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Novel Inhibitors for PRMT1 Discovered by High-Throughput Screening Using Activity-Based Fluorescence Polarization

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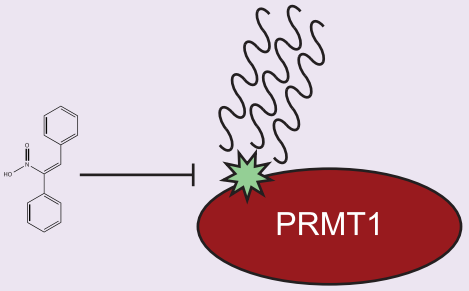
Supporting Information

ABSTRACT: Protein arginine methyltransferases (PRMTs) catalyze the posttranslational methylation of arginine using *S*-adenosylmethionine (SAM) as a methyl-donor. The PRMT family is widely expressed and has been implicated in biological functions such as RNA splicing, transcriptional control, signal transduction, and DNA repair. Therefore, specific inhibitors of individual PRMTs have potentially significant research and therapeutic value. In particular, PRMT1 is responsible for >85% of arginine methyltransferase activity, but currently available inhibitors of PRMT1 lack specificity, efficacy, and bioavailability. To address this limitation, we developed a high-throughput screening assay for PRMT1 that utilizes a hyper-reactive cysteine within the active site, which is lacking in almost all other PRMTs. This assay, which monitors the kinetics of the fluorescence polarization signal increase upon PRMT1 labeling by a rhodamine-containing cysteine-reactive probe, successfully identified two novel inhibitors selective for PRMT1 over other SAM-dependent methyltransferases.



Arginine methylation is a posttranslational modification with crucial functions in RNA splicing, transcriptional control, signal transduction, and DNA repair.[1-3] Methylation of arginine is catalyzed by a family of enzymes called the protein arginine methyltransferases (PRMTs).[1-3] Arginine methylation of proteins was first discovered in 1968, but only three decades later was the first PRMT family member, PRMT1, cloned and initially characterized.[4,5] Currently, the PRMT family is known to contain at least 11 enzymes that all use *S*-adenosylmethionine (SAM) as a methyl donor[1-3,5-13] and are divided into three classes based on methylation activity: Type I PRMTs (PRMT1, 3, 4, 6, and 8) catalyze the formation of asymmetric ω-N^G,N^G-dimethylarginine,[5-9,14] Type II PRMTs (PRMT5) form symmetric ω-N^G,N'^G-dimethylarginine,[6,10,12,14] and Type III PRMTs (PRMT7) form ω-N^G-monomethylarginine.[15]

PRMT1, a ubiquitously expressed Type I enzyme, is responsible for over 85% of the arginine methylation occurring in the cell and has a wide range of substrates involved in transcription, chromatin modification, and signal transduction.[1-3,16] PRMT1 has a preference for arginine residues flanked by glycines in what are termed glycine-arginine-rich regions.[14,17] Seven isoforms of PRMT1 have been cloned that contain a conserved enzymatic core but vary in their amino-termini.[18] PRMT1-deficient mice are embryonically lethal, and derived mouse embryonic fibroblasts (MEFs) lacking PRMT1 contain defects such as DNA damage, polyploidy, and cell cycle progression delays.[19] Therefore, a specific inhibitor would be valuable to further study the biological functions of PRMT1.

Many current inhibitors for PRMT1 are SAM analogues, such as sinefungin and *S*-adenosylhomocysteine (SAH), but they lack specificity and inhibit the activity of all methyltransferases that use SAM as a methyl donor, including lysine methyltransferases and the entire family of PRMTs.[7,8,12,20] Additional non-nucleoside compounds, including AMI-1 and AMI-408, have been discovered that retain more specificity to PRMT inhibition over lysine methyltransferases, but they still lack specificity across Type I PRMT members.[7,21] Other indirect inhibitors, including MDL 28,842 and adenosine dialdehyde (Adox),[7,8,12,20] have been developed to block the production of SAM by inhibiting SAH hydrolase, but these compounds are obviously not compatible with evaluating the specific function of PRMT1 in a context where multiple methyltransferases are present. Recently, inhibitors of PRMT1 consisting of modified substrate peptides have been developed,[22,23] although these still cross-react with other PRMTs, especially PRMT6.

