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Small Molecule Inhibitors of Protein Arginine Methyltransferases

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Abstract

Introduction—Arginine methylation is an abundant posttranslational modification occurring in mammalian cells and catalyzed by protein arginine methyltransferases (PRMTs). Misregulation and aberrant expression of PRMTs are associated with various disease states, notably cancer. PRMTs are prominent therapeutic targets in drug discovery.

Areas covered—The authors provide an updated review of the research on the development of chemical modulators for PRMTs. Great efforts are seen in screening and designing potent and selective PRMT inhibitors, and a number of micromolar and submicromolar inhibitors have been obtained for key PRMT enzymes such as PRMT1, CARM1, and PRMT5. The authors provide a focus on their chemical structures, mechanism of action, and pharmacological activities. Pros and cons of each type of inhibitors are also discussed.

Expert opinion—Several key challenging issues exist in PRMT inhibitor discovery. Structural mechanisms of many PRMT inhibitors remain unclear. There lacks consistency in potency data due to divergence of assay methods and conditions. Physiologically relevant cellular assays are warranted. Substantial engagements are needed to investigate pharmacodynamics and pharmacokinetics of the new PRMT inhibitors in pertinent disease models. Discovery and evaluation of potent, isoform-selective, cell-permeable and *in vivo*-active PRMT modulators will continue to be an active arena of research in years ahead.

Keywords

Arginine methylation; PRMT; Histone methylation; Inhibitor; Drug discovery; Chemical probe; Epigenetics

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1. Overview of PRMT function in biology and disease

Arginine methylation occurs ubiquitously as one of cellular posttranslational modifications in eukaryotic organisms [1]. It is mediated by the family of *N*-arginine methyltransferases (PRMTs). PRMTs impact on numerous essential biological pathways (e.g. epigenetic regulation, RNA processing, DNA repair, hormone-receptor signaling, etc.) by methylating different nuclear, cytoplasmic and membrane protein substrates. Misregulation or aberrant expression of PRMTs has been found in various pathological conditions, notably cancer.

During PRMT catalysis, the methyl group of the co-substrate *S*-adenosylmethionine (SAM or AdoMet) is transferred to a protein substrate and replaces a hydrogen atom on the ω-N^G of arginine, generating methylated arginine as a final product and leaving *S*-adenosyl homocysteine (SAH or AdoHcy) as the side product (Figure 1). There are mainly three types of methylated arginine products: monomethylarginine (MMA), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA). Currently, nine members of PRMTs have been identified with established arginine methylation activity in human cells [2]. They are classified into three types: type I, II and III. Type I enzymes (EC 2.1.1.319) convert arginine to MMA and further to ADMA [2, 3], including PRMT1, -2, -3, -4 -6 and -8. PRMT4 is also known as coactivator-associated arginine methyltransferase 1, or CARM1. Type II enzyme (EC 2.1.1.320) is comprised of PRMT5 [4] and -9 [5], which produce MMA and SDMA. PRMT7 is the only type III PRMT (EC 2.1.1.321) that generates MMA [6]. The global arginine levels in the mouse embryo fibroblast (MEF) cells are found to be 1500:3:2:1 for Arg:ADMA:MMA:SDMA [7]. PRMT1 accounts for 50% of ADMA formation [8], and PRMT5 is the predominant type II enzyme, responsible for the bulk of SDMA in MEF [9].

PRMTs have a wide range of protein substrates (Table 1). PRMT-1, -3, 5-, -6 and -8 prefer to methylate the glycine- and arginine-rich (GAR) region [7, 10]. There is a high degree of promiscuity in the substrate recognition by these PRMTs [1, 11]. No conserved sequences neighboring the methyl arginine sites are required [12], and the modification sites often occur in unstructured protein regions [13]. Structurally, the large binding surface for peptide substrates also supports the promiscuous nature of PRMTs [1, 11]. CARM1 tends to methylate proline-, glycine- and methionine-rich (PGM) motif [14, 15]; PRMT5 methylates GAR sequences as well as PGM regions [4]; PRMT7 was recently found to target R*X*R (*X* is a variable residue) in a lysine- and arginine-rich (KAR) motif [6] but not the previously reported GAR motif [16]; PRMT9 does not recognize GAR motif, and possesses very restricted activity on spliceosome-associated protein 145 (SAP145, also known as splicing factor 3b subunit 2, SF3B2) [5, 17].

A notable function of the PRMTs is that by depositing methyl marks onto the nucleosomal histone tails, they participate in the regulation of the dynamic transitions between transcriptionally active or silent chromatin states [18, 19]. PRMTs may act as either coactivators or repressors, depending on the modification site and status. As such, histone arginine methylation is an important component contributing to the complexity of the overall epigenetic marking system. Identified histone arginine methylation sites include H2AR3 (PRMT1, -5, -6), H2AR29/R31 (PRMT7), H3R2 (PRMT6), H3R8 (PRMT5), H3R17/R26

(CARM1), H4R3 (PRMT1, -5, -6), and H4R17/R19 (PRMT7) [3, 20–22], and there might be more to be uncovered. It is possible that a specific mark correlate to a specific chromatin state, despite the lack of understanding in their related pathways [3]. Binding of reader proteins to arginine methylation marks further recruits effectors of downstream signaling machinery, thus to mediate gene transcription, DNA replication and repair events [23, 24]. Arginine methylation can also block or promote other histone modifications or DNA methylation. The cross-talks among various epigenetic modifications further diversify the pathways of gene transcription and other fundamental DNA templated processes [21, 25, 26]. Grand challenges remain in deciphering the complex “histone codes.” Elaborating roles of PRMTs in histone modification will certainly fill in the knowledge gap.

PRMTs also have a broad spectrum of non-histone substrates, which are involved in crucial cellular regulations, such as transcription, RNA splicing, signal transduction, nuclear/cytoplasmic shuttling and DNA repair. Protein arginine methylation affects protein-protein interaction, protein-DNA or protein-RNA interaction, protein stability, subcellular localization, and enzymatic activity [3, 7, 27–29]. Selected PRMT substrates and functions are summarized in Table 1. For example, CARM1 methylates the C-terminal repeat domain (CTD) of RNA polymerase II for transcriptional control [30]. The cytoplasmic substrates of PRMT5, small nuclear ribonucleoproteins SmD1 and SmD3, are responsible for spliceosome assembly; spliceosome-associated proteins SAP49 and SAP145 are the substrates of CARM1 and PRMT9, respectively [5, 31]. PRMT1 methylates Sam68 (Src associated in mitosis, 68 kDa), an RNA-binding protein, and mediates signal transduction [32, 33]. PRMT1 also methylates the MRE11 (meiotic recombination 11) checkpoint protein for DNA damage checkpoint response and DNA repair [34]. PRMT5 takes part in a DNA damage responsive co-activator complex [35]. CARM1 methylates RNA-binding protein human antigen R (HuR) to affect its shuttling [36]. Despite the accumulating findings that reveal the molecular basis of PRMTs in the cell, our understanding of functions of this specific family of PTM enzymes remains to be further developed in depth, not to mention there is much of unknown to be discovered.

Mounting studies have pointed to multifaceted roles of PRMT misregulation in disease development. PRMT-related pathologies include virus-related diseases [37–39], inflammatory response [40, 41], cardiovascular disease [42, 43], renal disease [44, 45], diabetes [46], pulmonary disorders [47] and the most actively studied area, carcinogenesis [2, 3, 27, 48–53] (Table 1). Arginine methylation levels might be used as diagnostic biomarkers. For instance, the amount of ADMA is related to cardiovascular pathological conditions [42]; and in prostate cancer, the methylation of H4R3 predicts the risk of prostate cancer recurrence [54]. PRMTs are overexpressed in many types of cancers and some tumor types may overexpress several members of PRMTs, as listed in Table 1. Additionally, alternative splicing of PRMT isoforms may also play a critical role [55]: for example, seven PRMT1 isoforms (PRMT1v1-v7) have been found by alternative splicing in the 5′ region of its pre-mRNA [56], and PRMT1v1 is the most abundant variant. The overexpression of PRMT1v1 was observed colon cancer [57]; PRMT1v2 had the greatest increase in breast cancer and can promote the survival and invasiveness of breast cancer cells [58]. PRMT1 is a component of a mixed lineage leukaemia (MLL) transcriptional complex [59], and it is essential for the development of acute myeloid leukaemia (AML) [60]. A recent study shows that upregulated PRMT5 activity enhances AML growth [61]. PRMT5 is critical to B-cell lymphomagenesis and the maintenance of its malignant phenotype [37]. While it has been widely known that misregulation of PRMTs is involved in carcinogenesis and tumor progression, in most cases, the specific roles of these enzymes in cancer pathology are still poorly studied [2].

Along the line with biological studies on illuminating PRMT functions in physiology and pathology, developing potent and selective PRMT inhibitors has become a well-timed research endeavor in both academic laboratories and pharmaceutical industry. A number of PRMT inhibitors have been identified in the last decade through the efforts of researchers worldwide [62, 63]. Because multiple PRMT isoforms exist in the cell and each owns distinct functions, an important goal in the campaign of PRMT drug discovery is to find out effective lead compounds highly specific for one particular isoform enzyme. Pan-MTase inhibitors, such as simple SAM analogs sinefungin, SAH, methylthioadenosine (MTA), and AzaAdoMet (Figure 2), are often used as functional tools to change global methylation levels in cells and as cofactor ligands for determination of MTase X-ray structures [64]. However, due to their notorious promiscuity, pan-MTase inhibitors are not suited for use as chemical genetic tools for elucidating function of a particular PRMT isozyme in the proteomic context. In this regard, potent and isoform-selective small-molecule inhibitors will be much more desired. Encouragingly, quite a few small-molecule PRMT inhibitors have developed from different research laboratories and exhibit prominent isoform specificity, though at varying degrees. Below, we will first discuss biochemical assays used in the PRMT drug discovery, and then elaborate on different PRMT inhibitors according to their respective PRMT targets.

2. Biochemical assays in PRMT inhibitor study

Discovery of PRMT modulators relies on effective assays to quantitatively measure PRMT activities. Quite a few biochemical assays have been developed for PRMTs and protein lysine methyltransferases (PKMTs) (see the recent reviews [65–69]). The radiometric assays have been the gold standard for quantitating *in vitro* methyltransferase activity of PRMTs due to their high sensitivity. In a general procedure, the radioisotope-labeled methyl group from [^{3}H]-SAM or [^{14}C]-SAM is first enzymatically transferred into a peptide or protein substrate of PRMTs. Prior to quantitation by autoradiography or liquid scintillation counting, the methylated substrates have to be separated from unreacted SAM by using different approaches such as polyacrylamide gel electrophoresis (radiometric gel assay, RGA) [70–76], pipette chromatography (ZipTip assay) [77], and filtering on glass fiber or phosphocellulose paper discs (radiometric filter assay, RFA) [37, 73, 78–90]. To eliminate the washing step required for the above described radiometric assays, scintillation proximity assay (SPA) has been implemented [80, 91–101], in which the scintillation signals depend on the micrometer proximity between biotinylated substrates and streptavidin-coated scintillants (either FlashPlates or streptavidin-coated microscopic beads). As such, the SAM molecules present in the bulk solution fall off the SPA distance and thus do not produce scintillation signals.

Antibody-based immunosorbent assays represent another category of widely used methods for detection of PRMT activities, in which a methylated substrate is recognized by methyl-arginine specific antibody. A typical format of these assays is enzyme-linked immunosorbent assay (ELISA) [70, 102, 103], in which methylated substrates are adsorbed onto microplate wells and incubated with primary antibody that is further probed with horseradish peroxidase (HRP)-labeled secondary antibody. After wash-out of the unbound antibodies, chemiluminescence is developed from the HRP. Dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) [71, 104–106] is similar to ELISA, except that the antibody is labeled with a lanthanide probe instead of HRP. The lanthanide dissociates from the antibody by addition of an enhancement cocktail and gains amplified fluorescence under excitation at 340 nm. Lanthanide as fluorophore features large stoke shifts, long decay time and narrow emission spectrum, which together minimize the interference of background fluorescence. Other assay methods involving lanthanide labels are homogeneous (no-wash) technologies that include time-resolved fluorescence resonance energy transfer (TR-FRET) [107–110] and AlphaLISA [107, 111]. In TR-FRET assay, energy is transferred from the donor europium chelate to the acceptor fluorophore within FRET distance (~10 nm). For AlphaLISA, donor beads convert ambient oxygen to singlet oxygen upon excitation at 680 nm, which can diffuse about 200 nm in solution. Then, the europium in the acceptor bead within this range receives energy and emits fluorescence (Figure 3).

Quite a few assays have also been developed to detect SAH, the side product of the methylation reaction. These strategies are to convert SAH into derivatives with colorimetric, fluorescent, or luminescent properties by coupled enzymatic reactions. In the SAHH (SAH hydrolase)-coupled assay, SAH is hydrolyzed into adenosine and homocysteine, the latter of which subsequently reacts with ThioGlo or CPM, yielding strong fluorescence [112, 113] [96, 114]. In another approach, SAH is converted into adenine by SAH nucleosidase (SAHN) and then to hypoxanthine by adenine deaminase (ADA). The methylation process is monitored by the absorbance difference of adenine and hypoxanthine [115]. Additionally, strategies are also reported to convert adenine to ATP which can be monitored by luciferase-linked bioluminescence [116, 117]. Interested readers are referred to reviews [67, 68] for more discussions of methyltransferase assays.

3. PRMT1-specific inhibitors

PRMT1 is the major enzyme responsible for asymmetric dimethylation of protein arginine residues [118]. PRMT1 methylates histone H4 and many other non-histone proteins. Studies have linked PRMT to many crucial biological processes such as transcription, cell signaling, RNA splicing and DNA repair [50]. Overexpression of PRMT1 has been observed in breast, prostate, lung, colon, bladder cancer and leukemia [2]. It is also found to relate with pulmonary diseases [47], cardiovascular diseases [119], diabetes [120, 121] and renal diseases [122].

The recombinant PRMT1 is well expressed in bacteria and demonstrates robust enzymatic activity. Thus, it is no surprise that PRMT1 is the most widely used PRMT model to test arginine methylation inhibitors. In 2004, Bedford and coworkers identified AMI series of compounds, represented by compounds **1** (AMI-1) and **2** (AMI-8) (Table 2), as the first set of small molecules to target PRMTs [70]. Details about this work and related studies are discussed in Section 8.1.

