SUPPORTING INFORMATION

Pharmacophore-Based Screening of Diamidine Small Molecule Inhibitors for Protein Arginine Methyltransferases

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Experimental procedures

Peptide synthesis. All peptide substrates were synthesized using Fmoc [N-(9- fluorenyl) methoxycarbonyl]-based peptide synthesis protocol on a FOCUS XC peptide synthesizer (aapptec, Louisville, KY). The peptide sequences are as following: H4-20-biotin Ac-SGRGKGGKGLGKGGAKRHRK(biotin), Biotin-H3-20 Biotin-ARTKQTARKSTGGKAPRKQL, SMD1-biotin Ac-AGRGRGRGRGRGRG-biotin. Each amino acid was coupled to the solid phase with 4 equiv of amino acid/HCTU [O-(1H-6-

chlorobenzotriazole-1-yl)-1,1,3,3- tetramethyluronium hexafluorophosphate] (Novabiochem or Chempep). The Fmoc group was deprotected with 20% v/v piperidine/DMF, and the N-terminal amino acid was acetylated with acetic anhydride. The peptide was cleaved from the Wang resin by a cleavage solution consisting of 95% trifluoroacetic acid (TFA), 2.5% H2O, and 2.5% triisopropylsilane. It was then precipitated in cold ether and pelleted by centrifugation. Crude peptides were collected and purified using a Shimazu liquid chromatography instrument equipped with a C18 reversed-phase high performance liquid chromatography (RP-HPLC) column, where 0.05% TFA containing water and 0.05% TFA-containing acetonitrile were two mobile phases used in gradient purification. The identity of peptides was confirmed with MALDI-MS.

Computational study. The library used for the ligand-based virtual screening was the NCI Diversity set, which contained 260,071 compounds. The confirmed active inhibitor for PRMT1, DB75, was chosen to initiate the screening procedure. Up to 50 conformers for library molecules were generated with Omega[1] (2.5.1.4, OpenEye Scientific Software). The 3D shape based virtual screening was performed with ROCS[2, 3] (Rapid Overlay of Chemical Structures, version 3.2.1.4, OpenEye Scientific Software). EON[2] (version 2.2.0.5, OpenEye, Scientific Software) was then used to assess the similarity of the top 1000 compounds to DB75 from ROCS. We subsequently selected a total of 830 compounds by merging of the top 500 hits from the ROCS and EON screen. 406 of the top 830 compounds were available and tested for further biochemical screening. The compound k313 were then docked into the previous reported PRMT1 homology structure by using AUTODOCK4.2.[4] The best docking pose from AUTODOCK bound to PRMT1 were chosen for further molecular dynamics (MD) study. All energy minimizations and MD simulations were performed with AMBER16 in explicit solvent

(TIP3P water).[5] GAFF parameters were assigned for the ligand,[6] and the AMBER Parm14SB parameter set[7] was applied for the protein. The systems were then gradually brought up to 300 K and run for 50 ps in the NVT ensemble while keeping the protein backbone restrained. The equilibration was continued for another 2 ns in the NPT ensemble, and the harmonic restraints were gradually released. 50ns production simulation was performed in the NPT ensemble (1 atm and 300 K) with smooth particle mesh Ewald (SPME) electrostatics, 10 Å non-bonded cut-off and 2-fs time step. The free energy of binding for K313 to PRMT1 was estimated by using the MM-PBSA method and normal-mode analyses for entropy as the average over the last 20 ns (2000 frames, 10 frames for entropy) from the trajectories.[8] The normal-mode analyses for entropy to calculate the binding free energy for the protein complexes. Normal-mode analysis was used to calculate entropy. The interaction energies were decomposed into contributions from the ligands and PRMT1 residue pairs. We clustered the last 20 ns of MD trajectory of the PRMT1-k313 complex. The dominant k313 structure from the clustering was used as a template and a ligand-based virtual screening was performed with the same protocol above. A total 89 of compounds were available and tested for further biochemical screening. CPPTRAJ module of AMBERTOOLS16[9] and VMD[10] were used for the analysis of trajectories and structural visualization.

