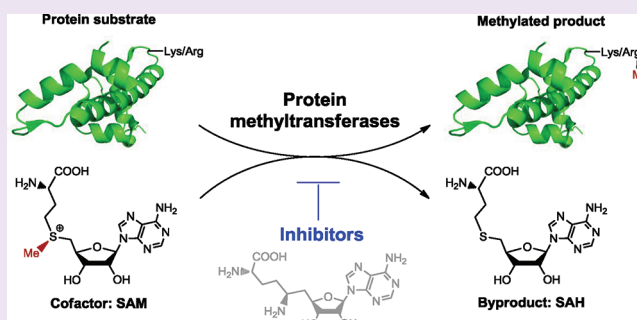


Current Chemical Biology Approaches to Interrogate Protein Methyltransferases

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ABSTRACT: Protein methyltransferases (PMTs) play various physiological and pathological roles through methylating histone and nonhistone targets. However, most PMTs including more than 60 human PMTs remain to be fully characterized. The current approaches to elucidate the functions of PMTs have been diversified by many emerging chemical biology technologies. This review focuses on progress in these aspects and is organized into four discussion modules (assays, substrates, cofactors, and inhibitors) that are important to elucidate biological functions of PMTs. These modules are expected to provide general guidance and present emerging methods for researchers to select and combine suitable PMT-activity assays, well-defined substrates, novel SAM surrogates, and PMT inhibitors to interrogate PMTs.



According to biochemical reactions and enzyme numerical classification (EC number), protein methyltransferases (PMTs), together with acetyltransferases, glycosyltransferases, and kinases, belong to the family of transferase enzymes (EC 2). The common feature of these enzymes is to transfer a functional group from a donor (cofactor or coenzyme) to an acceptor (Figure 1). For PMTs, the cofactor and acceptor are S-adenosylmethionine (SAM) and lysine or arginine side chains of protein substrates, respectively (Figure 1). The human genome encodes more than 60 PMTs including 9 known protein arginine methyltransferases (PRMTs) and >50 protein lysine methyltransferases (PKMTs) (Figure 2).¹

The 9-member human PRMTs (PRMT1–9) share a set of four conserved motifs (I, post-I, II, and III) and the characteristic THW loop for SAM binding.² With SAM as the methyl donor, PRMTs modify arginine's ω -guanidino nitrogen in a target-specific manner (Figure 2).² The three forms of arginine methylation products (MMA, ADMA, and SDMA for monomethylarginine, asymmetric dimethylarginine, and symmetric dimethylarginine, respectively) further distinguish PRMTs into three subtypes (Figure 2): Type I (MMA-then-ADMA products for PRMT1, 2, 3, 4, 6, 8), Type II (MMA-then-SDMA products for PRMT5, 7), and Type III (MMA product for certain targets of PRMT7).² The methylation pattern of PRMT9 remains to be characterized unambiguously.² With the exception of DOT1L, whose catalytic domain is similar to that of PRMTs, PKMTs harbor a canonical SET domain composed of 130 amino acids for SAM binding and enzyme catalysis.³ PKMTs methylate lysine's ϵ -amino group to specific degrees: mono-, di-, and trimethylation (Figure 2).^{4,5}

PRMTs and PKMTs methylate histone targets.^{4,5} For instance, PRMT1 and CARM1 methylate arginine 3 of histone

H4 (H4R3) and arginines 2/17/26 of histone H3 (H3R2/17/26), respectively.^{2,4,5} These events have been linked to transcriptional activation.^{2,4,5} In contrast, PRMT5 and PRMT6 modify H4R3 and H3R2. These methylation events are associated with transcriptional repression.^{2,4,5} This yin-yang type of switch has also been observed for PKMT-involved histone methylation. For instance, trimethylation of H3 lysine 4 (H3K4me3) and trimethylation of H3 lysine 36 (H3K36me3) and lysine 79 (H3K79me3) are the marks for active genes, whereas H3 lysine 9 di/trimethylation (H3K9me2/3) and H4 lysine 20 methylation (H4K20me1/2/3) are the marks for silenced genes.^{2,4–6}

Besides histones, PMTs also methylate diverse nonhistone targets.⁷ The majority of PRMT substrates are nonhistone targets including transcription factors STAT1, RUNX1, and FOXO1 (PRMT1 substrates);^{8–10} transcription coactivators p300 and CBP (CARM1 substrates);^{11,12} and RNA-binding proteins (substrates of PRMTs).¹³ Efforts over the past decade have led to the characterization of many PKMT nonhistone substrates as well (e.g., the tumor suppressor p53 as the substrate of SET7/9, SET8, SMYD2, G9a, and GLP).^{14–19} PMT-mediated histone and nonhistone methylation, together with other posttranslational modifications (e.g., acetylation, phosphorylation, sumoylation, and ubiquitination), can regulate binding partners (activators or repressors), localization, or stability of the PMT substrates.^{2,4,5,7} These modifications alone or in combination can modulate downstream signals in an epigenetic manner and thus render meaningful biological readouts.^{2,4,5,7}

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Reaction catalyzed by a transferase enzyme:



Reaction catalyzed by a protein methyltransferase (PMT):

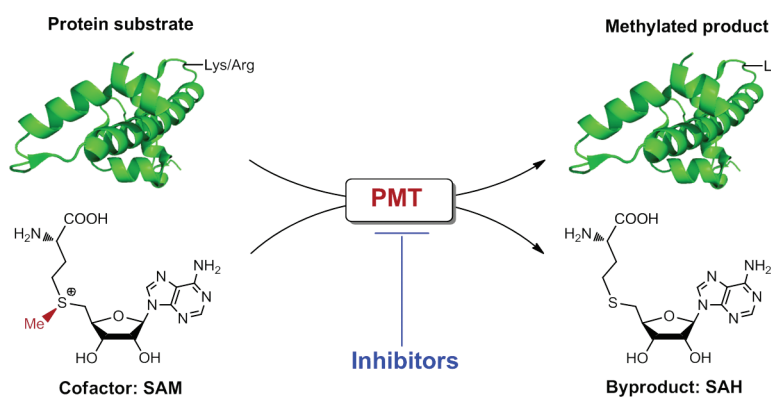


Figure 1. Reactions catalyzed by transferase enzymes and protein methyltransferases (PMTs). A transferase enzyme (X-transferase) has the ability to transfer a functional moiety (X) from a cofactor or coenzyme (A–X) to its substrates (B) and generate modified products (B–X) and a cofactor metabolite (A). In the case of PMTs, the functional moiety, the cofactor, substrates, products, and the byproduct are a methyl group, S-adenosylmethionine (SAM), specific Lys/Arg side chains of proteins, methylated products, and S-adenosylhomocysteine, respectively.

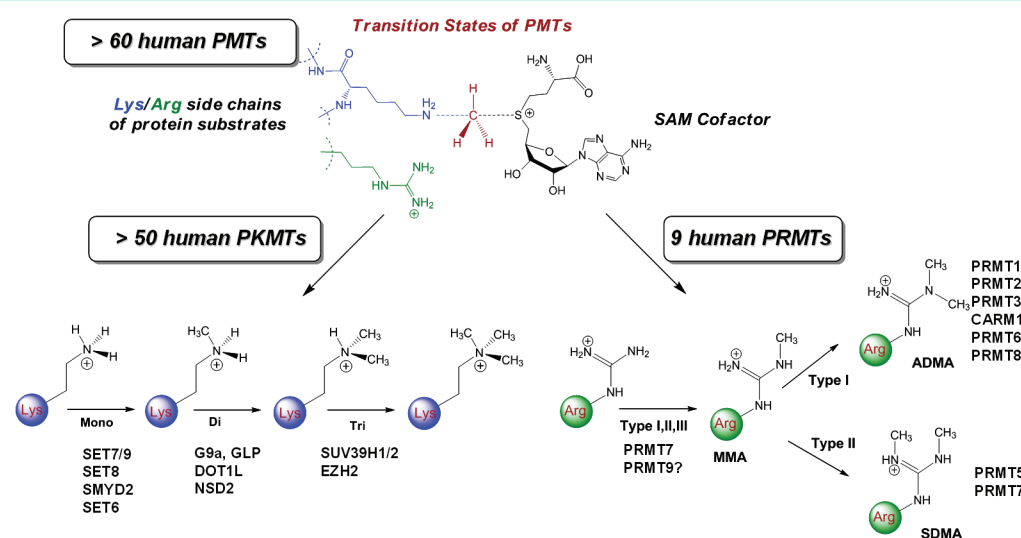


Figure 2. Human PMTs. The human genome encodes >60 PMTs, which diverge into protein lysine methyltransferases (PKMTs, >50) and protein arginine methyltransferases (PRMTs, around 9). PKMTs can mono/di/trimethylate their substrates in a processive or distributive manner. According to the three forms of arginine methylation products (MMA, ADMA, and SDMA for monomethylarginine, asymmetric dimethylarginine, and symmetric dimethylarginine, respectively), PRMTs can be categorized into three subtypes: Type I (MMA-then-ADMA products for PRMT1, 2, 3, 4, 6, 8), Type II (MMA-then-SDMA products for PRMT5, 7), and Type III (MMA product for certain targets of PRMT7). The methylation pattern of PRMT9 remains to be characterized unambiguously.

Apart from PMTs' roles in normal physiology, their dysregulation has been implicated in many diseases including cancer.²⁰ For instance, oncogenic properties of PMTs (e.g., EZH2, G9a, PRMT5, SUV39H1, and SMYD2) can rely on target methylation that destabilize or downregulate tumor suppressors.²⁰ PMTs can also be linked to cancer through aberrant upregulation of oncogenes.²⁰ For example, the enzymatic activities of DOT1L and PRMT1 were shown to be essential for downstream signals of mixed lineage leukemia (MLL) transcriptional complex. The constitutive recruitment of DOT1L and PRMT1 by MLL-fusion protein stimulates

hematopoietic transformation.^{21,22} Additionally, overexpression of PMTs such as GLP, SUV39H2, NSD2, NSD3, SMYD3, and PRDM14 has been reported in many primary tumors.²⁰ These findings further underscore the cancer relevance of PMTs.

Most PMT substrates were identified through a conventional candidate-based approach. In this approach, a proposed PMT substrate is tested against a panel of PMTs *in vitro* with [Me-³H]SAM as a cofactor. The radioactive methyl group is expected to be delivered to a *bona fide* substrate only by matched PMTs. To map the site(s) of the methylation, truncated or site-specifically mutated substrates are then

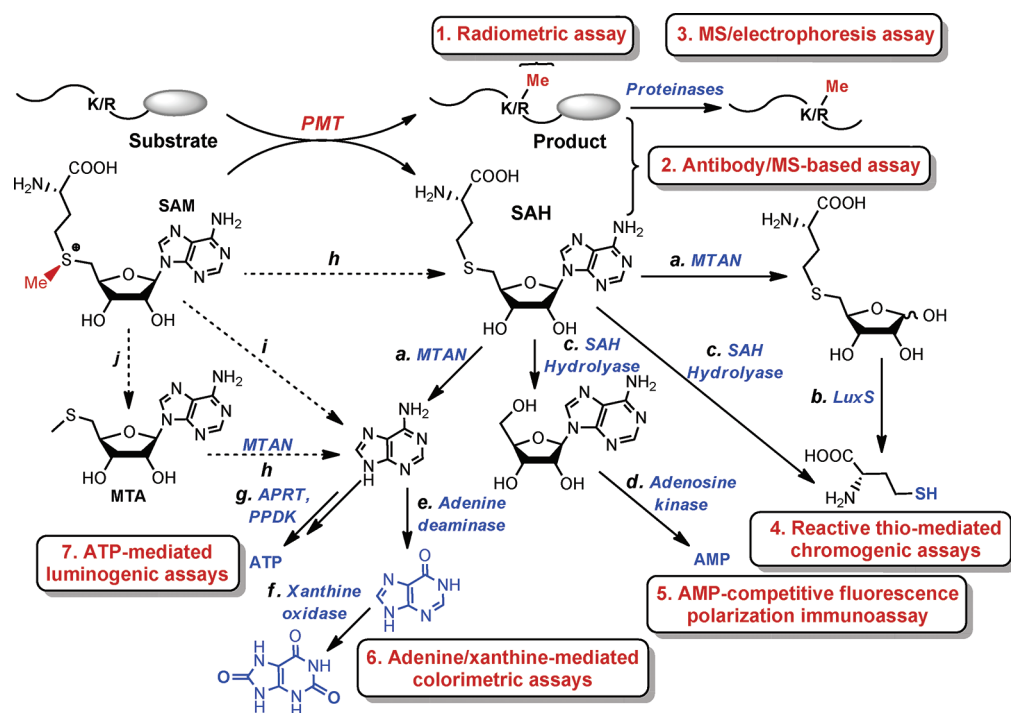


Figure 3. Schematic presentation of current PMT-activity assays and potential interfering factors. The current PMT-activity assays mainly rely on quantification of methylated protein products or the byproduct SAH. Methylated peptide/protein products can be quantified directly by radiometric and top-down mass spectrometric methods (Methods 1 and 2). The digested products of peptide/protein can be quantified by middle-down/shot-gun mass spectrometric or electrophoresis methods (Method 3). In contrast, the byproduct SAH can be quantified directly by *anti*-SAH antibody or MS (Methods 1 and 2) or indirectly by various colorimetric assays (Methods 4–7) with coupling enzymes (Pathways a–g): MTAN (*S*-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase), LuxS (*S*-ribosylhomocysteine lyase), SAH hydrolyase, adenosine kinase, adenosine deaminase, xanthine oxidase, APRT (adenine phosphoribosyl transferase), or PPK (pyruvate orthophosphate dikinase). SAM can spontaneously decompose into SAH, adenine, and MTA (Pathways h–j).

examined for either gain or loss of the methylation signal. The confirmed enzyme–substrate pair can then be validated in cellular contexts with other biochemical and genetic methods. After the methylation activities of PMT-substrate pairs have been validated *in vitro* and in cellular contexts, their upstream and downstream events can be further pursued with accurate disease or animal models.