Compound **3** (allantodapsone) is found from a virtual screening on a library of 1990 compounds based on a homology-modeled PRMT1 structure. It shows IC_{50} of 1.7 μM on PRMT1 in DELFIA assay [104]. This compound inhibits cellular H4R3 methylation to the same level as AMI-1 at the same concentration in hepatocellular carcinoma cell line HepG2 while the H3K4 methylation level is barely impacted. Based on the pharmacophore of **3**, compounds **4** and **5** are discovered by the same group from another round of virtual screening and show about 1/10 of the potency of **3** [105]. Later, a series of compounds are explored based on the structure of **3** and **4** [71]. Among these, **6** and **7** exhibit similar activity to their parental compounds in RGA assay and are demonstrated with reversible mode of inhibition. **6** is inactive to CARM1 and SET7/9 determined by DELFIA, while **7** shows 60% inhibition on SET7/9 at 50 μM and is inactive to CARM1. **6** inhibits the growth of breast cancer cell line MCF7a and prostate cancer cell line LNCaP with GI_{50} (i.e. concentration that produces 50% inhibition of growth) of 2 μM and 5 μM, respectively. **6** at 10 μM specifically reduces the level of androgen-dependent transcription to a significant degree. Intriguingly, compounds **3** – **7** structurally resemble the previously found AMI compound (AMI-8, compound **2**) and they possess comparable inhibitory activity toward PRMT1, indicating they may share very similar pharmacophore. Notably, the selectivity profile of compound **6** is improved compared with **2**.

Compound **8** (RM65) is another early-stage PRMT1 inhibitor discovered from virtual screening, with IC_{50} around 55 μM [106]. It is less active than AMI-1 (1.2 μM) and is inactive on SET7/9 at 50 μM. High dose (150 μM) of **8** is needed to achieve 50% inhibition of arginine methylation in HepG2 cells. It is of note that selectivity profile among PRMTs has not been reported for compounds **3** – **5** and **8**, so they cannot be considered as PRMT1-specific inhibitors. Besides, it is unlikely that compound **8** is useful in dissection of cellular PRMT1 function considering its relatively weak inhibitory activity.

Discovered from a structure-based virtual screening, compounds **9** and **10** were subsequently validated with better inhibitory activity on PRMT1 (IC_{50} = 18 μM and 26 μM, respectively) than AMI-1 (55 μM) using a TR-FRET assay. Attractively, **9** is inactive to CARM1 and PRMT6 at 6-fold of the IC_{50} on PRMT1, but **10** shows lower selectivity. At 100 μM, both compounds inhibit more than 40% of the proliferation of HepG2, MCF7 and leukemic monocyte cell line THP1, with **9** showing better activity [107].

We recently screened a serial carbocyanine compounds against PRMT1 using the RFA assay [78], among which compound **11** was discovered with promising potency (IC_{50} = 4.1 μM) compared to AMI-1 and compound **13** **(**stilbamidine) (137 μM and 105 μM, respectively, determined under the same assay condition [89]). This compound also inhibits PRMT3, -5 and -6 with slightly weaker potency but is totally inactive to CARM1. The GI_{50} of compound **11** on cervical cancer cell line HeLa is around 1 μM at 48 h of incubation. Fluorescent imaging shows that **11** is concentrated in the nucleus and arrests the cell growth in the S phase, suggesting that it may interfere with the cell growth by causing chromatin dysfunction. In the following study [95], an extensive SAR study was performed on structurally diversified cyanine compounds, which demonstrates that symmetric structure, halogen-substituted bulky aromatic groups caging the charged N atom and hydrophobic side chain on the N atom are all essential to the activity. Among the investigated compounds, compound **12** exhibits decent potency (IC_{50} = 3.4 μM) on PRMT1 compared to SAH (IC_{50} = 0.4 μM) by the SPA measurement. Tested under the balanced condition, the compound exhibits more than 6-, 10-, and 25-fold selectivity for PRMT1 over CARM1 (IC_{50} = 21.5 μM), PRMT5 (IC_{50} = 35.4 μM), and PRMT8 (IC_{50} = 84.9 μM). The fluorescence intensity of **12** increases about 6-fold upon binding to PRMT1, indicative of direct interaction between PRMT1 and **12**. The cellular activity of **12** was tested against chronic myelogenous leukemia cell line Meg01, acute myeloid leukemia cell line MOLM13 and erythroleukemia cell line HEL. Remarkably, **12** suppresses the cell proliferation at submicromolar level and is less sensitive in the HEL cells in which Janus kinase 2 (JAK2) has V617F mutation and is hence less dependent on PRMT1-mediated signaling pathway, indicating the anti-proliferation effect is achieved by interfering with PRMT-dependent pathway. Consistently, the global cellular methylation declines with 24h-treatment of **12** and the effect is significant at 100 nM of the compound for Meg01 and MOLM13 cells. One unique advantage of carbocyanine inhibitors is their strong fluorescent property, which can be utilized for subcellular tracking using fluorescence imaging microscopy [78].

Quite a few PRMT1 inhibitors found so far contain one or two amidine groups. The amidine moiety is a structural mimic of the side-chain guanidino moiety of substrate arginine: both of which are rigidly planar, highly basic, with two ω-nitrogen atoms. Although structurally not yet confirmed, the amidine could possibly act as a substrate-competitive functional group for PRMT binding in the active site. The first such inhibitor is compound **13** (stilbamidine), identified during a screening campaign of disclosing allantodapsone **3** [71]. It inhibits PRMT1 activity with an IC_{50} of 57 μM in the enzymatic assay. The inhibitory effect on the H4R3 methylation by stilbamidine is the same as AMI-1 and allantodapsone in HepG2 cells. Besides, stilbamidine shows better activity than AMI-1 on reducing the transcriptional activation of an estrogen-dependent gene in MCF-7-2a cells. Yu et al. designed a serial amidines based on an amidine hit from initial screening [111]. The SAR indicates at least one amidine functionality is required for the activity, with other substitutions dictating the activity as well. Tested by AlphaLISA assay, compound **14** exhibits a 10-fold better potency than AMI-1 (2.0 μM vs 22 μM) and promising selectivity for PRMT1 over CARM1, PRMT5 and PRMT6. The docking study suggests this compound occupies the substrate arginine binding site. The cancer cell viability tests show the colon cancer cell line DLD-1 is the most sensitive (GI_{50} = 4.4 μM), followed by bladder cancer cell line T24 and neuroblastoma cell line SH-SY-5Y. In contrast, AMI-1 is inactive for all the tested cell lines. In DLD-1 cells, **14** reduce the H4R3 methylation level significantly starting from 3 μM.

We screened a focused library of diamidine compounds against PRMT1 under the balanced assay condition [79]. The leading hit, compound **15 (**furamidine, also named DB75), shows inhibitory activity for PRMT1 at low micromolar potency. This study unravels some interesting SAR that is useful to guide further structure optimization. First, substitution by bulky group compromises the activity, which is probably caused by increased steric hindrance in the binding pocket assuming the amidino group mimics the structure of guanidine group in substrate arginine. Second, reducing the two amidino groups to one tampers the activity; perhaps the diamidine structure increases the probability of effective binding position of the amidino group. Third, the activity is reduced when the O atom in the furan is replaced by NH, especially by substituted NH, but replacing the O atom by S or Se atom was tolerated. DB75 is an old drug, originally developed for antiparasite treatment by targeting the minor groove of AT-rich DNAs. Its activity on PRMT inhibition, however, was unknown prior to our work. The IC_{50} of DB75 on PRMT1 is 2-fold lower than stilbamidine. DB75 shows a very favorable selectivity attribute for PRMT1 over the other PRMT members: 42-fold over CARM1, 30-fold over PRMT6, >15-fold over PRMT5. The selectivity property is also better than stilbamidine. The steady-state kinetic results show that DB75 is noncompetitive against SAM and has a strong nature of competitive inhibition with respect to the H4 peptide substrate. The competitive inhibition of DB75 versus the H4 substrate is also supported by competitive fluorescence anisotropy assay. Besides, surface plasmon resonance (SPR) assay has excluded any possibility of interaction between DB75 and H4 peptide, confirming the direct interaction between the inhibitor and enzyme. This diamidine molecule has a rigid, crescent, planar scaffold. Molecular modeling showed that the planar curving shape enables DB75 to fit the shape of the catalytic cavity of PRMT1, occupying partially the co-factor site and also spanning into the substrate arginine binding site. The computed binding free energy ΔG_b arises from the better shape complementarity of compounds DB75 for the PRMT1 pocket. By contrast, PRMT5 exhibits a larger, partially solvent exposed pocket and binds the ligands less tightly in agreement with the smaller computed affinity ΔG_b.

DB75 has been used as a tool compound to study PRMT1 function in different biological systems. Compound DB75 penetrated the plasma membrane of 293T cells (human embryonic kidney cell line) and inhibited cellular PRMT1 activity on ALY protein (also known as THO complex subunit 4, or THOC4) methylation, thereby regulating the turnover rate of ALY [79]. Treatment with DB75 (at 20 μM) for 72 hours led to significant inhibition of the growth of several leukemia cells. Intriguingly, cell lines derived from Down's syndrome patients and MLL-AF9 (a fusion oncogene seen in leukemia cells) patient seems more sensitive. Compound **16**, a close structural analogue of DB75, was used as a tool compound to inhibit PRMT1-mediated Smad6 methylation, revealing that the methyltransferase activity of PRMT1 is essential for bone morphogenetic proteins (BMP)-induced Smad1/Smad5 phosphorylation and downstream signaling activation [123]. Recently, we found that DB75 inhibits PRMT1-mediated putative RNA-binding protein 15 (RBM15) methylation at residue R578 [124]. Arginine methylation of RBM15 leads to its ubiquitination by the E3 ligase CNOT4 and subsequent degradation. Down-regulation of RBM15 changes alternative splicing of the downstream genes such as GATA1 (GATA-binding factor 1), C-MPL (MPL proto-oncogene) and RUNX1 (runt-related transcription factor 1 gene), which are critical for megakaryocyte differentiation. RBM15 binds to the SF3B1-containing complex in methylation-dependent manner. SF3B1 has been shown to be mutated in the myelodysplastic syndrome and other forms of leukemia. Thus, PRMT1-mediated methylation of RBM15 directly links the protein arginine methyltransferase to

RNA splicing pathways. Interestingly, Siboni et al. [125] recently found that DB75 impacts on RNA splicing in a myotonic dystrophy type 1 (DM1) model with equal efficacy and low toxicity compared to another diamidine compound pentamidine. Given our observation that DB75 modulates PRMT1-mediated RBM15-SF3B1 splicing regulators, it could be possible that DB75 also regulates RNA splicing in DM1 through PRMT1 inhibition. This hypothesis has to await future experimental verification.

Recently, inhibitors with core structure of **17** (Table 2) were disclosed as effective inhibitors for PRMT1 (as well as PRMT6 and PRMT8) in two patents filed by Epizyme [126, 127]. It is not clear how selective and how potent they are. However, out of this series, compounds with extended side chains at para-position (R1) of phenyl group were subsequently reported as selective PRMT6 inhibitors (see Section 7).

4. PRMT3-specific inhibitors

There are only a few non-histone substrates found for PRMT3 (Table 1). A major PRMT3 substrate is rpS2 (ribosomal protein S2), which interacts with the zinc finger of PRMT3 [128]. It has been found to play a role in ribosomal homeostasis [129]. Overexpression or enhanced activity of PRMT3 has been recognized in breast cancer [2], coronary heart disease [130] and chronic kidney disease [131].

Compound **18** (Table 3) is the first reported specific inhibitor for PRMT3 [96]. From a library of 16000 diverse compounds, **18** is identified as the most potent using SAHH-coupled assay. The IC_{50} is determined around 2 μM with histone H4 peptide or 40S ribosomal protein S2 (rpS2) protein as substrate. The K_d is determined as 9 μM by SPR. The crystal structure of **18** and PRMT3 reveals that **18** is an allosteric inhibitor bound into the dimer interface. Binding of **18** probably causes the disorder of αX-helix, which is essential for cofactor binding. Out of a panel of five PKMTs and four other PRMTs, only PRMT5 is weakly inhibited by **18**. Because the compound is metabolically unstable as tested from the *in vitro* assay with human and mouse liver microsomes, no cellular activity is reported for this compound. It is worthy to note that all the methylation assays were done under balanced condition, and hence the selectivity profile should be enzymatically meaningful. In addition, the inhibition mechanism was well characterized by a series of biophysics, enzymology, chemical biology and crystallography methods. All of these support that **18** is a *bona fide* PRMT3-specific inhibitor which is an ideal lead for further inhibitor development.

Later, a number of derivatives of compound **18** with diversified structures were subjected to SAR study [80]. The left-hand side region and the urea of **18** are crucial: replacing the two moieties by other ones causes total loss of the activity. However, the modifications of the right-hand side region are more tolerable. As a matter of fact, several compounds sharing the structure **19** have gained activity improvement by about 5 – 10 fold compared to **18**. Then, **19a** was picked out for the following selectivity profiling. Like **18**, **19a** also shows remarkable selectivity for PRMT3 against tested PKMTs, PRMTs and DNMT1, but no cellular activity is reported. The crystal structure of PRMT3-**19a** has confirmed the allosteric binding mode.

Afterwards, a great deal of efforts has been made by the same group to explore PRMT3 inhibitor with better cellular stability. Consequently, compound **20** (SGC707) was characterized as a promising lead [97]. The binding model and inhibition mechanism of SGC707 is the same as **18**. The K_d is determined as 0.053 μM and 0.085 μM respectively from SPR and ITC (isothermal titration calorimetry). It is much smaller than that of compound **18**, further validating that the intrinsic potency is enhanced by the structural optimization. Furthermore, it exhibits not only improved potency for PRMT3 (IC_{50} = 0.03 μM) but also outstanding selectivity against 31 PKMTs, PRMTs, DNMTs and RNA-methyltransferase as well as 250 non-epigenetic proteins. More importantly, SGC707 is proven to bind with PRMT3 overexpressed in HEK293 (human embryonic kidney cell line) and A549 (lung cancer cell line) cells by InCELL Hunter assay and inhibits the methylation of both endogenous and exogenous H4R3 in HEK293 cells (IC_{50} = 0.225 μM and 0.091 μM, respectively). No toxicity was observed after 24h-treatment of the compound. In addition, the pharmacokinetic study suggests the compound is feasible for *in vivo* in the future.

