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Recent progress in developing selective inhibitors of protein methyltransferases

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Abstract

Mounting evidence suggests that protein methyltransferases (PMTs), which catalyze methylation of histone as well as non-histone proteins, play a crucial role in diverse biological pathways and human diseases. In particular, PMTs have been recognized as major players in regulating gene expression and chromatin state. There has been an increasingly growing interest in these enzymes as potential therapeutic targets and over the past two years tremendous progress has been made in the discovery of selective, small molecule inhibitors of protein lysine and arginine methyltransferases. Inhibitors of PMTs have been used extensively in oncology studies as tool compounds, and inhibitors of EZH2, DOT1L and PRMT5 are currently in clinical trials.

Introduction

Histone methylation was first recognized in 2000 and is one of the most actively investigated posttranslational modifications.[1] Methylation of histones as well as non-histone proteins has been implicated in various cancers and numerous other diseases, as it is a dynamic process that plays a key role in the regulation of gene expression and transcription.[2,3] Given these key functions, there has been a steadily increasing interest towards assessing the potential of these enzymes as therapeutic targets. [4–8] Therefore, the discovery of selective small-molecule inhibitors of protein methyltransferases (PMTs) has become a very active and fast growing research area (Figure 1).[9–11] In this review we focus on selective, small molecule inhibitors of PMTs that are discovered in last two years.

Recent progress in discovery of inhibitors of PMTs

The first inhibitors of protein lysine methyltransferase (PKMT) and protein arginine methyltransferase (PRMT) were discovered in 2005 and 2004, respectively. In less than a decade, numerous PMT inhibitors with high potency and selectivity have been disclosed, some of which has entered the clinical trials emphasizing the rapid progress made in the field. For example, PKMT inhibitors, such as BIX-01294, UNC0638, UNC0642 (G9a/GLP),

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EPZ005687, GSK126, EI1, UNC1999, EPZ-6438, CPI-1205 (EZH2/EZH1), EPZ004777, SGC0946, EPZ-5676 (DOT1L), AZ-505 and LLY507 (SMYD2) are valuable chemical tools for further understanding biological functions of the targeted enzymes and have already been widely used in evaluating the therapeutic potential of these proteins (Table 1).[9,10] In addition, highly potent, selective, substrate-competitive PRMT inhibitors including MS023 (type I PRMTs), TP-064 (CARM1), EPZ015666 (PRMT5) and EPZ020411 (PRMT6) have been accomplished, suggesting that the substrate-binding grooves of PRMTs can also be successfully targeted (Table 1).[9,12,13] The discovery of the first allosteric PRMT3 inhibitor and the development of the PRMT3 chemical probe SGC707 have demonstrated that the allosteric binding site of PRMT3 can be exploited to yield potent, selective, and cell-active inhibitors, opening the door for discovering allosteric inhibitors of other PRMTs (Table 1).[14] Moreover, the discovery of the covalent SETD8 inhibitor MS453 has demonstrated that cysteine residues in active sites of PMTs can be selectively targeted (Table 1).[15] Furthermore, inhibitors that are disrupting the protein-protein interactions such as OICR-9429 (WDR5-MLL), MI-503 (MENIN-MLL) and A-395, EED226 (EED-PRC2) were also recently reported introducing yet another approach for the inhibition of the PMTs (Table 1).[16–19] The design, synthesis and biological studies of many of these aforementioned inhibitors have already been discussed in detail in literature.[9,10] The following sections will focus only on the potent, selective small-molecule PMT inhibitors that are discovered very recently (indicated in bold type in Table 1).

Inhibitors of H3K9 and H3K27 methyltransferases

The mono- and dimethylation of lysine 9 residue of histone 3 (H3K9) is primarily catalyzed by G9a and GLP, which share 80% sequence identity in their SET domains. Since the identification of the first G9a and GLP inhibitor, BIX-01294 in 2007, highly potent and selective inhibitors of G9a and GLP have been published.[9] All of these inhibitors however were highly potent for both enzymes. In 2017, MS012, a potent GLP selective inhibitor MS012 was discovered (Figure 2).[20] This inhibitor while still possessing quinoxaline scaffold as many of its predecessors is 140-fold selective for GLP over G9a. In addition, MS012 displayed selectivity for GLP over a broad range of other PKMTs, PRMTs, DNMTs, and RNMTs. Remarkably, X-ray structures show that this substrate-competitive inhibitor binds to GLP and G9a in virtually identical binding modes, underlining the challenges in structure-based design of selective inhibitors for these highly homologous enzymes.

