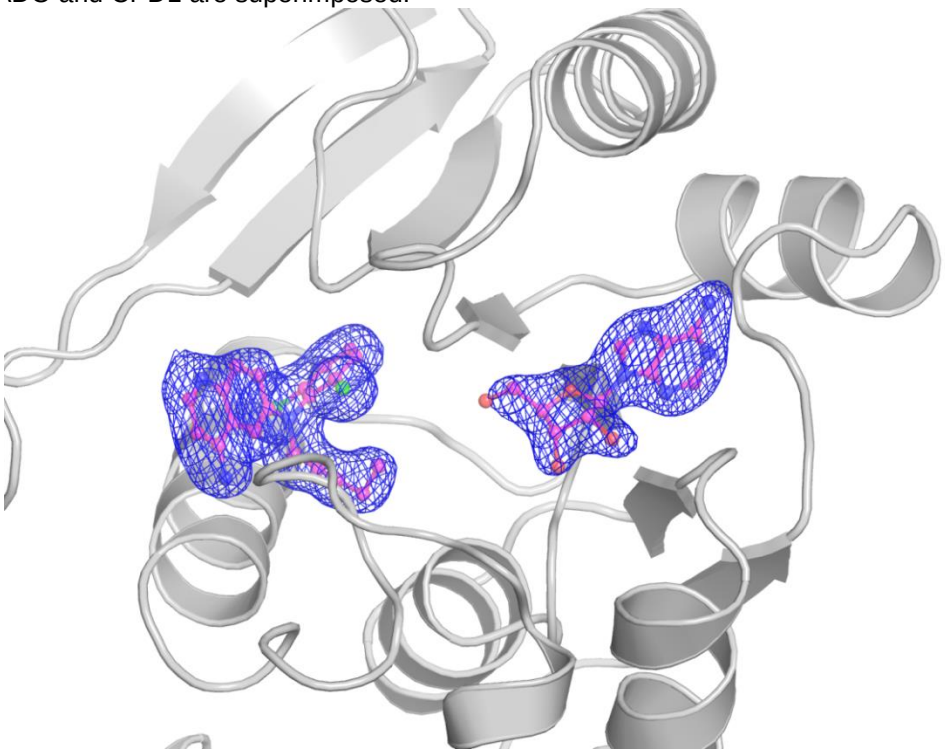


Figure S3: Unbiased difference electron density contoured at 3.7 sigma for 5MW3.pdb. The final refined models of CPD1 and CPD2 are superimposed.

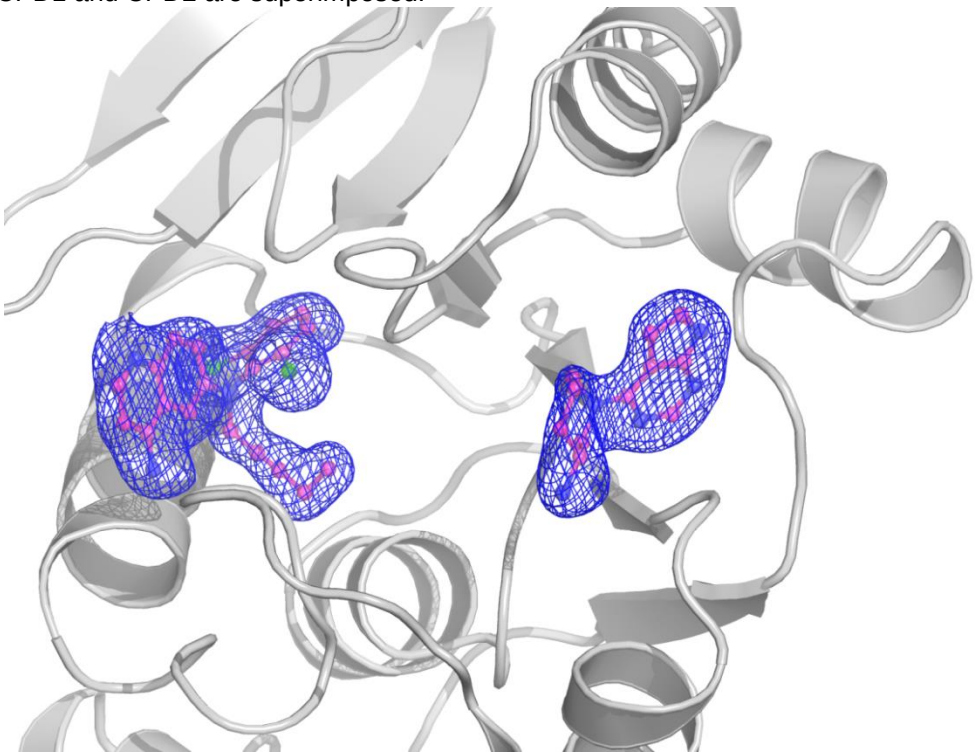
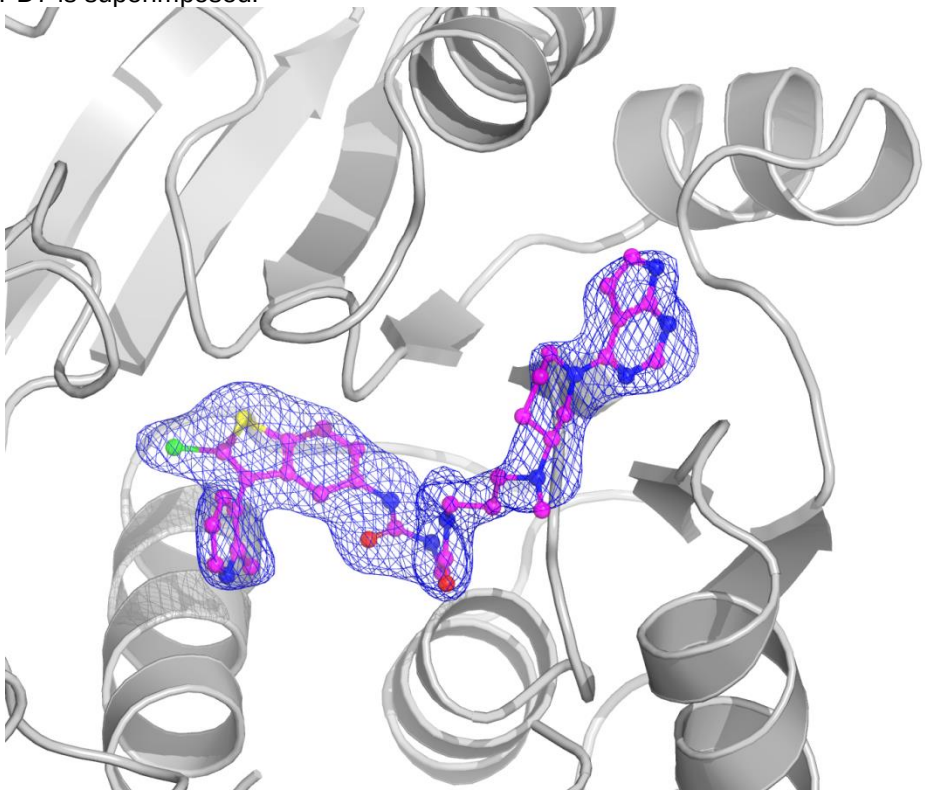