The structural basis of this set of inhibitors is the allosteric site binding, but this pocket can also be observed in other PRMTs (see Section 9 and review [132]). The high specificity is probably achieved by the variability of the sequence lining the pocket, implicating that the underlying rationale could also be applied to design specific inhibitors for other PRMTs. Of note is that compared with the SAH-bound PRMT3 [133], the residue R396 of the **18**-bound PRMT3 adopts a distinct orientation which creates an open space to allow the inhibitor binding. Therefore, if one attempts to perform structure-based virtual screening for inhibitors targeting this allosteric site of other PRMTs, it is better to use a structural model based on the compound **18**-bound PRMT3 structure instead of the SAH-bound crystal structure. In addition, the structure of PRMT dimer rather than monomer should be used since compounds **18** thru **20** are all shown to interact with both monomers.

5. CARM1-specific inhibitors

CARM1 is a transcriptional co-activator [134], and also involved in RNA splicing [15], cell proliferation [135] and cell differentiation [136]. It has a wide range of substrates including histones, transcriptional regulators, and splicing factors, as summarized in Table 1. CARM1 is overexpressed in breast cancer, prostate cancer and colorectal cancer [2]; and it also regulates pathology of human T-cell lymphotropic virus type 1 (HTLV1) [38].

During the screening for multivalent epigenetic inhibitors, a set of curcumin derivatives were found with inclination to inhibit CARM1 in the radiometric assay [137]. Later, one of the hits (**21**, Table 4) was chosen as a lead to develop inhibitor to selectively target CARM1. Various substitutions on the benzyl group in R group were explored. With RGA assay, **21** inhibited CARM1 with IC_{50} of 8.6 μM and is over 80-fold less sensitive to PRMT1 and SET7/9, and all the compounds show similar activity and selectivity to **21**, with **22** as the most potent one. Tested against a broader panel of PRMTs and PKMTs, **21** and **22** (at 100 μM) exhibit inhibition on PRMT3, -5 and -6 at different extents, but weaker than on CARM1. In HEK293 cells, **21** but not **22** suppressed the methylation of the transfected PABP1 (poly(A)-binding protein 1) protein. To investigate the inhibitory effect of the modulation of CARM1 on biological events, **21** and **22** were applied to treat LNCaP cells transfected with prostate-specific antigen promoter. The dose-dependent inhibition of the transcription level was observed starting from 4 μM for both compounds. At this concentration, **21** did not affect the cell viability [72].

Another class of extensively studied compounds for CARM1 is the Pyrazole inhibitors. The hit **23** was found with IC_{50} of 1.8 μM for CARM1. Modification on the R part (Table 4) can only be well tolerated if there is an aryl substitute in the structure. Besides, any analogues replacing the south end glycine decrease the activity except (S)-alanine which slightly improved the potency. From the optimization, compound **24** – **27** display IC_{50} at submicromolar level with **26** showing the best potency (IC_{50} = 0.08 μM) [82]. **26** is inactive on PRMT1 and -3 (IC_{50} > 25 μM). Because **26** showed only modest permeability by PAMPA (parallel artificial membrane permeability assay), the SAR is further explored [83]: 1) the amide was replaced with 1,3,4-oxadiazole and 1,2,4-oxadiazole and the former one (compound **28**) retained the activity and improved permeability against PAMPA; 2) the activity also declines by replacing the 3-trifluoremethyl pyrazole with other five-member heterocycle; 3) aryl groups are still tolerated for the R group. Finally, compounds **28** – **32** is found to show the nanomolar-leveled IC_{50}. In the following assays, the representative compound **31** showed satisfactory selectivity for CARM1 over PRMT1 and -3.

Based on **23**, structural optimization is performed by replacing the phenyl group with other aromatic ring and exploring more aryl groups for the R group by another lab [81]. From them, **33** and **34** are determined with similar IC_{50}. The selectivity of **33** and **34** are also found promising for CARM1 against PRMT1 and SET7/9 (both IC_{50} > 100 μM). However, no inhibition was observed for cellular methylation of H3R26 by the treatment of **34** at 5 μM for 48 h. In addition, **34** failed to show any significant activity in the hormone-dependent assays (IC_{50} > 10 μM), which is probably due to the poor cell permeability considering their structural resemblance to **23**. Envisaging that the alanine group (south end of 23) might be the cause, the lab then sought to improve the quality by replacing the alanine with other groups which generated compound **35** with a N^2-methyl-1,2-diamine group in south end showing IC_{50} of 0.2 μM [84]. Further modification of the R group yielded compound **36** – **39** (IC_{50} = 0.32 – 0.9 μM). However, substitution on the phenyl group of the core of **23** decreased the activity to different extent. Besides, compound **40**, a "N^2-methyl-1,2-diamine" version of **29**, exhibits similar activity (IC_{50} = 0.59 μM). **35** – **40** has indeed acquired enhanced pharmacokinetic profile (lower clearance, longer half-life and smaller volumes of distribution) compared with **33** while still retaining decent potency. Though selectivity property is yet to know for **35** – **40**, it could be deduced that they might be still selective for CARM1 over PRMT1 and -3 since they basically share the same type of structure with **23** – **34**.

Another structural class of CARM1 inhibitors (Benzo[*d*]imidazole inhibitors) represented by compounds **41** and **42** is identified [85] from screening campaign by the same lab who discovered **23** – **32**. Removing or modification of the 2-methylaminoethyl group of **41** results complete loss of the activity while substitution of the middle benzimidazole seems tolerable. The potency and selectivity of **41** and **42** are as good as **24** – **27**, but no further cellular activity is reported.

In the following work [86], the co-crystal structures with CARM1 are determined for the Pyrazole (**43**) and Benzo[d]imidazole (**44**) inhibitors**,** of which the potency and selectivity are comparable to **31** and **41**. They both bind to the substrate arginine binding pocket as seen in the ternary structures of CARM1, SAH (or sinefungin), and the inhibitor. In addition, cofactor binding is a prerequisite for the binding of the inhibitors according to the ITC experiment. This work not only verifies the binding pattern between inhibitors and CARM1 but also provides substantial information for rational tailoring the compound with better pharmacokinetic property without compromising the activity in the future. Of caution that the selectivity of all inhibitors (**23** – **44**) discussed under this category can only be considered as apparent selectivity because whether the assays were done under balanced condition is not clear.

In a recent study [98] releasing sinefungin derivatives as inhibitors for the PKMT enzyme SETD2 (SET domain containing 2), compound **45** (Pr-SNF) exhibited low micromolar inhibition on CARM1 (IC_{50} = 3 μM) though it was demonstrated a much better inhibitor for SETD2. The fact that Pr-SNF and sinefungin (Figure 2) possess very close potency for CARM1 indicates the attached propyl group on sinefungin is well tolerated by CARM1 and has the potential to be further tailored to generate CARM1-specific inhibitors.

6. PRMT5-specific inhibitors

PRMT5 is the predominant type II arginine methyltransferase [4], which participates in transcriptional repression [138], RNA splicing [139], signal transduction [140] and piRNA pathway [141]. PRMT5 is upregulated in gastric cancer, colorectal cancer, lung cancer, lymphoma, and leukaemia [2]. It is also overexpressed and dyslocalized in prostate cancer cells [142]. Furthermore, PRMT5 has found to be involved in renal and cardiovascular disease [143], Huntington’s disease [144] and Alzheimer’s disease [145].

Very recently, a few studies have reported inhibitors targeting PRMT5. Firstly, compound **46** (CMP5, or BLL-1 according to the patent [146], Table 5) is reported in delineating the role of overexpressed PRMT5 in Epstein-Barr virus (EBV)-associated B-cell lymphomagenesis [37]. **46** is discovered from a virtual screening based on a modeled PRMT5 structure. The inhibition on symmetric methylation of the H3R8 and H4R3 was tested in JeKo cells (a mantle cell lymphoma cell line). The study confirms that treatment with **46** reduces histone methylation by specifically inhibiting PRMT5 rather than PRMT1, CARM1 or PRMT7 through RFA assay. The selective inhibition of **46** (50 μM) on the symmetric arginine methylation is also confirmed by an immunofluorescence staining against D-9 cells (lymphoma cell line). The inhibition on viability of lymphoma cells treated with **46** for 24h became significant when concentration of compound went up to 20 – 40 μM. In contrast, the normal resting B cells was only mildly affected even treated for 72h at 100 μM of **46**, indicative of nontoxicity towards normal B cells. Further detailed work unravels the underlying mechanism of the cytotoxicity of **46**. Using **46** as a molecular tool, the study demonstrates that PRMT5 and p65 and HDAC3 constitute a transcriptional repressive complex on miR96 (microRNA 96) promoter which in turn enhances the expression of PRMT5 itself and hence the overexpression of PRMT5 is maintained. The overexpressed PRMT5 is then utilized by EBV to silence tumor suppressor gene ST7 (suppression of tumorigenicity 7) and PTPROt (protein tyrosine phosphatase receptor-type O truncated), which causes the B-cell immortalization.

Compound **46** is subjected to further structural optimization with multiple ligand simultaneous docking method [147]. Compound **47** (HLCL-61), in which the pyridine of **46** is replaced with an *ortho*-methoxylphenyl group, gains improved activity, perhaps due to an addition of hydrogen bond with enzyme. This compound (10 μM) exhibits about 80% inhibition on PRMT5 but no obvious inhibition on PRMT1, CARM1 and PRMT7. The cellular activity of **47** is tested against AML samples. It decreases the symmetric dimethylation level of H3R8 and H4R3 though the concentration used was not clear. Decreased cell viability is also detected after treatment of **47**, with the GI_{50} ranging 7 – 21 μM at 48h treatment. Further assays suggest compound **47** can promote apoptosis and induce differentiation. Next, **47** is used to dissect the function of PRMT5 in the myeloid leukemia growth and the authors find PRMT5 are critical to AML by silencing tumor suppressor microRNA miR29b while inducing transcription of a receptor tyrosine kinase gene, FLT3 (Fms-like tyrosine kinase 3).

Another report of PRMT5 inhibitors is from the research group in Epizyme [108, 110]. From several rounds of screening by a combination of various biochemical assays (TR-FRET and SPA) against a diversity library with 370000 small molecules, compound **48** (EPZ007345) was identified as a desirable inhibitor for PRMT5:MEP50 (methylosome protein 50) complex with IC_{50} around 0.3 μM. Iterative structural optimization was further performed to improve potency. The activity of **48** as well as its analogues was established for the inhibition on PRMT5 via the radiometric assay, decline of cellular methylation and suppression of cell proliferation (Z-138 cells). Any attempted modification on the tetrahydroisoquinoline part diminishes the potency due to a deleterious effect on the cation–π interaction between the ligand and enzyme, which is further confirmed by co-crystal structure of **48** and PRMT5:MEP50. Besides, amide replacing the alkoxy phenyl ring improves the activity by providing additional hydrogen bonds. After further optimization on the physiochemical property, compound **49** (EPZ015666) is obtained with desired clearance in human and mouse liver microsomes while retaining promising potency (IC_{50} = 0.022 μM). EPZ015666 shows outstanding selectivity against the other PRMTs (PRMT1, -3, -4, -6, -7 and -8; no inhibition at 50 μM) and a panel comprising 14 PKMTs, with all the assays being performed under the balanced condition. EPZ015666 acts as a substrate-competitor based on mechanism-of-inhibition study, which is also confirmed by the co-crystal structure of PRMT5:MEP50–SAM–EPZ015666. Interestingly, the binding of the cofactor SAM is prerequisite for the activity of this compound, as seen in the CARM1 inhibitors aforementioned. The global symmetric dimethylation (including the methylation on SmD3 protein) in Z-138 cells is found to be inhibited by EPZ015666, but not by an inactive analogue **50** (EPZ019896), which together support the cellular on-target effect. In a panel of five MCL cell lines, dose-dependent inhibition was observed for the symmetric dimethylation of SmD3 with IC_{50} ranging 4 – 340 nM. The cellular on-target binding of EPZ015666 was further confirmed by the cellular thermal shift assay. Longer period anti-proliferation (12 days) by EPZ015666 is observed for all the tested cell lines with IC_{50} ranging 60 – 900 nM. Excitingly, EPZ015666 shows antitumor effect in mice bearing Z-138 and Maver-1 xenografts. After 21 days of continuous oral dosing, 200 mg/kg of EPZ015666 resulted in significant tumor growth inhibition (> 90%, > 70% and > 45% for Z-138, Maver-1 and Granta-519 cell lines, respectively) with limited effect on the bodyweight. Accordingly, the symmetric methylation level in the xenograft tumors was decreased in a dose-dependent manner. A following study [109] reports that EPZ015666 exhibits low clearance in human, mouse and rat liver microsomes and higher clearance in dog liver microsomes as well as hepatocytes. This study provides important information about similarity of the metabolism profiles between different organisms and human, which may facilitate the transition from preclinical models to human test.

7. PRMT6-specific inhibitors

PRMT6 is a nuclear enzyme that primarily targets histone H3R2[148] and DNA polymerase β152 [149] (additional substrates are listed in Table 1). Studies show that PRMT6 can act as transcriptional repressor and activator through different pathways [150–152]. It is overexpressed in bladder and lung cancer cells, involved in pulmonary disorders [47] and can suppress HIV-1 activity [39].

