Discovery of Novel Dot1L Inhibitors through a Structure-Based Fragmentation Approach

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Supporting Information

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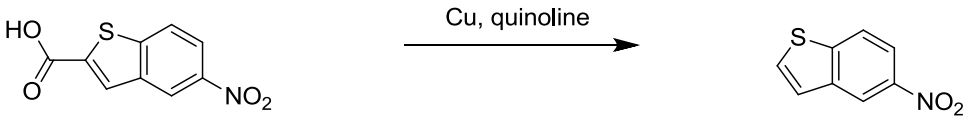
Experimental procedure for the synthesis of **12**

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 5 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 a 100x30 mm Waters Sunfire Prep C18 OBD™ 5 µm column with a flow of 30 mL/min and a solvent gradient of 5 to 100% B over 20 min, followed by 3 min at 100% B; solvent A: water with 0.1% trifluoroacetic acid, solvent B: acetonitrile.



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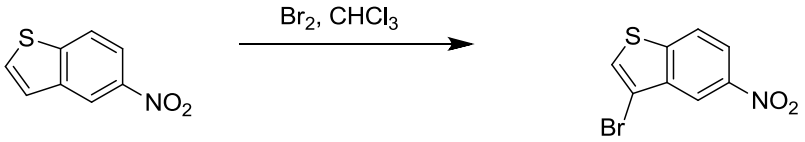
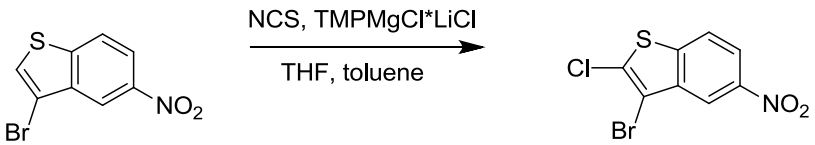
5-Nitrobenzo[b]thiophene. 5-Nitrobenzo[b]thiophene-2-carboxylic acid (**14**) (3.0 g, 13.31 mmol) was added to a mixture of Cu (0.846 g, 13.31 mmol) and Quinoline (50 mL) under N_2-atmosphere. After stirring for 2 h at 150 °C, the reaction mixture was diluted with 50 mL ethyl acetate and filtered. The filtrate was poured on ice and acidified with conc. HCl. Ethyl acetate 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 yield 5-nitrobenzo[b]thiophene 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 t_{ret} = 1.05 min.

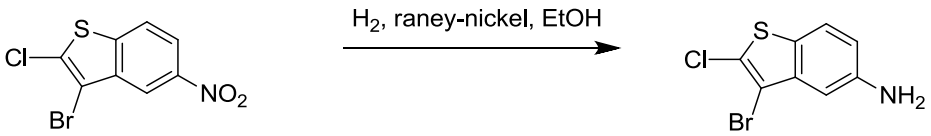


3-Bromo-5-nitrobenzo[b]thiophene. 5-Nitrobenzo[b]thiophene (1.94 g, 10.72 mmol) in chloroform (45 mL) was treated with bromine (0.773 mL, 15.01 mmol) and the mixture was stirred for 30 h at 60 °C. Then the solvent was evaporated, the residue was suspended in 6 mL ethyl acetate and 6 mL hexane and the crystals were filtered off to give 3-bromo-5-nitrobenzo[b]thiophene 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 t_{ret} = 1.18 min.