Although the well-established candidate-based approach demonstrated the feasibility for identifying and validating individual PMT targets, their application to proteome-wide profiling of PMT substrates is questionable. As exemplified with SET7/9, a PKMT initially characterized as a H3K4 methyltransferase, the efforts over the past decade have led to identification of a dozen SET7/9 nonhistone substrates, such as p53, TAF10, ER α , PCAF, NF- κ B, DNMT1, and HIV transactivator Tat.^{17,23–25} However, new SET7/9 targets keep emerging and give no sign to end the decade-long endeavor in searching SET7/9 targets.²⁶ In addition, target-recognizing patterns of PMTs cannot be readily rationalized because of the lack of consensus sequences. These challenges emphasize the need for new tools to elucidate how PMTs recognize structurally diverse substrates. Given the biological relevance of PMTs, it is equally important to develop tools to elucidate and manipulate the functions of PMTs in normal and disease contexts.

As chemical biology methods emerge to study transferase enzymes such as glycosyltransferases,²⁷ kinases,²⁸ and acetyltransferases,^{29,30} these approaches have been proven or show potential to be transformed for PMTs. Meanwhile, PMT-catalyzed reactions have been or can be investigated with PMT-

specific methods.^{31,32} This review focuses on providing the present status and additional perspectives on how chemical biology methods can be applied to interrogate PMTs. Given the features of the PMT-catalyzed transferase reaction, the review is organized into four discussion modules: assays, substrates, cofactors, and inhibitors. To minimize redundancy of the topics that have been covered by other excellent reviews,^{33,34} this article mainly deals with a collection of recently published literature and their chemical biology aspects. I apologize for the omission of many high-quality works because of space limitation.

■ PMT-ACTIVITY ASSAYS

In a PMT-catalyzed methylation reaction, the substrate (peptide/protein/protein complex) and SAM will be enzymatically processed into the methylated product and the byproduct *S*-adenosylhomocysteine (SAH), respectively (Figure 1). Because of PMTs' slow enzymatic turnovers, most PMT-activity assays were developed by detecting reaction products rather than measuring depletion of starting materials. Methylated products and SAH can be quantified either directly (*e.g.*, autoradiography, top/middle-down mass spectrometry (MS), and *anti*-methyllysine/arginine antibodies) or indirectly after processing them into various derivatives (*e.g.*, enzyme-coupled colorimetric assays and shot-gun MS) (Figure 3). The adaptability of these assays for high-throughput screening (HTS) will also be discussed below.

Radiometric Quantification of Substrate Methylation (Method 1 in Figure 3). For PMT-catalyzed methylation, the radiolabeled methyl group, from either [Me-³H]- or [Me-¹⁴C]-

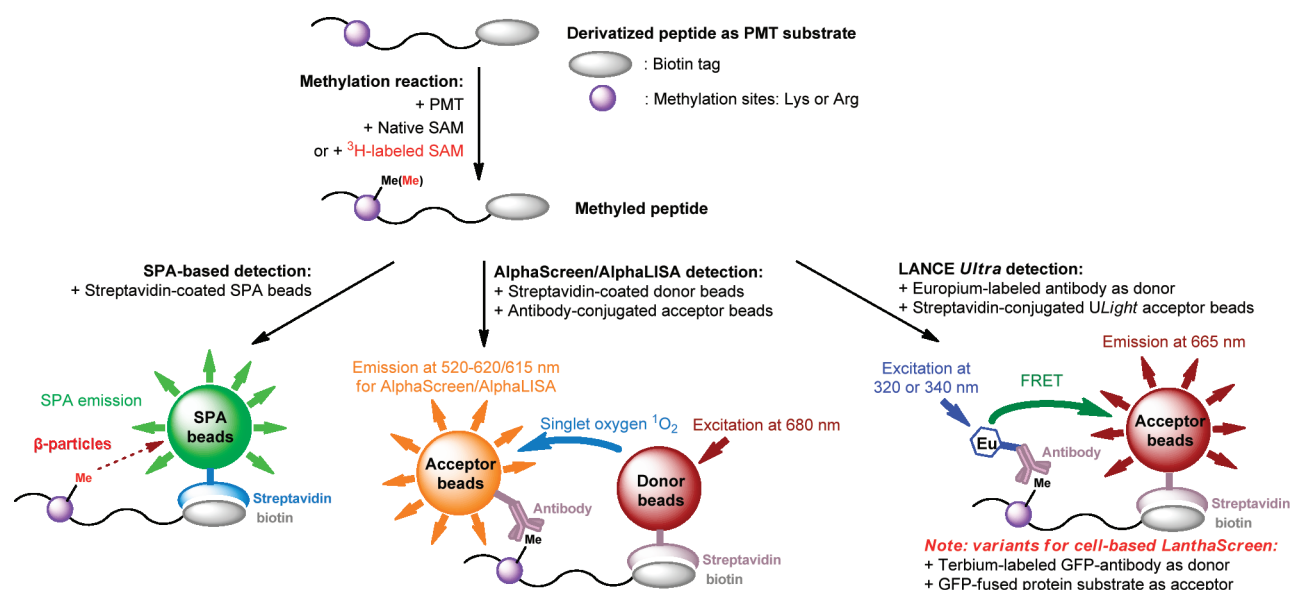


Figure 4. Emerging SPA-based and antibody-based homogeneous PMT-activity assays for HTS. The principles of SPA, AlphaScreen/LISA, LANCE Ultra, and LanthaScreen are briefly described below. In the reported SPA-based PMT-activity assay,³⁶ a biotinylated peptide substrate was radiolabeled by PMT with [Me- ^3H]-labeled SAM and then immobilized to streptavidin-conjugated SPA beads. The proximity between the β -particles from the immobilized peptide and SPA-coated scintillation fluid led to light emission. For the reported AlphaScreen and AlphaLISA PMT-activity assays,^{40,41} the methylated, biotin-tagged peptide product caused the proximity between streptavidin-coated donor beads and anti-methyllysine antibody-conjugated acceptor beads (or primary antibody-immunized secondary-antibody-conjugated acceptor beads). Exciting the donor beads at 680 nm led to emitting singlet oxygen ($^1\text{O}_2$), which excited the acceptor beads to generate emission at 520–620 nm (AlphaScreen) or at 615 nm (AlphaLISA). For the reported LANCE Ultra and LanthaScreen PMT-activity assays,^{42,43} europium/terbium-labeled anti-methyllysine antibodies were used as FRET donors and streptavidin-conjugated ULight (LANCE Ultra) or GFP-fused protein (LanthaScreen) as FRET acceptors. Exciting the donors at 320 or 340 nm led to FRET-mediated light emission of the acceptors.

SAM, can be enzymatically incorporated into PMT targets (Method 1 in Figure 3). After removing the unreacted SAM, the enzymatically incorporated radioactive moiety can be quantified by autoradiography or liquid scintillation counting. To separate radiolabeled products from residual SAM, the accepted practices are to use phosphocellulose filter paper to bind peptide or protein products, followed by washing and scintillation counting or SDS-PAGE separation, followed by autoradiography or gel extraction/scintillation counting.³⁵ Although these methods are favored for their straightforward protocols as well as facile access to reagents and instruments, they are laborious (multistep washing or transferring) and time-consuming ($\sim 6\text{ h} - 7\text{ d}$ for a single run).³⁵

To accelerate the assay turnover, the Hevel group found that radiolabeled protein products can be readily separated from unreacted SAM with ZipTip_{c4} pipet tips.³⁵ With the ZipTip protocol, the entire process can be completed within $\sim 2 - 14$ min. Alternatively, the Jeltsch and the Zheng laboratories adapted scintillation proximity assay (SPA) to rapidly quantify radiolabeled products.³⁶⁻³⁹ In the SPA-based assay, biotinylated peptides and [Me- ^3H]-labeled-SAM were used as PMT substrates and cofactor, respectively. After the [Me- ^3H]-labeled products were immobilized to avidin-conjugated plates or beads, the proximity between the β -particles from the immobilized ^3H -labeled peptide and SPA-plate/bead-coated scintillation fluid triggered an emission of scintillation signal (Figure 4). This SPA-based approach has been applied for measuring the activities of Dim5, G9a and PRMT1.³⁶⁻³⁹ In comparison with other radiometric methods, the homogeneous SPA approach features no separation of residual radioactive SAM and is thus adaptable for a mix-and-measure HTS format (The HTS application of SPA will be discussed later).³⁶

Antibody-Based Detection of Substrate Methylation (Method 2 in Figure 3).

Although radiometric assays are often used to study PMTs, their radioactive format is not environmentally friendly. In addition, positive radioactive signals only report the methylation activity, but not the degree of methylation (e.g., MMA/ADMA/SDMA for PRMTs or mono/di/trimethylation for PKMTs). However, these limitations can be addressed by antibody-based PMT-activity assays. Diverse primary monoclonal or polyclonal antibodies are available to recognize specific methylation epitopes for Western blot, CHIP, CHIP-on-chip and CHIP-seq analysis.⁶

In conjunction with several recent technologies, such as AlphaScreen (PerkinElmer), AlphaLISA (PerkinElmer), LANCE Ultra (PerkinElmer) and LanthaScreen (Invitrogen), anti-methyllysine(arginine) antibodies have demonstrated their use in homogeneous PMT-activity assays (Figure 4).⁴⁰⁻⁴³ These assays share a similar principle by pairing a PMT substrate and an anti-methyllysine antibody with donor and acceptor dyes (Figure 4).⁴⁰⁻⁴³ The anticipated interaction between the methylated product and the antibody brings the donor and acceptor dyes in a proximity. The excitation of the donor dye then leads to emission of the acceptor dye through either singlet oxygen ($^1\text{O}_2$) (AlphaScreen and AlphaLISA) or time-resolved fluorescence resonance energy transfer (TR-FRET) (LANCE Ultra and LanthaScreen).⁴⁰⁻⁴³ As the first application of PMTs, Quinn *et al.* reported chemiluminescence AlphaScreen immunoassay technology, combined with a polyclonal anti-methyl-H3K9 antibody, to examine G9a-catalyzed H3K9 methylation.⁴⁰ Gauthier *et al.* and Hauser *et al.* then developed an antibody-based AlphaLISA approach to monitor SET7/9-catalyzed H3K4 methylation and PRMT1-catalyzed H4R3 methylation, respectively.^{41,42} Gauthier *et al.*

also demonstrated a similar application combining LANCE *Ultra* technology and a europium-labeled antimethyllysine antibody.⁴¹ With terbium-labeled *anti*-methyl H3K9 antibody (the donor) and GFP-fused histone H3 (the acceptor), Machleidt *et al.* for the first time developed a LanthaScreen TR-FRET approach to visualize H3K9 dimethylation in cellular contexts.⁴³ The merit of these antibody-based homogeneous assays lies in their adaptability for HTS as discussed later.

Though the antibody-based approaches have the merit for the ready readouts, the specificity of the antibodies and the dynamic range of epitope concentrations need to be well-defined prior to their use in PMT-activity assays. Given the general narrow range of the latter, the antibody-based PMT-activity assays are not suitable to measure quantitative data such as K_m and k_{cat} (the PMT-activity assays for this purpose will be discussed later).

MS-Based Detection of Intact Protein Samples (Method 2 in Figure 3). When small peptides are used as PMT substrates, the reaction products can be analyzed by MS after simple workup.^{31,32} The level of methylation is directly reflected by corresponding mass shifts (*e.g.*, +14 Da for mono- or +28 Da for dimethylation). Since lysine/arginine methylation does not significantly alter the size and the charge of substrates, the peak ratio between unmodified and modified peptides is sometimes used for direct quantification.^{31,32,44} When small-to-medium-size proteins (*e.g.*, histones) are examined as PMT substrates, top-down MS is often used to monitor the level of methylation (*e.g.*, mono/di/tri) as well as map the site(s) of methylation. Combining top-down MS with metabolic labeling, Pesavento *et al.* were able to monitor cell-cycle-dependent dynamics of H4K20 methylation. Their work revealed that H4K20 methylation progressively accumulates on newly translated histones during G2, M and G1 phases, and reaches to a maximal degree within 2–3 cell cycles.⁴⁵ Combining top-down MS with heavy methyl-SILAC labeling, the Garcia laboratory was able to analyze systematically *in vivo* dynamics of multiple histone lysine and arginine methylations and showed that active-gene-associated histones are methylated faster than silenced-gene-associated histones.⁴⁶ A key advantage of using intact peptide/protein samples for MS analysis is the ability to unambiguously detect the methylation(s) together with other posttranslational modifications on a single target.