As mentioned in Section 2, compounds with the core structure of **17**, represented by compound **51** (Table 6), were found to show promising potency to inhibit PRMT1, -6 and -8 via the SPA measurement [99, 126, 127, 153]. Extension of the *para*-position of the phenyl group of **51** with alkoxy group retains the activity. Further extension of the length of the group at *para*-position of the phenyl group results retained activity to PRMT6 and decreased activity to PRMT1 and -8, while large substitution on the *meta*-position yields opposite effect: the activity is diminished toward PRMT6 but not PRMT1 and -8. During the structural optimization, some analogues even show low nanomolar level of potency (IC_{50} ranging 5 – 20 nM) and remarkable selectivity for PRMT6 [99]. Out of them is compound **52** (EPZ020411, IC_{50} = 10 nM) that exhibits desired pharmacokinetic property as well as excellent selectivity (> 100 fold) for PRMT6 over other tested PRMTs (PRMT3, -4, -5 and -7) and PKMTs. The crystal structure demonstrates EPZ020411 inhibits PRMT6 by occupying the putative substrate arginine pocket. In A375 cells with transiently overexpressed PRMT6, inhibition on H3R2 methylation by EPZ020411 was observed does-dependently with IC_{50} at 0.64 μM. In contrast, the inhibition on PRMT1-specific methylation mark was seen at the 10 fold of the IC_{50} for PRMT6. In addition, the pharmacokinetic study suggests the bioavailability of EPZ020411 in rat by subcutaneous dosing (5 mg/kg), but not by oral dosing, is satisfactory for further *in vivo* efficacy profiling.

Inspired by the discovery of EPZ020411, a serial derivatives were designed to target type I PRMTs lately, in which compounds were tested using SPA method under the balanced condition [100]. Modifications are introduced on the pyrazole ring as well as the substitution of the phenyl group and ethylenediamino of EPZ020411. As a replacement of the pyrazole core, pyrrole produces better activity towards all the type I PRMTs than 1,2,3-triazole ring. Besides, *para*-isopropoxy group improves the potency compared with *meta*-trifluoromethyl group. Slight modification on the ethylenediamino group, such as altering the distal NH_2 to OH or addition of a carbonyl group, totally abolishes the activity, supporting the authors' hypothesis that the ethylenediamino is critical for the activity. Among others, compound **53**

(MS023) has been extensively characterized at biochemical, biophysical and cellular levels. It shows nanomolar-leveled activity for tested type I PRMTs with best inhibition on PRMT6 and -8 (IC_{50} = 4 and 5 nM, respectively) but is inactive to type II and III PRMTs as well as a total of 28 PKMTs, DNMTs and histone lysine demethylases at up to 10 μM. The binding between PRMT6 and compound MS023 is confirmed by ITC (K_d = 6 nM) and differential scanning fluorimetry (DSF). In addition there is no binding between MS023 and nine tested methyllysine or methylarginine reader proteins as evaluated by DSF or differential static light scattering (DSLS). The co-crystal structure of PRMT6-SAH-MS023 reveals similar ligand-enzyme interactions as observed for EPZ020411, with the inhibitor occupying the arginine binding site. However, the mechanism of action (MOA) studies suggest compound MS023 is a noncompetitive inhibitor to both SAM and peptide substrate, which reminds that the active site-binding inhibitor does not warrant a competitive MOA [154]. After 48h-treatment, MS023 showed dose-dependent inhibition on endogenous PRMT1 activity on H4R3 in MCF7 cells (IC_{50} = 9 nM) and on overexpression PRMT6 activity on H3R2 in HEK293 cells (IC_{50} = 56 nM). In contrast, a structurally resembled but completely inactive analog, compound **54** (MS094), did not change the methylation status. The effect of MS023 on proliferation depends on exposure time and cell type: 4-day treatment of the inhibitor on HEK293 cells resulted great decrease of the cell growth at 1 μM while it required 10 μM and 10-day treatment of the inhibitor to affect cell growth significantly for the least sensitive MCF7 cells.

8. Other PRMT inhibitors

8.1 Promiscuous PRMT inhibitors

As aforementioned, AMI series are the first reported small molecule PRMT inhibitors. From an ELISA-based high throughput screening campaign against a library containing 9000 diverse chemicals, nine compounds (including AMI-1) stand out with low micromolar level inhibition for all tested PRMTs (PRMT-1, -3, -4 and -6) [70], but are inactive for PRMT5 [74]. The poor selectivity for PRMT1 may be related to the mechanism of inhibition (see Section 8.3). Among them, only AMI-1 (**1**) and AMI-8 (**2**) (Table 2) are inactive against the tested PKMTs. AMI-1 was able to inhibit the methylation level of exogenous nucleolar protein 3 (Npl3) and endogenous Sam68 protein in HeLa cells and suppress the effect of PRMT1 and CARM1 on nuclear receptor dependent transcriptional activation in MCF7 cells. Lately, AMI-1 was used to investigate the function of PRMT1 in chronic Ag-induced pulmonary inflammation (AIPI) in the E3 rat asthma model [40]. Similar to observation in PRMT1-knockdown rats, AMI-1-treated AIPI rats showed reduced COX2 (cyclooxygenase-2) production and humoral immune response, and abrogated mucus secretion and collagen generation.

Enlightened by AMI-1, **51** (AMI-6) and **52** (AMI-9), compounds **55 – 58** (Table 7) were designed and exhibited similar activities to their progenitors on inhibiting the PRMT1-mediated methylation. Though they are inactive to SET7/9, **55**, **56** [73] and **58** [74] still do not show selectivity among PRMTs (PRMT-1, 3, 4 or -6) while **57** [102] is only tested against PRMT1. Western blot analysis shows the arginine methylation level of exogenous Npl3p protein in HeLa cells was inhibited by **55** at 50 μM [73]. In VCaP cells (a prostate cancer cell line), dose-dependent inhibitory effect of **57** was observed on the expression level of two androgen-dependent genes [102]. In 293T cells, the methylation level of H3 protein Arg17 (H3R17, a major methylation site of CARM1 [155]) was decreased to 55% by **58** at 100 μM, in contrast to around 80% by AMI-1 of the same concentration [74]. Furthermore, **58** is demonstrated to reduce the secretion of interferon γ (IFNγ) and interleukin-4 (IL-4) by Type 1 T-helper (Th1) cells and Type 2 T-helper (Th2) cells, respectively, possibly due to interrupted association between PRMT1 and NIP45 (45 kDa NF-AT-interacting protein, a protein involved in transcription of IL-4 gene when methylated by PRMT1).

Since these inhibitors exhibit relatively low selectivity, more selective inhibitors are highly desired when it comes to the study of specific PRMT subtype. It is worth noting that the methylation assays done in these studies are not performed under balanced condition (see Section 9 for detailed discussion) [156]. Further studies are needed to validate their selectivity profiles. As a matter of fact, structurally related compounds (**3 – 7,** Table 2) are proved with improved selectivity.

8.2 Substrate-based inhibitors

Inhibitors based on the amino acid sequence of PRMT substrates presumably possess high affinity and specificity via extensive interaction between peptide and the targeted enzyme. Several sets of peptide inhibitors have been designed and studied, with some showing promising potency and selectivity [157–161]. Interested readers are recommended to refer to a recent review on PRMT peptide inhibitors for more detailed information [162]. Nonetheless, the substrate sequences of PRMT are usually highly charged (rich in Arg and Lys) in physiological pH, so the corresponding peptide inhibitors by themselves are neither orally available nor metabolically stable. From this point of view, the peptide inhibitors are more useful as chemical probes to study function and mechanism of PRMTs at biochemical and cellular levels.

Exploration of small bisubstrate analogue as inhibitor is a strategy that has been justified successful in achieving specificity between enzyme isoforms in the field of kinase [163, 164]. The cofactor SAM is the primary methyl donor for methyltransferase [165]. It is not surprising that simple SAM analogues, such as SAH, sinefungin, MTA (Figure 2) act as pan-inhibitors for methyltransferase and can result in global decrease of methylation [64]. However, structural studies shows that active sites of methyltransferases bear different degrees of plasticity and structural uniqueness [132]. Thus, it is possible to design SAM analogs for selective inhibition of a target methyltransferase, a successful example being DOT1L selective inhibitors [166, 167].

The SAM analogue AzaAdoMet (Figure 2) is a promiscuous methyltransferase inhibitor[168]. Dowden et al. [75, 76] synthesized several AzaAdoMet derivatives, and found that compound **59** and **60** (Table 7) inhibited PRMT1 with an IC_{50} value of 2.9 and 5.6 μM, respectively, and was inactive against CARM1 and SET7/9. The docking result suggests a bisubstrate binding mode and the four or five methylene linker is favored by PRMT1 but not by CARM1. The work has justified the strategy that combination of structural features from both substrate and cofactor could achieve selectivity not only between different methyltransferases families but also for different members within the PRMT family.

Following this methodology, compounds **61** and **62**, with shorter linkers between the two feature groups, are developed with IC_{50} at sub-micromolar level (0.12 and 0.15 μM, respectively) for CARM1 and the selectivity is at least more than 50-fold over PRMT1 and PRMT6. One of the compound (**63**) in the same series is found to be a selective PRMT6 inhibitor [103]. Interestingly, replacing the asterisked NH in R group (Table 7) with O or S atom totally abolished the activity to all the tested PRMTs. However, compound **61** and **62** did not show significant effect on the proliferation of two cancer cell lines even at the concentration of 100 μM, possibly due to the high polarity and poor membrane permeability.

A similar structure (compound **64**), rationally designed based on the ligand bound crystal structure of hPRMT5 and xlPRMT5, is found to be a potent PRMT5/PRMT7 dual inhibitor (IC_{50} = 6 μM for both enzyme), but inactive against a panel of 29 other human protein-, DNA-, and RNA-methyltransferases [101]. Being less polar than the compounds above, **64** exhibits dose-dependent inhibition on the methylation level of endogenic SmD and SmB proteins in breast carcinoma MB-MDA231 cells. The inhibition of **64** becomes significant when the concentration is larger than 10 μM. This study is encouraging because it suggests small bisubstrate inhibitors, in contrast to the peptide inhibitors, are capable to be tailored with appropriate hydrophobicity for penetrating cell membrane.

8.3 Histone-interacting inhibitors

Some PRMT inhibitors are found to possess capability of binding to the histone tail peptides, which include compound AMI-1 (**1**) and **65** – **69** (Table 7). Compound **65 (**TBBD, also known as ellagic acid) was found to be a specific CARM1 inhibitor (IC_{50} = 25 μM), which specifically inhibits methylation of H3R17 [88]. The ITC assay shows that **65** binds to a peptide sequence KAPRK of histone H3, highlighting it is a substrate-targeting inhibitor. Compound **66** (NS-1) was found to show more favorable IC_{50} (12 μM) than AMI-1 (137 μM) in PRMT1 inhibition. However, the interaction between **66** (as well as AMI-1) and the histone substrate was substantiated by mass spectrometry, UV, and fluorescence, suggesting the compound inhibits PRMT1 activity largely by binding with the substrate [89]. Unlike the recognition of TBBD for short peptide sequence, **66** has higher activity on H4(1-20) peptide than H4(1-11) peptide, suggesting the distal residues in the peptide sequence participate in the interaction of **66** with H4. **66** shows similar IC_{50} (21 μM) to inhibit the acetylation of H4(1-20) by p300 (an acetyltransferase), further supporting its substrate-targeting property. The same study also suggests that suramin (compound **67**), which is structurally similar to **66** and AMI-1, also acts by binding with the H4 substrate [169]. In addition, compound **68** (A9) and **69** (A36), discovered from pharmacophore-based virtual screening, are validated as histone-H4-tail-targeting inhibitors as well (IC_{50} = 41 μM and 12 μM) [90]. Because of their nature of interacting with the histone tails (and possibly other histone-like sequences), careful caution should be taken on interpreting the biological effects of these histone-targeting compounds in cells. This type of unique inhibitors could find use as complementary chemical probes together with enzyme inhibitors to study mechanism and function of nucleosome modifications.

Taken together, considerable success has been achieved in the discovery of PRMT inhibitors. The discovered inhibitors contain different structural scaffolds and act through different modalities, providing powerful and versatile molecular tools to understand PRMT mechanism and function. PRMTs are found to methylate hundreds to thousands of cellular proteins, symbolic of their diverse roles in myriad pathways. Inhibition of PRMTs via small molecule inhibitors is a powerful chemical genetic strategy for interrogating the pathophysiological function of PRMTs in various disease pathways. Validated leads will serve as potential drug candidates for pharmacological treatment of arginine methylation-involved diseases.

9. Expert opinion

Since the first report of small molecule inhibitors for arginine methylation by Bedford and coworkers in 2004, the field of PRMT drug discovery has been productive, which is evidenced by the appearance of several micromolar and submicromolar small molecule inhibitors targeting different PRMT members, such as DB75 (**15**) for PRMT1 [79], SGC707 (**20**) for PRMT3 [97] and EPZ015666 (**49**) for PRMT5 [108]. Such appreciable progress is made possible owing to multiple factors. A fundamental impetus lies in the biomedical community’s enthusiastic quest for novel disease targets and the improved understanding of PRMT functions in disease progression pathways. Although PRMT inhibitor discovery research until now still lags behind the area of PKMT inhibitor discovery [170–174], the scenario is constantly changing. During year 2014–2015, the investment on PRMT inhibitor discovery increased steadily and several patents were filed pertaining to PRMT inhibitors [87, 126, 127, 146, 153]. That said, great strides are in imperative demand in this field. We discuss three key challenging issues that need to be addressed for the field to leap forward.

First, structural mechanisms of the majority of PRMT inhibitors remain unclear. Although dozens of structurally diverse PRMT inhibitors have been available, co-crystal structures of PRMT-inhibitor complexes are significantly needed at this stage. Only a few PRMT-inhibitor complex structures were available [80, 86, 96, 97, 99, 108]. Co-crystal structures of PRMT-inhibitor complexes are of value to drug discovery for several reasons: structural determination is a straightforward means for target validation of inhibitors for PRMT binding and inhibition; structural information is useful to understand the MOA of PRMT inhibitors; structural insights can be obtained for inhibitor optimization and rational design to make more potent leads. For example, both sulphone inhibitors and furan inhibitors (represented by compounds **6** and DB75, respectively) have a similar crescent-shaped backbone scaffold and have terminal heteroatomtic groups. It would be tempting to postulate that these two types of compounds target PRMT1 activity with similar binding structural mode. Crystal or NMR structures of PRMT1 complexed with these inhibitors will be the key to approving or disapproving the proposition.