Competitive activity-based protein profiling (ABPP) has emerged as a powerful platform to identify inhibitors of enzymes from multiple mechanistic classes, including serine hydrolases, oxidoreductases, kinases, and protein arginine deiminases.[24-29] Briefly, activity-based probes typically contain a reactive chemical moiety that covalently labels the active site residues of enzymes and a tag (*e.g.*, a fluorophore, biotin, or click handle) to enable identification or enrichment of the labeled enzymes.[30,31] Competitive ABPP involves assaying the ability of a compound to block the labeling of an enzyme by an

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activity-based probe and is usually monitored using SDS-PAGE gels or mass spectrometry.[32,33] Unfortunately, these methods are not amenable to screening more than a few hundred compounds.[34] However, a recently developed version of competitive ABPP in which the interaction between a fluorescently labeled probe and an enzyme is monitored by the resulting increase in fluorescence polarization signal (FluoPol-ABPP) is compatible with high-throughput screening (HTS) of large compound libraries.[24] Here, we describe the development of a FluoPol-ABPP assay for PRMT1.

Unfortunately, classical activity-based probes do not yet exist for the PRMT enzyme family. Recently, however, Thompson and colleagues reported the use of a fluorescently conjugated chloroacetamide bearing a histone 4 tail substrate analogue as a probe capable of covalently interacting with PRMT1, though the target residue of this molecule is unknown.[23] Further, a previous screen for hyper-reactive cysteines in cell lysates identified cysteine 101 (Cys101) in PRMT1.[35] Interestingly, the homologous cysteine is absent in all other PRMTs except PRMT8 (Supplemental Figure 1). We hypothesized that this residue could form the basis of a screen involving the use of a "non-directed" activity-based probe that generally reacts with specific types of nucleophilic residues in proteins rather than a particular enzyme class.[36-38] Here, we report that a thiol-reactive maleimide probe conjugated to AlexaFluor488 covalently binds to Cys101 of PRMT1 and generates a strong fluorescence polarization signal. We adapted this interaction into a FluoPol-ABPP format and screened the Maybridge Hitfinder Collection for compounds that block the fluorescence polarization signal. Using this screen, we identified several novel and selective inhibitors for PRMT1.

RESULTS AND DISCUSSION

PRMT1 contains a hyper-reactive cysteine (Cys101) within its active site (Figure 1A).[35] Cys101 directly contacts SAM during catalysis of arginine methylation, although is not essential for activity.[35] In order to establish an HTS assay to screen for PRMT1 inhibitors, we reasoned that a "non-directed" activity-based probe that specifically labels hyper-reactive cysteine residues in proteins could provide a basis to monitor active PRMT1 protein. To evaluate this premise, we incubated the cysteine-reactive maleimide conjugated to AlexaFluor488 (maleimide-AF488) (5 nM) with purified wild-type or cysteine 101 alanine (C101A)-mutant PRMT1 (4 μM). After 45 min, the reactions were quenched, separated by SDS-PAGE, and quantified by in-gel fluorescence scanning (Figure 1B). The wild-type enzyme encouragingly gave a much stronger fluorescent signal by gel. Specificity of the signal from the maleimide probe binding to PRMT1 was determined by pre-incubating PRMT1 or the C101A mutant (4 μM) with unlabeled maleimide prior to addition of the fluorescently labeled probe (5 nM). As predicted, free maleimide competed with labeled maleimide for enzyme binding (Figure 1C).