Methylation of H3K27 is catalyzed by multisubunit protein complex polycomb repressive complex 2 (PRC2) and primarily functions to silence its target genes by trimethylation of H3K27.[21] Enzymatically active PRC2 complex consists of three subunits: catalytic subunit EZH2 or EZH1, VEFS domain containing SUZ12 and WD40 repeat containing protein EED. A number of highly potent and selective inhibitors of PRC2 have been reported since 2012 (Table 1).[9] These pyridone containing and co-factor competitive EZH2 inhibitors have been used in many studies in recent years targeting various cancer types as EZH2 is highly expressed in many types of cancers.[22] Recently the discovery of CPI-1205, displaying high potency, selectivity, and cellular activity was added to the list of pyridone containing inhibitors.[23] A co-crystal structure of an analog of CPI-1205 bound to human PRC2 was also obtained.[23] In addition, very recently, new series of pyridone

containing inhibitors, which led to the discovery of highly potent EZH2 inhibitor 1 (Figure 2) was reported.[24] Compound 1 displayed cellular potencies in cells that are comparable to previously reported EZH2 inhibitors. It is important to note that EPZ-6438, GSK126 and CPI-1205 are advanced into human clinical trials.

In late 2015, the first crystal structures of an active PRC2 complex from the yeast *Chaetomium thermophilum*, which contain EZH2, EED and SUZ12-VEFS in complex with inhibiting H3K27M peptide and SAH were reported.[25] Shortly after, in 2016, the structure of the human PRC2 complex was published.[26] Concurrently, the crystal structure of a small-molecule inhibitor in complex with the wild-type and Y641N-mutated PRC2 complex, consisting of human EED, human SUZ12-VEFS, and engineered American chameleon EZH2 subunits was also disclosed.[27] These crystal structures of the EZH2-EED-SUZ12 ternary complex revealed the molecular basis of the PRC2 core complex assembly and represent a landmark in this field. These structures show that the EED subunit is wrapped around by EZH2, and SUZ12 is sandwiched between EED and the SET domain of EZH2 forming a catalytically active complex. It was suggested that perhaps EED and SUZ12-VEFS allosterically activated the SET domain. The recognition of H3K27me3 by EED and the resulting increase of the methylase activity are believed to be crucial for the cellular function of PRC2 and thus, obstruction of this process would be a different approach in inhibiting PRC2 functions.

Very recently two inhibitors A-395 and EED226, which are targeting the EED H3K27me3 binding site and in turn preventing the allosteric activation of the PRC2 were reported. A-395 potently inhibited the catalytic activity of the trimeric PRC2 complex (EZH2-EED-SUZ12).[18] EED226 as well prevented the H3K27me3 binding to EED and therefore inhibited PRC2 activity when H3K27me0 peptide is used as substrate.[19] In addition both of these inhibitors reduced H3K27me2 and H3K27me3 levels in cells and inhibited proliferation of human cancer cell lines sensitive to EZH2 inhibition. Moreover they showed antitumor effects in a xenograft model *in vivo* and importantly, they were potent in cell lines resistant to EZH2 inhibitors. Crystal structures of these two inhibitors in complex with EED are also reported. In addition, recently peptidomimetic EED inhibitors that disrupt catalytic activity of PRC2 have been reported.[28]

Inhibitors of H3K4 and H3K36 methyltransferases

H3K4 and H3K36 methylation are hallmark of transcriptional activation.[29] SETD7, SMYD family proteins (SMYD1-3) and the MLL family proteins (MLL1-5) are some of the methyltransferases that are determined to be responsible for methylation of H3K4 in humans.[30] Studies have shown that these methyltransferases targets many non-histone proteins as well in some cases as their primary targets (Table 1). (*R*)-PFI-2 is the most potent, selective, and cell-active small-molecule inhibitor of SETD7 to date.[9] The major target of SMYD3 is determined to be MAP3K2 (also known as MEKK2) and highly potent, selective and cell active, small-molecule inhibitors of SYMD3, EPZ0330456 and EPZ031686 were reported and discussed elsewhere in greater detail.[9] MLL is a large multi-domain protein that is specific for H3K4 mono-, di-, and trimethylation.[30] Chromosomal rearrangements associated with MLL have been shown to cause acute

myeloid, acute lymphoblastic, or mixed lineage leukemia.[31] Small molecules that perturb protein-protein interactions (PPI) of MLL with its partners, such as WDR5 (OICR-9429) and menin (MI-503) have been discovered.[9,16,17]