It is a tremendous advance in the recent years that X-ray crystal structures of the majority of PRMTs have been resolved by research groups in America, Asia and Europe. These high-resolution structures undoubtedly deliver a thrust to speed up PRMT inhibitor discovery either by virtual screening or structure-based rational design. From the structural point of view, small molecule ligand targeting PRMT enzyme can bind to either the active site or an allosteric site. The deeply buried SAM binding pocket of PRMT is conserved among PRMTs and also share certain structural similarity with other methyltransferases, such as small molecule-, RNA-, and DNA-methyltransferases [22, 132, 175]. With regard to the substrate binding region, the surface grooves of PRMTs that likely accommodate substrate peptide backbones are shallow and wide. Besides, the substrate promiscuity of PRMTs suggests that the substrate binding grooves lack specificity regarding molecular recognition. Therefore, designing small molecules to target the substrate binding grooves would be highly challenging. On the other hand, exploration of distinct allosteric sites in PRMTs might be recommended as an alternative and effective strategy to identify small molecule inhibitors for specific PRMT members.

We analyzed the crystal structures of PRMTs using the Discovery Studio software to locate possible exosite binding pockets (Figure 4). Two major allosteric cavities are identified in the PRMT structures. Cavity 1 is located near the “double-E” loop that contacts the incoming arginine residue and is essential for catalytic activity. Cavity 1 of PRMT1, PRMT6 and PRMT8 have a volume in the range of 150 – 220 $Å^3$ and is located at almost the same location, with the loop between β13 and β14 sheets possessing the largest sequence diversity (Figure 4H, Cavity 1). The cavity 1 of PRMT3 [96] is located toward the β-barrel and can only be seen in the “inactive” form (PDB: 3SMQ) but not in the “active” form (PDB: 1F3L) [133], indicating that locking this cavity might interrupt the enzymatic activity though its volume is relatively smaller (64 $Å^3$). No such cavity is found in the CARM1 structure [86].

Cavity 2 is located underneath the dimerization arm (formed by αE, β7 and αH). Targeting this region could influence the dimerization and hence the enzyme activity. This conclusion is supported by recent molecular simulation study showing that the dimerization arm allosterically influences SAM binding and catalytic activity of PRMT1 [176]. Cavity 2 is shared by PRMT1, -3, -4, -6, -7 (C-terminal domain) and -8 (volume ranging 100 – 160 $Å^3$) but with varied sequence of αE-helix (Figure 4H, Cavity 2) and cavity shape. For PRMT8 [177], the sequence corresponding αE and αF helices in other PRMTs exists as coils (denoted as “αE”- and “αF”-coil, respectively), leaving a large gap between this motif and αG helix to form cavity 3. PRMT7 has two tandem PRMT core domains (denoted as N-terminal domain and C-terminal domain), possibly mimicking the canonical PRMT dimer architecture [178]. Cavities 2 and 3 are found in the C-terminal domain of mPRMT7 with a summed area of 263 $Å^3$ and the sequence of hPRMT7 lining this pocket is quite distinct from the other human PRMTs (not shown). There are additional unique cavities, such as a surface area (442 $Å^3$) near the middle of the C-terminal domain of PRMT7 (whether binding to this area is useful for interference of enzymatic activity is yet to discover) and a large area (2148 $Å^3$) between TIM-barrel (Figure 4D, green) and MEP50 (Figure 4D, yellow) in the PRMT5 complex structure [179]. The differences of the residue sequences lining the sidewalls of the allosteric sites between different PRMTs might render the specificity of inhibitor recognition for particular isoforms.

Second, caution should be taken on appropriate interpretation of reported potency values of PRMT inhibitors. The most common metric used in potency characterization is IC_{50}, i.e. the concentration of inhibitor that produces 50% inhibition of enzyme activity. The choices of assay methods and conditions for IC_{50} measurements often vary in one study from another, which sometimes yield large divergence in reported IC_{50}. For example, literature reported IC_{50} of AMI-1 (**1**) in PRMT1 activity inhibition vary substantially, from 1.2 μM [104], 8.8 μM [70], 22 μM [111], 67 μM [73], 77 μM [90], 92 μM [169], 137 μM [89], to 376 μM [89]. As a result of such divergence, the definition of "strong inhibition" could be interpreted differently by individual researchers. One should keep in mind that IC_{50} values directly depend on assay methods, reagent sources and concentrations, and other experimental conditions. IC_{50} of a single inhibitor can vary largely under different experimental conditions. Depending upon its working mechanism, IC_{50} of an inhibitor can change in different manners as the concentration of substrate increases: it can either increase (for competitive inhibitors), decrease (for uncompetitive inhibitor), or remain unaffected (for classic noncompetitive inhibitors). In contrast, inhibition constant (K_i) or binding affinity constant (K_d,), which represents the intrinsic affinity of a ligand for a specific enzyme, is independent of substrate concentration. Thus, K_i is more stable in comparison to IC_{50} with regard to experimental condition changes. Measurement for K_i or K_d, however, is neither necessary nor simple for every hit in the initial round of screening. Practically, inclusion of a standard PRMT inhibitor (e.g. sinefungin or SAH) as a proper control is highly recommended in PRMT inhibition assays in order to achieve meaningful potency comparison for the IC_{50} data obtained by different laboratories. Because the exact IC_{50}—[S] relationship is determined by the action mechanism of an inhibitor, we recommend that PRMT inhibitor screening and IC_{50} measurement be carried out in the initial velocity phase and under the balanced assay condition, in which the concentration of the selected substrate is equal or close to its Michaelis constant, K_m. The balanced assay condition is an experimental choice to identify structurally diversified inhibitors of enzyme that may act through different mechanisms, which can minimize the pitfall of missing hits in library screening. The balanced assay condition is also important in evaluating the selectivity profile of an inhibitor against one PRMT versus the other PRMT isoforms. In selectivity profiling, if inappropriate substrate concentrations are chosen for different PRMTs, the drawn conclusion about inhibitor selectivity could be inaccurate and biased. The reason is that individual PRMT isoforms have different K_m for their substrates. For instance, for a substrate-competitive inhibitor, relationship of IC_{50} to substrate concentration follows the equation of $IC_{50}=K_i(1+\frac{[S]}{K_m})$. As such, IC_{50} is affected by both K_m and substrate concentration. Under the balanced assay condition ($[S] = K_m$ and $IC_{50} = 2K_i$), the ratio of IC_{50} is a faithful representation of the inhibition constant K_i, thus can be compared between different PRMTs. Interested readers can read more details from the reference [156].

Third, extensive studies should be invested on target validation of new PRMT inhibitors in both *in vitro* and *in vivo* models. A good lesson is learnt from the mechanism study of PRMT1 inhibition by AMI-1 (**1**): although originally expected to be a PRMT1-binding inhibitor, later biophysical experiments show strong interaction existing between the inhibitor and the histone substrate, which likely is the main reason for observed PRMT1 inhibition [89]. Amongst the reported PRMT inhibitors, dozens may have chemical susceptibility of being pan-assay interference compounds (PAINS). PAINS refer to those compounds which are reactive to amino acids in protein and therefore inhibit enzyme non-specifically. Increasing advocacy has been made to filter off PAINS in the early stage of drug discovery [180–182]. Besides, overly hydrophobic compounds that are prone to form aggregates could cause non-specific enzyme inhibition [183].

Target validation of PRMT inhibitors *in cellulo* and *in vivo* is particularly important and needed. In a cellular setting in which the inhibitor is exposed to the entire proteome and complex metabolic networks, its effective binding to the protein target becomes much more challenging than in biochemical assays. Since it is hardly possible for a drug candidate to have a sole molecular target, the selectivity is critical for confirming the correlation between the inhibition of targeted PRMT and corresponding phenotype [184, 185]. Many PRMT inhibitors demonstrate anti-tumor effects in cellular assays. Whether such phenotypic effects are a direct result of PRMT inhibition is an important issue. Therefore, following discovery of PRMT inhibitors, extensive pharmacological examination of those inhibitors *in cellulo* and in animal models should be conducted. It would be necessary to develop effective and physiologically relevant cellular assays for individual PRMTs and their chemical modulators. However, this is quite challenging because different PRMTs have different function and substrate profile, and different cellular systems may respond differently to a single PRMT inhibitor. Specific cellular reporters will be useful for assaying PRMT inhibitors. Wei et al. implemented a GFP-PABP1 reporter to study CARM1 modulators [186]. In this approach, GFP-PABP1 substrate protein is transiently expressed in MCF7 cells, and the methylation event is captured by methyl-PABP1-specific primary antibody and terbium-labeled secondary antibody in the cell lysate, leading to the formation of the antibody/substrate complex. Upon excitation of terbium by light at 340 nm, the proximity between terbium and GFP enables TR-FRET to occur, which reflects the methyltransferase activity of CARM1. In principle, similar assay platform can be created to study activities of other PRMT enzymes. Of further notion is that chemical proteomics in combination with mass spectrometry has gained increased popularity in inhibitor selectivity profiling and *in vivo* study of drug-proteome interactions [187]. It remains to see if chemoproteomic assays can be designed and applied for target validation of new PRMT inhibitors.

The majority of PRMT enzymes still lack nanomolar potent inhibitors. There is no PRMT2, -7, -8 and -9 specific inhibitors reported yet. Functions of PRMT7 and PRMT9 are studied only recently. PRMT inhibitors thus far are largely evaluated in biochemical and cellular models. A pressing issue is the inadequate study of pharmacodynamics and pharmacokinetic attributes of PRMT inhibitors in animal disease models. Most importantly, a big gap exists for PRMT inhibitors to translate into clinical drug leads. Thus authentication of PRMT inhibitors as an effective therapeutic strategy warrants further investigation. We foresee that generation and evaluation of potent, isoform-selective, pharmacokinetically amenable, and *in vivo*-active molecules will continue to be an active arena of research in years ahead.

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Article highlights

- PRMTs are crucial players in the regulation of fundamental cellular processes and disease pathways.
- PRMTs are emerging disease targets for drug discovery.
- PRMT modulators are powerful tools to elucidate PRMT function in physiology and pathology, and are potential therapeutic agents for the treatment of various diseases, notably cancer.
- Specific inhibitors have been developed for PRMT1-, -3, -4, -5 and -6.
- Caution should be taken on possible off-target effects of new PRMT inhibitors.
- Breakthroughs are needed to translate PRMT inhibitors into clinical candidates.

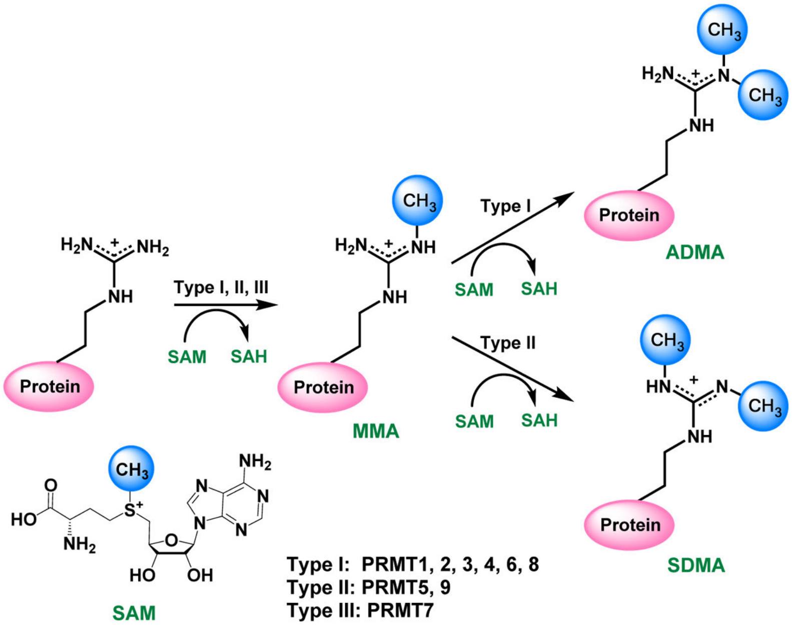

Protein
Type I, II, III
SAM
SAH
Protein
MMA
Type I
SAM
SAH
Protein
ADMA
Type II
SAM
SAH
Protein
SDMA
SAM
Type I: PRMT1, 2, 3, 4, 6, 8
Type II: PRMT5, 9
Type III: PRMT7

Figure 1.

Protein arginine methylation regulated by PRMTs.

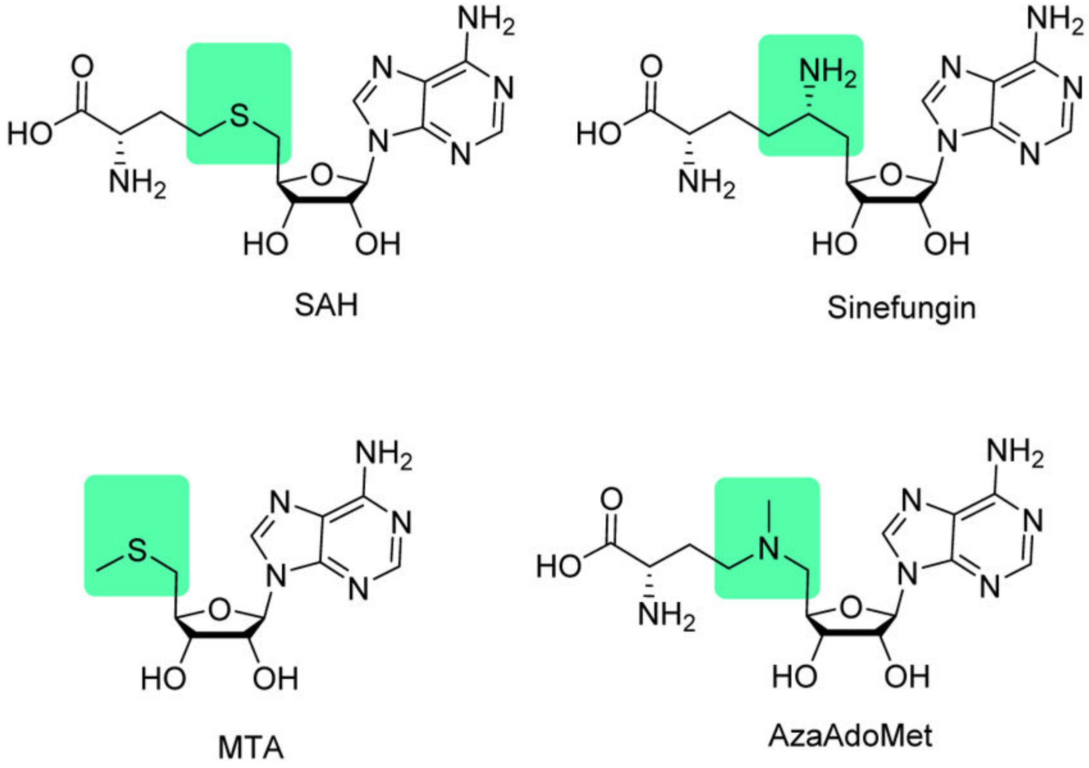

NH2
O
HO
NH2
S
N
N
N
N
O
HO
OH
SAH
Sinefungin
MTA
AzaAdoMet

Figure 2.