High throughput screening (HTS) of small molecule libraries. Screening compounds were kept at 10 μM and 100 μM in the assay. The HTS by scintillation proximity assay (SPA) method was performed on 96-well plate format. The DMSO solution of the inhibitors were at 10 or 20 mM, then diluted with ddH_2O to 50 or 500 μM. Diluted inhibitor solutions (3 μL) were incubated with 9 μL mixture containing H4-20-Biotin peptide, [^{3}H]-SAM and 2X reaction buffer, then the enzyme (3 μL) was added to initiate the reaction. The reaction buffer contains 50

mM HEPES, 10 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT) at pH 8.0. The final concentrations of the enzyme, [^{3}H]-SAM, and H4-20-Biotin are 0.02, 0.5, and 1 μM, respectively. The concentrations of [^{3}H]-SAM, and biotinylated H4 peptide in the assay were kept at balanced condition (≈ Km) to indiscriminately identify competitive, uncompetitive and noncompetitive inhibitors. The mixtures were incubated at room temperature for 8 min before it was quenched with15 μL isopropanol. After mixing with 5 μL of 20 mg/mL streptavidin-coated SPA beads, the plates were incubated in the dark for 30 min, and detected by a Microbeta2 scintillation counter (Perkin Elmer). The positive control was carried out with the corresponding DMSO dilute surrogate, and the background control only contained [^{3}H]-SAM, H4-20-biotin peptide and DMSO. The reported data was based on the average of two experiments.

Biochemical selectivity assay. The single point screening and IC_{50} values of inhibitor hits over a panel of methyltransferases were determined. The reaction condition for each enzyme is listed in Table S1. The reaction time was controlled under initial rate conditions for reaction yields of less than 10%. The reaction buffer contains 50 mM HEPES pH 8.0, 10 mM NaCl, 0.5 mM EDTA, and 0.5 mM DTT for all the PRMTs. The reaction buffer contains Tris 50 mM pH 9.0, 5 mM $MgCl_2$, 4 mM DTT for lysine methyltransferases (G9a). The inhibitor solutions were made at 10 μM for single point screening or as a series of dilution for IC_{50} determination. For the methylation reaction using biotinylated peptides, the procedure was similar to the SPA method described above. The methylation reaction using protein substrates was carried out by radioactive filter plate assay in a 96-well format. Protein substrate and [^{3}H]-SAM were preincubated in the reaction buffer for 2 min prior to initiation of the methyl transfer reaction by adding the enzyme (30 μL total volume). The reaction was quenched by 10 μL of 30% TCA, followed by spotting the reaction mixture on a 96-well 0.2 μm filter plate (Multiscreen filter

plates, Millipore cat. Number: MSFBN6B10). Then 40 μL of 10% TCA was added for protein precipitation and the plate was incubated at 4 ºC for 20 min. After the plate were washed with 100 μL of 10% TCA for 4 times and 100 μL of 100% ethanol once, they were immersed in 50 μL of liquid scintillation mixture (Microscint PS, PerkinElmer). Scintillation counting was performed by a Microbeta2 to measure the amount of methylated products. The positive control was carried out with the corresponding DMSO dilute surrogate under the same condition, and the background control only contained [^{3}H]-SAM, protein substrate and DMSO. IC_{50} values were obtained by the quantification of formed product at various concentrations of inhibitors, and fit with equation 1. Relative activity of protein in the presence of the inhibitor is normalized to the value of product formation without inhibitor present, n is hill coefficient. The reported data was based on the average of two experiments.

$$Relative\ Activity = 1/(1 + ([Inhibitor]/IC50)^n) \quad (1)$$

Inhibition mode studies. Experimental conditions for the SAM or peptide competitions in a 30-μL total volume in 96-well plate format were similar to those for the IC_{50} experiments. K313 concentration was in two-fold dilutions from 10,000 to 312.5 nM. [^{3}H]-SAM was serially diluted three-fold a top concentration of 4.5 μM. Reactions were initiated by the addition of 20 nM enzyme and 1 μM peptide. For peptide titration assay, H4-20-biotin was serially diluted three-fold with a top concentration of 4.5 μM. Reactions were initiated by the addition of 20 nM enzyme and 0.5 μM [^{3}H]-SAM. Reactions were incubated at room temperature for 10 min, and then quenched by the addition of 30 μL per well of isopropanol.