As an initial test whether this assay could detect PRMT1 inhibition, we added nonspecific methyltransferase inhibitors (100 μM) to either PRMT1 or PRMT1-C101A (4 μM) prior to incubation with maleimide-AF488. The SAM analogue sinefungin reduced the fluorescence signal of only the wild-type enzyme (Figure 1D). 4-Hydroxynonenal (HNE), previously shown to reduce labeling of wild-type but not PRMT1-C101A by a thiol-reactive iodoacetamide probe,[35] encouragingly also reduced maleimide-AF488 binding in this assay (Figure 1d). However, the small molecule inhibitor AMI-408 interfered with

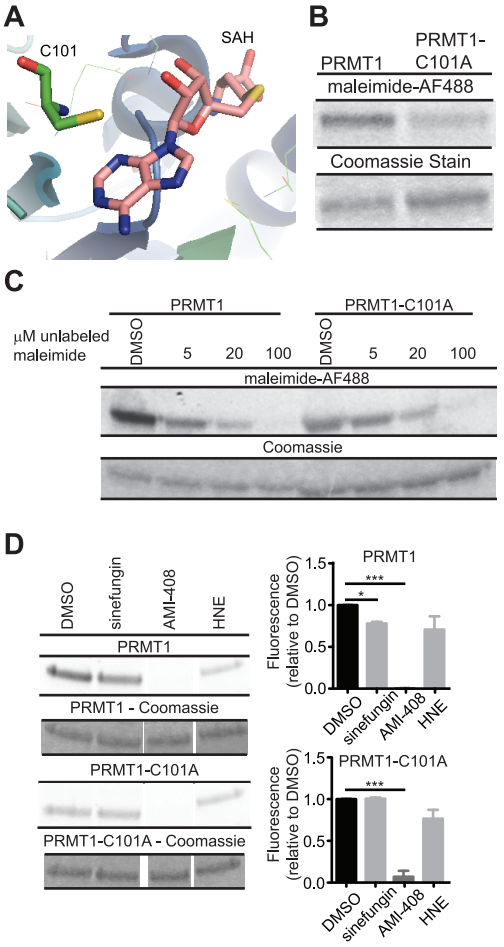

Figure 1. Maleimide-AF488 binds PRMT1 at Cys101. (A) Crystal structure of PRMT1 (PDB: 1ORH). Cys101 is shown binding to SAH (*S*-adenosyl-homocysteine) at the enzymatic site. (B) PRMT1 and PRMT1-C101A (4 μM) labeled with maleimide-AF488 (5 nM) for 1 h at RT and then separated by SDS-PAGE. After imaging on a flat-bed fluorescent scanner, gels were stained with Coomassie to determine protein load. (C) PRMT1 and PRMT1-C101A incubated with unlabeled maleimide prior to the addition of maleimide-AF488 for 30 min and then imaged as in panel B. (D) Fluorescence after sinefungin (20 μM), AMI-408 (100 μM), and HNE (100 μM) incubated for 30 min with PRMT1 or PRMT1-C101A prior to addition of maleimide-AF488. Densitometry is on right. Data is representative of at least two separate experiments. Errors bars are SEM; $*p < 0.01$, $***p < 0.0001$.

probe binding to both PRMT1 and PRMT1-C101A (Figure 1d), indicating AMI-408 may interact with PRMT1 independent of C101.

We next monitored the fluorescence polarization signal upon incubation of maleimide-AF488 with the PRMT1 proteins and again observed a significantly stronger signal with the wild-type enzyme compared with the C101A mutant (Figure 2A). This indicates that the hyper-reactive cysteine 101 is indeed labeled by the probe and that this signal should be adaptable to a FluoPol-ABPP format. However, since maleimide-AF488 reactivity with PRMT1-C101A was still detected by fluorescence polarization (Figure 2A), we next titrated the enzyme to determine an optimal protein concentration to maximize the signal-to-noise ratio. We discovered that the fluorescence polarization signal generated by the interaction of maleimide-AF488 with PRMT1C101A was reduced to similar levels as