Figure S4: Unbiased difference electron density contoured at 3.3 sigma for 5MW4.pdb. The final refined model of CPD7 is superimposed.



HMT selectivity panel for compound **7**

Enzyme assay	Compound 7 IC_{50} (μM)	Assay with enzyme complex
EZH2/PRC2	>100	EZH2/SUZ12/EED/AEBP2/RbAP48
EZH1/PRC2	>100	EZH1/SUZ12/EED/AEBP2/RbAP48
MLL	>100	MLL/WDR5/RBBP5/ASH2L
SETD6	>100	
SMYD3	>100	
SMYD2	>100	
Set7/9	>100	
SetD8	>100	
Suv39H2	>100	
G9a	>100	
ESET	>100	
NSD2	>100	
NSD1	>100	
NSD3	>100	
SETD2	>100	
Dot1L	<0.046	
PRMT1	>100	
PRMT3	>100	
CARM1	>100	
PRMT5/MEP50	>100	
PRMT8	>100	
DNMT1	>100	

All HMT reactions were performed as described previously.[7]

References

(1) Copeland, R. A. Evaluation of enzyme inhibitors in drug discovery: A guide for medicinal chemists and pharmacologists. Wiley 2005, ISBN-13: 978-1118488133.

(2) Kabsch, W. XDS. *Acta Cryst.* **2005***, D66*, 125-132.

(3) McCoy, A.J.; Grosse-Kunstleve, R.W.; Adams, P.D.; Winn, M.D.; Storoni, L.C.; Read, R.J. Phaser crystallographic software. *J. Appl. Crystallogr.* **2007**, *40*, 658-674.

(4) Min, J.; Feng, Q; Li, Z.; Zhang, Y.; Xu, R.M. Structure of the catalytic domain of human DOT1L, a non-SET domain nucleosomal histone methyltransferase. *Cell* **2003**, *112*, 711-723.

(5) Bricogne, G.; Blanc, E.; Brandl, M.; Flensburg, C.; Keller, P.; Paciorek, W.; Roversi, P.; Sharff, A.; Smart, O.S.; Vonrhein, C.; Womack, T.O. BUSTER version 2.11.4. Cambridge, United Kingdom: Global Phasing Ltd. **2011**.

(6) The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.

(7) Qi, W.; Chan, H.; Teng, L.; Li, L.; Chuai, S.; Zhang, R.; Zeng, J.; Li, M.; Fan, H.; Lin, Y.; Gu, J.; Ardayfio, O.; Zhang, J. H.; Yan, X.; Fang, J.; Mi, Y.; Zhang, M.; Zhou, T.; Feng, G.; Chen, Z.; Li, G. Yang, T.; Zhao, K.; Liu, X.; Yu, Z.; Lu, C. X.; Atadja, P.; Li, E. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 21360-21365.