Leukaemia cell studies. Cell viability was measured by CellTiter-Glo viability kit (Promega, Madison WI). 1,000 cells were seeded in individual wells of 96-well plates with 100 μL culture volume per well. All these leukemia cell lines were grown in RPMI medium supplemented with 10% fetal bovine serum. Indicated concentrations of K313 or the same amount of DMSO were

added to the culture. At 72 and 96 h after drug treatment, 100 μL of CellTiter-Glo reagent was added to each well. Luminescence signals, which are proportional to the viable cell numbers in each well, were measured by microplate reader (Biotek, Winooski, VT). For the detection of asymmetric dimethylation level in cells, 4 × 105 cells were cultured with indicated concentrations of K313 or DMSO. Cells extracts were harvested after 24 h of treatment and resolved by SDS−PAGE. Total protein arginine methylation level was determined by Western blotting of Asym 24 (Millipore,Germany) antibody.

Table S1. Reaction conditions for the methyltransferase panel.

Protein	Substrate	Cofactor	Reaction time
PRMT1, 20 nM	H4-20-biotin, 1 uM	SAM, 0.5 uM	8 min, RT
PRMT3, 20 nM	SMD1-biotin, 1 uM	SAM, 0.5 uM	10 min, RT
CARM1, 20 nM	H3.3 protein, 0.4 uM	SAM, 0.5 uM	120 min, RT
PRMT5, 20 nM	H4-20-biotin, 1 uM	SAM, 0.5 uM	8 min, RT
PRMT6, 40 nM	SMD1-biotin, 1 uM	SAM, 0.5 uM	30 min, RT
PRMT7, 40 nM	SMD1-biotin, 1 uM	SAM, 0.5 uM	120 min, RT
PRMT8, 20 nM	H4-20-biotin, 1 uM	SAM, 0.5 uM	8 min, RT
G9a, 20 nM	Biotin-H3-20, 1 uM	SAM, 0.5 uM	30 min, RT