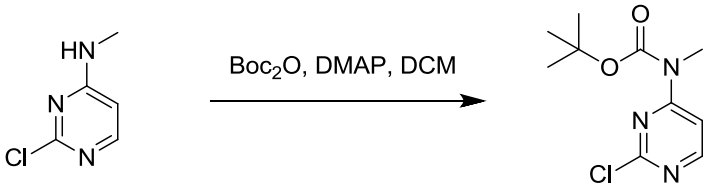
15

3-Bromo-2-chloro-5-nitrobenzo[b]thiophene (15). 3-Bromo-5-nitrobenzo[b]thiophene (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 TMPMgCl·LiCl in toluene / THF (215863-85-7) (7.59 mL, 7.59 mmol) was added dropwise in 10 min. After stirring for further 30 min at -75 °C, *N*-chlorosuccinimide (1.04 g, 7.59 mmol) in 5 mL THF was added dropwise. The reaction mixture was stirred for further 10 min at -75 °C, then was quenched with 10 mL NH_4Cl-solution and 10 mL H_2O and transferred to a separatory funnel with 40 mL H_2O and washed twice with 100 mL ethyl acetate. The organic layers were washed with 50 mL water and 50 mL brine, dried (Na_2SO_4) and evaporated. The crude product was purified with silica chromatography using 100% hexane to 100% TBME as eluent to yield 3-bromo-2-chloro-5-nitrobenzo[b]thiophene (**15**) 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 t_{ret} = 1.32 min.



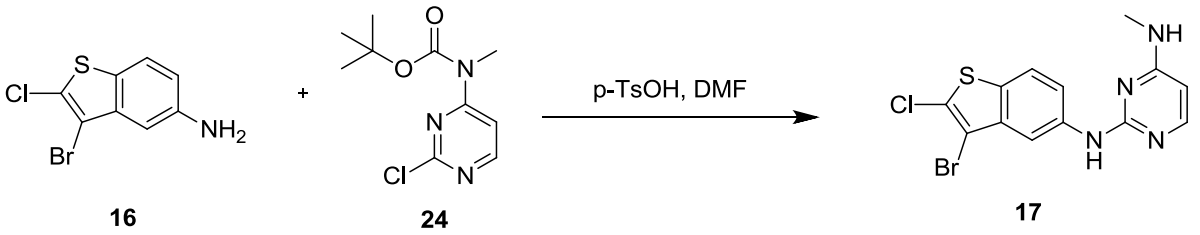
15 **16**

3-Bromo-2-chlorobenzo[b]thiophen-5-amine (16). 3-Bromo-2-chloro-5-nitrobenzo[b]thiophene (**15**) (700 mg, 1.91 mmol) was dissolved in EtOH (20 mL) and transferred to 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 (**16**) 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 t_{ret} = 1.13 min.

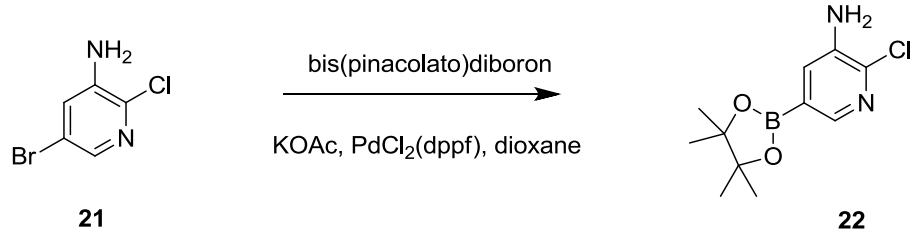


23 **24**

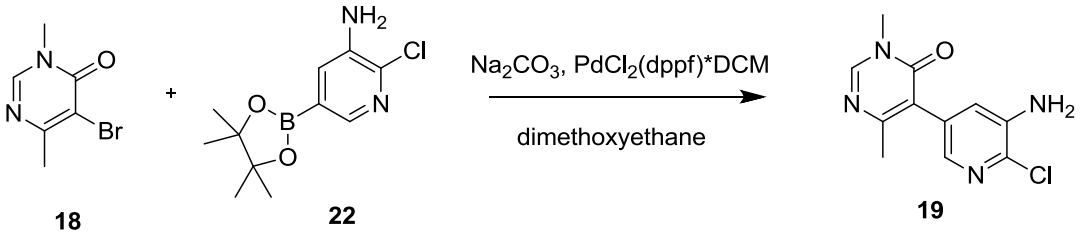
***Tert*-butyl (2-chloropyrimidin-4-yl)(methyl)carbamate (24).** 2-Chloro-*N*-methylpyrimidin-4-amine (**23**) (940 mg, 6.42 mmol) was given into a flask with DCM (30 mL), then Boc_2O (2.24 mL, 9.62 mmol) and DMAP (157 mg, 1.28 mmol) were added and the reaction mixture was stirred for 3 h at ambient temperature. Then the reaction mixture was evaporated and the residue was transferred into a separatory funnel with 50 mL ethyl acetate and 30 mL water. The aqueous layers were washed with 50 mL EtOAc. The organic layers were washed with 40 mL H_2O and 40 mL brine, dried (Na_2SO_4) and evaporated. The crude product was purified with silica chromatography using hexane /ethyl acetate as eluent (gradient of 0 to 10% ethyl acetate) to yield *tert*-butyl (2-chloropyrimidin-4-yl)(methyl)carbamate (**24**) in 98% yield. ^{1}H NMR (600 MHz, DMSO-d6) δ 8.51 (d, J = 6.0 Hz, 1H), 7.94 (d, J = 5.9 Hz, 1H), 3.32 (s, 3H), 1.51 (s, 9H). LC-MS t_{ret} = 1.17 min, m/z = 244.1/246.1 $[M+H]^+$.



