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Review

Histone lysine methyltransferases as anti-cancer targets for drug discovery

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Post-translational epigenetic modification of histones is controlled by a number of histone-modifying enzymes. Such modification regulates the accessibility of DNA and the subsequent expression or silencing of a gene. Human histone methyltransferases (HMTs) constitute a large family that includes histone lysine methyltransferases (HKMTs) and histone/protein arginine methyltransferases (PRMTs). There is increasing evidence showing a correlation between HKMTs and cancer pathogenesis. Here, we present an overview of representative HKMTs, including their biological and biochemical properties as well as the profiles of small molecule inhibitors for a comprehensive understanding of HKMTs in drug discovery.

Keywords: histone methyltransferases; adenosylmethionine; small molecule inhibitors; anti-cancer drugs; epigenetic modification; drug discovery

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Introduction

Post-translational epigenetic modifications of histones are controlled by histone-modifying enzymes, including histone methyltransferases (HMTs), histone demethylases, histone acetyltransferases, histone deacetylases (HDACs), ubiquitin ligases, and other specific kinases phosphorylating serine residues on histones. Such modifications regulate the accessibility of DNA and the subsequent expression or silencing of a gene[1, 2]. Histone methyltransferases include a large family of dozens of histone lysine methyltransferases (HKMTs) and histone/protein arginine methyltransferases (PRMTs), many of which have been found to play critical roles in cell differentiation, gene regulation, DNA recombination and damage repair[3–6].

The methylation of histone lysine residues was first discovered in the 1960s[7, 8] and was believed to be irreversible in the following decades until the discovery of a lysine demethylase in 2004[9]. Recently, increasing evidence indicating a correlation between HKMTs and cancer pathogenesis has emerged[3–6]. Therefore, a number of small molecule inhibitors of histone lysine methyltransferases have been identified and exhibited selective cancer cell killing activities *in vitro* and *in vivo*, suggesting the possibility of using HKMTs as targets for cancer therapy. Here, we discuss individual histone lysine methylation with the currently available small molecule inhibitors and their applications in cancer treatment.

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HKMTs-catalyzed methylation

Histone lysine methyltransferases (HKMTs) transfer the methyl group from the cofactor adenosylmethionine (AdoMet), which contains a highly reactive methylthiol group, to the tailed nitrogen of the substrate lysine residue, producing mono-, di-, or tri-methylated products and its analogue adenosylhomocysteine (AdoHcy) (Figure 1)[10, 11]. AdoHcy is structurally similar to AdoMet, binding to methyltransferases in the same orientation with a similar K_d value, but it lacks the reactive methyl group and acts as a competitive inhibitor with respect to AdoMet[12]. Additionally, it cannot be used as a probe in cell biology or *in vivo* because of its quick degradation to adenosine and homocysteine by SAH hydrolase[13].

Catalytic active sites in HKMTs

Two pockets are involved in the catalytic active sites: a SAM-binding pocket and a lysine acceptor channel, which are on opposite sides of the protein surface facilitating the entrance of two substrates to the active sites from opposite sides of the enzyme surface[14]. The structures of cofactor binding sites are conserved in different SAM-dependent methyltransfer-