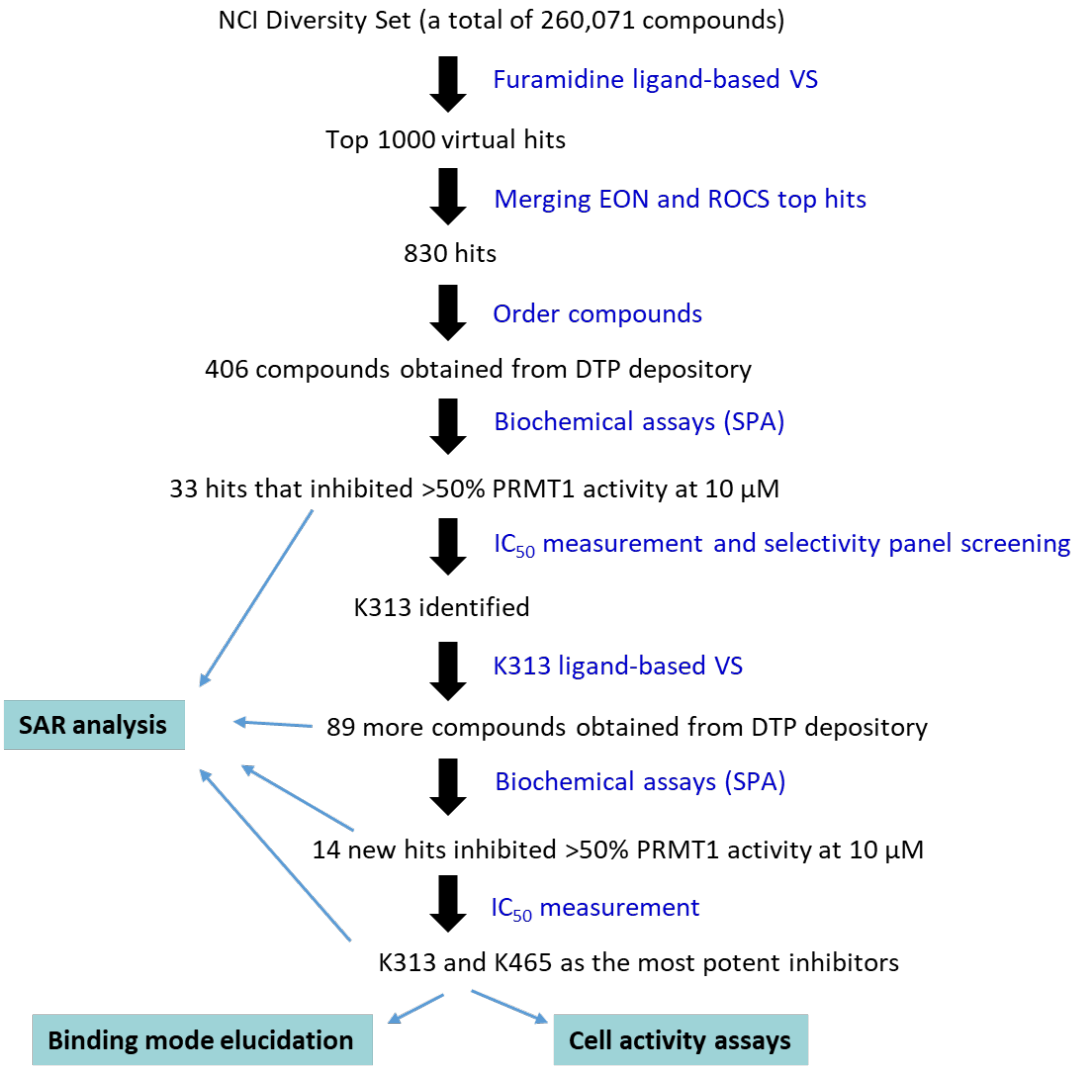


Figure S1. Flow chart of PRMT1 inhibitor discovery.

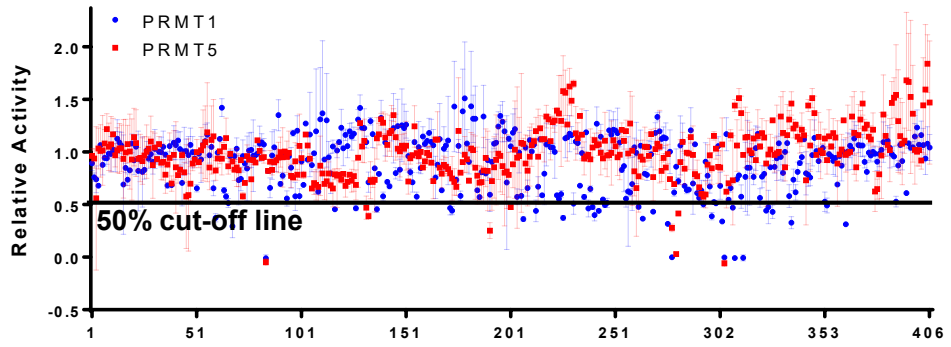


Figure S2. Biochemical activity screening of the 406 hits from virtual screening against PRMT1 and PRMT5 tested at single concentration 10 µM. The final concentrations of the enzyme, [3H]-SAM, and H4-20-Biotin are 0.02, 0.5, and 1 µM, respectively.