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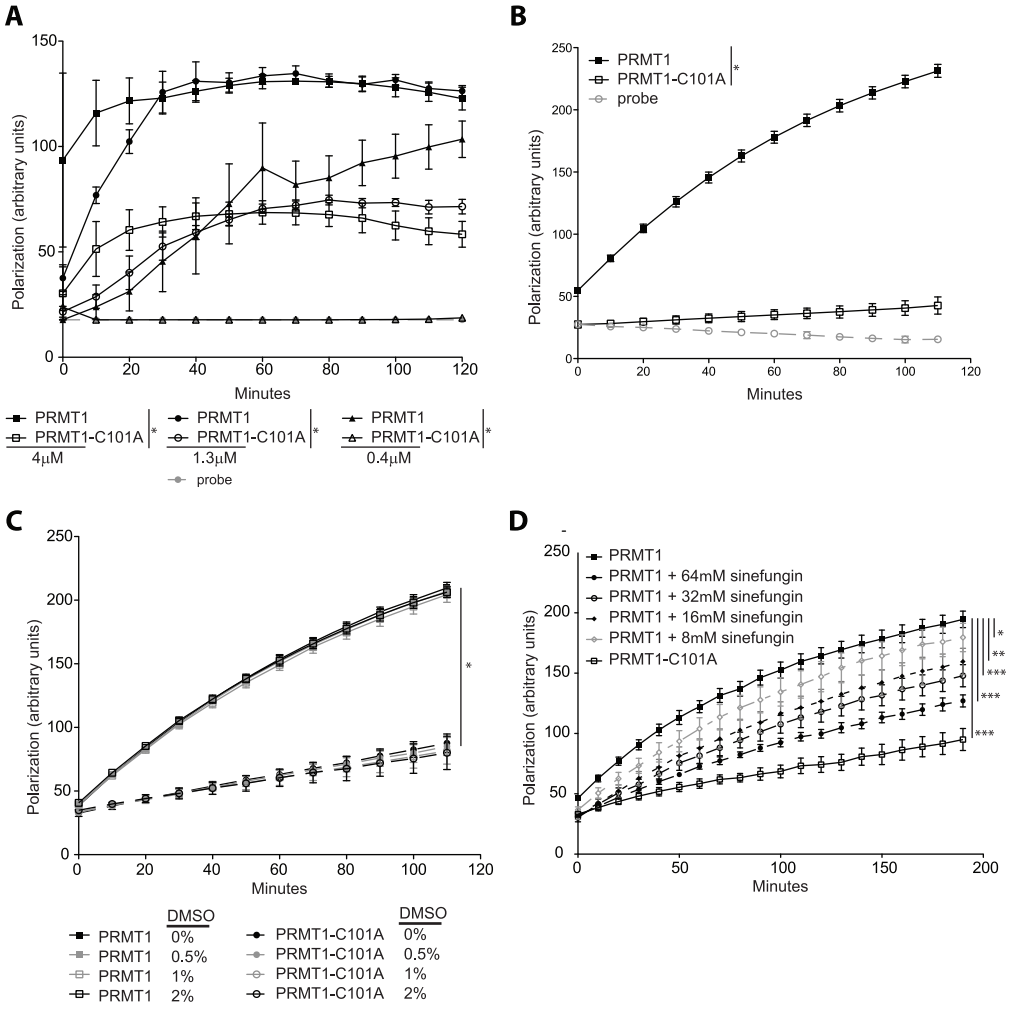

Figure 2. Different binding between PRMT1 and PRMT1-C101A sufficient for HTS. (A) Fluorescence polarization of dilutions of PRMT1 and PRMT1-C101A with maleimide-AF488 (5 nM). (B) Fluorescence polarization of PRMT1 and PRMT1-C101A and maleimide-AF488 as dispensed by BioRAPTR FRD. (C) Fluorescence polarization maleimide-AF488 binding to PRMT1 and PRMT1-C101A in the presence of 0.5%, 1%, or 2% DMSO. Data is representative of at least two independent experiments. Errors bars are SEM; $*p < 0.0001$. (D) Fluorescence polarization after sinefungin incubated for 30 min prior to addition of maleimide-AF488. Data is representative of two independent experiments. Errors bars are SEM; $***p < 0.0001$, $**p < 0.005$, $*p < 0.01$.