Simple SAM analogues.

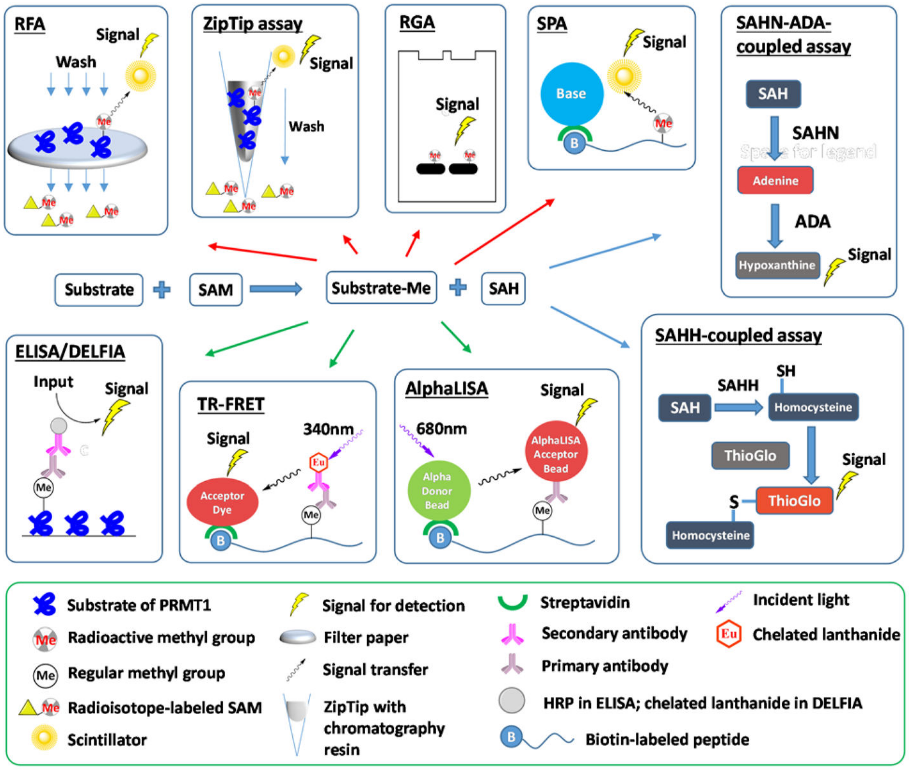

RFA
Signal
Wash
ZipTip assay
Signal
Wash
RGA
Signal
SPA
Signal
Base
B
SAHN-ADA-coupled assay
SAH
SAHN
Adenine
ADA
Hypoxanthine
Signal
Substrate
SAM
Substrate-Me
SAH
ELISA/DELFIA
Input
Signal
TR-FRET
Signal
340nm
Acceptor Dye
Eu
AlphaLISA
Signal
680nm
Alpha Donor Bead
AlphaLISA Acceptor Bead
SAHH-coupled assay
SAHH
SH
SAH
Homocysteine
ThioGlo
Signal
S
ThioGlo
Homocysteine
Substrate of PRMT1
Radioactive methyl group
Regular methyl group
Radioisotope-labeled SAM
Scintillator
Signal for detection
Filter paper
Signal transfer
ZipTip with chromatography resin
Streptavidin
Secondary antibody
Primary antibody
HRP in ELISA; chelated lanthanide in DELFIA
Biotin-labeled peptide
Incident light
Chelated lanthanide

Figure 3.

Biochemical assays for PRMT activity detection. Top blocks denote radiometric assays, bottom blocks denote antibody-based assays, and right blocks denote enzyme-coupled assays. RFA: radiometric filter assay. RGA: radiometric gel assay. SPA: scintillation proximity assay. SAHN: SAH nucleosidase. ADA: adenine deaminase. ELISA: enzyme-linked immunosorbent assay. HRP: horseradish peroxidase. DELFIA: dissociation-enhanced lanthanide fluorescent immunoassay. TR-FRET: time-resolved fluorescence resonance energy transfer. SAHH: SAH hydrolase.

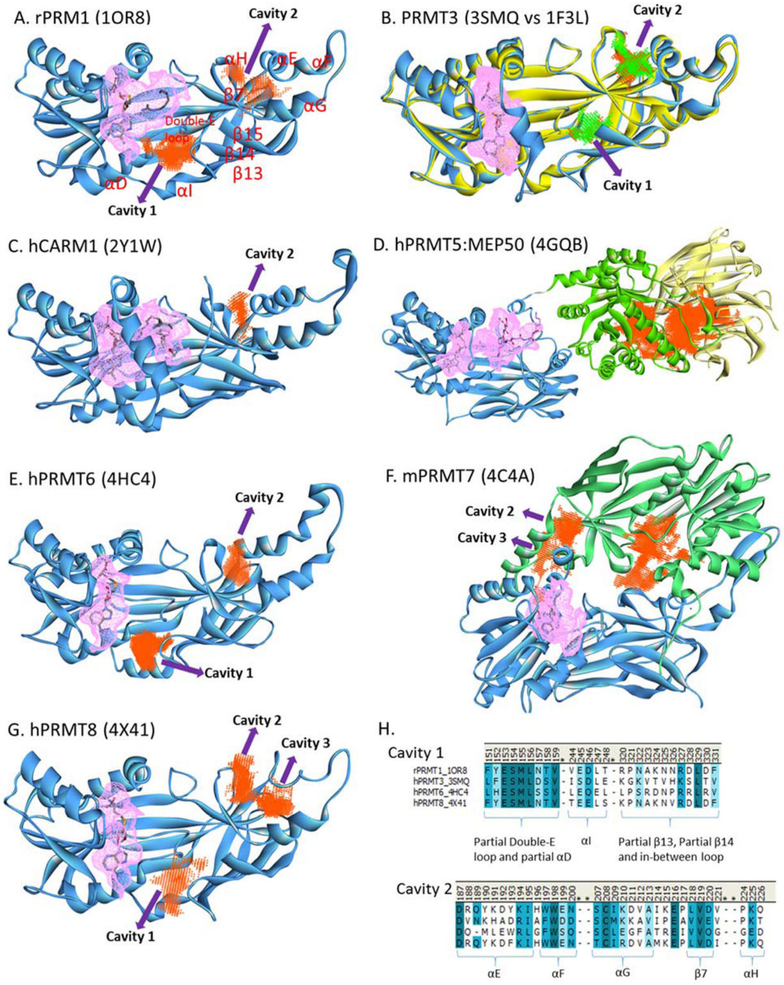

A. rPRM1 (1OR8)
Cavity 2
αH
αE
αF
β7
αG
Double-E loop
β15
β14
β13
αD
αI
Cavity 1
B. PRMT3 (3SMQ vs 1F3L)
Cavity 2
Cavity 1
C. hCARM1 (2Y1W)
Cavity 2
D. hPRMT5:MEP50 (4GQB)
E. hPRMT6 (4HC4)
Cavity 2
Cavity 1
F. mPRMT7 (4C4A)
Cavity 2
Cavity 3
G. hPRMT8 (4X41)
Cavity 2
Cavity 3
Cavity 1
H.
Cavity 1
151 152 153 154 155 156 157 158 159 • 244 245 246 247 248 • 320 321 322 323 324 325 326 327 328 329 330 331
rPRMT1_1OR8 FYESMLNTV-VEDLT-RPNAKNNRDLDF
hPRMT3_3SMQ LFESMLDSV-ISDLE-KGKVTVHKSLTV
hPRMT6_4HC4 LHESMLSSV-LEQEL-LPSRDNPRRLRV
hPRMT8_4X41 FYESMLNTV-TEELS-KPNAKNVRDLDF
Partial Double-E loop and partial αD
αI
Partial β13, Partial β14 and in-between loop
Cavity 2
187 188 189 190 191 192 193 194 195 196 197 198 199 200 • • 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 • • 224 225 226
DRQYKDYKIHWWEN--SCIKDVAIKEPLVDV--PKQ
DVNKHADRIAFWDD--SCMKKAVIPEAVVEV--PKT
DQ-MLEWRLGFWSQ--SCLEGFATREIVVQG--GED
DRQYKDFKIHWWEN--TCIRDVAMKEPLVDI--PKQ
αE
αF
αG
β7
αH

Figure 4. Illustration of possible allosteric cavities in PRMTs

The pink colored mesh represents the catalytic pocket. The allosteric cavities are identified using the Discovery Studio software. Numbering of α-helices and β-sheets follows the orders of secondary structures in PRMT1. Panel H shows the alignment of the sequences in PRMT1, -3, -6 and -8 lining the two allosteric cavities.

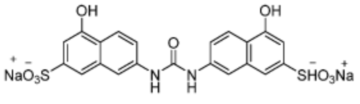
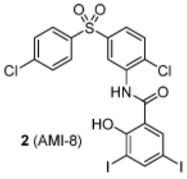
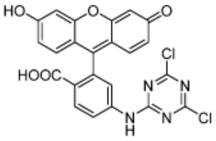
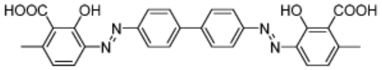
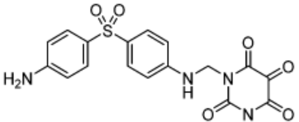
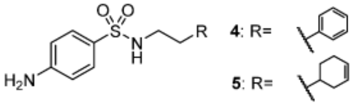
Table 1

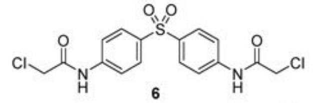
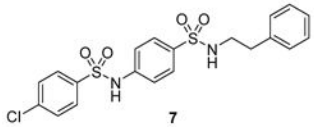
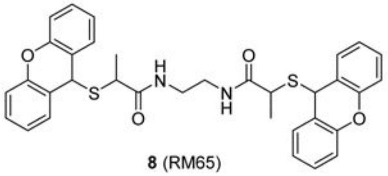
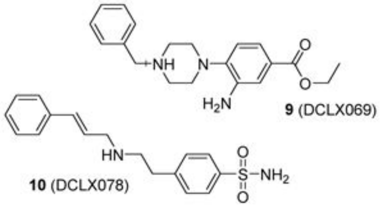
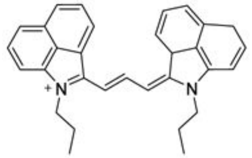
Primary substrates, functions and disease relevance of PRMTs.

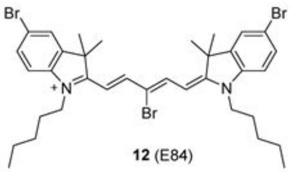
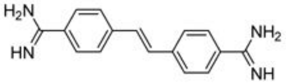
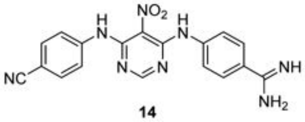
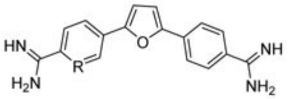
PRMT	Substrate	Function	Disease relevance
PRMT1	H4R3[188] hnRNP A1[189], BTG1[190], TIS2[190], IFNα/β[191], ILF3[192], SPT5[193], SAF-A[194], p53[195], MRE11[34], FMRP[196], Sam68[33], SLM[33], ERα[197], RUNX1[198], TAF15[199], BCR[200], CF Im59 and Im68[201], Ash2L[202], nuclear poly(A)-binding protein (PABP1)[203]	Transcriptional coactivator[134], signal transduction[197, 204], RNA splicing[205] and DNA repair[34]	Overexpressed or aberrant in breast, prostate, lung, colon, bladder cancer and leukemia.[2] Overexpressed or aberrant in pulmonary diseases: pulmonary fibrosis, pulmonary hypertension, chronic obstructive pulmonary disease (COPD) and asthma. [40, 47, 206] Play regulatory roles in cardiovascular disease[119, 207], diabetes[120, 121] and renal disease[44, 45, 122, 131].
PRMT2	H3R8[208] ERα[209], Glutathione transferase[210]	Transcriptional coactivator and androgen/estrogen receptor coactivator[211]	Overexpressed or aberrant in breast cancer [212, 213]. Overexpressed in pulmonary inflammation [206, 214].
PRMT3	FMRP[196], rpS2[128], PABP1[203]	Ribosomal homeostasis[129]	Enhanced activity in breast tumors [2]. Overexpressed in coronary heart disease [130] and chronic kidney disease [215].
CARM1	H3R2, H3R17, H3R26[216] PABP1[14]; SAP49 (CBP)/p300[217]; FMRP[196]; Sox9[218]; CA150, SmB, U1C and SF3b4[15]	Transcriptional coactivator[134], RNA splicing[219], cell proliferation[135], cell differentiation[136]	Overexpressed in breast, prostate and colorectal cancer [2]. Regulate human t-cell lymphotropic virus type 1 (HTLV1) [38].
PRMT5	H2AR3, H3R8, H4R3[138]; H3R2[220]; MBP (Myelin basic protein) [221]; LSm4, Sm D1 and Sm D3[31]; EBNA-2[222]; SPT5[193]; EBNA-1[223]; p53[200]; CBP-1[224]; CF Im68[201]; Ash2L[202]; PDCD4[225]; HoxA[41]; NFkB[226]	Transcriptional repressor[138], RNA splicing[139], signal transduction[140] and piRNA pathway[141]	Overexpression/increased activity in gastric, colorectal, lung cancer, lymphoma, and leukaemia [2]; mis-localized in prostate cancer cells [142]. Play regulatory roles in renal and cardiovascular disease [143], Huntington's disease [144], Alzheimer's disease [145]
PRMT6	H2AR29[227], H3R2[148] PRMT6[228, 229]; HIV Tat[230]; HMGA1a[231], DNA polymerase β[149], PABP1[203]	Transcriptional repressor[150, 151] and activator[152]	Overexpressed in bladder and lung cancer [2] Suppress HIV-1 activity [39] Overexpressed or aberrant in pulmonary fibrosis, COPD and asthma [47]
PRMT7	H4R3 and H2AR3[232], H3R2[220], Fibrillarin [233]	DNA damage[232], embryonic stem cell pluripotency[234], male germline gene imprinting[235, 236]	Involved in breast cancer metastasis [237]
PRMT8	H2A, H4[238] MBP, PRMT8[238] EWS (Ewing sarcoma)[239]	Brain specific functions[240]	Somatic mutations were found in ovarian, skin and large intestine cancer [2]
PRMT9	SAP145 (SF3B2)[9]	RNA splicing[5]	lymphoma, melanoma, testicular, and pancreatic cancers [241]

Table 2

PRMT1-specific inhibitors.