NSD family proteins (NSD1-3), SETD2, SETD3, ASH1L, SETMAR, and SMYD2 are responsible for H3K36 methylation.[30] SMYD2 can methylate a variety of non-histone substrates implicating effects on diverse biological processes.[30] Small molecule inhibitors of SMYD2; AZ-505, A-893 and LLY-507 have already been reported and previously reviewed.[9,10] Very recently, in 2016, a screening campaign and optimization studies yielded in the discovery of enantiomerically pure (*S*)-BAY-598 (Figure 2) as a cell- and *in vivo*-active inhibitor of SMYD2.[32] (*S*)-BAY-598 shows >10-fold selectivity for SMYD2 over SMYD3 and >100-fold selectivity over 31 other methyltransferases. This inhibitor is also highly selective against kinases and other primary molecular targets, including several CNS targets and competitive with the peptide substrate, but uncompetitive with cofactor.

Inhibitors of H4K20 methyltransferases

Methylation of H4K20 is catalyzed by the methyltransferases SUV420H1, SUV420H2, and SETD8.[30] SETD8 is the sole methyltransferase that catalyzes monomethylation of H4K20 and methylates many non-histone substrates, including the tumor suppressor p53 and proliferating cell nuclear antigen.[30] SETD8 has been shown to be overexpressed in various types of cancers.[33]

The first reported inhibitor of SETD8 was a marine natural product, nahuic acid A, which was a co-factor competitive inhibitor.[34] In 2014, the first substrate-competitive, selective inhibitor of SETD8, UNC0379 was reported (Figure 2).[35]. In 2016, a more potent inhibitor, MS2177 (Figure 2), was obtained via addition of an aminoalkyl group to the 7-position of UNC0379.[15] MS2177 is competitive with the H4 peptide, but non-competitive with the cofactor *S*-5'-adenosyl-L-methionine (SAM). A cocrystal structure of MS2177 complexed with SETD8 revealed a cysteine residue (C311) in close proximity to the inhibitor binding site, presenting an opportunity to develop a covalent inhibitor of SETD8. Therefore, MS453 (Figure 2) was designed as a covalent inhibitor that modified C311, without effecting other cysteine residues of SETD8.[15] No covalent adduct was observed with PMTs such as PRC2, SMYD2, and SMYD3 upon incubation with MS453 suggesting specificity to SETD8. Furthermore, MS453 was highly selective for SETD8 over 29 other methyltransferases and the crystal structure of MS453 in complex with SETD8 confirmed covalent modification of C311.

SUV420H1 and SUV420H2 are highly homologous methyltransferases that di- and trimethylate H4K20.[30] A-196 (Figure 2) was recently discovered as the first potent, selective, and cell-active inhibitor of SUV420H1 and SUV420H2.[36] A-196 inhibits SUV420H1 and SUV420H2 potently in a peptide competitive manner and is >100-fold selective for SUV420H1 and SUV420H2 over other methyltransferases and a broad range of non-epigenetic targets. A-196 reduced the H4K20me3/me2 marks while elevated of H4K20me1 levels throughout the cell cycle in human cells without any observed toxicity. It inhibited 53BP1 foci formation in response to ionizing radiation, end reduced

nonhomologous end joining (NHEJ) mediated DNA-break repair and it did not induce resistance/compensation effects, as H4K20me_{2/3} remained reduced after 20 population doublings.

Inhibitors of H3K79 methyltransferases

Highly potent, selective and cell active inhibitors of DOT1L: EPZ004777 SGC0946 and EPZ-5676 were disclosed between 2011 and 2013.[9] These inhibitors are SAM derivatives and competitive with the co-factor. In addition, a SAM derived covalent inhibitor was also reported.[9] In 2012, EPZ-5676 (also known as pinometostat) has become the first PMT inhibitor advanced to the clinic, and the first major breakthrough in the PMT inhibitor field.