detected by probe alone at an enzyme concentration of 0.4 μM. Importantly, the wild-type enzyme still gave a robust signal at this concentration (Figure 2A). To determine the assay quality at these concentrations, we next performed reactions using automated liquid handlers and monitored polarization over 2 h (Figure 2B). We observed that the Z'-factor increases over time and plateaus around 0.8 at 1 h, indicating that the assay is compatible with HTS (Table 1). Further, the assay is largely insensitive to DMSO, as little change in polarization was observed up to concentrations of 2% (Figure 2C). Finally, we observed that sinefungin inhibited the fluorescence polarization increase by wild-type PRMT1 in a dose-dependent manner (Figure 2D), and thus potential reversible inhibitors would not be out-competed by the covalent binding of the maleimide probe, as has been shown previously in other ABPP screens.[24] Therefore, this FluoPol-ABPP assay was deemed to provide a high-quality, simple format to screen for compounds that occlude the active site of PRMT1.

We applied the FluoPol-ABPP PRMT1 assay to screen the 16,000 compounds in the Maybridge Hitfinder Collection. The activity of the compounds (at 10 μM) was determined by the percent of inhibition of PRMT1 polarization compared to DMSO-treated wild-type enzyme (0% inhibition) and PRMT1-C101A (100% inhibition) (Figure 3); 789 (4.9%) compounds screened had greater than 50% inhibition. Although this hit rate

Table 1. Z'-Factor of PRMT1 HTS Plateaus at 60 min and Is Sufficient for High-Throughput Screening

min	Z'-factor
0	0.46
10	0.71
20	0.76
30	0.77
40	0.79
50	0.79
60	0.82
70	0.82
80	0.82
90	0.83
100	0.82
110	0.81

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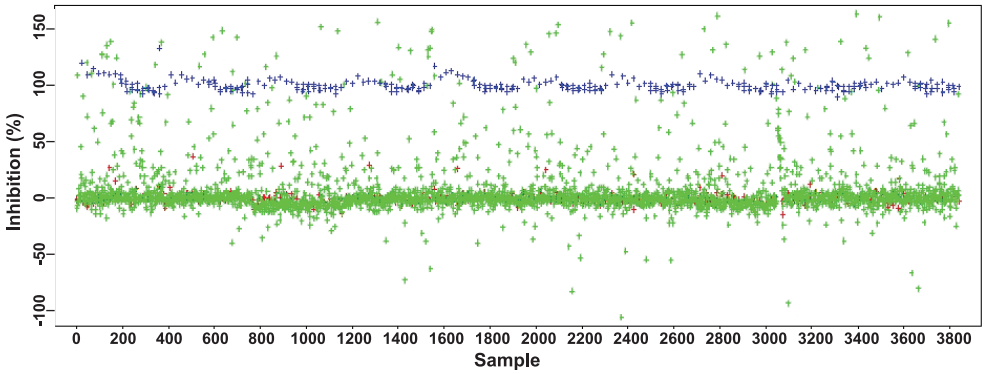

Figure 3. Screen of Maybridge Hitfinder Collection identifies potential inhibitors. Representative samples of Maybridge screen as percent inhibition of maleimide-AF488 polarization at 60 min after pre-incubation of PRMT1 with compounds for 30 min. PRMT1 controls are in red, C101A controls are in blue, and compounds screened are in green.

number is higher than what is typically observed in high-throughput screens, it is similar to rates observed with enzymes previously known to be sensitive to inhibition by generally thiol-reactive compounds.[24] The addition of the detergent F-127 prevents limits false positives that act as aggregators.[39,40] As a counter-screen to identify false positives, PRMT1 HTS results were compared to two screens using the identical maleimide-AF488 probe with different proteins containing active-cysteines to which maleimide-AF488 binds.[41] This counter-screen identified false positives, cross-reactive compounds, and/or thiol-reactive compounds, which were discarded from further analysis. The remaining 98 compounds (0.6% of total) were re-tested in triplicate to confirm inhibition. From these compounds, the top 19 inhibitors with greater than 50% inhibition were selected for further screening.