PMT-Activity Assays Using Digested Protein Samples (Method 3 in Figure 3). Top-down MS approach is largely limited to small-size, high-quality protein samples such as histones.⁴⁶ In contrast, middle-down/shot-gun MS using digested protein samples is more generally applicable. One general application of middle-down/shot-gun MS analysis is to map protein methylation sites. For example, after confirming SMYD2's activity on pRb with a radiometric assay, Addict *et al.* were able to rely on the shotgun MS and tandem MS (MS/MS) approach to conclude readily that the methylation occurs at Lys 860 but not at adjacent Lys 844/847.⁴⁷ The La Thangue laboratory was able to use the same approach to identify Lys 810 of pRb as the methylation site for SET7/9.⁴⁸ Compared with the laborious radiometric approach with truncated or site-specifically mutated proteins to map protein methylation, the shotgun proteomic approach avoids the need to test multiple samples and thus significantly simplifies the mapping process (Method 3 in Figure 3).

Although peptide samples are generally subject to MS analysis without protease digestion, the Janzen laboratory reported a microfluidic capillary electrophoresis using endo-

proteinase-digested peptides to quantify PMT-catalyzed reactions (Method 3 in Figure 3).⁴⁹ The authors relied on a methylation-sensitive endoproteinase, which cleaves unmethylated peptide but not methylated peptide.⁴⁹ The resultant digested (unmethylated substrate) peptide and undigested peptide (methylated product) were resolved by microfluidic capillary electrophoresis according to their different charge-to-mass ratios. With G9a as a model PMT, the authors demonstrated that the approach is highly quantitative and suitable for characterizing the kinetics of PMT-catalyzed reactions.⁴⁹

PRMTs generate three types of arginine methylation products (MMA, ADMA and SDMA) (Figure 2).² To distinguish the three types of products, [Me-³H/¹⁴C]-SAM-labeled substrate samples can be subjected to acid hydrolysis to yield MMA, ADMA and SDMA amino acids, which can be further characterized by column/thin-layer chromatography or MS analysis. With the acid-hydrolysis approach, Branscombe *et al.* and Lee *et al.* were able to detect the SDMA products of PRMT5 and PRMT7, and categorized the two enzymes as Type II PRMTs.^{50,51} With the same approach, the Frankel laboratory was able to experimentally define PRMT2 as a Type I PRMT.⁵² The Wang laboratory further demonstrated a MALDI-TOF MS/MS approach to differentiate MMA, ADMA and SDMA at the peptidic level.⁵³ The MMA-, ADMA- and SDMA-containing peptides showed characteristic neutral losses of (–56 Da, –31 Da), (–45 Da) and (–70 Da, –30 Da), respectively.⁵³

Direct Quantification of SAH with MS or *anti*-SAH Antibody (Method 2 in Figure 3). MS- and antibody-based approaches have also been used to measure the byproduct SAH in PMT-catalyzed reactions (Method 2 in Figure 3). The Frankel lab reported a tandem MS/MS approach to quantify SAH.⁵⁴ With this assay, they were able to quantify the sources causing SAH background in PRMT1-catalyzed reactions and concluded that, besides the SAH from the contamination in commercial SAM and from SAM's nonenzymatic decomposition (Pathway **h** in Figure 3), automethylation of PRMT1 accounts for a portion of the observed SAH background.⁵⁴

The byproduct SAH in PMT-catalyzed reactions can also be quantified by antibody-based competitive assays (Method 2 in Figure 3). Capdevila *et al.* first reported a competitive immunoassay using SAH-BSA conjugate and *anti*-SAH antibody (~150-fold preference to SAH over SAM) to quantify SAH in plasma.⁵⁵ In this assay, SAH competes with microplate-coated SAH-BSA to bind *anti*-SAH antibody and thus reduces ELISA signal from the microplate-immobilized antibody. Graves *et al.* developed a similar competitive assay with fluorescein-SAH and *anti*-SAH antibody.⁵⁶ In Graves's approach, SAH is quantified by competing fluorescein-SAH to bind the antibody and thus cause the loss of fluorescence polarization signal. The assay has demonstrated its feasibility for catechol-*O*-methyltransferase and is likely applicable to PMTs, given their shared byproduct SAH.⁵⁶ However, one should be cautious to use the SAH-based fluorescence polarization because the readout is linear only in a narrow range of SAH concentration (10 nM – 500 nM).⁵⁶

PMT-Activity Assays through SAH Derivatives (Methods 4–7 in Figure 3). Many SAH-based quantification assays were developed for small-molecule methyltransferases such as salicylic acid methyltransferase⁵⁷ and catechol-*O*-methyltransferase.⁵⁸ The Zhou laboratory reported an enzyme-coupled chromogenic assay for salicylic acid methyltransferase.⁵⁷ This

assay relied on two coupling enzymes MTAN (5'-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase) and LuxS (*S*-ribosylhomocysteine lyase) to convert SAH into homocysteine (Method 4, Pathway a+b in Figure 3). Homocysteine can then be quantified with Ellman's reagent (UV change at 412 nm). The Hrycyna laboratory reported a comparable fluorogenic assay for catechol-*O*-methyltransferase (Method 4, Pathway c in Figure 3).⁵⁸ This assay relies on the coupling enzyme SAH hydrolase to process SAH into homocysteine, which is then quantified by a free-thiol-activated dye fluorescein-cystamine-methyl red. The Trieval laboratory developed the first SAH-based quantification assay for PMTs.⁵⁹ Although Trieval's assay also relied on SAH hydrolase as a coupling enzyme (Method 4, Pathway c in Figure 3), it was improved by using a more sensitive free-thiol-reactive dye ThioGlo 1 for better signal and a cysteine-free SAH hydrolase for lower background.⁵⁹ Our laboratory noticed that replacing ThioGlo 1 with another dye, 7-diethylamino-3-(4'-maleimidyl-phenyl)-4-methylcoumarin, further improves signal-to-noise separation.⁶⁰ In comparison with the radiometric, antibody- or MS-based assays as reviewed above, most SAH-based chromogenic assays are valuable because of their capacity to tolerate a broad concentration range of PMT substrates and cofactors, and thus are more suitable for measuring the kinetics of PMTs (e.g. k_{cat} and K_{m}).^{59,60}

To enhance the detection threshold of SAH-based quantification assays, our laboratory developed an ultrasensitive luminescence assay (Method 7, Pathway a+g in Figure 3).⁶⁰ In this assay, SAH is sequentially converted into adenine, adenosine monophosphate,⁶¹ and then adenosine 5'-triphosphate (ATP) by three coupling enzymes: MTAN, adenine phosphoribosyl transferase and pyruvate orthophosphate dikinase. The resultant ATP is quantified with a sensitive luciferin/luciferase kit. This assay is ultrasensitive and is able to detect 0.3 pmol of SAH (30-fold better than prior SAH-based colorimetric assays) and has been validated by measuring the kinetics of SET7/9.⁶⁰ To adapt a SAH-based colorimetric assay in a continuous format, the Hevel laboratory used MTAN and adenine deaminase as coupling enzymes to convert SAH into hypoxanthine (Method 6, Pathway a+e in Figure 3).⁶² The amount of SAH was then quantified by the change of the UV absorption at 265 nm. The authors demonstrated the merit of the continuous assay by determining the kinetic parameters of PRMT1. G-Biosciences commercialized a methyltransferase assay kit with three coupling enzymes: MTAN, adenine deaminase and xanthine oxidase to convert SAH into highly chromogenic xanthine derivatives (Method 6, Pathway a+e+f in Figure 3). This format is an extended version of Hevel's continuous assay and is expected to be applicable to other PMTs, given that the byproduct SAH is shared by all SAM-dependent methyltransferases (Compare Pathway a+e with Pathway a+e+f for Method 6 in Figure 3). Klink *et al.* developed another generic PMT assay by converting SAH into adenosine and then AMP by two coupling enzymes SAH hydrolase and adenosine kinase (Method 5, Pathway c+d in Figure 3).⁶³ The resultant AMP can be quantified by Transcreener AMP/GMP assay kit (TransBellBrook Laboratories). As will be discussed later, the assay was developed in a HTS format.

To compare SAH-dependent chromogenic PMT-activity assays, several interfering factors should be considered (Pathways h, i, j in Figure 3). The cofactor SAM can decompose spontaneously through three main pathways

(Pathways h, i, j in Figure 3): hydrolysis of methyl-sulfonium bond to SAH, cleavage of N-ribosyl bond to adenine and intramolecular $S_{\text{N}}2$ lactonization to methylthioadenosine (MTA).⁶⁰ The SAM-to-SAH decomposition can interfere with all SAH-mediated PMT-activity assays (Pathways h, i in Figure 3).^{54,60,64} The Frankel laboratory found that this degradation occurs at a slow rate and its effect can be mitigated by using Tris buffer rather than Hepes buffer and freshly purified SAM.⁵⁴ SAM's degradation also affects the PMT-activity assays that rely on MTAN as one coupling enzyme and adenine or its derivatives as readouts. Since MTAN is promiscuous toward SAH and MTA, all nonenzymatic SAM-degrading products (SAH, MTA and adenine) will contribute signal readouts as enzymatic adenine production (Pathways h+a, i, j+h in Figure 3).⁶⁴ With the ATP-mediated luminogenic assay as a model, our laboratory evaluated the effect of three SAM-degrading products and found that SAH, MTA and adenine together gave 2-fold higher background than SAH alone.⁶⁴ The spontaneous decomposition of SAM to SAH, MTA and adenine therefore restricts the use of the SAH-dependent chromogenic assays for PMTs of low-activity.

In many SAH-based chromogenic assays, SAH is degraded *in situ* by coupling enzymes (Pathways a,c in Figure 3). The lack of accumulation of SAH is expected to be beneficial by releasing potential SAH inhibition of PMTs. However, our laboratory showed that SAH-based chromogenic assays can be carried out in an uncoupled format by allowing SAH accumulation followed by SAH quantification.⁶⁴ The potential SAH inhibition will not be dominant if the examined PMTs have low affinity to SAH or a high concentration of SAM is used.⁶⁴ In addition, reactive-thiol-based chromogenic PMT-activity assays should be carried out under conditions free of reducing reagents such as DTT and β -mercaptoethanol, because these reagents interfere with the assays by reacting with the dyes directly (Method 4 in Figure 3). Cysteines of PMTs and coupling enzymes are another source of high background in reactive-thiol-based PMT-activity assays. This effect can be minimized by using cysteine-free coupling enzymes.⁵⁹

HTS Adaptability of PMT-Activity Assays. PMT-activity assays have caught increasing attention for their potential medium-/high-throughput screening of PMT inhibitors (these inhibitors will be discussed later). As an early effort toward HTS of PRMT inhibitors, the Bedford laboratory formulated an antibody-based ELISA PMT-activity assay and applied it to identify a suite of PRMT inhibitors (e.g., AMI1, 5, 6, 9, 18) from a 9,000-compound library;⁶⁵ the Imhof laboratory applied a radiometric filter-binding assay to a pooled mixture of 2,976 compounds (eight compounds per pool) and identified an SU(VAR)3-9 inhibitor chaetocin;⁶⁶ Purandare *et al.* developed a similar radiometric filter-binding assay and identified a pyrazole-based CARM1 inhibitor.⁶⁷ The medium throughput format of these assays, though feasible for a small library of compounds, is not efficient to handle current HTS compound libraries, which generally contain >100K entities.

Kubicek *et al.* developed the first HTS assay for PMTs (Figure 4).⁶⁸ In this dissociation enhanced lanthanide fluoroimmunoassay (DELFA), N-terminal biotinylated H3 1-20 amino-acid peptide was dimethylated by G9a at H3K9 and then immobilized onto a neuroavidin-coated 384-well microtiter plate. After multiple-step washing, the microtiter-plate-immobilized H3Kme2-epitopes were probed by primary rabbit α -H3Kme2 antibody followed by secondary europium-

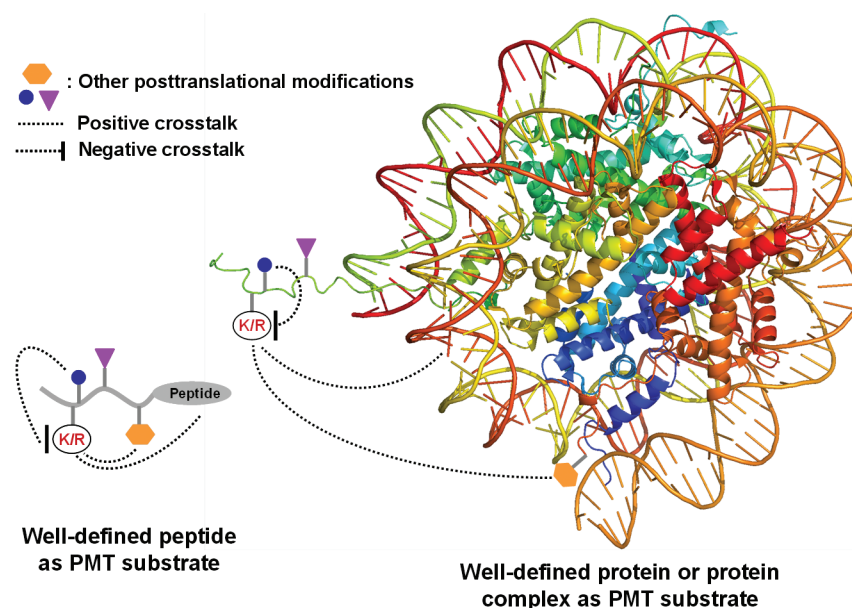


Figure 5. Well-defined homogeneous peptides and proteins as PMT substrates. PMT-substrate specificity can be affected by amino acid sequences that are adjacent to or remote from methylation sites. In a similar manner, PMT-substrate specificity can be affected by other posttranslational modifications or allosteric factors that are adjacent to or remote from methylation sites. The crosstalk can only be examined with well-defined peptide or protein substrates.