In June 2016, a new series of DOT1L inhibitors that differ structurally from all previously published SAM-based inhibitors was reported.[37] These new inhibitors interact with an induced pocket adjacent to the SAM binding site, but do not bind the SAM binding site. A weak fragment-based screening hit that displayed suboptimal interactions in binding pocket was developed into a highly potent inhibitor of DOT1L, **2** (Figure 2) via careful analysis of the co-crystal structure and elegant structure-based optimization. These inhibitors were identified as SAM-competitive because, upon binding, they engage the lid loop of the SAM binding pocket and form a conformation preventing SAM binding. In a subsequent report, another structurally novel DOT1L inhibitor series that targets the same induced pocket was discovered.[38] Again a fragment based approach is utilized and resulted in the discovery of potent DOT1L inhibitors **3** and **4** (Figure 2), which displayed very high potencies in biochemical assays. The same research group realized that some of the identified fragments binding to the induced pocket extended into the methionine pocket of SAM binding site. However, the high throughput and fragment based screening methods used in the earlier studies, failed to find the fragments that occupy the SAM binding site. As already mentioned inhibitors **2-4** function in SAM-competitive manner. Interestingly, however, adenosine could bind to DOT1L in the presence of the induced pocket binders. With this observations in mind, a ternary X-ray crystal structure of adenosine and an induced pocket binder was obtained.[39] In this structure the lid loop of the SAM binding pocket in the SAM-bound state, is still folded over and collapsed onto induced pocket ligand as before. In addition, adenosine does not interact with the flexible loop in the ternary complex but otherwise forms the same contacts with DOT1L as in its binary complex. With these information in hand, the screening of a fragment-based second-site via NMR (i.e with the induced pocket already occupied) and also knowledge-based virtual screening of fragment library resembling kinase hinge binders that satisfy an adenosine-based pharmacophore were conducted. All hits discovered from both screening approaches were submitted to co-crystallization experiments with DOT1L in the absence and presence of induced pocket ligands. As a result, a pyrolopyrimidine fragment was obtained as weak adenosine-like fragment inhibitor of DOT1L. Eventually, two ligands (adenosine-like pyrolopyrimidine fragment and induced pocket binding fragment) that perfectly lined up for linking were identified. Linked fragments were further optimized resulting in the discovery of a highly potent inhibitor **5** with IC₅₀ in picomolar range.[39] Impressively, inhibitor **5** performed equivalent or better than EPZ-5676 in cellular assays.

Inhibitors **2-5** displayed high selectivity against a panel of PKMTs and PRMTs, showing no inhibitory activity at up to 50 μ M. Importantly, they potently decreased H3K79me2 levels and reduced the activity of the HoxA9 promoter in cellular assays. Moreover, they efficiently inhibited the proliferation of MV4-11 cells carrying the oncogenic *MLL-AF4* fusion with nanomolar IC₅₀ values. Overall, these novel series (compounds **2**, **3/4** and **5**) of potent, selective, SAM-competitive DOT1L inhibitors are exciting. In addition, Compounds **3** and **4** are useful chemical tools for cellular and *in vivo* studies.

Inhibitors of protein arginine methyltransferases

CARM1 (PRMT4) is responsible for the asymmetric dimethylation of H3R17 and H3R26, with preference for the former. CARM1 also methylates a variety of non-histone proteins. [40] Several HTS campaigns and SAR studies resulted in the identification of CARM1 inhibitors with limited selectivity.[41–43] Cocrystal structures of the CARM1 catalytic domain in complex with these inhibitors reveal that they are anchored in the PRMT arginine-binding channel through a basic alkyl-diamino or alanine-amide tail.[44]

