Published in final edited form as:

J Med Chem. 2012 September 27; 55(18): 7978–7987. doi:10.1021/jm300521m.

Pharmacophore-Based Virtual Screening and Biological Evaluation of Small Molecule Inhibitors for Protein Arginine Methylation

Juxian Wang^{a,#}, Limin Chen^{b,#}, Sarmistha Halder Sinha^{a,#}, Zhongjie Liang^{b,e}, Huifang Cai^c, Sakthivel Muniyan^f, Yu-Wei Chou^f, Chao Yang^a, Keqin Kathy Li^d, Ming-Fong Lin^f, Hualiang Jiang^b, Yujun George Zheng^{a,*}, and Cheng Luo^{b,e,*}

^aDepartment of Chemistry, Program of Molecular Basis of Diseases, Georgia State University, PO Box 4098, Atlanta, Georgia 30302

^bDrug Discovery and Design Center, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China

^cDepartment of Pharmacy, Guiyang College of Traditional Chinese Medicine, Guiyang 550002, China

^dState Key Laboratory of Medical Genomics, Shanghai Institute of Hematology, Rui Jin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China

^eCenter for Systems Biology, Soochow University, Jiangsu 215006, China

^fDepartment of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska 68198, USA

Abstract

Protein arginine methyltransferases (PRMTs) are proved to play vital roles in chromatin remodeling, RNA metabolism and signal transduction. Aberrant regulation of PRMT activity is associated with various pathological states such as cancer and cardiovascular disorders.

Development and application of small molecule PRMT inhibitors will provide new avenues for therapeutic discovery. We combined pharmacophore-based virtual screening methods with radioactive methylation assays, six hits were identified as inhibitors against the predominant arginine methyltransferase PRMT1 within micromolar potency. Two potent compounds, **A9** and **A36**, exhibiting the inhibitory effect by directly targeting substrate H4 other than PRMT1 and displayed even higher inhibition activity than the well-known PRMT inhibitors AMI-1 and stilbamidine. **A9** significantly inhibits proliferation of castrate-resistant prostate cancer cells. Together, **A9** may be a potential inhibitor against advanced hormone-independent cancers and the work will provide clues for the future development of specific compounds that block the interaction of PRMTs with their targets.

*Correspondence: Cheng Luo and Y. George Zheng, Tel: For C.L., +86-21-50271399; Fax: +86-21-50807088. For Y.G.Z.: 1-404-413-5491; Fax: 1-404-413-5505, cluo@mail.shnc.ac.cn or yzheng@gsu.edu.

#These authors contributed equally to this work

Supporting Information Available: Additional experimental data, figures, and graphs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

arginine methylation; PRMT1; inhibitor; pharmacophore; virtual screening

Introduction

In eukaryotic cells, histones were subject to various posttranslational modifications such as methylation, acetylation, phosphorylation, sumoylation, and ubiquitinylation.¹ In particular, the amino-terminal tails of the four core histone proteins are rich in arginine and lysine residues many of which are found to be heavily methylated. The reaction of methylation is specifically catalyzed by protein arginine methyltransferases (PRMTs).^a In addition to histones, a large number of non-histone proteins involved in signal transduction and cell proliferation are also shown to contain methyl arginine markers.² The influence of protein arginine N-methylation on cell function is becoming widely appreciated, which involves in a host of biological processes including protein trafficking, signal transduction, DNA transcriptional regulation, DNA repair, RNA maturation, and embryonic development.^{3–7}

PRMTs can monomethylate and dimethylate the terminal nitrogen atom of guanidinium side chains within arginine residues of proteins using *S*-adenosyl-*L*-methionine (SAM or AdoMet) as methyl donor. PRMT1 accounts for 85% of cellular PRMT activity in mammalian cells which is a predominant PRMT.⁸ Abnormal activity of PRMT1 is associated with various diseases, including mixed lineage leukemia (MLL) and hormone-dependent cancers. PRMT1 is an essential component for oncogenic transformation induced by a MLL complex.³ Direct fusion of MLL with PRMT1 promotes self-renewal of primary hematopoietic cells. On the contrary, specific knockdown of PRMT1 expression suppresses MLL-mediated transformation. PRMT1 is associated with the up-regulation of serum (ω -N^G,N^G-asymmetric dimethylarginine) ADMA, which is an endogenous nitric oxide synthase (NOS) inhibitors, blocks the production of NO and causes many cardiovascular implications such as diabetes and hypertension,⁹ and other implications such as inflammatory reactions.¹⁰

PRMT-mediated arginine methylation plays key roles in various biological processes and diseases. However, compared with histone deacetylases (HDACs), the development of arginine methyltransferase inhibitor is still at a slow development stage. In addition to cofactor analogs such as sinefungin¹¹, only a few potent arginine methyltransferase inhibitors were reported^{2, 12–20} and rarely have been widely recognized. Moreover, current methyltransferase inhibitors, with low specificity, target many other enzymes that use SAM as cofactor. The sequences among PRMTs throughout evolution are highly conserved, increase the difficulty of developing potent and specific PRMT inhibitors.