labeled goat α -rabbit antibody, which has characteristic fluorescence emission at 620 nm. The hits were identified by observing the loss of the signals. After screening a library of 125K compounds, Kubicek *et al.* identified seven G9a inhibitors including BIX-01294 (the inhibitor will be discussed later).⁶⁸

The so-far reviewed medium- to high-throughput PMT assays, though feasible for compound screening, require multiple-step washing and therefore have certain limitations for a broader application. The PMT-activity assays in a homogeneous mix-and-measure format have their merit in HTS automation (Figure 4).^{36,40–43,63} The new technologies such as AlphaScreen, AlphaLISA, LANCE *Ultra*, and LanthaScreen (discussed above) have been explored as potential HTS platforms for PMTs (Figure 4).^{41–43} A key statistical parameter of their HTS adaptability is to evaluate signal-to-background separation by Z' factors ($Z' = 1 - [(3\delta_+ + 3\delta_-)/(\mu_+ - \mu_-)]$), where δ_+ , δ_- , μ_+ , and μ_- denote standard deviations (δ) and average values (μ) for the high (+, the maximal signal) and low (–, the background signal) controls, respectively).⁶⁴ Assays with Z' value greater than 0.5 are suitable for HTS. Gauthier *et al.* and Machleidt *et al.* evaluated the Z' factors of AlphaLISA for *in vitro* SET7/9-catalyzed H3K4 monomethylation and LanthaScreen TR-FRET assay for cellular H3K9 dimethylation, respectively (discussed above).^{41,43} The excellent Z' values (>0.7) of both the assays demonstrated their HTS adaptability. Klink *et al.* also measured the Z' of their AMP-competitive fluorescence polarization immunoassay (discussed above).⁶³ Although it only has a modest Z' of 0.59, the assay has merit in being generic for multiple PMTs by quantifying SAH-derivatized AMP (Figure 3). HTS adaptability of other PMT assays remains to be evaluated.

Another major consideration for HTS adaptability is a low false-positive hit rate. In the course of searching for SMYD2 inhibitors, Ferguson *et al.* developed an AlphaScreen HTS PMT assay (Figure 4).⁶⁹ The authors point out that the AlphaScreen assay intrinsically has a high false-positive hit rate.

The false-positive hit rates in enzyme-coupled PMT-activity assays are also expected to be high, given potential false inhibition of coupling enzymes. To rapidly triage false-positive hits, a secondary orthogonal assay is necessary. Ferguson *et al.* described a radiometric SPA-based approach as a robust secondary assay to validate the hits of SMYD2 after the primary AlphaScreen.⁶⁹ To identify PRMT1 inhibitors, the Zheng laboratory independently reported the feasibility of using the radiometric SPA approach as a primary HTS assay.³⁶ The radiometric SPA HTS is expected to be robust because of its simple detection format by involving only radiolabeled SAM, biotinylated substrate, a PMT, and streptavidin-coated SPA beads (Figure 4). In terms of reagents, the SPA HTS approach is more generic in comparison with the antibody-based HTS assays because the latter require high-quality antibodies in individual assays (Figure 4). However, the SPA approach, which generates radioactive wastes, can raise environmental concerns given the amount of radioactive SAM needed in any typical HTS of 100–500K compounds. The HTS merits of the radiometric SPA approach versus antibody-based or coupling-enzyme-based assays therefore need to be evaluated case by case.

General Guidance in Selecting PMT-Activity Assays.

With so many PMT-activity assays available, general guidelines may help select PMT-activity assays for specific research purposes. Here I summarized the Rule of Six followed by our laboratory as a quick reference: (1) use filter-radiometric binding/scintillation counting or SDS-PAGE/autoradiography assays to demonstrate and validate new PMT activities; (2) apply top-down/middle-down/shotgun MS analysis to map methylation sites (straightforward); otherwise use the radiometric assays for this purpose; (3) develop sequence-specific *anti*-methyllysine/arginine antibodies or quantitative MS approach to probe cell-based methylation events; (4) use SAH-based MS or colorimetric assays to measure kinetics of high-turnover PMTs; (5) use radiometric medium-throughput PMT-activity assays to measure kinetics of low-turnover PMTs;

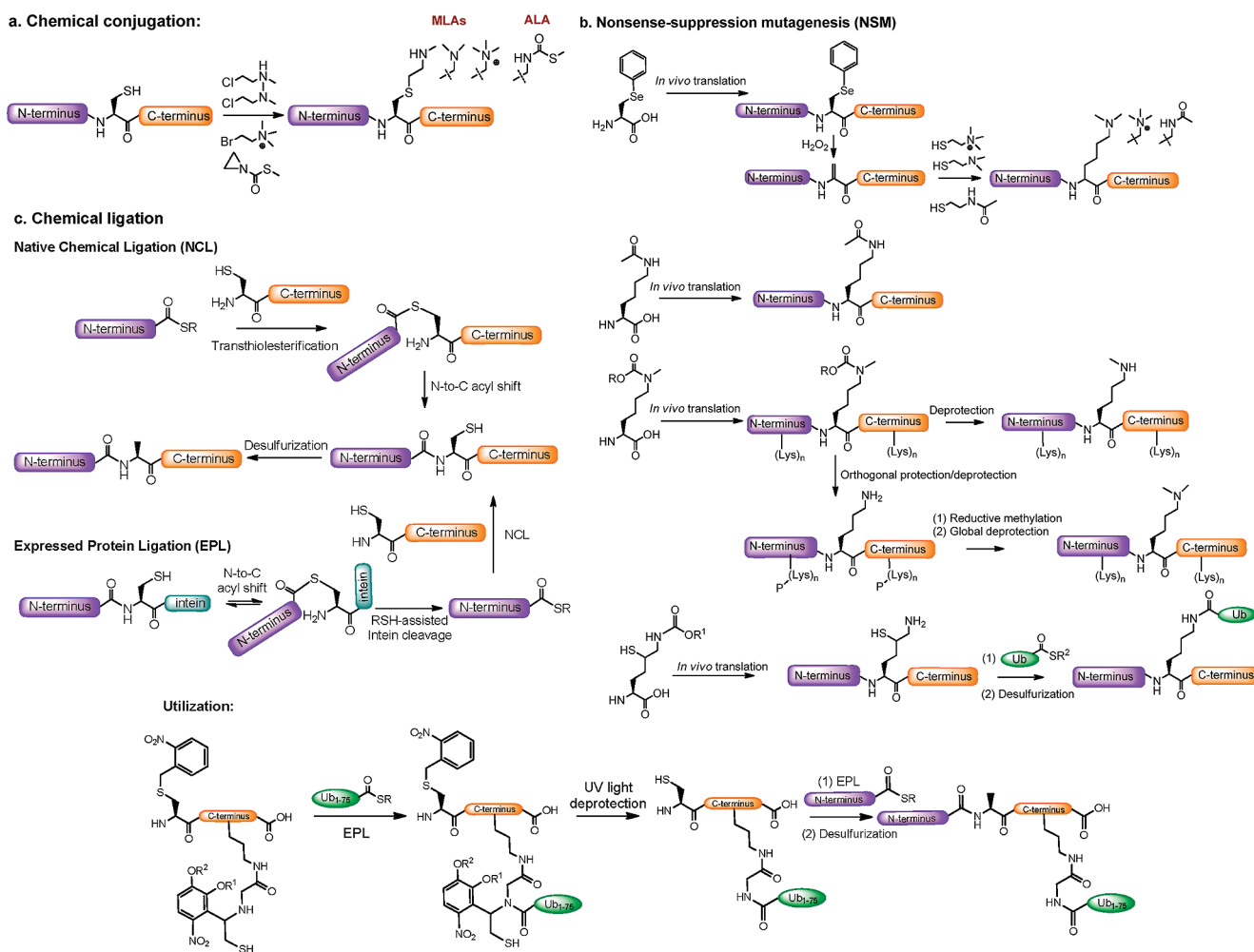


Figure 6. Emerging chemical biology approaches to prepare PMT protein substrates containing well-defined posttranslational modifications: (a) chemical conjugation, (b) nonsense-suppression mutagenesis (NSM), and (c) chemical ligation. These approaches alone or in combination have been applied to prepare histones containing mono/di/trimethyllysine, acetyllysine, ubiquitin, or their mimics. A utilization of these approaches to prepare K120-ubiquitinated H2B is described as well.

(6) apply mix-and-measure homogeneous SPA or antibody-based assays for HTS.

SUBSTRATES OF PMTS

It remains challenging to identify substrates of designated PMTs and map their methylation sites solely based on their primary sequences. The adjacent or remote residues (or other posttranslational modifications) of a PMT target can positively or negatively modulate its reactivity as a substrate (Figure 5). Current chemical biology approaches allow many PMT substrates to be synthesized or even arrayed with well-defined structures. The studies using these homogeneous substrates and arrayed libraries have shed light on how PMTs recognize their targets.

Peptides as PMT Substrates. Many PMTs can recognize protein substrates as well as the corresponding peptides (Figure 5). Since peptides and their variants can be readily prepared through solid-phase peptide synthesis, they have been widely used as *in vitro* substrates to characterize PMTs. With PRMT1 as an example, the Thompson laboratory used various N-terminal H4 peptide to examine PRMT1's substrate specificity.⁷⁰ The detailed kinetic analysis on these peptide substrates revealed that although PRMT1 has comparable H4R3

methylation activities (k_{cat}/K_m) on histone H4 and N-terminal H4 1–21 peptide, its activities on N-terminal H4 1–18 peptide and the corresponding R19A peptide drop 200-fold. This difference therefore indicated that a long-distance interaction between PRMT1 and a remote positively charged region of the substrate is essential for substrate recognition (a step affecting K_m). With the same N-terminal H4 1–21 peptide as well as its R3-methylated variant as substrates, the Thompson laboratory further demonstrated that PRMT1 catalyzes H4R3 dimethylation in a partially processive manner.⁷¹ Interestingly, when examining PRMT1 with a different substrate eIF4A1 R362 peptide, the Hevel laboratory found that PRMT1-mediated dimethylation occurs in a dissociative manner.⁷² The discrepancy argues the importance of the PMT substrates in the course of characterizing PMT-catalyzed methylation.

Examining crosstalk between methylation and other posttranslational modifications is also benefited from using well-defined homogeneous peptides as PMT substrates (Figure 5). With an N-terminal H3 peptide and its posttranslationally modified variants as substrates, the Pradhan laboratory examined how Ser10 phosphorylation and Thr11 phosphorylation affect G9a-catalyzed H3K9 methylation.⁷³ The kinetic analysis showed that S10 phosphorylation decreased k_{cat} and K_m of the methylation for more than 10-fold in comparison

with only 2-fold decrease of k_{cat}/K_m by T11 phosphorylation. Yamagata *et al.* demonstrated that PRMT1 methylates FOXO1 at R248 and R250.⁹ The two methylations inhibited Akt-mediated phosphorylation of S253, but the S253 phosphorylation does not inhibit the methylation of R248/R250. Upon reviewing this work as well as other crosstalk involved with RXXXS/T motif, Rust and Thompson proposed a dozen proteins including B-Raf, EZH2 and FOXG1 as highly probable PRMT1 substrates.⁷⁴ This prediction is expected to be tested readily after obtaining the corresponding peptides.

The Zheng laboratory recently reported an approach using a fluorescent peptide as a chemical probe to study the transient kinetics of PMT catalysis.^{75,76} In Zheng's work, Leu10 of a H4 N-terminal peptide was replaced by a fluorescein moiety. The resultant fluorescent H4 peptide showed comparable kinetics to native H4 peptide as a PRMT1 substrate. As reflected by fluorescence change, the fluorescein-labeled peptide displayed multiple-phase kinetics upon binding PRMT1. After dissecting the kinetics, the authors concluded that PRMT1 catalyzes H4 methylation *via* a multiple-step process including an ultra fast substrate-binding step, a modestly fast formation of the ternary PRMT1-SAM-substrate complex, and then the rate-limiting methylation.⁷⁵ This exemplifies an elegant utilization of substrate-type chemical probes to characterize PMTs.

Proteins or Protein Complexes as PMT Substrates.

The target specificity of PMTs can be altered dramatically depending on the nature of their substrates (Figure 5). For instance, NSD2 methylates H3K36 if nucleosomes are provided as substrates but acts on H4K44 if histone octamers as the substrates.⁷⁷ In these cases, full-length proteins or protein complexes are more relevant as *in vitro* substrates of PMTs. Using *in vitro* reconstituted chromatin templates as substrates of PRMT1, p300, and CARM1, the Roeder laboratory was able to study the p53-dependent crosstalk between the three (co)activators.⁷⁸ The authors showed that PRMT1-involved H4R3 methylation, p300-involved H3/H4 acetylation, and CARM1-involved H3R2/17/26 methylation can occur in a sequentially stimulated manner. Daujat *et al.* showed a similar crosstalk on the pS2 promoter, where CBP-mediated H3K14/18 acetylation stimulates the tight association of CARM1 with chromatin and the resultant H3R17 methylation.⁷⁹ Besides the *cis*-crosstalk of posttranslational modifications, which occurs in the same peptide, *trans*-crosstalk of posttranslational modifications has also been implicated in multiple biological contexts. For example, the ubiquitination of H2K120 often precedes the methylation of H3K79 for transcriptional activation.^{61,80} These substrate-dependent target preferences and *cis/trans*-crosstalk therefore underscore the relevance of using proteins or protein complexes as substrates to elucidate PMTs' functions.