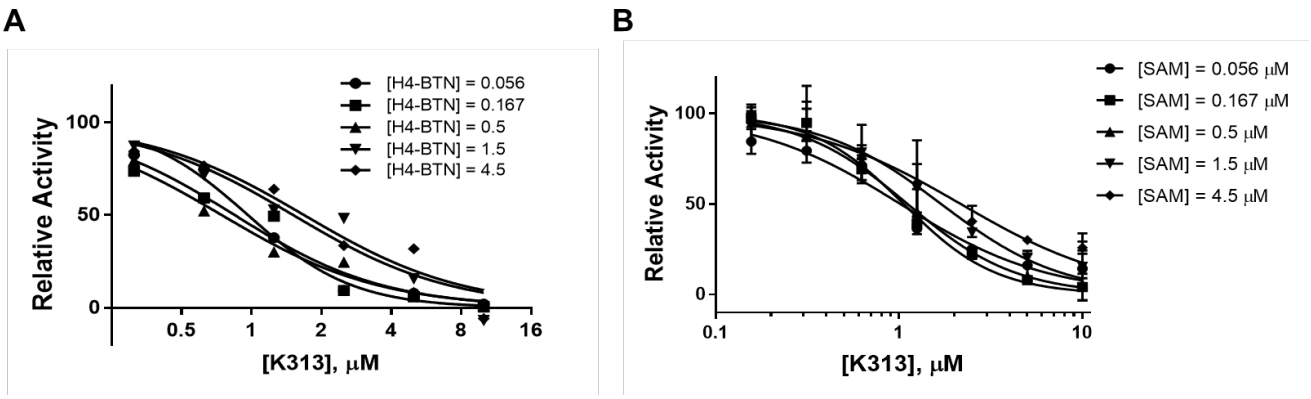
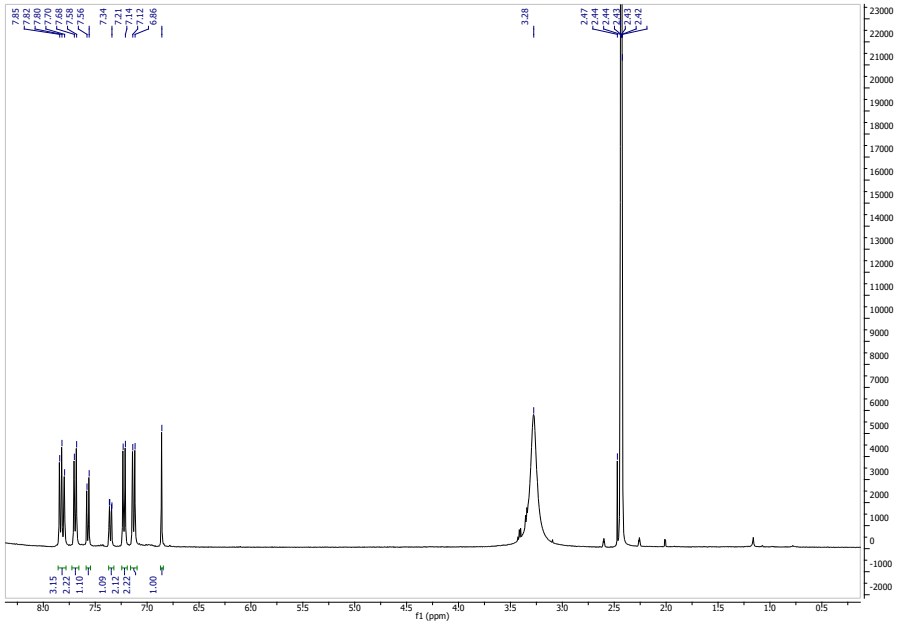


Figure S3. Dose-dependent inhibition curves of K313 at various concentrations (0.3125 µM - 4.5 µM) of the substrate peptide (A) or cofactor SAM (B), at [PRMT1] = 20 nM.



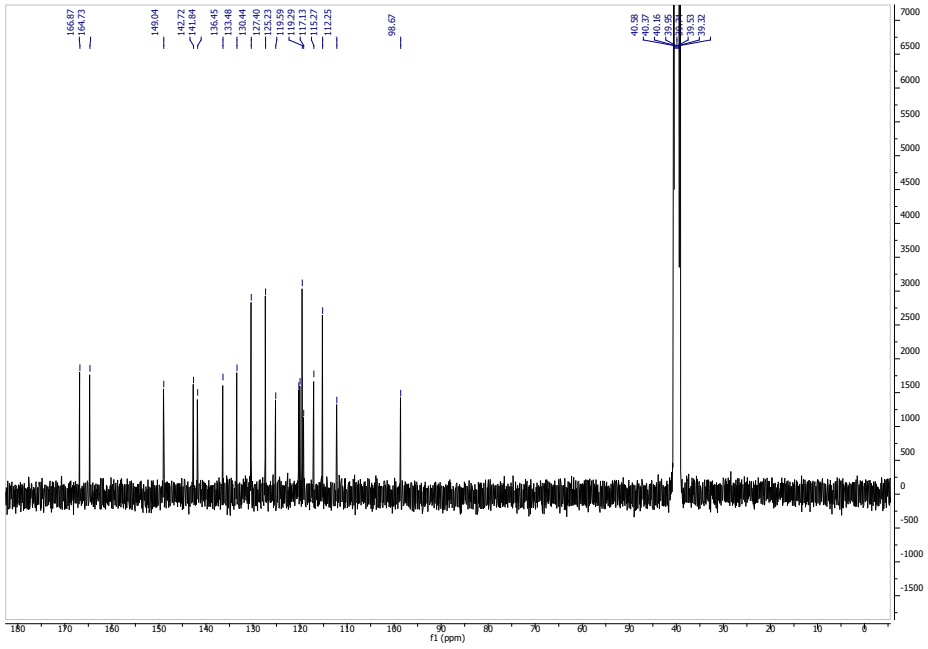


Figure S4. ^{1}H-NMR and ^{13}C-NMR spectra of K313.

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