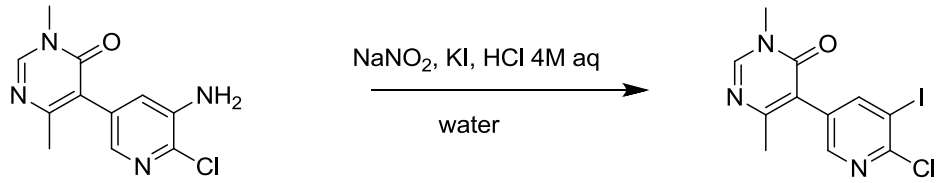
N2-(3-bromo-2-chlorobenzo[b]thiophen-5-yl)-N4-methylpyrimidine-2,4-diamine (17). To 3-bromo-2-chlorobenzo[b]thiophen-5-amine (**16**) in DMF (5 mL) was added *tert*-butyl (2-chloropyrimidin-4-yl)(methyl)carbamate (**24**) (223 mg, 0.914 mmol) and *p*-toluenesulfonic acid monohydrate (362 mg, 1.90 mmol) under Ar-atmosphere and the reaction mixture was stirred for 15 h at 80 °C. The reaction mixture was transferred to a separatory funnel with 20 mL $NaHCO_3$-solution and 20 mL H_2O. The aqueous layers were washed twice with 40 mL ethyl acetate. The organic layers were washed with 20 mL H_2O and 20 mL brine, dried (Na_2SO_4) and evaporated. The crude product was purified with silica chromatography using 100% hexane to 100% ethyl acetate as eluent to yield N2-(3-bromo-2-chlorobenzo[b]thiophen-5-yl)-N4-methylpyrimidine-2,4-diamine (**17**) in 66% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.30 (s, 1H), 8.70 (br, 1H), 7.80 (d, *J* = 8.8 Hz, 2H), 7.64 (br, 1H), 7.21 (br, 1H), 5.95 (d, *J* = 5.8 Hz, 1H), 2.92 (s, 3H). LC-MS t_{ret} = 0.94 min, m/z = 368.9/371.0 $[M+H]^+$.



2-Chloro-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-amine (22). 5-Bromo-2-chloropyridin-3-amine (**21**) (2.0 g, 9.54 mmol) was given into a flask under N_2-atmosphere with dioxane (40 mL) and KOAc (2.34 g, 23.86 mmol), then bis(pinacolato)diboron (3.15 g, 12.41 mmol) and $PdCl_2$(dppf) (0.419 g, 0.573 mmol) were added and the reaction mixture was stirred for 3 h at 100 °C. The reaction mixture was diluted with 60 mL ethyl acetate and 60 mL water and transferred to a separatory funnel. The aqueous layers were washed with 40 mL EtOAc. The organic layers were washed with 20 mL brine, dried (Na_2SO_4) and evaporated. The crude product was purified with silica chromatography using hexane/ethyl acetate as eluent (gradient 0-30% ethyl acetate) to yield 2-chloro-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-amine (**22**) in 80% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 7.73 (d, J = 1.6 Hz, 1H), 7.37 (d, J = 1.9 Hz, 1H), 5.56 (s, 2H), 1.27 (s, 12H). LC-MS t_{ret} = 1.00 min, m/z = 255.1 $[M+H]^+$.