In the last several decades, virtual screening has emerged as a powerful tool for identifying novel compounds with structural diversity,^{21–24} which is a complementary approach to high throughput screening. It allows the discovery of novel bioactive compounds from very large compound databases through either the information about a set of active ligands or the protein active pocket. If the crystal structure of target protein is available, molecular docking approach is usually carried out. However, the pharmacophore method is an alternative choice when the structure of interest target is unknown. The pharmacophore model

interprets the interaction between a receptor and a ligand, which is a successful computational drug design approach.²⁵ Typically, a pharmacophore model was constructed according to the common structural features of existing active compounds. Pharmacophore-based virtual screening has been successfully carried out in the search of novel leads for several targets.^{26–28}

In this study, pharmacophore-based virtual screening was employed for searching novel PRMT1 inhibitors within SPECS database containing more than 30,000 compounds. Pharmacophore models were firstly constructed based on reported active compounds. Then the top scored compounds through the pharmacophore-based virtual screening were subjected to cluster analysis. 102 compounds were selected and purchased for further PRMT1 inhibitor bioactivity assay. Experimental results demonstrated that six potential compounds can inhibit PRMT1 activity on the micromolar scale. Two most potent compounds, **A9** and **A36** with similar structures, are found to be more efficacious than previously reported PRMT1 inhibitors, AMI-1 and stilbamidine. Further experimental studies demonstrate that compound **A36** is noncompetitive versus both peptide substrate and SAM, whereas **A9** is competitive versus peptide and noncompetitive versus SAM. Interesting, both **A9** and **A36** exhibit inhibitory potency for PRMT1 through targeting the substrates. **A9** significantly inhibits proliferation of prostate cancer cells both in regular culture media and in a hormone-depleted media. Taken together, this work provides new lead molecules and structural clues to develop inhibitors that regulate PRMT1-mediated arginine methylation, and **A9** may be a potential therapeutic for treating advanced castration-resistant prostate cancer.

Results and Discussion

Pharmacophore-Based Virtual Screening

The process for virtual screening in this study is shown in Figure 1. First of all, nine pharmacophore models (Table 1) were constructed based on training set including 17 reported compounds^{8, 29} (Supporting Information, Table S1) with a large scale PRMT1 inhibitive bioactivity and structural diversity in Accelrys Discovery Studio 2.1 (Accelrys Inc., San Diego, CA). Typically, two index values were used to evaluate the statistical significance of hypotheses: the margin between null cost and fixed cost, and the margin between null cost and total cost (cost). The margin between the null cost and the fixed cost should be greater than 80. The value indicates the chance of finding useful hypotheses. In this study, the difference between null cost and fixed cost was 87.462. A good hypothesis may usually be obtained when cost is more than 60. cost for all the nine hypotheses generated was greater than 85, indicating 90% statistical significance of these hypotheses (Table 1). What's more, the correlation coefficient, higher than 0.9, is often considered good for alignment that stands for the alignment of the training set compounds onto the hypothesis. The correlation coefficient for all the nine hypotheses generated is greater than 0.91 (Table 1). Furthermore, this training-set contained two wellrecognized PRMT1 inhibitor, AMI-1 and allantodapsone²⁹ (Supporting Information, Table S1). AMI-1 and allantodapsone can map all the nine pharmacophore models well (fit value is greater than 4.6), and a few examples of AMI or allantodapsone fitting the models were shown in Figure

S1 of Supporting Information. Taken these information together demonstrated that the nine hypotheses generated with statistical significance would be suitable for further virtual screening.

To validate the reliability of the nine constructed pharmacophore models, the pharmacophore models were employed to screen the testing-set database, respectively. The nine models can efficiently recover the known inhibitors of PRMT1. Model 2–5 got higher enrichment ratio than that of model 6–10 (Supporting Information, Figure S2A). In the meantime, these models were well clustered according to the pharmacophore features (Supporting Information, Figure S2B). Taking the information together, models 2–5 (colored red in Figure S2B of Supporting Information) from two classes were identified as rational models for virtual screening. The model 2 and 5 with similar features consist of one HBA, one HBD, and one RA. The features of model 3 and 4 were consisted of two HBA and one RA. AMI-1 could map on model 2 and 5 well, and allantodapsone could map on model 3 and 4 well (Supporting Information, Figure S1). AMI-1 and allantodapsone are two most potent compounds among training set, we concluded that identified hits based on the four models would have similar pharmacophore features with AMI-1 or allantodapsone.

Next, the four rational models were chosen as queries against theoretical soluble SPECS database that contains 90,000 small molecule compounds. The amounts of the candidate compounds predicted by each of the four pharmacophore models were summarized in Figure S3A of Supporting Information. The numbers of candidate compounds acquired from each pharmacophore model were almost equal. Because the four models share several similar features, some compounds screened by different models may be the same. Figure S3B of Supporting Information is the alignment of the screening results from different pharmacophore models. After deleting the duplicates, 798 unique candidate compounds were obtained for further research.

Cluster analysis demonstrated that the 798 compounds were divided into 40 groups according to structural features. Compounds in every group were arranged respectively based on fit values which were results from pharmacophore-based virtual screening, 3 top-ranked compounds were selected from every group. 120 candidate compounds were selected. However, only 102 compounds were available from SPECS database for biochemical assay.

Identification of A9 and A36 as PRMT1 inhibitors by using recombinant enzyme inhibition assay

The 102 *in silico*-screened compounds were tested for inhibitory activity against PRMT1, the predominant arginine methyltransferase in mammalian cells by using the carbon-14 labeled radioactive methylation assay. In the screening inhibition assay, recombinant histidine-tagged PRMT1 was used as the enzyme and an amino-terminal 20-amino-acid H4 peptide (H4-20) was used as the substrate. The methylation products were bound to P81 filter paper and then quantified by liquid scintillation counting. The degree of the decrease in product formation was used as a parameter to evaluate the potency of each compound in inhibiting methyltransferase activity of PRMT1.