Utilizing a fragment-based approach, a commercially available diverse fragment library of compounds mimicking basic amino tails was tested against PRMT6, resulting in the discovery of fragment hit **6** that inhibits CARM1, PRMT6, and PRMT8 (Figure 3).[45] Recently, highly potent, selective, and cell-active dual CARM1 and PRMT6 inhibitor, MS049 (Figure 3), was discovered via SAR studies based on the aforementioned fragment hit **6**. [46] The same research group also reported a potent and selective inhibitor of CARM1, compound **7** (Figure 3), based again on the fragment hit **6**. [47] Concurrent with the two studies described above, another potent and selective CARM1 inhibitor SGC2085 displaying good selectivity was discovered via virtual screening.[48] However, this inhibitor is inactive in cell-based assays, likely due to its poor cell membrane permeability. Recently, the first potent, selective, and cell-active inhibitor of CARM1, TP-064, was discovered but detailed report of this inhibitor has not yet been published (Figure 3).[49]

In 2016, a potent PRMT5 inhibitor GSK3326595 (structure is not yet disclosed in literature), potently inhibits tumor growth in cellular and animal models, has entered phase I clinical trials (NCT02783300). Moreover, LLY-283, the first potent and selective SAM-competitive chemical probe for PRMT5 was recently discovered (Figure 3).[50] LLY-283 potently inhibits PRMT5, is >100-fold selective for PRMT5 over other methyltransferases and non-epigenetic targets, and shows activity in cellular assays.

Conclusion

Remarkable progress has been made in the discovery of selective, small molecule inhibitors of PMTs over the past two years. These inhibitors have been used and continue to be used extensively in studies as tool compounds to decipher biology and disease relations of these enzymes. While there has been significant progress, there is still much to be achieved in the PMT inhibitor field since, many individual targets and subgroups of targets on the PMT phylogenetic tree lack selective inhibitors, including MLL family, MMSET (NSD-2), and PRDMs (Figure 1). Potent, selective, and cell-active inhibitors of PMTs are invaluable

chemical tools to better understand biological functions of PMTs and test therapeutic hypotheses concerning these proteins.

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Highlights

- Inhibitors that selectively target EED component of PRC2 and GLP were discovered.
- First-in-class, selective, cell active SUV420H1/H2 inhibitor was disclosed.
- A covalent inhibitor of SETD8 has recently been reported.
- Novel DOT1L inhibitors differing from known SAM-based inhibitors were discovered.
- New inhibitors of protein arginine methyltransferases CARM1 and PRMT5 were published.