Structure and numbering	Activity		year[ref]
	Biochemical	Cellular	
 **1** (AMI-1)  **2** (AMI-8)  **51** (AMI-6)  **52** (AMI-9)	Assay: ELISA; RGA. Compound **1**, **2**, **51** and **52**: IC_{50} 0.3–8 μM (PRMT1); 100% inhibition (@ 5 μg) for PRMT-3, -4 and -6; Compound **1:** inactive on PRMT5 Compound **1** and **51** (@ 5 μg): inactive for Suv39H1, Suv39H2, SET7/9 and DOT1	Compound **1**: **1** Dose-dependent inhibition of arginine methylation of both Npl3 protein (exogenous, by western blot) and Sam68 protein (endogenous, by in vivo radiometric methylation assay) in HeLa cells; 50% inhibition at 120 μM; **2** Suppress PRMT-mediated nuclear receptor dependent transcriptional activation (being apparent at 5 μM and leveled off at 100 μM); **3** Reduce COX2 production and humoral immune response, and abrogate mucus secretion and collagen generation by suppressing PRMT1 on E3 rat asthma model.	2004[70], 2010[74], 2015[40]
 **3** (Allantodapsone)	Assay: DELFIA IC_{50} on PRMT1: 1.7 μM, compared to 1.2 μM of AMI-1	In HepG2 cells, inhibition on H4R3 methylation level > 70% (@ 50 μM) determined by DELFIA, the same as AMI-1.	2007[104]
	Assay: DELFIA IC_{50} 13–17 μM (PRMT1)	—	2009[105]

Structure and numbering	Activity		year[ref]
	Biochemical	Cellular	
 **6**  **7**	Assay: RGA; DELFIA Both: reversible inhibition Compound **6**: IC$_{50}$ 1.5 μM (PRMT1); inactive on CARM1 and SET7/9 Compound **7**: IC$_{50}$ 5 μM (PRMT1); inactive on CARM1; 60% inhibition (@ 50 μM) on SET7/9	Compound **6**: **1** GI$_{50}$ 2 μM (MCF7a cells) and 5 μM (LNCaP cells); **2** Significantly reduce the level of androgen-dependent transcription determined by androgen-driven luciferase assay and qRT-PCR (@ 10 μM).	2011[71]
 **8** (RM65)	Assay: DELFIA IC$_{50}$ 55 μM (PRMT1), compared with 1.2 μM of AMI-1	150 μM to achieve 50% inhibition of arginine methylation in HepG2 cells determined by DELFIA.	2007[106]
 **9** (DCLX069) **10** (DCLX078)	Assay: TR-FRET, AlphaLISA Compound **9:** IC$_{50}$ 18 μM (PRMT1); Inhibition < 15% on CARM1 and PRMT6 (@ 100 μM) Compound **10**: IC$_{50}$ 26 μM (PRMT1); 45% inhibition on CARM1 (@ 50 μM); 18% inhibition on PRMT6 (@ 100 μM) Note: IC$_{50}$ 55 μM of AMI-1 (PRMT1)	Both: More than 40% of the proliferation of three cancer cell lines (HepG2, MCF7 and THP1), with compound **9** showing better activity.	2014[107]
 **11** (MHI-21)	Assay: RFA IC$_{50}$ 4.1 μM (PRMT1), 8–13 μM (PRMT3, -5, and -6), 100 μM (CARM1)	**1** GI$_{50}$ 1 μM (HeLa cells); **2** Concentrated in or around nucleus and arrest cell in S phase.	2012[78]

Structure and numbering	Activity		year[ref]
	Biochemical	Cellular	
 **12** (E84)	Assay: SPA; RFA More than 6-, 10-, and 25-fold selectivity for PRMT1 (3.38 μM) over CARM1 (21.5 μM), PRMT5 (35.4 μM), and PRMT8 (84.9 μM) Note: all the methylation assays were done under balanced condition	**1** GI_{50} of 48h treatment: 26 nM (Meg01 cells), 22nM (MOLM13 cells), 171nM (HEL cells); **2** Inhibit global cellular methylation significantly at 100 nM after 24h treatment determined by western blot.	2015[95]
 **13** (Stilbamidine)	Assay: DELFIA IC_{50} 57 μM (PRMT1), compared to 1.2 μM of AMI-1	**1** In HepG2 cells, inhibition on H4R3 methylation level > 70% (@ 50 μM), the same as AMI-1; **2** In MCF-7-2a cells, reduce receptor activation by 75% (@ 150 μM), compared with 25% of AMI-1 (@ 150 μM).	2007[104]
 **14**	Assay: AlphaLISA IC_{50} 2.0 μM (PRMT1), 10 μM (CARM1), >100 μM (PRMT5 and -6)	**1** GI_{50} 4.4 μM (DLD-1 cells), 13 μM (T24 cells), 11 μM (SH-SY-5Y cells), 51 μM (MCF-7 cells), 45 μM (UMUC-3 cells), >100 μM (A375 cells); **2** Decrease H4R3me2a level in DLD-1 cells significantly (starting @ 3μM) from western blot	2015[111]
 **15** (DB75): R = CH; **16**: R = N	Assay: RFA IC_{50} on PRMT1: **15**, 9.4 μM; **16**, 7.2 μM; Stilbamidine (**13**), 15 μM. Selectivity: Both preferred PRMT1 over CARM1 (>42-fold), PRMT5 (>18-fold), and PRMT6 (>30-fold) Note: all the methylation assays were done under balanced condition	Compound **15**: **1** Cell permeable and inhibit methylation of ALY protein in 293T cells; **2** 20 μM inhibit cell growth for most of the tested leukemia cell lines except HEL cells which have JAK2V617F mutations; **3** Cell lines derived from Down's syndrome patients and MLL-AF9 patient are more sensitive; **4** inhibit PRMT1-mediated putative RBM15 methylation at residue R578, linking PRMT to RNA splicing pathways; **5** Impact on RNA splicing in a DM1 model. Compound **16**: Inhibit PRMT1-mediated Smad6 methylation, revealing that the methylase activity of PRMT1 is was essential for BMP-induced Smad1/Smad5 phosphorylation and downstream signaling activation	2013[123], 2014[79], 2015[124], 2015[125]

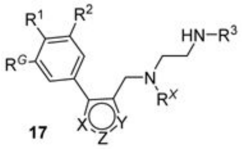
Structure and numbering	Activity		year[ref]
	Biochemical	Cellular	
 R¹ R² R^G HN−R³ N R^X X Y Z 17	—	—	2015[126, 127]

Table 3

PRMT3-specific inhibitors.

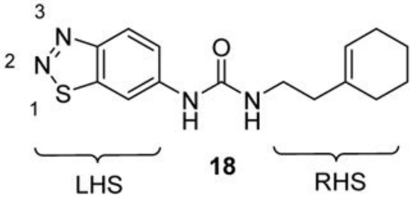
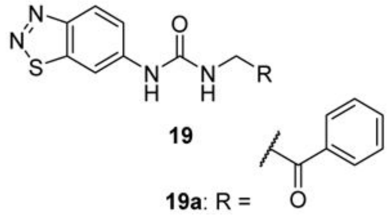
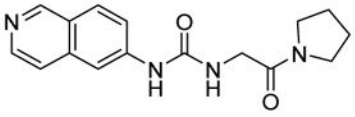
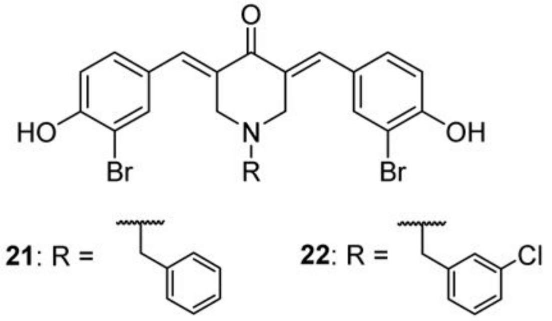
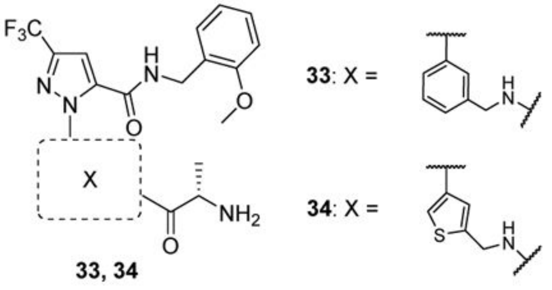
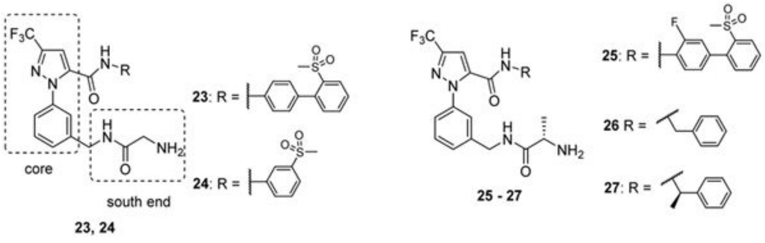
Structure and numbering	Activity		year[ref]
	Biochemical		
 **18**	Assay: SAHH-coupled assay; SPA; RFA **1** IC_{50} 1.6 μM (with H4-24 peptide as substrate), 2 μM (with rpS2 protein as substrate); K_d = 9 μM; inactive to five HKMTs (G9a, EHMT1, SUV39H2, SETD7, and SETD8), three PRMTs (-1, -4, and -8), weakly inhibit PRMT5; **2** Allosteric binding, crystal structure available; **3** Not metabolically stable enough for cell-based assays; Note: all the methylation assays were done under balanced condition.		2012[96]
 **19** **19a**: R =	Assay: SPA; RFA **19a**: **1** IC_{50} 0.48 μM on PRMT3; 40-fold selective for PRMT3 over G9a, GLP, and SUV39H2; totally inactive against PRMT5, SETD7, PRC2, SETD8, SETDB1, SUV420H1, SUV420H2, MLL1, SMYD3, SMYD2, DOT1L, and DNMT1; **2** Crystal structure available.		2013[80]
	Biochemical	**Cellular & Animal model**	
 **20** (SGC707)	Assay: SPA **1** IC_{50} 0.03 μM (PRMT3); outstanding selectivity against 31 HKMTs, PRMTs, DNMTs and RNA-methyltransferase as well as 250 non-epigenetic proteins; **2** Crystal structure available; **3** K_d = 0.053 μM and 0.085 μM, determined by ITC and SPR assay, respectively.	**1** Bind with PRMT3 overexpressed in HEK293 and A549 cells (EC_{50} = 1.3 μM and 1.6 μM, respectively) determined by InCELL Hunter assay; **2** Inhibit endogenous and exogenous H4R3me2a with (IC_{50} = 0.225μM and 0.091μM, respectively) from western blot; **3** No toxicity at 24h; **4** In vivo PK: The plasma level at 6 h post injection was 208 nm, more than 2-fold higher than its IC_{50} value in the cellular assay and the half-life was about 1 h.	2015[97]

Table 4

CARM1-specific inhibitors.

Structure and numbering	**Activity**		**year[ref]**
	Biochemical	**Cellular**	
 O HO OH Br Br N R 21: R = 22: R = Cl	Assay: RGA **1** IC$_{50}$ around 8 μM (CARM1 with PABP1 as substrate); **2** Certain inhibition on PRMT3, -5 and -6, but weaker than on CARM1; **3** Inactive to tested HKMTs (SET7/9, G9a, DOT1L, Suv39H1).	**1** Compound **21** suppressed the methylation of the transfected PABP1 protein in HEK293 cells; **2** From androgen-driven luciferase assay, both compounds showed dose-dependent inhibition of the transcription level was observed starting from 4 μM.	2011[72]
 F$_3$C N N H N O O X NH$_2$ O 33, 34 33: X = 34: X = H N S	Assay: RFA Both: **1** IC$_{50}$ 0.06 μM for (CARM1); **2** Inactive to PRMT1 and SET7/9 (IC$_{50}$ > 100 µ)	Compound **34**: **1** No inhibition was observed for cellular methylation of H3R26 at 5 μM for 48 h; **2** No significant activity in the hormone-M) dependent assays (IC$_{50}$ > 10 μM).	2009[81]
		Biochemical	
 F$_3$C N N H N R O core H N NH$_2$ O south end 23, 24 23: R = 24: R = 25 - 27 25: R = F 26 R = 27: R =		Assay: RFA IC$_{50}$ (CARM1): Compound **23**, 1.8 μM; Compound **24–27**, 0.08–0.23 μM; Compound **26**: inactive to PRMT1 and -3 (IC$_{50}$ > 25 μM); moderate permeability against PAMPA.	2008[82], 2009[83]

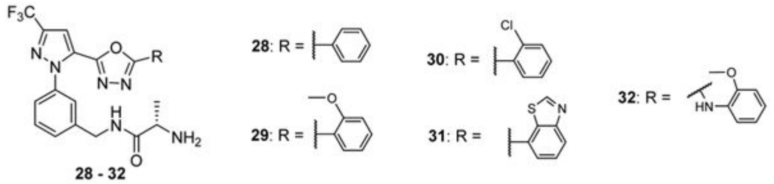
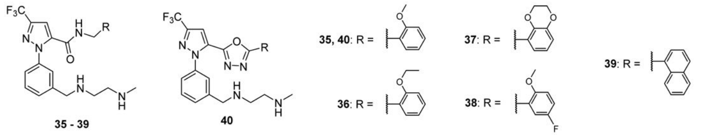
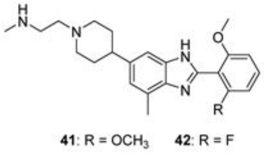
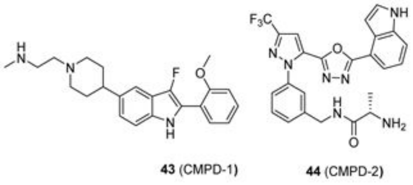
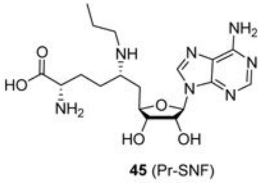
Structure and numbering	Activity		year[ref]
	Biochemical	Cellular	
 28 - 32 28: R = 29: R = 30: R = 31: R = 32: R =		Assay: RFA IC_{50} (CARM1): compound **28–32**, 0.04–0.06 µM; Compound **31**: inactive to PRMT1 and -3 (IC_{50} > 25 µM); improved permeability.	2009[83]
 35 - 39 40 35, 40: R = 36: R = 37: R = 38: R = 39: R =		Assay: RFA 1 IC_{50} = 0.2 – 0.9 µM; 2 Better pharmacokinetics profile (lower clearance, longer half-life and smaller volumes of distribution) compared with compound **33.**	2009[84]
 41: R = OCH_3 42: R = F		Assay: RFA Compound **41:** IC_{50} 0.07 µM (CARM1); Inactive to PRMT1 and -3 (IC_{50} > 25 µM); Compound **42:** IC_{50} 0.12µM (CARM1);	2009[85]
 43 (CMPD-1) 44 (CMPD-2)		Assay: RFA 1 IC_{50} around 0.03 µM (CARM1); 2 Inactive to PRMT1 and -3 (IC_{50} > 25 µM); 3 Crystal structures available	2011[86]
 45 (Pr-SNF)		Assay: SPA IC_{50} 0.80 µM for SETD2, 2.2 µM for SET7/9, 3.0 µM for CARM1, 9.5 µM for PRMT1, >100 µM for SET8, G9a, and GLP.	2015[98]

Table 5

PRMT5-specific inhibitors.