From the enzyme inhibition assay, we identified six compounds that inhibited PRMT1 activity by more than 40% at the concentration of 100 μ M (Table 2). The high number of hits validates the accuracy of our pharmacophore-based virtual screening strategy. The hits identified by two kind pharmacophore models with great structural similarity. Based on their structures, these identified inhibitors are characterized as two groups, namely, group I and group II. The group I compounds are comprised of **A9** and **A36** that each contain naphthalene aromatic rings and one or two phenolic hydroxyl groups (Table 2). The group II compounds contain **A18**, **A44**, **A56** and **A69** that all share a heterocyclic thiazole motif (Table 2). Group I compounds were identified by model 2 or 5, but group II compounds were identified by model 3 or 4. However, group I compounds can inhibit PRMT1 more efficiently than group II compounds. For example, **A9** and **A36** inhibited 65% and 79% of PRMT activity at the tested concentration. In a more quantitative analysis, we measured the methyltransferase activity of PRMT1 at a range of concentrations of **A9** and **A36** and the IC_{50} values were derived from the dose response curve as 41.7 μ M and 12.0 μ M, respectively. For comparison, we measured IC_{50} under the same experimental condition of stilbamidine and AMI-1, two previously reported PRMT1 inhibitors. It is clear that both **A9** and **A36** showed stronger inhibition for PRMT1 (Table 3). In particular, the potency of **A36** is 11 fold that of stilbamidine and 6 fold that of AMI-1. The remarkable potency of **A9** and **A36** warrants further investigation of their inhibitory activities and mechanisms.

Inhibition Pattern of compound **A9** and **A36**

To provide a molecular basis of PRMT1 inhibition by group I compounds **A9** and **A36**, we performed steady-state kinetics characterization to determine the inhibition pattern of these compounds in PRMT1 catalysis. The initial velocities of PRMT1 were measured at several selected concentrations of each inhibitor over a range of varied concentrations of one substrate while fixing the concentration of the other. The data were plotted in the double reciprocal format with $1/\text{velocity}$ versus $1/[\text{concentration of the varied substrate}]$ (Figure 2). As can be seen from the double-reciprocal plots, in the presence of **A9**, a series of straight lines intersected on the $1/\text{velocity}$ ordinate when the concentrations of H4-20 are varied, while the intersecting point moved to the western side of the ordinate when concentrations of SAM are varied. These data demonstrate that **A9** is competitive versus the peptide substrate and noncompetitive versus the methyl donor SAM (Figure 2A and 2B). We fitted the primary velocity-vs-peptide-concentration data in the presence of different concentrations of **A9** to the nonlinear kinetic equation³⁰ and derived the inhibition constants $K_{is} = 8.4 \pm 1.8 \mu\text{M}$ and $K_{ii} > 257 \mu\text{M}$. K_{is} is significantly smaller than K_{ii} which validates the competitive nature of **A9** against H4 substrate. For **A36** inhibition, the intersecting points in both $1/v \sim 1/[\text{SAM}]$ and $1/v \sim 1/[\text{H4-20}]$ double-reciprocal plots were found to be in the fourth quadrant, suggesting **A36** is a noncompetitive inhibitor in respect to peptide and substrate (Figure 2C and 2D). A more strict analysis of the primary velocity vs H4 concentration plots yielded $K_{is} = 11.3 \pm 1.8 \mu\text{M}$ and $K_{ii} = 29.7 \pm 0.8 \mu\text{M}$. Given that K_{is} is less than K_{ii} , we conclude that **A36** is primarily competitive in respect to the H4 substrate. Such a mechanism of action for both **A9** and **A36** is consistent with that of AMI-1. For **A36**, the K_{ii} component cannot be neglected and it likely suggests that **A36** also targets a weaker binding site in PRMT1.

Inhibition mechanism of compound A9 and A36

A9 and **A36** bear certain similar pharmacophore features with the previously reported inhibitors AMI-1 and NS-1 that both target the substrate of PRMT1 rather than the enzyme to execute inhibitory effect³¹. Furthermore, the kinetic pattern of PRMT1 inhibition by **A9** and **A36** suggest that these compounds may compete with peptide substrate. To elucidate the inhibition mechanism, we attempted to test whether **A9** and **A36** also target the substrate of PRMT1 to execute the inhibitory effect. In this set of experiments, we directly probed the possible binding interaction between **A36** and PRMT1 or with the substrate by carrying out spectroscopic measurements. For inhibitor-substrate interaction, we collected the fluorescence emission spectra of a fluorescein-labeled H4 peptide, namely H4-(1–20)FL with the sequence of Ac-SGRGKGGKGDpr(FL)GKGGAKRHRK, in the presence of different concentrations of the inhibitor **A36**. It was found from the spectra of H4-(1–20)FL that, the addition of increasing concentrations of **A36** significantly quenched the fluorescence emission of H4-(1–20)FL (Figure 3A and 3B). To analyze the enzyme-inhibitor interaction, we measured the intrinsic fluorescence of PRMT1 in the addition of increased concentration of the inhibitor (Figure 3C and 3D). In contrast, the fluorescence emission of the enzyme changed very little by the inhibitor. These data strongly suggest that the interaction between the **A36** and the H4-(1–20)FL is much stronger than the **A36** — enzyme (if there is any). From the fluorescence binding measurement K_d of **A36** with the substrate was obtained as 23.8 μ M, which is in the close range as its IC_{50} value. We also tried several other biophysical methods to further confirm the binding between **A9/A36** and H4 substrate, including ESI-MS, isothermal titration calorimetry (ITC), and surface plasma resonance (SPR). Unfortunately none of these assays were sensitive enough for detecting the interaction (data not shown).