We next tested these 19 compounds using an orthogonal *in vitro* methylation assay in which a radiolabeled SAM substrate is used for the methylation of PRMT substrates by PRMT1. Of these 19 compounds, all but four at 100 μM (2729237, 2745438, 2810812, and 2815040) showed detectable inhibition of PRMT1 activity (Figure 4A and Supporting Information 2b). Four of the top compounds, 2806087, 2811408, 2818500, and 5380390, were selected for further characterization, along with two negative controls, 2797621 and 2729237 (Figure 4B). To confirm their activity in the methylation assay, titration curves of these compounds were generated for inhibition of PRMT1. As seen in Figure 4C, the strongest inhibition was observed with 5380390 and 2818500, with apparent IC_{50} values of 23 and 11 μM, respectively. The other four compounds tested had limited activity below the 100 μM used in Figure 4A. Additionally, as the activity of PRMT1 is reported to be affected by amino-terminal tags,[42] we performed titration experiments using an untagged PRMT1 and found similar IC_{50}'s (data not shown). The activity of the two hits, 5380390 and 2818500, is unlikely to be due to a covalent interaction between the compounds and the substrate, since Histone4 lacks cysteine residues. Therefore, novel PRMT1 inhibitors were discovered using our PRMT1 HTS assay.

Although a determination of their mechanism of action must await further studies, it is interesting to note that four of the five compounds identified contain electrophilic groups and both of the high-affinity inhibitors (5380390 and 2818500) are nitroalkenes. It is therefore expected that these compounds compete with the maleimide probe for modification of Cys101. Consistent with this hypothesis, these two compounds had no effect on the methylation activities of both CARM1, a Type I PRMT family member that does not contain a cysteine homologous to PRMT1 Cys101 in the SAM binding site (Figure 5A), and the SAM-dependent lysine methyltransferase Set7/9 (Figure 5B). Contrariwise, 5380390 and 2818500 did inhibit the activity of PRMT8 as well as PRMT1, the only two Type I PRMTs that contain a SAM-binding cysteine residue. The IC_{50} values obtained from the dose–response studies of Figure 4C reflect only the apparent inhibitory power of these molecules under the experimental conditions employed. Since they are likely to be covalent inhibitors, their potency can be affected by several factors that we have not yet explored, including their intrinsic electrophilicity, ability to access the Cys101 site, and rates of competitive decomposition by attack of solvent or other nucleophiles. The possibility also remains that these two compounds have the potential to cross-react with other cysteine-dependent proteins outside the PRMT class. The possibility will be further investigated using competitive ABPP on whole cell lysates with cysteine-reactive probes.[35]

In conclusion, we have developed a high-throughput fluorescence polarization assay to screen for novel PRMT1 inhibitors based on a specific SAM-binding cysteine absent in other SAM-dependent methyltransferases except PRMT8. Using this screen, we have indentified two novel inhibitors specific for PRMT1 and PRMT8 over other SAM-dependent methyltransferases. We anticipate that these inhibitors may serve as lead compounds to probe cellular functions of PRMT1 and more generally that the assay described herein can be readily adapted to screen much larger compound libraries to identify new lead inhibitors of this important enzyme.

METHODS

Materials. AlexaFluor488-C5-maleimide was purchased from Invitrogen (no. A10254). Sinefungin (no. 567051) and 4-hydroxynonal (no. 393204) were purchased from EMD Chemicals. AMI-408 was a generous gift from M. G. Finn and colleagues.[21] Recombinant proteins were acquired as follows: CARM1 from Upstate (no. 14-575), Set7/9 from Enzo Life Sciences (no. ALX-201-178), and Histone4 and Histone3.1 from New England Biolabs (nos. M2504S and M2503S, respectively).