Homogenous proteins or protein complexes with well-defined posttranslational modifications cannot be prepared readily from cell lysates or *via in vitro* enzymatic reactions. In contrast, they can be accessed efficiently through emerging chemical biology approaches.³³ This review will briefly highlight three such approaches (Figure 6): (a) chemical conjugation, (b) nonsense-suppression mutagenesis (NSM), and (c) chemical ligation (see Chatterjee's recent review for more details about the semisynthetic approaches).⁸¹ These approaches alone or their combination allow scientists to access various recombinant proteins containing well-defined posttranslational modifications (Figure 6). This collection of recombinant proteins serves as an unprecedented substrate

repertoire to study PMTs and their crosstalk with other posttranslational modifications.

a. Chemical Conjugation. The free-thiol position of site-specifically introduced cysteine is an ideal warhead for chemical conjugation. To exploit this chemistry, the Shokat laboratory first reported the approach to conjugate an *N*-methyl aminoethyl moiety to proteins (Figure 6).⁸² The resultant *N*-methylated aminoethylcysteine proved to be an excellent methyllysine analogue (MLA), which can be recognized by α -methyllysine antibodies, methyllysine-binding protein HP1 α , and multiple PMTs.⁸² As one application, Margueron *et al.* relied on this MLA approach to prepare a series of MLA-containing histones and used them as substrates to examine the crosstalk between PRC2-EZH2/EED (a PMT complex that methylate H3K27) and histone methylation marks (*e.g.*, H3K27, H3K36, and H3K9).⁸³ This work showed that the EED subunit of PRC2 complex strongly interacts with nucleosomes containing H3K27me3 and H3K9me1/2/3 MLAs but not H3K36me1/2/3 MLAs. Together with other biochemical evidence, the authors concluded that this interaction leads to the allosteric elevation of EZH2's methyltransferase activity and suggested that PRC2 complex self-propagates to nearby chromatin by interacting with its own methylation product. To incorporate an acetyllysine analogue (ALA) into histones (Figure 6), the Cole laboratory explored similar cysteine-S-alkylation chemistry using methylthiocarbonyl-aziridine as an electrophile.⁸⁴ The chemical conjugation approach is restricted to incorporation of only one type of posttranslational modifications and has only been demonstrated with MLA and ALA on histones so far. There is thus a need to extend the approach to other posttranslational modifications as well as nonhistone targets.

b. Nonsense-Suppression Mutagenesis (NSM). NSM allows unnatural amino acids to be introduced site-specifically into a recombinant protein (Figure 6). Once orthogonally engineered tRNA/tRNA synthetase pairs are available, matched amino acid analogues can be introduced readily into proteins by supplying them to a cell-free translational system or to *E. coli*, yeast, mammalian cells, or animals.⁸⁵ The incorporation of posttranslational modifications into recombinant proteins has been demonstrated in several recent NSM applications (Figure 6). For instance, the Schultz laboratory was able to prepare recombinant proteins containing racemic methyllysine and acetyllysine mimics through site-specific phenylselenocysteine chemistry (Figure 6).⁸⁶ To access recombinant proteins containing enantiomerically pure methyllysine, Chin/Schultz/Liu laboratories developed NSM by incorporating *N*-protected-methyllysine into a recombinant protein, followed by deprotection (Figure 6).⁸⁷⁻⁹⁰ With a similar NSM, The Chin and Liu laboratories can also access enantiomerically pure acetyllysine with high efficiency (Figure 6).⁹¹⁻⁹³ To use NSM to prepare recombinant proteins containing dimethyllysine, the Chin laboratory developed a multiple-step orthogonal protection/deprotection strategy (Figure 6).⁸⁷ The Chin group recently demonstrated an NSM approach for site-specific ubiquitination of recombinant proteins using δ -thio-L-lysine as a building block, which was later used as an anchor for native chemical ligation followed by desulfurization (a strategy as discussed later) (Figure 6).⁹⁴ The Chin and Liu laboratories also developed the strategies using a quadruplet-decoding ribosome (ribo-Q1) and the ochre stop codon UAA, respectively, to incorporate two amino acid analogues into multiple sites of a recombinant protein.^{95,96} The combined

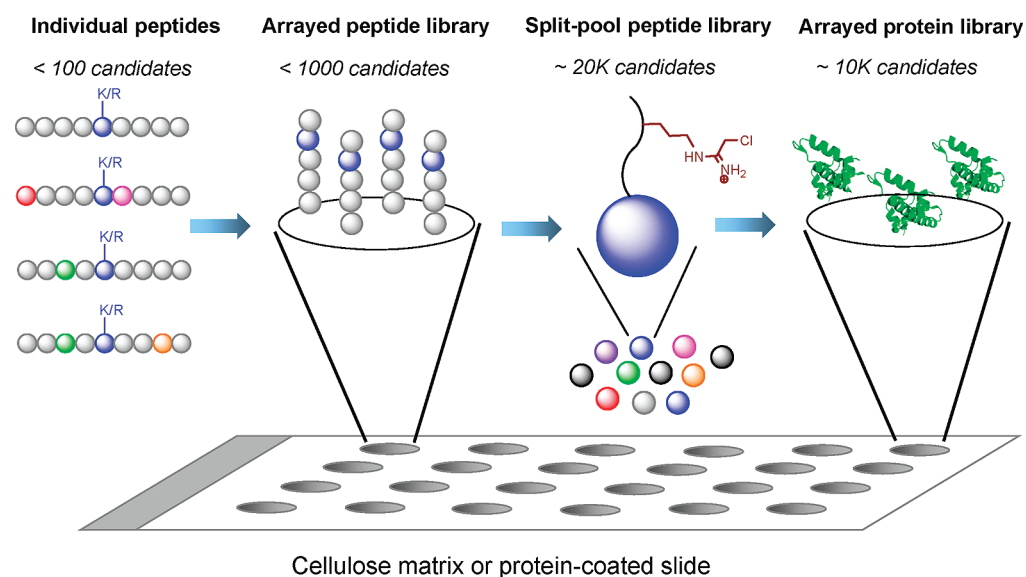


Figure 7. Substrate candidates that can be used to identify novel PMT targets. The conventional approach relies on laborious screening of <100 individual peptides to identify novel PMT substrates. The peptide array approach allows several hundred distinct peptides to be examined in a single run. Split-pool peptide library allows several thousand candidates to be examined in a single run. The protein array approach maintains comparable capability of the split-pool library but presents structurally relevant proteins as substrate candidates.

efforts of the Schultz/Chin/Liu laboratories therefore allowed the current NSM strategies to generate recombinant histone H3 containing mono/di/trimethyllysine, acetyllysine, ubiquitin, or their mimics alone or in combination (Figure 6).

c. Chemical Ligation. In comparison with site-specific chemical conjugation and NSM, chemical ligation is featured by its ability to assemble a target protein from well-defined peptide fragments (Figure 6). The approach is expected to be a powerful method for introducing complex patterns of posttranslational modifications to protein targets. Native chemical ligation (NCL) and expressed protein ligation (EPL) are by far the most widely employed technologies in chemical ligation (Figure 6).^{33,34} The residual cysteine in both NCL and EPL can be optionally converted into alanine through desulfurization. Multistep sequential ligation, combined with chemical protection/deprotection and chemical conjugation, has also been developed to access targets that harbor distantly separated posttranslational modifications or branched ubiquitination (Figure 6).^{97–99}

As an application of chemical ligation to PMTs, the Muir laboratory relied on the chemical ligation strategy to access H2BK120-ubiquitinated nucleosome (Figure 6).^{33,98} Using the nucleosome as a substrate, they were able to study the crosstalk between H2BK120 ubiquitination and H3K79 methylation, which are catalyzed by RNF20 E3 ligase and DOT1L, respectively. The first step in Muir's approach was to conjugate a short Cys117-protected, K120-modified H2B 117–125 peptide with a recombinant C-terminal intein-fused ubiquitin *via* an EPL-like auxiliary-facilitated chemical ligation. After removing the auxiliary and the Cys117-protecting group through UV irradiation, the resultant fragment was then connected to the N-terminal 1–116 fragment of H2B *via* NCL, and the resultant cysteine was desulfurized. By combining chemical ligation and chemical conjugation, the Muir laboratory later developed a simplified strategy to access disulfide-linked analogues of H2BK120ub.^{33,99} With the aid of these ubiquitinated histones/nucleosomes as substrates, they were able to show that H2BK120ub is sufficient to stimulate

DOT1L-mediated H3K79 methylation.^{97–99} This observation presented direct *in vitro* evidence that H2BK120-ubiquitination is an immediate upstream event of DOT1L-mediated H3K79 methylation.

Identifying PMT Targets *via* Consensus Sequences and Peptide Array. Although efforts over the past decade have led to identification and characterization of hundreds of PMT targets, dissecting target profiles for individual PMTs is still a formidable task. For the conventional candidate-based approach, novel targets of designated PMTs were identified from the peptide library generated based on the known substrate sequences. As an example, to explore the substrates of PRMT1 beyond the classical RGG sequence, the Hevel laboratory used a focused peptide library (*ca.* 20 peptides) derived from the PRMT1 substrate fibrillar.⁷² From this peptide collection, they were able to confirm 11 new PRMT1 substrate sequences.

To expand the candidate-based approach, the Jeltsch laboratory transformed a SPOT synthesis method to array peptide substrate candidates onto functionalized cellulose membrane (Figure 7).^{26,100,101} With Dim5, G9a, and SET7/9 substrate peptides as lead sequences, the Jeltsch laboratory designed a peptide library by systematically replacing each amino acid with the other 19 amino acids. The resultant peptides were SPOT-synthesized and arrayed on cellulose membrane. The membrane was then incubated with recombinant PMTs and radiolabeled SAM, followed by autoradiography to map hot spots. With these peptide-array libraries, the authors were able to study the substrate-specificity of Dim-5, G9a, and SET7/9 and conclude that Dim-5 recognizes R8-G12 of H3 tail with T11 and G12 being most important for the substrate recognition, but Arg8 and Lys9 most important for G9a's substrate recognition.^{26,100,101} Through proteome-wide search on the basis of the consensus sequences of active peptide substrates, the authors were able to report and validate a dozen novel proteins including CDYL1, WIZ, ACINUS, and G9a (automethylation) as G9a targets and AKA6, CENPC1,

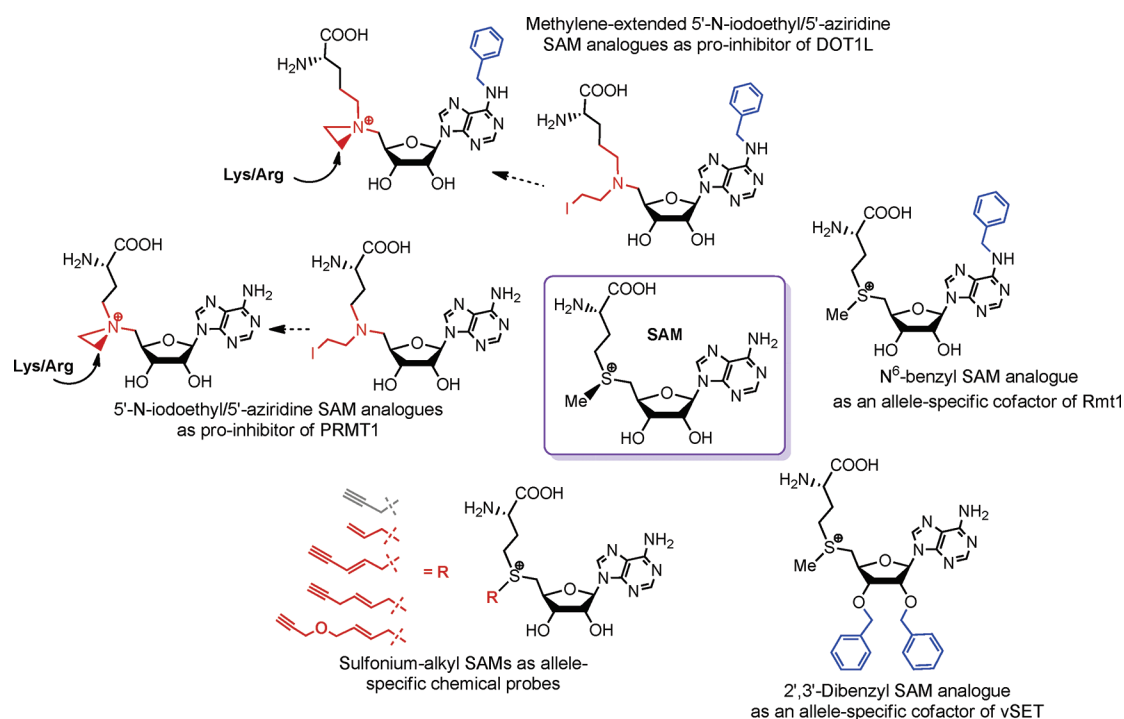


Figure 8. SAM analogues as cofactor surrogates or chemical probes for PMTs. Clockwise: N^6 -benzyl SAM analogue as allele-specific cofactor and inhibitor of Rmt1; 2',3'-dibenzyl SAM analogue as an allele-specific cofactor of vSET; sulfonium-alkyl SAM as cofactor surrogates and allele-specific chemical probes; 5'-N-iodoethyl/5'-aziridine SAM analogues as precursors of bisubstrate inhibitors of PRMT1 and DOT1L.