Inhibition of Histone Acetyltransferase (HAT) Activity of p300 by A36

The observed inhibition of PRMT1 via targeting the substrate indicates that **A36** may inhibit the other enzymes that modify the amino-terminal tail of histone H4. To investigate this possibility, we tested the activity of **A36** on the acetylation of H4-20 by p300, a well-known HAT enzyme which is able to acetylate the N-terminal tail of H4 at multiple sites³². The measurement was carried out with 0.03 μ M p300, 5 μ M [¹⁴C]-acetyl CoA, and 10 μ M H4-20, at varied concentration of **A36**. The dose-dependent assay yielded IC_{50} of 15.2 ± 1.7 μ M (Figure 4). Such potency in HAT inhibition is very similar to that of PRMT1 inhibition by **A36**. These data provide further evidence that **A36** binds to H4 directly, preventing H4 being recognized by histone modifying enzymes p300 and PRMT1. Thus, **A36** targets substrate rather than the enzyme for the inhibition, in the same manner as certain previously reported arginine methylation inhibitors.³¹

Enzymatic Selectivity of A36

To investigate whether **A36** is a selective inhibitor against PRMT1, we compared its inhibitory activity towards PRMT1, PRMT4 and PRMT5 using the radioactive methylation assay. Each reaction mixture contained specified concentrations of recombinant PRMT, [¹⁴C] SAM, and peptide substrate and was incubated at 30°C in the presence or absence of 30 μ M **A36**. For both PRMT1 and PRMT5 assays, H4-20 was used as the substrate while for

PRMT4 assay H3(1–20) was used as the substrate. The retained activity of each PRMT in the presence of **A36** was normalized using its respective controls (no inhibitor) and presented in Figure 5. It is seen that **A36** inhibited PRMT4 activity at potency 7.5-fold weaker than PRMT1 and inhibited PRMT5 at 2 fold potency weaker than PRMT1. Though PRMT1 and PRMT5 have similar substrate specificity, they undergo different types of methylation. So the minor difference in activity of **A36** for PRMT1 and PRMT5 is likely caused by the structural difference between the two proteins. On the other hand, PRMT4 have distinct substrate specificity from PRMT1, and PRMT4 preferentially methylates histone H3, whereas PRMT1 preferentially methylates histone H4. Such variation in inhibition potency may be caused either by structural differences between PRMT1 and PRMT4 or by the distinct nature of the substrates used. To sum up, **A36** exhibits stronger potency for PRMT1 than PRMT5 and PRMT4.

Effect of **A9** and **A36** on prostate cancer cell proliferation

Since compounds **A9** and **A36** were identified and characterized as two most potent compounds for inhibiting PRMT1 activity from the screening study, we further investigated whether these compound have any effect on proliferation of disease cells. LNCaP C-81 cells exhibit many biochemical properties of the advanced prostate cancer phenotype, including functional androgen receptor (AR) expression and prostate-specific antigen (PSA) secretion with rapid growth in the steroid-deprived condition.^{33, 34} In regular culture medium, **A9** can significantly inhibit the proliferation of LNCaP C-81 cells by up to 70%, following a dose-dependent manner (Figure 6A). Since prostate cancer growth is regulated by steroids, we next investigated whether **A9** can also inhibit the proliferation of LNCaP C-81 cells in a steroid-reduced condition, mimicking the clinical androgen-ablation state. As shown in Figure 6B, 10 μ M **A9** have significant inhibitory effect on the proliferation of LNCaP C-81 cells by 30%. The structure of **A36** is similar to that of **A9**; however, the effect of **A36** on the proliferation of LNCaP C-81 cells is much weaker than that of **A9**, only about 32% in regular medium (data not shown). Large molecular weight is likely to contribute to the poor biological activity of **A36** on cells.

Conclusion

Arginine methylation catalyzed by PRMTs regulates various cellular processes, such as DNA repair, RNA processing, nucleosomal remodeling, etc. However, the research on developing effective PRMT inhibitors is relatively slow in comparison to HDAC inhibitor discovery work. Most PRMT inhibitors reported so far have low efficacy and specificity. In this study, by combining pharmacophore-based virtual screening and biochemical methylation assays, we have successfully identified six small molecules that inhibit PRMT1 activity at micromolar potency. Group I compounds, **A9** and **A36** exhibit the best inhibitory effect with IC₅₀ value of 41.7 μ M and 12.0 μ M respectively, which are more potent than AMI-1 and stilbamidine under the same reaction conditions (Table 2). We further conducted steady-state kinetic characterization to determine the inhibition pattern of the **A9** and **A36** in PRMT1 catalysis. Interestingly, the biochemical data demonstrates that the **A9** compound is competitive versus peptide substrate and noncompetitive versus SAM, whereas the compound **A36** is noncompetitive versus both peptide and SAM. The structures and kinetic

patterns of **A9** and **A36** are similar to that of previous identified PRMT1 inhibitors AMI-1 and NS-1. Indeed, further close examinations showed that **A36** also binds to the histone substrate, preventing recognition of the substrate by the enzyme which is important for the enzymatic activity. **A36** also inhibited the activity of p300 that uses H4-20 as a substrate, with an IC₅₀ value of 15.2 μM. This is consistent with the conclusion that **A36** binds to the substrate to exhibit enzyme activity. **A9** significantly inhibited the proliferation of prostate cancer LNCaP C-81 cells; the effect of **A36** is weaker than that of **A9**. Because PRMT1 catalyzes arginine methylation of diverse proteins, inhibition of the activity of PRMT1 overwhelmingly results in extensive inhibition of methylation of multiple target substrates other than a particular substrate sequence, potentially causing adverse side effects and complications. In this study, we discover novel inhibitors that target the PRMT1 substrate and regulate PRMT1-mediated histone arginine methylation. Developing new modules of therapies targeting specific genes or gene products are a prominent aim of modern cancer biology. It is of high merit to discover small molecule probes that effectively modulate PRMT-mediated methylation by non-canonical mechanisms. Recently, small molecules targeting histones were shown to block cancer cell growth both in vitro and in vivo. Thus, **A9** that inhibits PRMT1 activity by targeting the histone substrate and shows a strong blocking effect on the proliferation of prostate cancer cells may be of therapeutic potential for treating advanced castration-resistant prostate cancers. Overall, the discovered histone binders **A9** and **A36** act as new chemical leads to develop improved inhibitors that block the interaction of PRMTs and their histone targets.