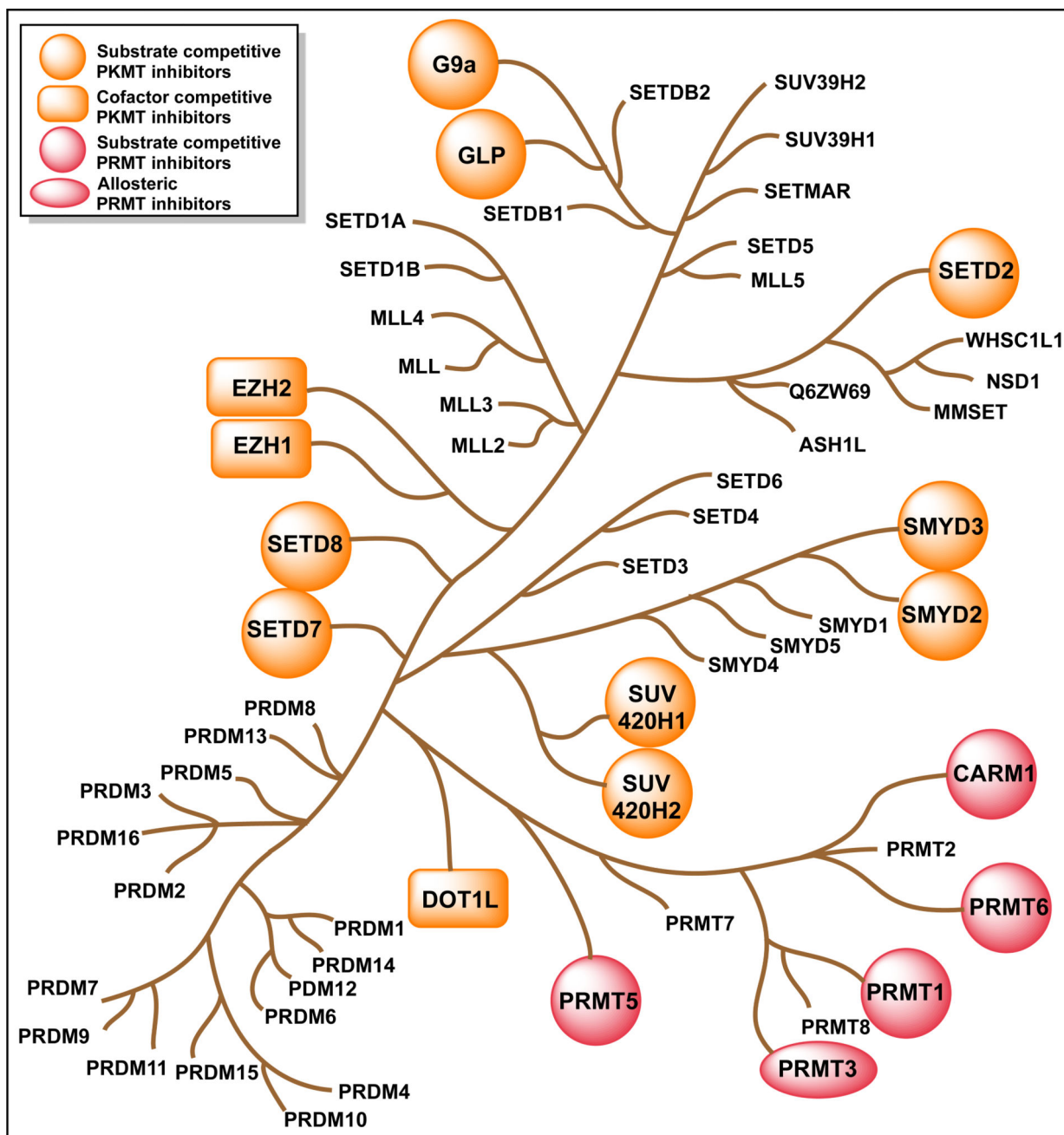


Figure 1. Phylogenetic tree of protein methyltransferases
 The methyltransferases with known inhibitors are marked and their mechanism of inhibition indicated.