5-(5-Amino-6-chloropyridin-3-yl)-3,6-dimethylpyrimidin-4(3H)-one (19). 5-Bromo-3,6-dimethylpyrimidin-4(3H)-one (**18**) (667 mg, 3.25 mmol) was given into a vial with DME (12 mL) under Ar-atmosphere, then Na_2CO_3 aq. (4.07 mL, 8.13 mmol), 2-chloro-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-amine (**22**) (900 mg, 3.25 mmol) and $PdCl_2$(dppf).DCM adduct (159 mg, 0.195 mmol) were added and the reaction mixture was heated to 80 °C for 1 h. The resulting suspension was filtered and filtrate was transferred to a separatory funnel with 40 mL ethyl acetate and 40 mL water. The aqueous layers were washed with 40 mL ethyl acetate. The organic layers were washed with 20 mL brine, dried (Na_2SO_4) and evaporated. The crude product was purified with silica chromatography using hexane:EtOAc:MeOH 80:18:2 to 0:90:10 as eluent to yield 5-(5-amino-6-chloropyridin-3-yl)-3,6-dimethylpyrimidin-4(3H)-one (**19**) in 68% yield. ^{1}H NMR (600 MHz, DMSO-d6) δ 8.41 (s, 1H), 7.45 (d, J = 2.1 Hz, 1H), 7.01 (d, J = 2.1 Hz, 1H), 5.60 (s, 2H), 3.40 (s, 3H), 2.09 (s, 3H). LC-MS t_{ret} = 0.50 min, m/z = 251.1/253.1 $[M+H]^+$.



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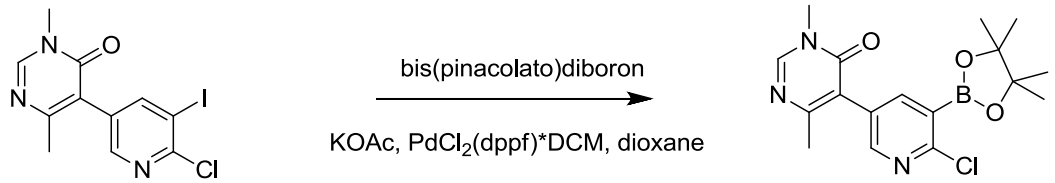
6'-Chloro-5'-iodo-1,4-dimethyl-[3,3'-bipyridin]-2(1H)-one. 5-(5-Amino-6-chloropyridin-3-yl)-3,6-dimethylpyrimidin-4(3H)-one (**19**) (310 mg, 1.22 mmol) was given into a flask with HCl 4 M aq (6 mL), then the reaction mixture was cooled down to 5 °C and $NaNO_2$ (101 mg, 1.460 mmol) in water (3 mL) was added dropwise. After stirring for 10 min at 5 °C, KI (283 mg, 1.70 mmol) in water (3 mL) was added dropwise at 5 °C and the suspension was stirred for 10 min at 5 °C, then the suspension was allowed to warm to ambient temperature. The suspension was transferred to a separatory funnel with 120 mL ethyl acetate, 50 mL water and 50 mL Na_2CO_3-solution. The aqueous layers were washed with 100 mL ethyl acetate. The organic layers were washed with 60 mL brine, dried (Na_2SO_4) and evaporated. The crude product was purified with silica chromatography using hexane:EtOAc:MeOH 80:18:2 to 0:90:10 as eluent to yield 6'-chloro-5'-iodo-1,4-dimethyl-[3,3'-bipyridin]-2(1H)-one in 36% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.44 (s, 1H), 8.36 – 8.27 (m, 2H), 3.41 (s, 3H), 2.12 (s, 3H). LC-MS t_{ret} = 0.82 min, m/z = 360.9/362.9 $[M+H]^+$.