Recombinant Protein Purification. Full-length PRMT1 cDNA and PRMT1-C101A subcloned into the pET45b(+) vector (Novagen) was a gift from the Cravatt laboratory.[35] His-tagged PRMT8 was generated by subcloning full-length human PRMT8 cDNA into pET29a(+) vector (Novagen). For recombinant expression, Over-

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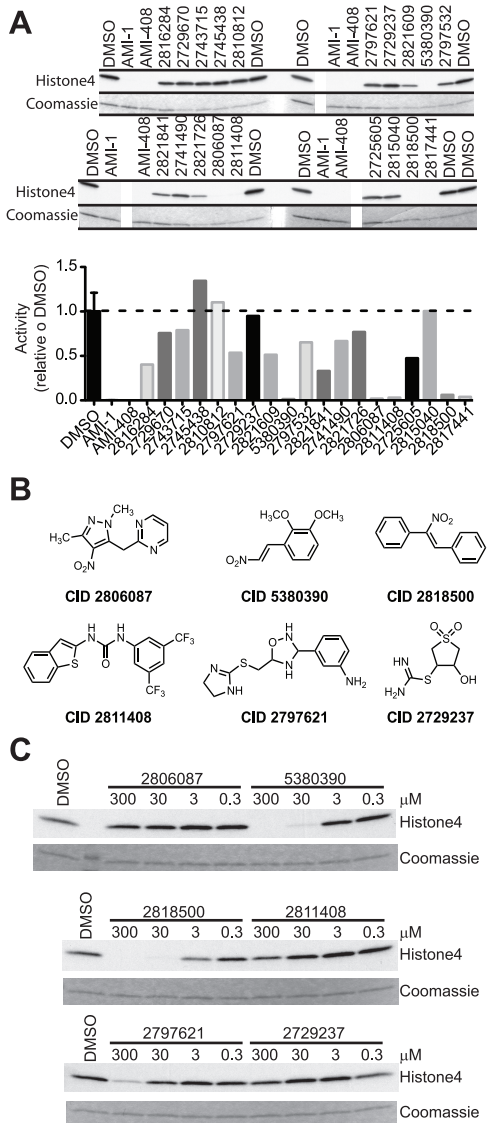

Figure 4. 5380390 and 2818500 inhibit PRMT1 methyltransferase activity. (A) *In vitro* methylation of PRMT1 on Histone4 after incubation with 100 μM concentration of potential inhibitors. Densitometry is below. (B) Chemical structures of top four inhibitor scaffolds (2806087, 5380390, 2818500, and 2811408) and two negative controls (2797621 and 2729237). (C) *In vitro* methylation of PRMT1 on Histone4 after incubation with titration of potential inhibitors. Data is representative of three independent experiments.

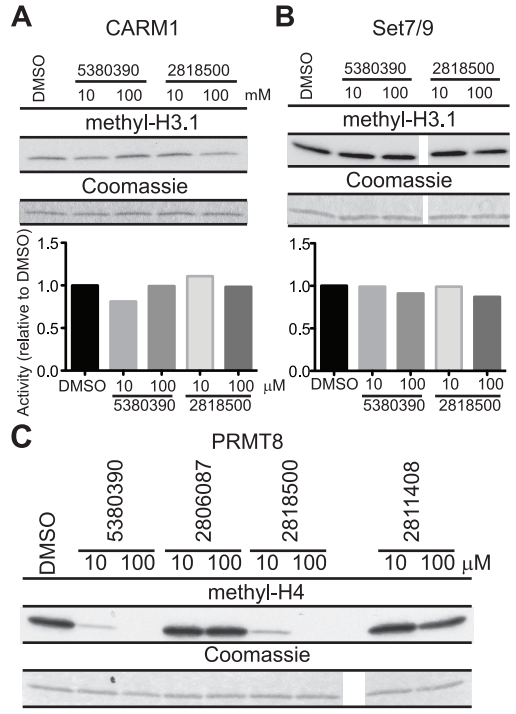

Figure 5. 5380390 and 2818500 do not inhibit SAM-dependent methyltransferases absent the SAM-binding cysteine. (A) *In vitro* methylation of CARM1 on Histone3.1 after incubation with 10 and 100 μM concentrations of potential inhibitors. (B) *In vitro* methylation of Set7/9 on Histone3.1 after incubation with 10 and 100 μM concentrations of potential inhibitors. (C) *In vitro* methylation of PRMT8 on Histone4 after incubation with potential inhibitors. Data is representative of at least two independent experiments.