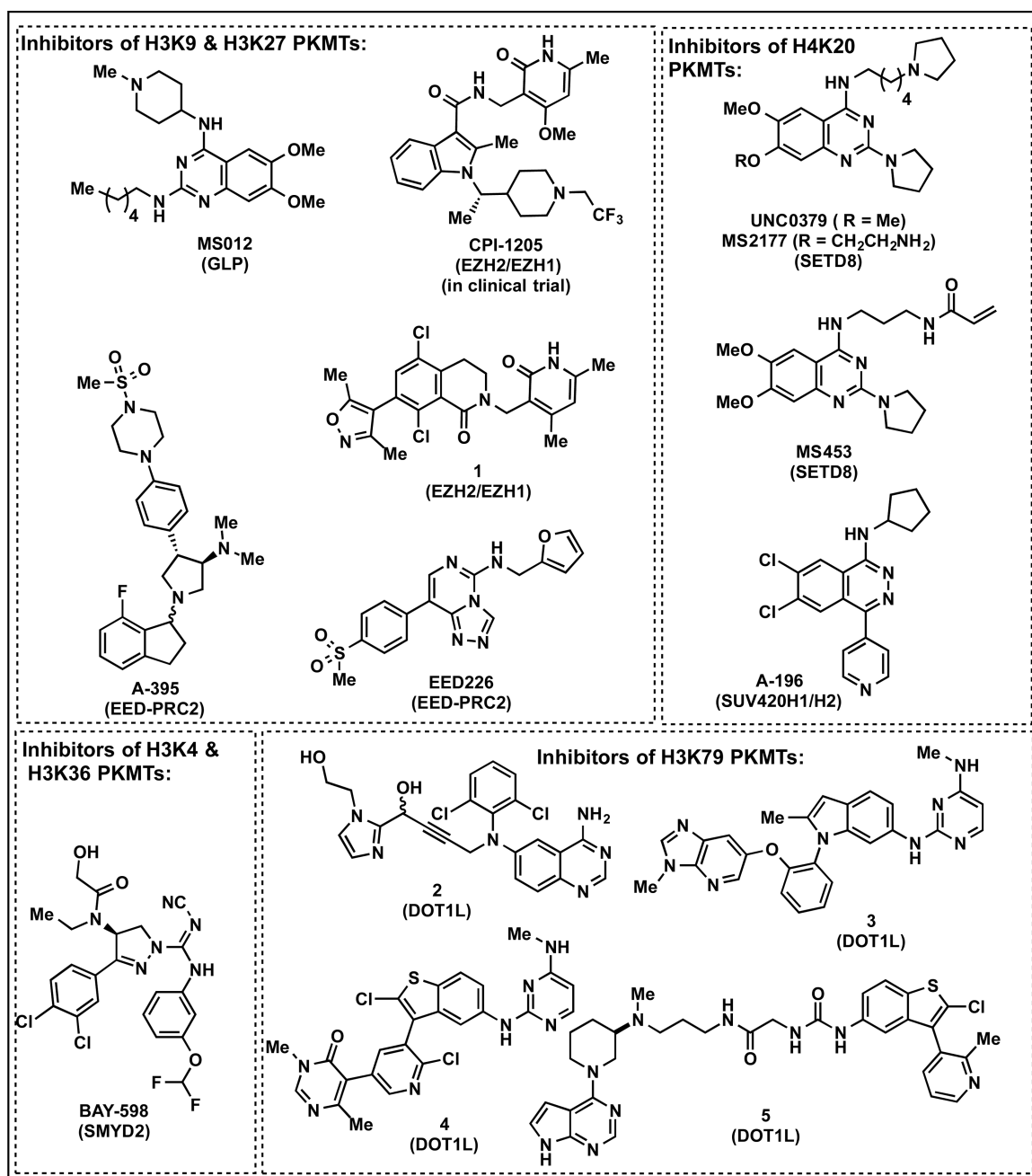


Figure 2. Structures of inhibitors of PKMTs

The structures of recently reported inhibitors of EZH2/EZH1, EED-PRC2, SMYD2, SETD8, SUV420H1/H2 and DOT1L