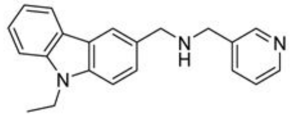
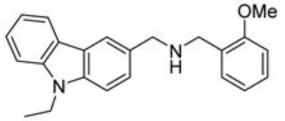
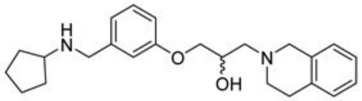
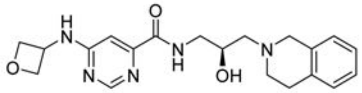
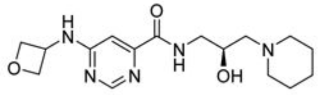
Structure and numbering	Activity		year[ref]
	Biochemical	Cellular & Animal model	
 **46** (CMP5 or BLL-1) Structure is drawn according to patent WO2011079236 A1	Assay: RFA 10–100 μM of compound **46** decreased histone methylation by inhibiting PRMT5 rather than PRMT1, CARM1 or PRMT7	**1** Selectively inhibit symmetric methylation in D-9 cells; **2** Selectively inhibit lymphoma cells significantly (24h, starting from 20–40 μM), while only mildly decline the normal resting B cells (72 h, 100 μM); **3** Mechanism for cytotoxicity: cause loss of the repressive complex (PRMT5/p65/HDAC) on miR96 promoter, re-expression of miR96 and down-regulation of PRMT5 level as well as restore of the tumor suppressor expression.	2011[146], 2015[37]
 **47** (HLCL-61)	Assay: RFA 10 μM compound exhibited about 80% inhibition on PRMT5 but no obvious inhibition on PRMT1, CARM1 and PRMT7.	**1** Significantly decrease symmetric dimethylation level of H3R8 and H4R3 (concentration not reported) in AML cells; **2** Inhibit AML cell growth (IC_{50} ranging 7 – 21 μM at 48h); **3** Mechanism for cytotoxicity: reverse the effect of PRMT5 on miR29b and FLT3.	2014[87], 2015[147]
 **48** (EPZ007345)  **49** (EPZ015666)  **50** (EPZ019896)	Assay: SPA; TR-FRET Compound **48:** IC_{50} 0.4 μM (PRMT5 : MEP50) Compound **49:** **1** IC_{50} 0.022 μM (PRMT5 : MEP50); inactive to other PRMTs (PRMT1, -3, -4, -6, -7 and -8; no inhibition at 50 μM) and fourteen HKMTs; **2** Substrate-competitive, crystal structure available; **3** K_d on PRMT5:MEP50 complex: 171 nM (SAH-bound); < 1 nM (SAM-bound or Sinefungin-bound) Note: all the methylation assays were done under balanced condition	Compound **49:** **Cellular activity** on MCL cell lines (Z-138, Maver-1, Mino, Granta-519 and Jeko-1): IC_{50} 4 – 340 nM for inhibition on SmD3 dimethylation; IC_{50} 60 – 900 nM for anti-cell-growth effect. **In vivo activity:** **1** 200 mg/kg dose resulted in tumor growth inhibition (> 90%, > 70% and > 45% for Z-138, Maver-1 and Granta-519 cell line, respectively) with limited effect on the bodyweight; **2** Reduced SDMA level in the xenograft tumors; **3** Favorable PK property. Low clearance in human, mouse and rat liver microsomes and higher clearance in dog liver microsomes as well as hepatocytes.	2015[108–110]

Table 6

PRMT6-specific inhibitors.

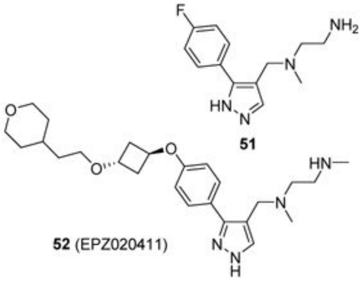
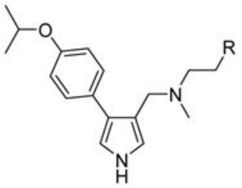
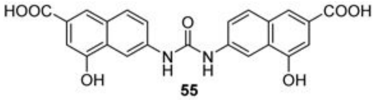
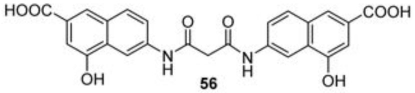
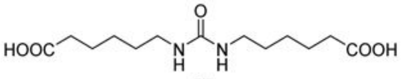
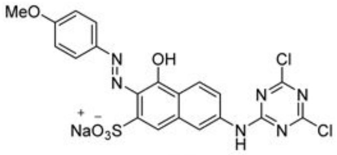
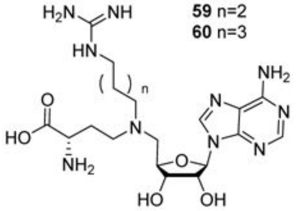
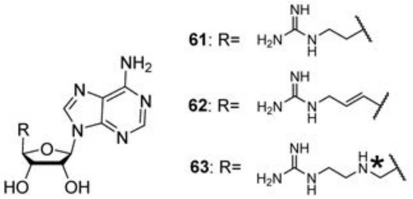
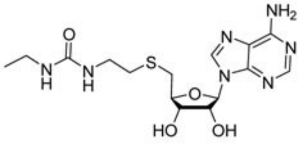
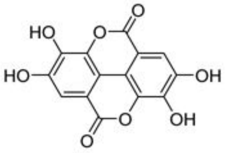
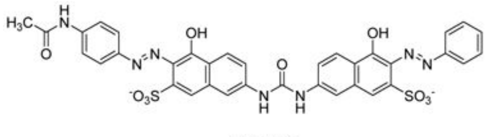
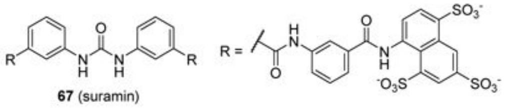
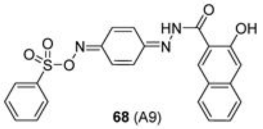
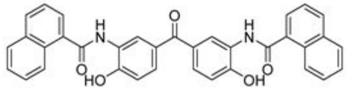
Structure and numbering	Activity		year[ref]
	Biochemical	**Cellular**	
 **51** **52** (EPZ020411)	Assay: SPA Compound **52**: **1** IC_{50} 10 nM (PRMT6); 223 nM (PRMT8); 119 nM (PRMT1); **2** Occupy the putative substrate arginine pocket, crystal structure available; Note: all the methylation assays were done under balanced condition	Compound **52**: **1** Inhibit H3R2 methylation does-dependently with IC_{50} at 0.64 μM; 10 fold selective for PRMT6 over PRMT1 in A375 cells; **2** Bioavailability in rat by subcutaneous dosing (5 mg/kg), but not by oral dosing, is satisfactory for further in vivo efficacy profiling	2015[99]
 **53 (MS023)** R = NH_2 **54 (MS094)** R = OH	Assay: SPA Compound **53:** **1** Nanomolar-leveled activity for tested type I PRMTs with best inhibition on PRMT6 and -8 (IC50 = 4 and 5 nM, respectively); inactive to type II and III PRMTs as well as a total of 28 PKMTs, DNMTs and histone lysine demethylases at up to 10 μM; **2** No binding on methyllysine or methylarginine reader by DSF or DSLS **3** K_d = 6 nM (PRMT6) tested by ITC; **4** Occupy the putative substrate arginine pocket, crystal structure available; **5** Non-competitive to both SAM and peptide substrate. Note: all the methylation assays were done under balanced condition	Compound **53**: **1** dose-dependent inhibition on endogenous PRMT1 activity on H4R3 in MCF7 cells (IC_{50} = 9 nM) and on overexpression PRMT6 activity on H3R2 in HEK293 cells (IC_{50} = 56 nM); **2** The dose to achieve significant effect on proliferation depended on exposure time and cell type: 4-day treatment of 1 μM on HEK293 cells; 10-day treatment of 10 μM on MCF7 cells.	2015[100]

Table 7

Other PRMT inhibitors.

Structure and numbering	Activity		year[ref]
	Biochemical	Cellular	
1) Promiscuous PRMT inhibitors			
 **55**  **56**	Assay: RFA; western blot; RGA Compound **55**: IC_{50} 112μM (PRMT1); similar activity to AMI-1 against PRMT-1,-3 and -4; Compound **56**: IC_{50} 272 μM (PRMT1) Note: IC_{50} of AMI-1 was 92 μM (PRMT1)	Compound **55**: Inhibition on methylation of transiently transfected Npl3p start to show at 50 μM	2010[73]
 **57**	Assay: ELISA IC_{50} 6.1 μM (PRMT1); 15% – 20% inhibition on PRMT5 (@ 100 μM)	Dose-dependent inhibitory effect on the expression level of androgen-dependent genes (TMPRSS2 and FKBP5); 50% of the maximum inhibition was achieved at 25μM.	2013[102]
 **58**	Assay: RGA Better activity than AMI-1 on PRMT1, -3, -4, -5, -6 and -8; IC_{50} 4.2 μM (PRMT1); IC_{50} 2.7 μM (CARM1); Inactive on SET7/9	**1** Inhibit H3R17 methylation to 55% (@100 μM) and 35% (@300 μM), compared with around 80% by AMI-1 of the same concentration; **2** Inhibit secretion of IFNγ by Th1 cells (starting @ 10μM) and IL-4 by Th2 cells (starting @ 0.1μM); **3** No effect on viability of Th cells **4** Interfere with IL-4 expression at transcription level; **5** Interrupt association of PRMT1 and NIP45.	2010[74]
2) Bisubstrate inhibitors			

Structure and numbering	Activity		year[ref]
	Biochemical	Cellular	
 **59** n=2 **60** n=3	Assay: RGA IC_{50} 3–6 µM (PRMT1); IC_{50} > 100 µM (CARM1); IC_{50} > 100 µM (SET7/9)	—	2010[75], 2011[76]
 **61**: R= **62**: R= **63**: R=	Assay: ELISA IC_{50} of compound **61** and **62**: IC_{50} 0.1–0.2 µM (CARM1); > 5 µM (PRMT1, PRMT6); IC_{50} of compound **63**: 3.2 µM (PRMT6); > 50 µM (PRMT1, CARM1)	—	2015[103]
 **64** (DS-437)	Assay: SPA IC_{50} 6 µM (PRMT5/PRMT7); inactive to other PRMTs Note: all the methylation assays were done under balanced condition	Start to decrease the methylation level of SmD and SmB proteins in MBMDA231 cells at 10 µM.	2015[101]
3) Inhibitors with substrate-targeting property			
 **65** (TBBD)	Assay: RFA; western blot Bind histone H3 instead of CARM1 as determined by ITC, targeting residue sequence KAPRK Specifically inhibit methylation of H3R17 by CARM1 (IC_{50} around 25 µM); no effect to acetylation of H3K14 and H3K18 by p300.	Affect H3R17 methylation but not H3K14 and H3K18 acetylation in HeLa cells; Repress the expression of p53-responsive gene, p21, by decreasing H3R17 methylation	2010[88]

Structure and numbering	Activity		year[ref]
	Biochemical	Cellular	
 **66** (NS-1)  **67** (suramin)	Assay: RFA Compound **66**: IC_{50} 12 μM (PRMT1 with H4(1-20) peptide), 7.1 μM (PRMT3 with R4 peptide), 2 mM (CARM1 with H3(1-31) peptide), 39 μM (PRMT6 with H3(1-31) peptide), 21 μM on (p300 with H4(1-20) peptide) Bind H4(1-20) peptide as validated by uv, fluorescence and mass spectra . Note: : IC_{50} of AMI-1 is 137 μM on PRMT1 with H4(1-20) peptide as substrate	—	2010[139]
 **68** (A9)  **69** (A36)	Assay: RFA Compound **68**: IC_{50} 41 μM (PRMT1 with H4(1-20) peptide) Compound **69**: **1** IC_{50} 12 μM (PRMT1 with H4(1-20) peptide), 15 μM (p300 with H4(1-20) peptide; **2** Targeting peptide as determined by fluorescence spectrum Note: : IC_{50} of AMI-1 is 77 μM on PRMT1 with H4(1-20) peptide as substrate	Compound **68**: 10 μM achieve 70% inhibition on proliferation of LNCaP C-81 Cells at in regular medium and 30% inhibition in steroid-reduced condition	2012[242]