Experimental Section

Pharmacophore models generation and validation

17 reported compounds with a large scale inhibitive activity against PRMT1 and structural diversity were chosen to generate pharmacophore models in Accelrys Discovery Studio 2.1. During the process of generating pharmacophore models, four chemical features were selected, including HB_ACCEPTOR, HB_DONOR, HYDROPHOBIC and RING_AROMATIC; CAESAR conformation generation method was chosen; activity uncertainty was set at 3.0; and other control parameters were set at default values.

To validate the accuracy of the pharmacophore models, they were employed to screen the test-set database that was comprised of 27 compounds with PRMT1 inhibition activity and another 183 compounds with structural diversity randomly selected from SPECS database within Discovery Studio 2.1, respectively. In the meantime, these models were underwent cluster analysis with Discovery Studio 2.1.

Database preparation and pharmacophore-based screening

The SPECS database from ZINC database containing more than 300,000 small molecule compounds was filtered with log S > -6 to construct theoretical soluble SPECS database. Four rational pharmacophores were conducted to screen the theoretical soluble SPECS database with flexible search method within Discovery Studio 2.1 one by one. During the screening process, the CAESAR conformation generation method was used, and the

maximum number of conformers generated was set to 250, and other control parameters were set as default values.

Small molecule compounds

Small molecule organic compounds were purchased from SPECS Corporation (The Netherlands).

Peptide substrates

Peptides were synthesized following the standard solid phase peptide synthesis (SPPS) protocols, purified with C-18 reversed phase HPLC, and confirmed with MALDI-MS as previously described.³⁵ The structural sequence of the NH₂-terminal peptide of histone H4, H4-20, is Ac-SGRGKGKGLGKGGAKRHRK. The structural sequence of the NH₂-terminal peptide of histone H3, H3(1–20), is Ac-ARTKQTARKSTGGKAPRKQL.

Protein expression and purification

His6x-tagged PRMT1 was expressed from the pET28b vector. GST-PRMT1 is expressed from the PGEX4T1 vector. The proteins were expressed in *E. coli* BL21 (DE3). His6x-tagged protein was purified on Ni-NTA beads. Protein concentrations were determined using the Bradford assay.

Radioactive methylation assay

The inhibition effect of small molecule compounds was tested using the carbon-14 labeled radioactive methylation assay as previously reported.³⁶ The assays were carried out in 0.6 mL plastic tubes at 30 °C in a reaction volume of 30 µL. The reaction buffer contained 50 mM HEPES (pH=8.0), 10mM NaCl and 1 mM DTT. In a typical inhibition assay, 2 µM of peptide substrate, 5 µM of [¹⁴C]-SAM and varied concentrations of inhibitor were pre-incubated in the reaction buffer for 5 min at 30 °C prior to the initiation of the reaction by the addition of enzyme (0.1 µM final). After incubating for an appropriate period of time (8 min), the reaction was quenched by spreading the reaction mixture onto the surface of P81 filter paper (Whatman). The paper disc was then washed with 50 mM NaHCO₃ (3×300 mL), dried for 2 hours. The amount of methylated products was quantified by liquid scintillation counting.

The inhibition pattern analysis of A9 and A36 was determined by measuring initial velocities of PRMT1 at varied concentrations of one substrate, a fixed concentration of the other substrate, and selected concentrations of the inhibitors. 0.1 mM of His6x-PRMT1 was used in all these assays. The data were displayed in double reciprocal formats and fitted to competitive or noncompetitive kinetic equations.

Selectivity test of A36 with different PRMTs

To know whether A36 is selective towards PRMT1 than other PRMT member, radioactive methylation assay was performed. His6x-rPRMT1, -4, -5 proteins were expressed in *Escherichia coli* BL21 (DE3). In this assay for three set of reactions, H4-(1–20) for PRMT1 & PRMT5 (2 µM), H3 (1–31) for PRMT4 (400 µM) and [¹⁴C]-S-Adenosyl-L-methionine (5

μM) were preincubated in the reaction buffer for 5 min prior to the initiation by the addition of PRMT1 (0.05 μM final), PRMT5 (1 μM final), PRMT4 (0.3 μM final), in absence of inhibitor. The reaction time used for PRMT1 was 20 min, for PRMT5 was 6 h 30 min and for PRMT4 was 2 h. At the appropriate time, the reaction was quenched by spreading the reaction mixture onto P81 filter paper discs (Whatman). The paper disk was washed with 50 mM NaHCO₃ (3×300mL), and dried in air for 2 h. The amount of methylated products was quantified by liquid scintillation. Another three set of reaction were done in presence of 30 μM of A36 and DMSO (2%) by following the same procedure as described above.

Fluorescent Binding Assay

The fluorescence intensity of fluorescein-labeled peptides was measured on a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon). The buffer was the same as that for the radioactive assay. The excitation and emission wavelength were selected at 498 and 524 nm, respectively. Fluorescence intensity changes of H4(1–20)FL and PRMT1 at different concentrations of A36 were measured to detect their interaction. 1μM concentration of H4(1–20)FL and PRMT1 at 30° was titrated with increasing concentration of A36 (1–100 μM for H4(1–20)FL and 1–50 μM for enzyme interaction) in different set of experiment.

Inhibition of p300 by A36

Recombinant p300HATdomain (1287–1666) was a gift from Dr. Philip Cole at Johns Hopkins University, and its expression was described in an earlier report.³² The enzymatic activity of p300 and its inhibition by A36 was measured by radioactive acetylation assays. A reaction mixture of 10 μM H4-20, 5 μM [¹⁴C]-acetyl CoA, 0.03 μM p300, and increasing concentrations of the inhibitors were incubated in the reaction buffer [50 mM HEPES (pH 8.0), 50 mM NaCl, 0.5 mM DTT, and 1 mM EDTA] at 30 °C for 10 min, and the reaction was quenched by loading the mixture onto p81 filter paper. The radioactive products were quantified by liquid scintillation, and the fractional activity of p300 was plotted with respect to the concentration of inhibitors.