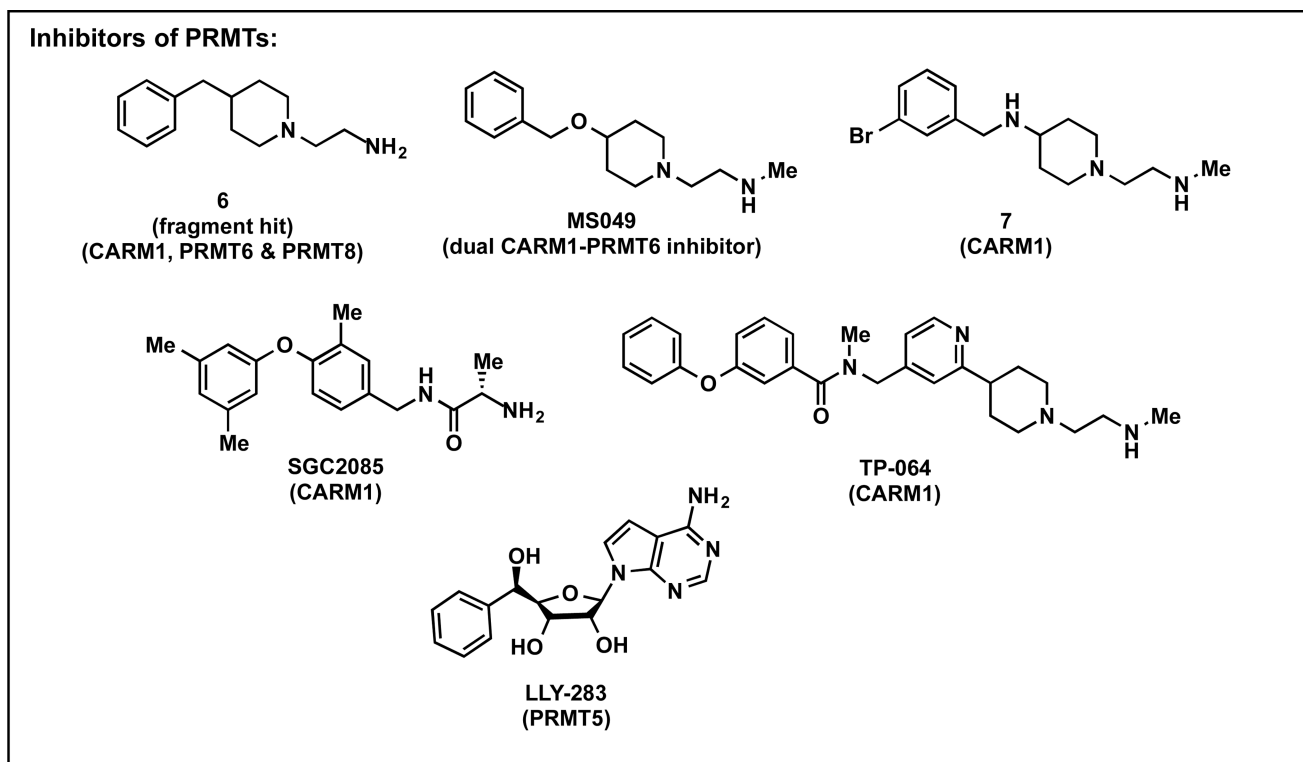


Figure 3. Structures of inhibitors of PRMTs

The structures of recently reported inhibitors of CARM1 and PRMT5.

Table 1
List of known selective, small molecule inhibitors of PMTs and their mechanism of action (MOA)

Recently discovered inhibitors that are discussed in this review are shown in bold type.

PMT	Histone target ^a	Non-histone target(s) ^a	Inhibitor(s) ^b [ref]	MOA
G9a/GLP	H3K9	p53	BIX-01294; UNC0224; E72; UNC0638; BRD4770 ^c ; UNC0642; A-366	Substrate competitive
GLP	H3K9	p53	MS012 [20]	Substrate competitive
EZH2/EZH1	H3K27	-	EPZ005687; GSK126; EI1; UNC1999; EPZ-6438; EPZ011989; ZLD1039; CPI-1205 [23]; Compound 1 [24]	Co-factor competitive
EED-PRC2	H3K27	-	A-395 [18]; EED226 [19]	Allosteric/PPI-disruption
SETD7	H3K4	p53, p65, DNMT1	PFI-2	Substrate competitive
SMYD3	H3K4, H4K5	MAP3K2	EPZ031686; EPZ030456	Substrate competitive
MENIN-MLL	H3K4	-	MI-503	PPI-disruption
WDR5-MLL	H3K4	-	OICR-9429	PPI-disruption
SMYD2	H3K4, H3K36	p53, Rb	AZ-505; A-893; LLY507; BAY-598 [32]	Substrate competitive
SETD2	H3K36	p53	Pr-SNF	Substrate competitive
SETD8	H4K20	p53, PCNA	Nahuoic Acid A; UNC0379 [35]; MS2177 ; MS453 [15]	Substrate competitive
SUV420H1/SUV420H2	H4K20	-	A-196 [36]	Substrate competitive
DOT1L	H3K79	-	EPZ004777; SGC0946; EPZ-5676, Compounds 2-5 [37–39]	Co-factor competitive
PRMT1	H4R3	NPL3p, MRE11, 53BP1, ASH2L	AMI-1; NS1; A36; MS023 (Type I PRMT inhibitor)	Substrate competitive
PRMT3	H4R3	rpS2 , PABPN1	SGC707	Allosteric
CARM1	H3R17 , H3R26	CBP/p300, PABP, HuR, HuD, CA150, SAP49, SmB, U1C	MS049 ^d [46]; Compound 7 [47]; SGC2085 [48]; TP064 [49]	Substrate competitive
PRMT5	H2AR3, H4R3 , H3R2, H3R8	p53, NF- β	EPZ015666; GSK591; LLY-283 [50]	Substrate competitive
PRMT6	H4R3 , H3R2	-	EPZ020411	Substrate competitive

^aHistone and non-histone targets of the PMTs (not a comprehensive list). Main targets of the enzymes are shown in bold type.

^bKnown inhibitors of PMTs.[9] The inhibitors that are focus of this review are shown in bold type.

^cThe inhibitory activity of BRD4770 against GLP was not reported and it was reported to be co-factor competitive.

^dMS049 is a selective, dual CARM1 and PRMT6 inhibitor.