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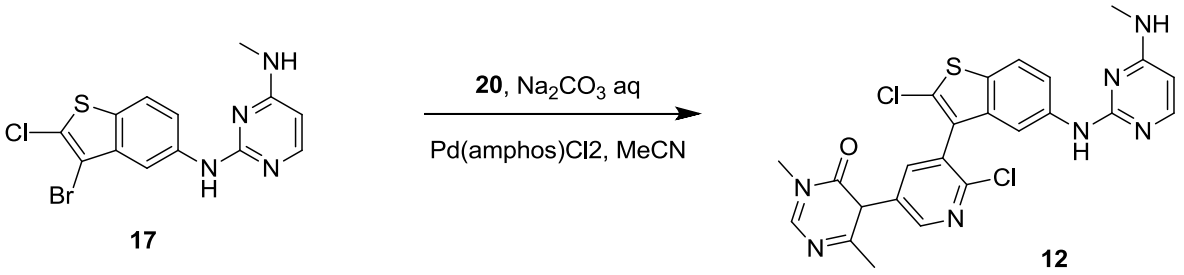
5-(6-Chloro-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)-3,6-dimethylpyrimidin-4(3H)-one (20). 6'-Chloro-5'-iodo-1,4-dimethyl-[3,3'-bipyridin]-2(1H)-one (540 mg, 1.449 mmol) was given into an argon-flushed vial with dioxane (15 mL) and KOAc (498 mg, 5.07 mmol), then bis(pinacolato)diboron (736 mg, 2.90 mmol) and $PdCl_2$(dppf)·DCM adduct (177 mg, 0.217 mmol) were added and the suspension was stirred for 10 h at 110 °C. The reaction mixture was filtered and the filtrate was evaporated. The crude

product was purified with silica chromatography using hexane/ethyl acetate as eluent (gradient 30-100% EtOAc) to yield 5-(6-chloro-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)-3,6-dimethylpyrimidin-4(3H)-one (**20**) in 49% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.42 (s, 1H), 8.35 (d, J = 2.5 Hz, 1H), 7.91 (d, J = 2.4 Hz, 1H), 3.38 (d, J = 19.4 Hz, 3H), 2.10 (d, J = 4.5 Hz, 3H), 1.30 (s, 12H). LC-MS t_{ret} = 0.43 min, m/z = 280.3 $[M+H]^+$ (boronic acid).



5-(6-Chloro-5-(2-chloro-5-((4-(methylamino)pyrimidin-2-yl)amino)benzo[b]thiophen-3-yl)pyridin-3-yl)-3,6-dimethylpyrimidin-4(3H)-one (12). To N2-(3-bromo-2-chlorobenzo[b]thiophen-5-yl)-N4-methylpyrimidine-2,4-diamine (**17**) (25 mg, 0.064 mmol) in an Ar-flushed vial was added MeCN (1.0 mL), Na_2CO_3 aq (0.080 mL, 0.161 mmol), 5-(6-chloro-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)-3,6-dimethylpyrimidin-4(3H)-one (**20**) (58 mg, 0.064 mmol) and Pd(amphos)Cl_2 (CAS 887919-35-9) (4.55 mg, 6.42 µmol), then the reaction mixture was heated to 80 °C for 30 min. The resulting suspension was filtered and evaporated. The crude product was purified with silica chromatography using hexane:EtOAc:MeOH 90:9:1 -> 0:90:10 as eluent. Because the product was only ~90% pure, it was further purified on a Gilson prepHPLC; The product-containing fraction was adsorbed on a PoraPak® 6cc cartridge from Waters. After washing the column with 10 mL MeOH, the product was eluted with 10 mL MeOH:NH_3 (7 M in MeOH) 9:1 and the filtrate was evaporated to yield 5-(6-chloro-5-(2-chloro-5-((4-(methylamino)pyrimidin-2-yl)amino)benzo[b]thiophen-3-yl)pyridin-3-yl)-3,6-dimethylpyrimidin-4(3H)-one (**12**) in 25% yield. ^{1}H NMR (600 MHz, DMSO-d6) δ 9.25 (s, 1H), 8.53 (d, J = 2.3 Hz, 1H), 8.45 (s, 2H), 7.96 (d, J= 2.3 Hz, 1H), 7.82 (d, J = 8.8 Hz, 1H), 7.72 (d, J = 15.4 Hz, 1H), 7.51 (s, 1H), 7.11 (s, 1H), 5.85 (d, J = 5.8 Hz, 1H), 3.43 (s, 3H), 2.36 (d, J = 20.2 Hz, 3H), 2.19 (s, 3H). LC-MS t_{ret} = 0.86 min, m/z = 524.1/526.1 $[M+H]^+$.