MeCP2, MINT, PPARBP, ZDH8, Cullin1, and IRF1 as SET7/9 targets.^{26,100,101}

To further enhance the throughput of the peptide-based approach for PMT target identification, the Thompson laboratory reported a randomized screening using a combinatorial peptide library (Figure 7).¹⁰² The one-compound-one-bead split-pool peptide library utilizes a Cl-acetamidine warhead at the Arg site of the PRMT1 target. Prior to this work, the Thompson laboratory had demonstrated that the Cl-acetamidine moiety in the context of substrate covalently interacts with PRMT1.¹⁰³ The active PRMT1 substrates containing the chemical moiety are expected to immobilize the enzyme onto the beads. Upon screening a +3 to -3 region of H4R3 using a pool of 21,000 peptides and with a fluorescein isothiocyanate labeled PRMT1 as a probe, the authors were able to identify 57 distinct hits as potential PRMT1 targets.

Although a few novel PMT targets were identified through the sequence-guided peptide-array strategy, these targets only account for a small portion of PMT substrates. Many PMT targets lack consensus sequences and there is no simple rule to generalize the substrate-recognition pattern of PMTs. These observations suggest that factors besides the sequences adjacent to methylation sites can be essential for PMTs' substrate recognition.

Identify PMT Targets with Protein Array Libraries. In contrast to peptides, full-length proteins have more merit as PMT substrates, since certain PMTs function only in the context of full-length proteins (Figure 7). The Gozani laboratory recently demonstrated the feasibility of using a protein-array approach to identify PMT substrates.¹⁰⁴ In this study, the commercially available ProtoArray glass slide (coated with 9,500 proteins) was used for proteome-wide identification of SETD6 substrates. After the on-chip methyltransferase reaction, the hits were identified either by fluorescence signals

when primary pan-*anti*-methyllysine antibody and secondary Alexa Fluor 647-conjugated antibody were used for readouts or through autoradiography when radiolabeled SAM was used as the cofactor. From 9,500 proteins arrayed on the glass slide, 118 proteins were identified as hits by the fluorescence method and 114 by the radiometric method with 26 proteins overlapped. Six proteins were cherry-picked for validation and were shown to be SET6 targets *in vitro*. Two of them were further validated as physiological substrates. In this work, however, detecting on-chip methylation with either antibody or autoradiography did not seem to be robust, because overlap analysis showed that each detection method favors a subset of targets with only 20% overlap. It is likely that the radiometric method is relatively robust but less sensitive and therefore can only detect more active substrates. In contrast, the antibody-based assay is more sensitive for slow substrates but may be restricted by the epitopes that the antibodies can recognize. The Gozani laboratory showed that the quality of commercial antibodies varies dramatically.¹⁰⁴ In order to improve this protein-array approach, more effort can be made to increase the quantity of arrayed proteins as well as improve detection methods.

Profiling PMT Targets from Cellular Proteomes.

Although novel PMT targets can be identified from arrayed peptide or protein libraries, the *in vitro* assay conditions frequently do not reflect those occurring in cellular contexts or *in vivo*. PMTs often associate with other binding partners *in vivo* to form multimeric complexes,^{78,83} and identification of authentic PMT targets may therefore rely on the native contacts. Some PMT-mediated methylations also depend on specific cellular or *in vivo* stimulation (e.g., methylation of Reptin by G9a only under hypoxic conditions and p65 by SET7/9 under TNF α stimulation).^{105,106} These observations

therefore argue the importance to profile PMT targets in their native contexts.

To profile PMT targets in a cellular context, Frankel *et al.* incubated recombinant enzymes with whole cell extracts in the presence of radiolabeled SAM, followed by autoradiography.¹⁰⁷ The substrates can be labeled in the presence of matched PMTs. With this *in vitro* approach, the authors were able to radiolabel the targets of PRMT1, CARM1, and PRMT6. The different labeling patterns between the three closely related PRMTs indicated their distinct substrate preference.¹⁰⁷ To identify substrates of PRMT3 in a cellular context, the Bedford laboratory developed a comparable *in vivo* labeling approach by culturing cells in methionine-free medium and then supplying L-[methyl-³H]methionine.¹⁰⁸ After the radiolabeled methionine was transported into the cells and processed into SAM (presumably by endogenous SAM synthetase), PMTs utilized the radiolabeled SAM to label substrates in the native cellular context. Because of the presence of protein synthesis inhibitors cycloheximide and chloramphenicol, radiolabeled methionine was not directly translated into proteins.¹⁰⁸

Although the radiometric approach allows the PMT targets to be visualized by autoradiography, it does not provide direct information for target identification. As a complementary approach, the Richard laboratory generated ADMA- and SDMA-specific antibodies for proteome-wide profiling of PRMT targets.¹⁰⁹ These antibodies allowed ADMA/SDMA-containing substrates to be pulled down from HeLa cell lysate. The reagents combined with shot-gun MS analysis enabled the Richard group to identify several hundreds of potential PRMT targets.¹⁰⁹ However, this approach cannot assign the substrates to specific PRMTs (a bioorthogonal approach to label the substrates of individual PMTs in a cellular context will be discussed later).

COFACTORS OF PMTS

SAM ranks after ATP as the second most commonly used enzyme cofactor.¹¹⁰ The cofactor reactivity is harbored around the sulfonium center in most SAM-involved biochemical transformations. For instance, the sulfonium carbon bond in SAM's thio-adenosyl moiety undergoes an enzyme-catalyzed homolytic cleavage to form a 5'-deoxyadenosyl radical, a key intermediate for canonical radical SAM enzymes.¹¹¹ The sulfonium carbon bond in SAM's homocysteine moiety can also undergo noncanonical homolytic cleavage to generate the 3-amino-3-carboxypropyl radical.¹¹² The same sulfonium carbon bond can also be subject to intra- and intermolecular heterolytic cleavage, which provides the building blocks for biosynthesis of acylhomoserine and polyamine, respectively.⁶⁰ Despite the diverse reactivity of SAM as a cofactor, the most ubiquitous role of SAM remains its use as a biological methyl donor for SAM-dependent methyltransferases. As reviewed below, several efforts have been made over the past decade to develop SAM analogues as cofactor surrogates or chemical probes for PMTs (Figure 8).

***N*⁶-Benzyl SAM Analogues As Allele-Specific Cofactor and Inhibitor of PRMTs (Figure 8).** Lin *et al.* designed a series of *N*⁶-substituted SAM analogues and examined their activity as cofactors of Rmt1 (a yeast PRMT) and its variants.¹¹³ Using a "bump-and-hole" approach guided by the structure of Rmt1 (the only available PRMT structure at that moment), the authors were able to identify an Rmt1 mutant (E117G) that can utilize *N*⁶-benzyl-SAM as a cofactor. This analogue is preferentially processed by E117G Rmt1 at the rate

67-fold faster than by native Rmt1. Following the same trend, *N*⁶-benzyl-SAM is an allele-specific inhibitor to the mutant with 20-fold increased selectivity versus the wild-type enzyme. The active enzyme-cofactor pair can be used for allele-specific labeling of Rmt1 targets. This was the first effort toward manipulating PMTs with SAM analogue cofactors.

2',3'-Dibenzyl SAM Analogue As Allele-Specific Cofactor of PKMT (Figure 8). Besides *N*⁶-substituted SAM analogues, the Zhou laboratory explored 2'- or 3'-substituted SAM analogues as potential SAM surrogates of engineered PMTs.¹¹⁴ The authors focused on vSET, a viral SET-domain-containing PKMT. Like human EZH2, the enzymatic component of PRC2, vSET methylates H3K27 *in vivo*. Guided by the structure of vSET, the Zhou laboratory located two residues that are expected to be sensitive to SAM's 2'- or 3'-substituent. Upon mutating them followed by screening against 2'- or 3'-substituted SAM analogues, the Zhou laboratory were able to identify vSET L116A mutant and its matched 2',3'-dibenzyl SAM cofactor. The enzyme-cofactor pair showed comparable k_{cat}/K_m to that of native vSET and SAM. Since the authors only examined a small number of SAM analogues and vSET mutants, more active mutant-cofactor pairs may exist. These active enzyme-cofactor pairs can be used for vSET-specific labeling.

5'-*N*-Iodoethyl/5'-Aziridine SAM Analogues as Precursors of Bisubstrate Inhibitors of PMTs (Figure 8). 5'-*N*-Adenosylaziridine and its SAM-like derivatives were reported to be active cofactors of bacterial DNA and small-molecule methyltransferases.¹¹⁰ The Thompson laboratory first examined whether PMTs can act on a 5'-aziridine SAM analogue.¹¹⁵ With PRMT1 as a model system, the authors demonstrated that the 5'-aziridine SAM analogue rapidly reacts with an N-terminal H4 peptide in an enzyme-dependent manner. H4R3 of the peptide (the methylation site of PRMT1) conjugates with the 5'-aziridine SAM analogue *in situ* to form a bisubstrate analogue inhibitor of PRMT1. This inhibitor showed a modest IC₅₀ and 4.4-fold preference to PRMT1 over CARM1. The Song laboratory then examined the 5'-aziridine SAM analogue against DOT1L, G9a, and SUV39H1. Only a modest IC₅₀ against DOT1L was observed.¹¹⁶

In the course of developing DOT1L inhibitors, the Song laboratory noticed that, unlike PRMTs and other SET-domain-containing PKMTs, DOT1L has a relatively spacious binding site for SAM's 6-NH₂ group.¹¹⁶ By introducing the *N*⁶-benzyl-substituent to the 5'-aziridine SAM analogue (Figure 8), the authors observed a 15-fold improvement of IC₅₀ against DOT1L but not other PMTs (*e.g.*, PRMT1, CARM1, G9a, and SUV39H1). In addition, the authors reasoned that since C–N bonds (~1.47 Å) in the 5'-aziridine SAM analogue are slightly shorter than C–S bonds (~1.82 Å) in SAM and SAH, extending one more methylene in the 5'-aziridine SAM analogue would further improve the potency. The resultant methylene-extended 5'-aziridine-*N*⁶-benzyl SAM analogue (Figure 8) showed an IC₅₀ of 110 nM against DOT1L and >1000-fold selectivity over PRMT1, CARM1, G9a, and SUV39H1.¹¹⁶ Although the authors did not further characterize the mechanism of the inhibition, the DOT1L inhibitor is expected to behave much like the *N*-adenosylaziridine through the substrate-participating formation of a bisubstrate analogue inhibitor (Figure 8).^{115,116} However, since aziridine SAM analogues are not stable under physiological pH, their broad application within biological contexts remains to be investigated.

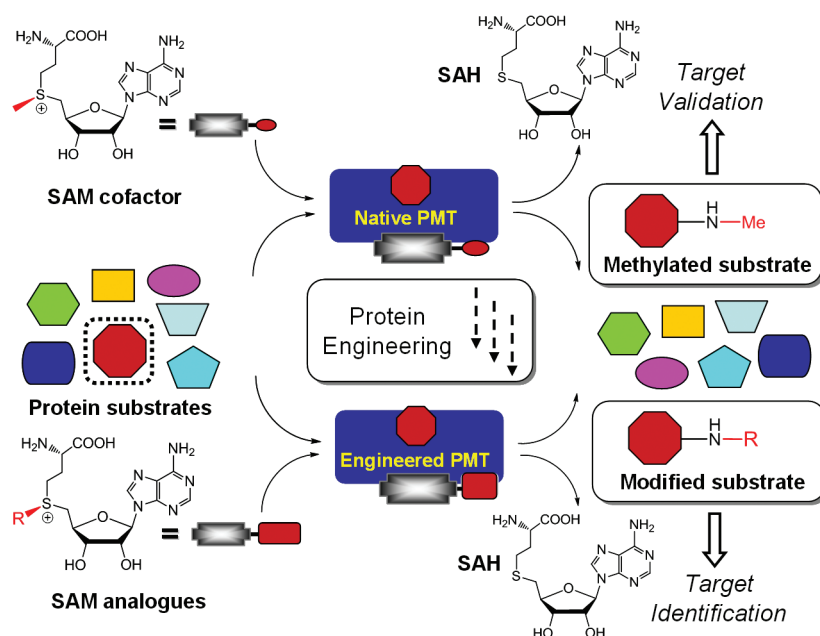


Figure 9. Bioorthogonal profiling approach to label PMT substrates. Native PMTs use SAM as the cofactor to methylate their targets. In contrast, PMTs can be engineered to accommodate SAM derivatives as cofactors and label their substrates with distinct chemical groups. Since bulky SAM analogues are inert to native PMTs, the resultant distinctly modified substrates can be assigned to a single, designated PMT.