Cell Culture and Cell Proliferation assay

RPMI 1640 medium, gentamicin and trypsin/EDTA reagents were purchased from Invitrogen Corporation (Carlsbad, CA). Regular and Charcoal/dextran-treated, certified FBS were from Atlanta Biologicals (Lawrenceville, GA, USA). Human prostate carcinoma cell line LNCaP was originally purchased from the American Type Culture Collection (Rockville, MD). LNCaP cells were routinely maintained in the regular medium, *i.e.*, phenol red-positive RPMI 1640 medium supplemented with 5% FBS, 2 mM glutamine and 50 μg/ml gentamicin. The LNCaP PCa cell progression model was described originally by Lin *et al.*³³ and further characterized by Igawa *et al.*³⁴ with passage numbers between 80 and 120 as C-81. For steroid-reduced (SR) medium, cells were maintained in phenol red-free RPMI 1640 medium containing 5% charcoal/dextran-treated FBS (v/v), 2 mM glutamine, 50 μg/ml gentamicin and 1nM dihydrotestosterone (DHT). The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in a CO₂ incubator. A9 and A36 were dissolved in DMSO as 1000×concentrated stock solutions, stored at –20°C, and diluted in the respective

culture media at the time of use. Control cells were treated with media containing an equal concentration of DMSO.

For cell proliferation assay, in regular medium, LNCaP C-81 cells were plated at a density of 2×10^3 cells/well in 6-well for 3 days and then treated with different concentrations of A9 or A36 for 3 days. The cell numbers were counted using a Cellometer Auto T4 Image-based cell counter (Nexcelom Bioscience). The ratio of cell growth was calculated by normalizing the cell number to that of the control cells. In SR medium condition, C-81 cells were plated as described as above for 3 days, and then steroid-starved for 2 days in a SR medium. After fed with fresh SR medium, cells were treated with different concentrations of A9 or A36 for 3 days. Control cells were treated with solvent alone. The cell numbers were counted and the ratio of cell growth was calculated by normalizing the cell number to that of the control cells. Results shown are an average of three sets of independent experiments performed in triplicates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge financial support from National Institutes of Health (NIH), American Heart Association and Georgia Cancer Coalition Distinguished Cancer Scholar Award to YGZ. This study was also supported in part by CA88184 (NIH) to MFL. We also gratefully acknowledge financial support from the State Key Program of Basic Research of China grant (2009CB918502), the National Natural Science Foundation of China grants (20972174 and 91029704), Shanghai Committee of Science and Technology grants (10410703900) and the Chinese Academy of Sciences (XDA01040305) to CL and HJ.

Abbreviation

PRMT	protein arginine N-methyltransferase
SAM/AdoMet	S-adenosyl-L-methionine
MLL	mixed lineage leukemia
ADMA	ω -N _G , N _G -asymmetric dimethylarginine
NOS	nitric oxide synthase
HDAC	histone deacetylases
HAT	Histone Acetyltransferase
AR	androgen receptor
PSA	prostate-specific antigen

References

- Schäfer S, Jung M. Chromatin Modifications as Targets for New Anticancer Drugs. *Archiv der Pharmazie*. 2005; 338:347–357. [PubMed: 16041839]
- Bedford MT, Richard S. Arginine Methylation: An Emerging Regulator of Protein Function. *Molecular cell*. 2005; 18:263–272. [PubMed: 15866169]