Express C41(DE3) pLysS Chemically Competent Cells (Lucigen) were transformed with the above constructs and grown on LB media + antibiotic agar plates. Selected colonies were grown in 2X YT media to OD_{600} = 0.8 and induced with 0.25 mM IPTG for 4 h at 37 °C. Cell pellets were resuspended in Lysis Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) supplemented with 1 mg mL^{-1} lysozyme (Sigma), sonicated, and centrifuged at 17,000*g* for 30 min. The clarified lysates were collected and incubated overnight with Ni-NTA slurry (Qiagen) at 4 °C. The Ni-NTA beads were collected at 1000*g* for 5 min and washed twice in Lysis Buffer + 30 mM imidazole for 15 min. Protein was eluted with Lysis Buffer + 300 mM imidazole 3 times for 1 h at 4 °C. PD-10 columns (GE Healthcare) were used to size-exclude contaminants and exchange the buffer to PBS, and protein-containing fractions were concentrated by Amicon centrifugal filter (Millipore). Protein concentration was determined by BCA kit (Pierce). The purification yielded >95% purity (by SDS-PAGE).

Fluopol-ABPP Assays. In a 384-well Greiner Low Volumes plate (no. 788076/1 V), 10 μL of PRMT1 or PRMT1-C101A (0.44 μM final unless otherwise specified) in assay buffer (100 mM HEPES pH 7.0, 100 mM NaCl, 0.05% Pluronic F-127 (Invitrogen)) was added to each well by BioRAPTR FRD. Compounds were then dispensed by Pintool (50 nL; final concentration 10 μM) to test wells and DMSO to control wells. The plates were incubated at RT for 30 min. Then 10 μL of maleimide-AF488 (5 nM final unless otherwise specified) in assay buffer was added to all wells, and the plates were centrifuged briefly and incubated at RT for another 60 min. Plates were then read on an Envision microplate reader (PerkinElmer, Turku, Finland) using a FITC FP filter set (excitation = 480 nm, emission = 535 nm) and a FITC Dual enhanced FP dichroic mirror.

Counter-screens. Data for the counter-screens came from maleimide-AF488 binding experiments to the protein mitoNEET, which contains reactive cysteines (P. Jennings and S. Brown, personal communication). Incubation time of library compounds and concentrations of compounds of counter-screen were identical to those for PRMT1 HTS.

Gel-Based ABPP Assays. Gel-based ABPP experiments were performed similarly as FluoPol-ABPP assays with the following exceptions: Total reaction volume for each sample was 30 μL. After the final RT incubation, the reactions were quenched with 5 μL of 6X SDS-PAGE loading buffer and boiled for 5 min at 95 °C. Samples were separated by SDS-PAGE, and fluorescence was imaged on a flat-bed fluorescence scanner (Hitashi). Subsequently, the gels were stained for 30 min with Coomassie stain (0.25% Brilliant Blue, 50% MeOH, 10% acetic acid) and distained overnight in 50% MeOH, 10% acetic acid. Gels were dried and stored at RT.

***In Vitro* Methylation Assays.** Recombinant protein (0.4 μM unless otherwise stated) and compounds were incubated for 60 min at RT in 20 mM Tris-HCl pH8, 200 mM NaCl and 0.4 mM EDTA. Subsequently, 1 μg of substrate protein (H4 or H3.1) and 2 μL of *S*-

adenosyl-[*methyl*-^{3}H]methionine (2 μCi) (Perkin-Elmer) were added for an additional 60 min at RT. Reactions were stopped with 6X SDS Loading Buffer, boiled for 5 min at 95 °C, and separated by SDS-PAGE. Gels were stained by Coomassie as above and subsequently incubated with Amplify (GE Healthcare no. NAMP100) for 30 min. After drying, gels were exposed to HyBlot CL film at −80 °C.

Z'-Factor Calculations.

$$Z'\text{-factor} = \frac{3SD_{\text{PRMT1}} + 3SD_{\text{PRMT1-C101A}}}{AVE_{\text{PRMT1}} - AVE_{\text{PRMT1-C101A}}}$$

Compound Library Screening. Compounds are from the Maybridge Hitfinder Collection version 4.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interest.

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