Sulfonium-alkyl SAM as Cofactor Surrogates and Allele-Specific Chemical Probes (Figure 8). The Weinhold laboratory explored the use of sulfonium- β -sp²/sp¹-doubled-activated SAM analogues as cofactors for bacterial DNA/RNA methyltransferases for target labeling (Figure 8).¹¹⁰ However, the implementation of these SAM analogues to label PMT substrates had not been reported until recently. Peters *et al.* developed (*E*)-pent-2-en-4-ynyl-SAM (EnYn-SAM) as an SAM surrogate and showed that the SAM analogue can be utilized by Dim-5 for target labeling under basic conditions (pH = 9).¹¹⁷ The authors also demonstrated that the same SAM analogue can be utilized by native MLL4 and ASH2-MLL complex to some degree. Binda *et al.* developed a propargyl-SAM analogue for PMT target labeling (Figure 8).¹¹⁸ With a clickable FLAG probe coupled to a sensitive *anti*-FLAG antibody, Binda *et al.* showed that SETDB1 but not SET7/9, SMYD2, PRMT1, CARM1, PRDM8, -10, and -16 can utilize the propargyl-SAM analogue. Interestingly, the Weinhold laboratory noticed that the propargyl-SAM analogue suffers a rapid decomposition at neutral and basic conditions.¹¹⁷ This discrepancy may be rationalized if SETDB1 can rapidly process the SAM analogue before decomposition.

Although the prior cases demonstrated the feasibility of using the SAM analogue cofactors to label PMT substrates, the activities of native PMTs on these synthetic cofactors are generally low. A limitation in the prior approaches is that they cannot unambiguously assign the labeled targets to designed PMTs in cellular contexts because other promiscuous PMTs may be present to label their own substrates with these cofactors. To address these limitations, our laboratory aimed at developing SAM analogue cofactors that are inert toward native PMTs but can be recognized by engineered PMTs.^{31,64} We envisioned that this bioorthogonal approach would allow the labeled substrates to be assigned to engineered enzymes in an unambiguous manner (Figure 9). Toward this goal, we developed (*E*)-hex-2-en-5-ynyl-SAM (Hey-SAM) and 4-propargyloxy-but-2-enyl-SAM (Pob-SAM), respectively, to profile

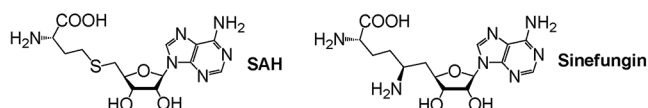
the substrates of G9a and PRMT1 (Figure 9).^{31,64} The two SAM analogues are inactive with native PMTs but can be processed efficiently by engineered G9a and PRMT1. Furthermore, Pob-SAM was demonstrated to be an excellent SAM surrogate for labeling PRMT1 substrates in a complex cellular milieu.

With the aid of a reformulated fluorogenic assay, our laboratory systematically evaluated the activities of native PMTs (PRMT1, PRMT3, CARM1, SUV39H2, SET7/9, SET8, G9a, and GLP) on a panel of SAM analogues (allyl-SAM, propargyl-SAM, (*E*)-pent-2-en-4-ynyl-SAM (EnYn-SAM), (*E*)-hex-2-en-5-ynyl-SAM (Hey-SAM), and 4-propargyloxy-but-2-enyl-SAM (Pob-SAM)).⁶⁴ Among the examined 8 × 5 pairs of PMTs and SAM analogues, only native SUV39H2, G9a, and GLP show slight activity toward allyl-SAM. The bulky SAM analogues, such as EnYn-, Hey-, and Pob-SAM are inert toward the screened native PMTs. This finding is also consistent with the observed low activity of native MLL4 or ASH2-MLL on EnYn-SAM. These results therefore argue that the SAM-binding pocket of native PMTs needs to be tailored to accommodate bulky SAM analogues for efficient substrate labeling. The suitability of these SAM analogues to other engineered PMTs is being investigated in our laboratory.

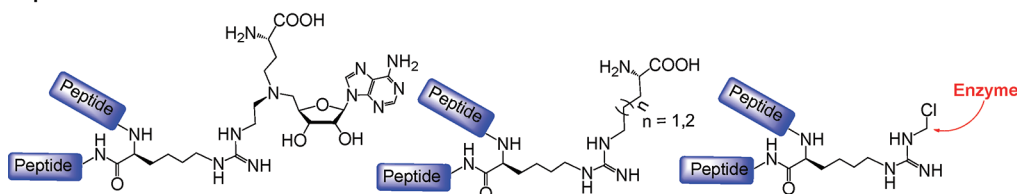
■ INHIBITORS OF PMTs

Given that the methylation activities of PMTs associate with diverse cellular processes and their dysregulation is implicated in many diseases including cancer,²⁰ many efforts have been made in academia and industry to develop PMT inhibitors as chemical probes and therapeutic reagents. However, the success in finding lead compounds is still limited, and many of those have not been fully characterized. Because all PMTs have one of two types of highly conserved SAM-binding pockets and utilize less-structured substrate-binding regions, it remains challenging to develop selective and potent PMT inhibitors for these enzymes. At present, rational design, HTS and *in silico* screening are three mainstream approaches in developing PMT

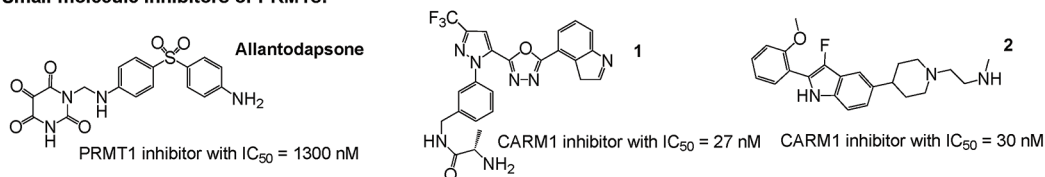
Pan-inhibitors of PMTs:



Peptidic inhibitors of PRMTs:



Small-molecule inhibitors of PRMTs:



Small-molecule inhibitors of PKMTs:

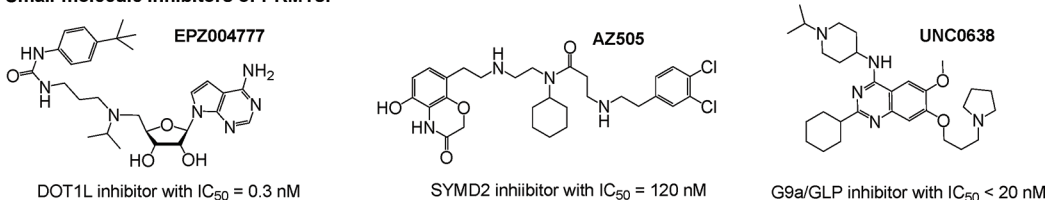


Figure 10. Representative inhibitors of PMTs. SAH and sinefungin are the best characterized pan-inhibitors of PMTs. Peptidic inhibitors were designed on the basis of the sequence of PMT substrates with their Arg residue conjugated with some moiety of the SAM or replaced with a functional group. Allantodapson is a potential PRMT1 inhibitor with $IC_{50} = 1300$ nM. Compounds 1 and 2 are so far the most potent CARM1-selective inhibitors. EPZ004777 and UNC0639 are so far the most potent and best-characterized inhibitors of DOT1L and G9a/GLP, respectively. AZ505 is so far the most potent SYMD2 inhibitor.

inhibitors. The successful implementations and potential pitfalls of these approaches will be discussed in this section.

Principles To Define High-Quality PMT Inhibitors.

Sinefungin and SAH are SAM analogue inhibitors that have been claimed as pan-inhibitors of PMTs (Figure 10). The former is a natural product available from Sigma. The latter is the metabolite byproduct of SAM-dependent methylation reactions. To achieve high intracellular concentrations of SAH, a common practice is to treat cells with adenosine dialdehyde,¹¹⁹ an irreversible SAH hydrolase inhibitor that blocks SAH hydrolase-mediated SAH degradation and thus causes its intracellular accumulation. However, caution should be taken with these SAM analogues as pan-inhibitors of PMTs because IC_{50} of these inhibitors can alter by 2 orders of magnitude for different PMTs (e.g., $K_i > 25$ μ M for sinefungin against EZH2 versus 0.10 μ M against CARM1).³ Therefore, the activity profile of the PMT pan-inhibitors needs to be defined carefully prior to their uses in biological contexts.

PMT inhibitors can be developed either as chemical genetic probes to modulate the activities of PMTs or as potential drugs to treat patients. Although the rules applied to the former are less strict, several common principles still need to be fulfilled for high-quality PMT chemical genetic probes. In the article "The art of the chemical probe", Frye introduced five simple principles for general development of chemical probes.¹²⁰ Here I have rephrased these principles in the specific context of PMTs as the following: (1) a high-quality chemical genetic probe should show sufficient *in vitro* potency (inhibit one or

several designated PMT(s)) and selectivity (does not inhibit irrelevant PMTs and other targets); (2) such PMT chemical genetic probes should show decent *in vivo* or at least cellular-level potency and selectivity that correlates with the *in vitro* data; (3) the inhibition mechanism should be clear and consistent *in vitro* and *in vivo* or in a cellular-level context (e.g., SAM-competitive, substrate-competitive, or irreversible inhibitors; identity of the active species as intact chemical probes versus well-characterized derivatives); (4) high-quality chemical genetic probes of designed PMTs should show at least one proved utilization (e.g., the treatment of a chemical genetic probe should recapitulate the biological readouts anticipated by genetically disrupting the corresponding PMT target); (5) as a bonus point, an ideal chemical genetic probe should be accessible either through commercial vendors or synthetically *via* well-described chemical methods. The recent advancement in PMT chemical genetic probes will be discussed according to these criteria (some of them listed in Figure 10).

Rationally Designed PMT Inhibitors. Based on the amino acid sequences of PMT substrates, several peptidic PMT inhibitors were reported recently (Figure 10). As one example described above, the Thompson laboratory showed the H4R3 can react with the 5'-aziridine SAM analogue in a PRMT1-dependent manner. The resultant bisubstrate inhibitor of PRMT1 can be generated *in situ* with its IC_{50} in the range of single-digit micromolar.¹¹⁵ The Thompson laboratory also developed substrate-based, irreversible PRMT inhibitors containing the Cl-acetamidine warhead and implemented

them as activity-based probes (described above, Figures 7, 10).^{103,121} In contrast, the Martin and Frankel laboratory prepared partial-bisubstrate PRMT inhibitors that contain the peptidic fragments of PRMTs' substrates and the α -amino carboxylic acid moiety of SAM.¹²² Though some of these peptidic PRMT inhibitors showed decent IC_{50} and were utilized as chemical probes *in vitro*, their utilization *in vivo* remain to be tested given general undesirable pharmacological properties of peptidic molecules. Since peptidic bisubstrate inhibitors have been only reported for PRMTs so far, examining whether a similar strategy can be applied to PKMTs can be interesting.

Thus far, known rationally designed small-molecule PMT inhibitors were developed either by conjugating a moiety of PMT substrates with an azo-SAM analogue (bisubstrate-type inhibitors) or by exploring distinct SAM-binding pockets of specific PMTs. For example, the Ward laboratory reported efforts in developing PRMT-specific bisubstrate-type inhibitors by connecting a guanidium moiety with the azo-SAM analogue *via* various linkers.^{123,124} The series of compounds showed modest *in vitro* single-digit micromolar values of IC_{50} against PRMTs (only PRMT1 and CARM1 were tested) and >10-fold selectivity over SET7/9. The Hirano laboratory reported similar efforts in developing bisubstrate-type inhibitors of PKMTs by linking the azo-SAM analogue with various N^2 -alkyl-aminoethyl moieties, which resemble the lysine side chain in a PKMT-catalyzed reaction.¹²⁵ Surprisingly, their best inhibitors showed only modest *in vitro* IC_{50} values of 10–100 μM against SET7/9, the only PKMT that was tested. The *in vitro* IC_{50} of these PMT bisubstrate-type inhibitors against other PMTs remains to be measured. More mechanistic studies may help the design of bisubstrate-type PMT inhibitors to achieve better potency and selectivity.

An alternative approach to design rationally target-specific PMT inhibitors is to explore the difference of SAM-binding sites in PMTs. One of the most successful examples is the DOT1L-specific inhibitor EPZ004777 (Figure 10).²² Daigle *et al.* reported EPZ004777 as a SAM-competitive inhibitor with an *in vitro* K_i of 0.3 nM, a cellular-level sub-micromolar EC_{50} , and >3000-fold selectivity over 9 other examined PMTs. Because DOT1L is an oncoprotein in several subtypes of mixed lineage leukemia (MLL), EPZ004777's efficacy was also validated in the context of the relevant leukemia cells and with a mouse MLL xenograft model.²² In addition to this work, the Song laboratory reported a suite of 5'-*N*-iodoethyl (5'-aziridine)-based SAM analogues as potent DOT1L inhibitors (described above, Figure 8).¹¹⁶ Although the Song laboratory did not perform biological validation of their DOT1L inhibitors, their work shed light on how EPZ004777 achieves high selectivity for DOT1L versus other PKMTs. They noticed that, because DOT1L-bound SAM (unlike the SAM bound by other PKMTs) adapts an open conformation, extending the 5'-region by a methylene moiety significantly enhanced the potency of their 5'-*N*-iodoethyl (5'-aziridine) SAM analogue inhibitors.¹¹⁶ The same rationale may be applicable to EPZ004777, whose 5'-linker may mimic the length and extended conformation of DOT1L-bound SAM. Although EPZ004777 was demonstrated to be a high-quality chemical genetic probe, its synthesis remains to be disclosed.