3. Cheung N, Chan LC, Thompson A, Cleary ML, So CWE. Protein arginine-methyltransferase-dependent oncogenesis. *Nat Cell Biol.* 2007; 9:1208–1215. [PubMed: 17891136]
4. Cheng D, Yadav N, King RW, Swanson MS, Weinstein EJ, Bedford MT. Small Molecule Regulators of Protein Arginine Methyltransferases. *Journal of Biological Chemistry.* 2004; 279:23892–23899. [PubMed: 15056663]
5. Dery U, Coulombe Y, Rodrigue A, Stasiak A, Richard S, Masson J-Y. A Glycine-Arginine Domain in Control of the Human MRE11 DNA Repair Protein. *Mol. Cell. Biol.* 2008; 28:3058–3069. [PubMed: 18285453]
6. Yu Z, Vogel G, Coulombe Y, Dubeau D, Spehalski E, Hebert J, Ferguson DO, Masson JY, Richard S. The MRE11 GAR motif regulates DNA double-strand break processing and ATR activation. *Cell Res.* 2011
7. Torres-Padilla ME, Parfitt DE, Kouzarides T, Zernicka-Goetz M. Histone arginine methylation regulates pluripotency in the early mouse embryo. *Nature.* 2007; 445:214–218. [PubMed: 17215844]
8. Heinke R, Spannhoff A, Meier R, Trojer P, Bauer I, Jung M, Sippl W. Virtual Screening and Biological Characterization of Novel Histone Arginine Methyltransferase PRMT1 Inhibitors. *ChemMedChem.* 2009; 4:69–77. [PubMed: 19085993]
9. Sasser JM, Moninka NC, Cunningham MW, Croker B, Baylis C. Asymmetric dimethylarginine in angiotensin II-induced hypertension. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology.* 2010; 298:R740–R746.
10. Chen MF, Xie XM, Yang TL, Wang YJ, Zhang XH, Luo BL, Li YJ. Role of asymmetric dimethylarginine in inflammatory reactions by angiotensin II. *J Vasc Res.* 2007; 44:391–402. [PubMed: 17551258]
11. Vedel M, Lawrence F, Robert-Gero M, Lederer E. The antifungal antibiotic sinefungin as a very active inhibitor of methyltransferases and of the transformation of chick embryo fibroblasts by rous sarcoma virus. *Biochemical and Biophysical Research Communications.* 1978; 85:371–376. [PubMed: 217377]
12. Spannhoff A, Heinke R, Bauer I, Trojer P, Metzger E, Gust R, Schüle R, Brosch G, Sippl W, Jung M. Target-Based Approach to Inhibitors of Histone Arginine Methyltransferases. *Journal of Medicinal Chemistry.* 2007; 50:2319–2325. [PubMed: 17432842]
13. Spannhoff A, Machmur R, Heinke R, Trojer P, Bauer I, Brosch G, Schüle R, Hanefeld W, Sippl W, Jung M. A novel arginine methyltransferase inhibitor with cellular activity. *Bioorganic & Medicinal Chemistry Letters.* 2007; 17:4150–4153. [PubMed: 17570663]
14. Ragno R, Simeoni S, Castellano S, Vicidomini C, Mai A, Caroli A, Tramontano A, Bonaccini C, Trojer P, Bauer I, Brosch G, Sbardella G. Small Molecule Inhibitors of Histone Arginine Methyltransferases: Homology Modeling, Molecular Docking, Binding Mode Analysis, and Biological Evaluations. *Journal of Medicinal Chemistry.* 2007; 50:1241–1253. [PubMed: 17323938]
15. Mai A, Cheng D, Bedford MT, Valente S, Nebbioso A, Perrone A, Brosch G, Sbardella G, De Bellis F, Miceli M, Altucci L. Epigenetic Multiple Ligands: Mixed Histone/Protein Methyltransferase, Acetyltransferase, and Class III Deacetylase (Sirtuin) Inhibitors. *Journal of Medicinal Chemistry.* 2008; 51:2279–2290. [PubMed: 18348515]
16. Cheng D, Yadav N, King RW, Swanson MS, Weinstein EJ, Bedford MT. Small molecule regulators of protein arginine methyltransferases. *J Biol Chem.* 2004; 279:23892–23899. [PubMed: 15056663]
17. Li KK, Luo C, Wang D, Jiang H, Zheng YG. Chemical and biochemical approaches in the study of histone methylation and demethylation. *Med Res Rev.* 2010
18. Bissinger E-M, Heinke R, Spannhoff A, Eberlin A, Metzger E, Cura V, Hassenboehler P, Cavarelli J, Schüle R, Bedford MT, Sippl W, Jung M. Acyl derivatives of p-aminosulfonamides and dapson as new inhibitors of the arginine methyltransferase hPRMT1. *Bioorganic & Medicinal Chemistry.* 2011; 19:3717–3731. [PubMed: 21440447]
19. Li KK, Luo C, Wang D, Jiang H, Zheng YG. Chemical and biochemical approaches in the study of histone methylation and demethylation. *Medicinal Research Reviews.* 2010 n/a-n/a.

20. Zheng YG, Wu J, Chen Z, Goodman M. Chemical regulation of epigenetic modifications: Opportunities for new cancer therapy. *Med Res Rev.* 2008; 28:645–687. [PubMed: 18271058]
21. Stahl M, Guba W, Kansy M. Integrating molecular design resources within modern drug discovery research: the Roche experience. *Drug Discov Today.* 2006; 11:326–333. [PubMed: 16580974]
22. Liang Z, Zhang D, Ai J, Chen L, Wang H, Kong X, Zheng M, Liu H, Luo C, Geng M, Jiang H, Chen K. Identification and synthesis of N'-(2-oxoindolin-3-ylidene)hydrazide derivatives against c-Met kinase. *Bioorganic & Medicinal Chemistry Letters.* 2011; 21:3749–3754. [PubMed: 21561768]
23. Li N, Thompson S, Schultz DC, Zhu W, Jiang H, Luo C, Lieberman PM. Discovery of Selective Inhibitors Against EBNA1 via High Throughput *In Silico* Virtual Screening. *PLoS ONE.* 2010; 5:e10126. [PubMed: 20405039]
24. Luo C, Xie P, Marmorstein R. Identification of BRAF Inhibitors through *In Silico* Screening. *Journal of Medicinal Chemistry.* 2008; 51:6121–6127. [PubMed: 18783202]
25. Guner O, Clement O, Kurogi Y. Pharmacophore modeling and three dimensional database searching for drug design using catalyst: recent advances. *Curr Med Chem.* 2004; 11:2991–3005. [PubMed: 15544485]
26. Dube D, Periwal V, Kumar M, Sharma S, Singh T, Kaur P. 3D-QSAR based pharmacophore modeling and virtual screening for identification of novel pteridine reductase inhibitors. *Journal of Molecular Modeling.* 2011:1–11.
27. Tanneeru K, Guruprasad L. Ligand-based 3-D pharmacophore generation and molecular docking of mTOR kinase inhibitors. *Journal of Molecular Modeling.* 2011:1–14.
28. Chen Z, Tian G, Wang Z, Jiang H, Shen J, Zhu W. Multiple Pharmacophore Models Combined with Molecular Docking: A Reliable Way for Efficiently Identifying Novel PDE4 Inhibitors with High Structural Diversity. *Journal of Chemical Information and Modeling.* 2010; 50:615–625. [PubMed: 20353193]
29. Spannhoff A, Heinke R, Bauer I, Trojer P, Metzger E, Gust R, Schule R, Brosch G, Sippl W, Jung M. Target-based approach to inhibitors of histone arginine methyltransferases. *J Med Chem.* 2007; 50:2319–25. [PubMed: 17432842]
30. Wu J, Xie N, Wu Z, Zhang Y, Zheng YG. Bisubstrate Inhibitors of the MYST HATs Esa1 and Tip60. *Bioorg Med Chem.* 2009; 17:1381–1386. [PubMed: 19114310]
31. Feng Y, Li M, Wang B, Zheng YG. Discovery and Mechanistic Study of a Class of Protein Arginine Methylation Inhibitors. *Journal of Medicinal Chemistry.* 2010; 53:6028–6039. [PubMed: 20666457]
32. Thompson PR, Kurooka H, Nakatani Y, Cole PA. Transcriptional Coactivator Protein p300. *Journal of Biological Chemistry.* 2001; 276:33721–33729. [PubMed: 11445580]
33. Lin M-F, Meng T-C, Rao PS, Chang C, Schönthal AH, Lin F-F. Expression of Human Prostatic Acid Phosphatase Correlates with Androgen-stimulated Cell Proliferation in Prostate Cancer Cell Lines. *Journal of Biological Chemistry.* 1998; 273:5939–5947. [PubMed: 9488733]
34. Igawa T, Lin F-F, Lee M-S, Karan D, Batra SK, Lin M-F. Establishment and characterization of androgen-independent human prostate cancer LNCaP cell model. *The Prostate.* 2002; 50:222–235. [PubMed: 11870800]
35. Xie N, Elangwe EN, Asher S, Zheng YG. A dual-mode fluorescence strategy for screening HAT modulators. *Bioconj Chem.* 2009; 20:360–6. [PubMed: 19146394]
36. Feng Y, Li M, Wang B, Zheng YG. Discovery and mechanistic study of a class of protein arginine methylation inhibitors. *J Med Chem.* 2010; 53:6028–39. [PubMed: 20666457]