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. 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 BiacoreTM T100 (GE Healthcare) or 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. 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 resulting in K_D 100 nM under these conditions. Curve fitting was performed using the Biacore T100 Evaluation or Proteon Manager software.

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 starting concentrations of either 10 µM or 3 µM. Plates were incubated for 8 days at 37 °C, 5% CO_2 and were imaged daily for well confluence by a Clone Select Imager (Molecular Devices, Sunnyvale CA, US) to control for cell morphology and density. 8 days post treatment cell viability was assessed by quantification of cellular ATP using the luminescent 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.

PAMPA data of **3** and **12**, rat PK of **12**

PAMPA: The HDM-PAMPA assay was done according to Wohnsland and Faller but using a modified protocol LC-MS/MS to quantify.[6]

PAMPA	logPAMPA [cm/s]	Calculated FA [%]	$logP_e$ pH4 [cm/s]	$logP_e$ pH6.8 [cm/s]	$logP_e$ pH8 [cm/s]	permeability classification
3	-5.5	19	<-6.5	<-6.5	<-6.5	low
12	-4.5	88	<-5.9	-5.1	-4.6	high

logPAMPA: Overall highest permeability (sum of trans and paracellular contribution)
FA: Calculated fraction absorbed
P_e: Effective permeability calculated from the measured concentrations at different pH
High permeability: Calculated FA ≥ 75%, corresponding to logPAMPA ≥-4.8
Medium permeability: Calculated FA ≥ 35%, but <75%, corresponding to logPAMPA ≥-5.3 but <-4.8
Low permeability: Caculated FA <35%, corresponding to logPAMPA <-5.3

Pharmacokinetic: Male Sprague Dawley rats were obtained from Charles River Laboratories (Iffa Credo, L'Arbesque, France). The animals were housed under controlled environmental conditions (ambient temperature 21 °C, humidity 60%, 12 h light/dark cycle) with ad libitum access to standard food (NAFAG pellets 890; Provimi Kliba AG, Kaiseraugst, Switzerland) and tap water before dosing and during the entire experimentation period. All animal experimental procedures comply with Swiss animal welfare regulations and were approved by the Cantonal Veterinarian Office of Basel-City, Switzerland (Permit No. 1587 and 458). The body weight of the Sprague Dawley rats used in this study was on average 256 g.

Oral and intravenous pharmacokinetic studies were performed with three male rats for each route of administration. The animals were weighed before treatment and the dose was calculated for each rat. For the oral dose (15 mg/kg), **12** was suspended in MEPC5 / water (10:90, v/v). The oral formulation was prepared the day before the administration, stirred overnight, then administered by gastric intubation (2.5 mL/kg) in the next morning. For the intravenous dose (1 mg/kg), **12** was dissolved freshly in a mixture of N-1-methylpyrrolidone (NMP)/polyethylenglycol 200 (30:70, v/v) and administered into the surgically exposed jugular vein (0.5 mL/kg). Blood samples were collected from the second surgically exposed jugular vein into EDTA-coated Eppendorf tubes at 5 min (i.v. only), 15 min, 0.5, 1, 2, 4, 7, 24 and 48 h post-dose. For blood sampling, the awake rats were gently manipulated and restrained (i.e. using a bag or a blanket for the immobilization). Immediately after collection, these whole blood samples were frozen on dry-ice and then stored at −20 °C until LC/MS/MS analysis for parent drug determination.

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