Recent structural and chemogenetic analysis on a dozen of human PMTs reveal that closely related PMTs can bind to SAM, SAH or sinefungin preferentially.^{3,126} Many human PMTs have distinct SAM-recognizing motifs as well.^{3,126} The

chemogenetic and structural information therefore present a general roadmap to further explore the differences between these SAM-binding sites for more potent and selective SAM analogue inhibitors of PMTs.

PMT Inhibitors from HTS Leads. HTS is so far the most powerful approach for identifying PMT inhibitors. As discussed in a previous section (PMT-Activity Assays), several HTS assays have been developed to facilitate the HTS-based identification of PMT inhibitors. From a collection of 9000 compounds, the Bedford laboratory identified a series of PRMT inhibitors including AMI-1 (will be discussed later), AMI-5 (eosin, a SAM-competitive nonspecific inhibitor), AMI6, AMI9 and AMI18.⁶⁵ After optimizing AMI-5, the Bedford laboratory reported a CARM1-specific AMI-5 derivative, which shows a decent *in vitro* IC_{50} and a cellular-level EC_{50} of around 10 μM with >100-fold *in vitro* selectivity over PRMT1 and SET7/9.¹²⁷ By focusing on the PRMT-specific inhibitor AMI-6 and nonspecific PMT inhibitor AMI-9, Bonham *et al.* merged their pharmacological components to invent the hybridized inhibitor.¹²⁸ This compound showed a decent IC_{50} *in vitro* of 2–4 μM against PRMT1 and CARM1 and a cellular-level EC_{50} of 100–300 μM for CARM1-mediated H3R17 methylation. The authors showed that this compound modulates T-helper-cell function at a dose of >50 μM , which turns out to be lower than their cellular-level EC_{50} . Further studies are still needed to validate its use as a PRMT1/CARM1 chemical genetic probe and elucidate how the AMI inhibitors interact with their targets.

Purandare *et al.* reported a pyrazole-based CARM1-specific inhibitor with an *in vitro* IC_{50} of 1.8 μM .⁶⁷ Optimization of the lead compound led to a potent and selective CARM1 inhibitor (compound 1 in Figure 10) with an *in vitro* IC_{50} of 27 nM and >500-fold *in vitro* selectivity over PRMT1 and PRMT3 (Figure 10).¹²⁹ Sack *et al.* recently released the structure of a new indole-type CARM1 inhibitor (compound 2 in Figure 10) with a potent *in vitro* IC_{50} of 30 nM (Figure 10).¹²⁹ Although the *in vitro* IC_{50} values of the two CARM1 inhibitors are more promising than those of the AMI-derived CARM1 inhibitors, no *in vivo* or cell-based efficacy of the two compounds has been reported (as discussed below).

From a collection of 2,976 compounds, the Imhof laboratory identified chaetocin as the first PKMT inhibitor, which has an *in vitro* IC_{50} and a cellular-level EC_{50} around 0.8 μM against *Drosophila melanogaster* SU(VAR)3-9.⁶⁶ Unfortunately, the natural product lacks selectivity because it also inhibits G9a and DIM5 with *in vitro* IC_{50} of 2.5 and 3 μM , respectively. A following cell-based characterization showed that chaetocin can block histone H3K9 trimethylation (a target of SUV39H1, a human homologue of SU(VAR)3-9).¹³⁰ However, given the complex synthesis of chaetocin and its derivatives,¹³¹ use of chaetocin as a general chemical probe may be limited.

From a 125K-compound library, Kubicek *et al.* identified the first G9a inhibitor BIX-01294, which has an *in vitro* IC_{50} of 2.7 μM and does not inhibit SUV39H1 and PRMT1.⁶⁸ The following lead optimization led to a series of derivatives with improved potency and selectivity.^{132–135} At this point, the best characterized BIX-01294 derivative is UNC0638 (Figure 10), a substrate-competitive inhibitor with ~20 nM *in vitro* and cellular-level IC_{50} values for G9a and GLP (closely related homologue of G9a), >3000-fold selectivity over other so-far-examined PMTs.¹³² Treatment with UNC0638 can reactivate silenced genes by reprogramming H3K9me2 and DNA methylation in mouse embryonic stem cells. This observation recapitulates the anticipated phenotype of genetic disruption of

G9a and GLP. Other important properties of UNC0638 include no significant degradation in cellular contexts and low cellular toxicity. According to the five rules in Frye's "the art of the chemical probe",¹²⁰ UNC0638, which is available from Sigma, is arguably a high-quality chemical genetic probe (Figure 10). However, UNC0638 displays a fast clearance rate in animals, which may limit its use as a therapeutic reagent.

Using the AlphaScreen HTS assay, Ferguson *et al.* reported AZ505, an inhibitor of SMYD2 with an *in vitro* IC₅₀ of 0.12 μ M and >800-fold selectivity over other PMTs including the closely related SMYD3 (Figure 10).⁶⁹ However, the compound was characterized to be a substrate-competitive, SAM-uncompetitive inhibitor, a mechanism that requires the formation of a SAM-inhibitor-enzyme ternary complex to satisfy the observed high potency (a Hook effect of SAM).⁶⁹ Given the uncertainty of intracellular concentrations of SAM,^{136,137} the cellular-level inhibition of AZ505 remains to be tested.

PMT Inhibitors Identified through *in Silico* Screening, Intuition, and Serendipity. Besides rational design and HTS, virtual screening is another complementary approach to identify inhibitors of PMTs. As the first effort of *in silico* screening for PMT inhibitors, the Jung and Sippl laboratories docked the NCI diversity-set compound library into RmtA (a fungal homologue of human PRMT1) for the primary screening and then into PRMT1 for validation.^{138,139} The authors were able to identify and validate multiple PRMT1 inhibitors including allantodapsone (Figure 10), C-7280948, RM65, and stilbamidine with *in vitro* IC₅₀ values of 1.3, 12.8, 55.4, and 56.0 μ M, respectively.^{138–141} In cellular contexts, a C-7280948 derivative, allantodapsone, RM65, and stilbamidine showed EC₅₀ values around 25–50 μ M.^{138–141} Mechanisms of these inhibitors against PRMT1 remain to be examined. Although the current *in silico* screening still focuses on PRMT1, this approach is expected to be transferable to other PMTs, given that around 20 distinct structures of human PMTs have been deposited into the PDB database.

The aforementioned HTS performed by the Bedford laboratory also led to the discovery of a set of polyphenol-type PRMT inhibitors such as AMI-18, which are structurally related to xenoestrogens.¹⁴² Driven by this intuition, Cheng and Bedford tested a few xenoestrogens and were able to identify tamoxifen as a CARM1-specific inhibitor with a modest *in vitro* and cellular-level EC₅₀ of around 30–50 μ M.¹⁴² In contrast to Cheng and Bedford's intuition, pure serendipity led Selvi *et al.* to identify a substrate-uncompetitive CARM1 inhibitor.¹⁴³ In the course of purifying the active ingredients of pomegranate extract, Selvi *et al.* found that one component, ellagic acid, inhibits CARM1 as well as p300. Ellagic acid was then characterized as a substrate-uncompetitive CARM1 inhibitor that depends on the substrate's "KAPRK" motif at H3R17 region to interact with the enzyme.¹⁴³ The formation of the dead enzyme-substrate-inhibitor ternary complex (the Hook effect of substrate) accounts for the observed inhibition of CARM1-mediated H3R17 methylation. The intuition- and serendipity-based findings surely enriched our tool box and contributed to the urgent need for PMT inhibitors.

Pitfalls of PMT Inhibitors. Lessons learned from previous experiences are valuable to avoid the pitfalls of PMT inhibitors. AMI-1 was identified through HTS as a PRMT-specific inhibitor.⁶⁵ When examining the fluorescein-conjugated H4 N-terminus peptide (a PRMT1 substrate), the Zheng laboratory noticed that AIM-1 preferentially interacts with the histone peptide rather than the enzyme.¹⁴⁴ This interaction

with the peptide, likely native histones, accounts for the observed PRMT1 inhibition. This scenario resembles that of sanguinarine, which inhibits PMT-mediated histone methylations by interacting with core histones rather than enzymes themselves.¹⁴⁵

Another pitfall of certain PMT inhibitors are SAM-, SAH-, or substrate-uncompetitive inhibitors, as exemplified by the pyrazole- or indole-based CARM1 inhibitors (compounds 1 and 2) and the SMYD2 inhibitor AZ505.^{69,129} Kinetic analysis and inhibitor-substrate-enzyme structures suggest that the three inhibitors are substrate-competitive, SAM/SAH-uncompetitive inhibitors.^{69,129} The tight binding of these inhibitors to their targets requires the presence of uncompetitive SAM or SAH to form the ternary enzyme-inhibitor-SAM/SAH dead complex (aforementioned Hook effect of SAM/SAH). Characterizing these inhibitors in cellular contexts and *in vivo* can be complicated by the uncertainty of concentrations of SAM and SAH in different cell types.^{136,137} Although using a low concentration of SAM in HTS assays can minimize the Hook effect of SAM or SAH, the issue seems to be unavoidable for SMYD2 because of its high affinity to SAM ($K_d = <1$ nM).³ It is also possible to identify substrate-uncompetitive inhibitors (the Hook effect of substrate), such as Ellagic acid as exemplified above. To avoid the pitfall of substrate-uncompetitive inhibitors, Ferguson *et al.* recommended using a low concentration of substrate to run HTS.⁶⁹

With these experiences in mind, it is thus important to use enzymatic kinetics or other complementary tools to elucidate and validate the inhibition mechanisms of potential PMT inhibitors at the early stage. For instance, if it is known that a PMT inhibitor is substrate-competitive, it is worth testing its potency against several PMT substrates to avoid a situation where the PMT inhibitor could compete only with weak-binding but not tight-binding substrates. In contrast, if a PMT inhibitor is SAM competitive, more efforts should be made to examine how intracellular concentrations of SAM affect the EC₅₀ of the inhibitor and to define potential cross-activity against other methyltransferases. For any irreversible (covalently interacting) inhibitor, lack of off-target effects should be addressed vigorously. Although the initial characterization consumes extra time and resources, the effort will be repaid by narrowing the focus on well-behaving leads for optimization. The key here is to be aware of Frye's five principles of chemical probes.

■ SUMMARY AND PERSPECTIVE

During the past decade, PMTs have caught significant attention because of their roles in epigenetics and diseases. Academic and industrial laboratories are highly engaged in developing tools to elucidate and manipulate PMT-involved methylation. This article has reviewed the current available chemical biology approaches for PMTs. These tools were further categorized into four modules: assays, substrates, cofactors and inhibitors. Herein I reviewed how the current chemical and biochemical assays can be applied to study PMTs. In particular, reliable HTS assays are still needed for identifying PMT inhibitors. In terms of PMT substrates, examining PMTs in the context of well-defined proteins and protein complexes will surely shed light on how PMTs behave in biological contexts. The current focus on this aspect still lies in histones or nucleosomes but should be extended to nonhistone proteins. Emerging SAM analogues and PMT inhibitors surely diversify our tools to interrogate PMT functions. However, more efforts need to be

put into characterizing these inhibitors in details and in particular how they interact with PMT targets. Few efforts have been made over the past decade to experimentally characterize the transition state structures of PMT-catalyzed reactions. Elucidating the transition state structures of PMT-catalyzed reactions can provide meaningful guidance in designing novel PMT inhibitors. These chemical biology approaches have infiltrated many aspects of PMT-related research and will contribute to our understanding of PMT biology.

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ABBREVIATIONS

ADMA, asymmetric dimethylarginine; ALA, acetyllysine analogue; AMI, arginine methyltransferase inhibitor; APRT, adenine phosphoribosyl transferase; CARM1 or PRMT4, coactivator-associated arginine methyltransferase 1; CBP, CREB (cAMP-response element-binding protein)-binding protein; CHIP, chromatin immunoprecipitation; DELFIA, dissociation enhanced lanthanide fluoroimmunoassay; DNMT1, DNA methyltransferase 1; ER α , estrogen receptor alpha; ELISA, enzyme-linked immunosorbent assay; EPL, expressed protein ligation; FOXO1, Forkhead box protein O1; HTS, high-throughput screening; LuxS, S-ribosylhomocysteine lyase; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight; MMA, monomethylarginine; MLA, methyllysine analogue; MLL, mixed lineage leukemia; MS, mass spectrometry; MTAN, 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase; NCL, native chemical ligation; NSM, Nonsense-suppression mutagenesis; PCAF, p300/CBP associated factor; PKMT(s), protein lysine methyltransferases; PMT(s), protein methyltransferase(s); PPK, pyruvate orthophosphate dikinase; PRMT(s), protein arginine methyltransferases; RUNX1, Runt-related transcription factor 1; SAM, S-adenosylmethionine; SDMA, symmetric dimethylarginine; SILAC, stable isotope labeling with amino acids; SPA, scintillation proximity assay; STAT1, signal transducer and activator of transcription 1; TAF10, transcription initiation factor TFIID subunit 10; THW loop, threonine-histidine-tryptophan loop; TR-FRET, time-resolved fluorescence resonance energy transfer

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