Figure 1.
The flowchart for virtually screening PRMT1 inhibitors.

Figure 2.

Kinetic analysis of PRMT1 inhibition by A9 and A36. (A) Double-reciprocal plotting of initial velocities versus varied concentrations of H4 (1–20) at fixed [¹⁴C]-SAM (5 μM) and varying A9: 0 μM (●), 20 μM (◆), 40 μM (–), 60 μM (▲) and 80 μM (■). (B) Double-reciprocal plotting of initial velocities versus varied concentrations of [¹⁴C]-SAM at fixed H4-20 (2 μM) and varying A9: 0 μM (◆), 20 μM (■), 40 μM (▲) and 80 μM (●). (C) Double-reciprocal plotting of initial velocities versus varied concentrations of H4-20 at fixed [¹⁴C]-SAM (5 μM) and varying A36: 0 μM (◆), 10 μM (▲), 20 μM (■) and 30 μM (●). (D) Double-reciprocal plotting of initial velocities versus varied concentrations of 23 [¹⁴C]-SAM at fixed H4-20 (2 μM) and varying A36: 0 μM (◆), 10 μM (▲), 20 μM (■), 30 μM (●), and 40 μM (*).

Figure 3.

Effect of A36 on the fluorescence spectra of H4(1–20)FL and PRMT1. (A) Fluorescence emission spectra of H4(1–20)FL (1 μ M) at different concentrations of A36 (0–100 μ M). The excitation wavelength was 498 nm. (B) Fluorescence intensity of H4(1–20)FL at 523 nm as a function of A36 concentration. (C) Fluorescence emission spectra of PRMT1 (1 μ M) at different concentrations of A36 (0–50 μ M). The excitation wavelength was 285 nm. (D) Fluorescence intensity of H4(1–20)FL at 338 nm as a function of A36 concentration.

Figure 4. Inhibition of p300 HAT activity by A36. The fractional activity of p300 was plotted with respect to the concentration of A36.

B

	Peptide substrate	Remaining activity of the respective protein
PRMT1	H4(1-20)	5%
PRMT4	H3(1-31)	38%
PRMT5	H4(1-20)	20%

Figure 5.

(A) Comparative activity of A36 for PRMT1, PRMT4, and PRMT5. (B) Quantification of remaining activity of respective PRMT.

A **B** **Figure 6.**

Antiproliferative effect of A9 on prostate cancer cells. (A) The dose dependent effect of A9 on LNCaP C-81 cells in regular medium. (B) The dose dependent effect of A9 on LNCaP C-81 cells in steroid-reduced medium. The results presented were mean $\pm$ SD of three sets of independent experiments in triplicates. $p < 0.05$, $p < 0.001$ are statistically significant difference from controls.

Table 1

Results of Statistical Significance, Correlation Coefficients, and Features of Nine Hypotheses^d

hypothesis	total cost	RMS ^b	correlation	cost ^c	features ^{d,e}		
					HB ^f	HBD	RA
2	80.413	0.385	0.930	86.192	*	*	*
3	80.423	0.388	0.930	86.182	* ²		*
4	80.453	0.392	0.928	86.152	* ²		*
5	80.520	0.401	0.925	86.085	*	*	*
6	80.666	0.423	0.916	85.939	* ²		*
7	80.722	0.414	0.923	85.883	* ²		*
8	80.836	0.433	0.915	85.769	* ²		*
9	80.933	0.433	0.917	85.672	* ²		*
10	81.047	0.438	0.916	85.558	* ²		*

^aFixed cost of nine hypotheses is 79.143, and null cost value is 166.605.

^bRMS, root mean square. For RMS value, the smaller, the better.

^ccost= (null cost)–(total cost).

^dHB^f, HB_ACCEPTOR; HBD, HB_DONOR; RA, RING_AROMAIC.

^eThe asterisk (*) indicated that these features were present in the hypothesis.

^fNumber of the feature presenting in the hypothesis.

Table 2

Chemical structures of identified PRMT1 inhibitors that show inhibitive activity against PRMT1.

Compound ID	SPECS product ID	compound	Inhibition (%)
A9	AE-848/32000014		65%
A36	AE-848/34542007		79%
A18	AA-516/12432333		48%
A56	AE-848/15341269		51%
A44	AA-516/33240027		42%
A69			52%

Table 3

Comparison of the inhibition activity of A9, A36 with NSC35605 stilbamidine, AMI-1.

Compound ID	IC ₅₀ (μM)
A9	41.7 ± 6.3
A36	12.0 ± 0.2
NSC35605 (stilbamidine)	137.6 ± 7.2
AMI-1	76.9 ± 15.4