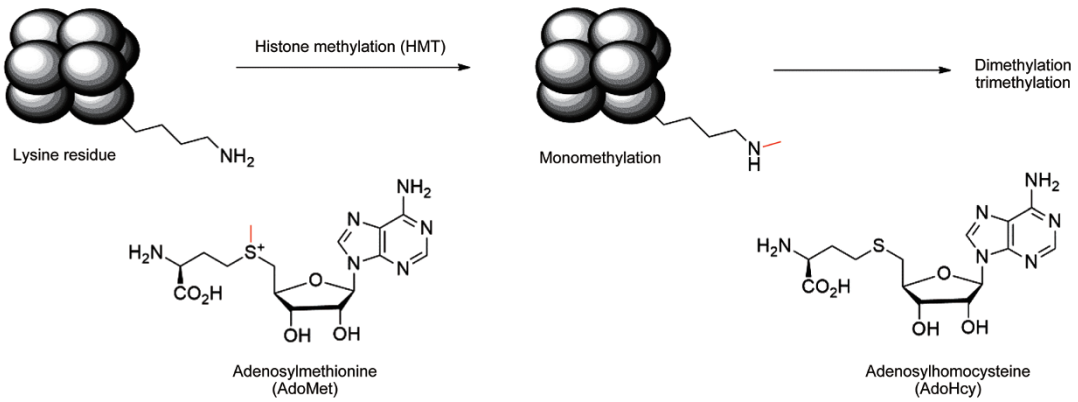


Figure 1. Methylation of lysine residues by histone methyltransferases utilizing adenosylmethionine (AdoMet) as a cofactor.

ase classes and are represented by three motifs located in the P loop, G loop and part of strand β4[15]. The co-crystallized structures of SET domain-containing HKMTs[16] (except DOT1L) with SAM or SAH show that the cofactor adopts a 'U-shaped' configuration in contrast to DOT1L, which adopts an extended configuration[17] similar to that of PRMTs. In the 'U-shaped' configuration, the methylsulfonium cation of SAM forms a juxtaposition to the ε-amino group of the acceptor lysine residue within the narrow lysine channel, which was induced by an H-bond function between the conserved Asp or Glu residue and the hydroxyl groups of ribose as well as a salt bridge between a positively charged lysine and the carboxylate group of SAM. A 'tyrosine (Y)-phenylalanine (F) switch', a key determinant for mono-, di-, and tri-methylation of lysine residue in the SET domain-containing HKMTs, also exists, which is demonstrated by the mutants of DIM5^{F281Y} and SETD7^{Y305F} [18]. The location of substrate also varies among different HKMTs. For instance, the substrates of SET domain-containing HKMTs are located in the tail of unordered histone, while H3K79, the substrate of DOT1L, is located at the core structure of ordered histone octamer[19, 20].

Representative HKMTs and their inhibitors

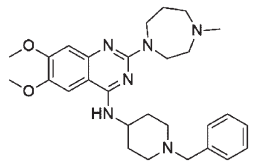
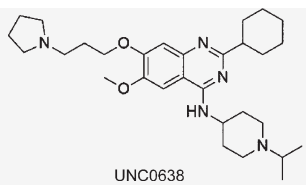
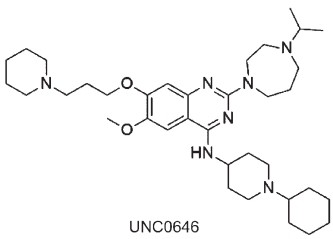
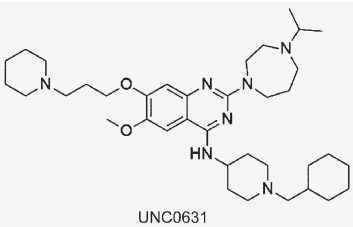
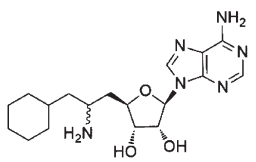
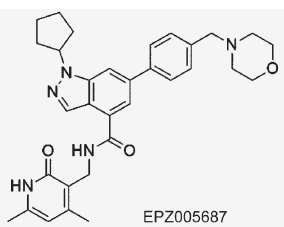
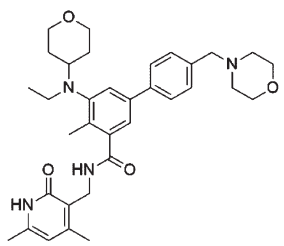
DOT1L

DOT1L, the only HKMT belonging to class I methyltransferases, mono-, di-, and tri-methylates H3K79 and plays a role in embryonic development and hematopoiesis, *etc*[20–22]. Compared with many other SET-domain HKMTs, DOT1L is a distributive enzyme[20], which releases SAH, and mono-, di- and tri-methylated products at the same time after one catalytic cycle. Several studies[19–22] indicated DOT1L as a target for acute leukemia with MLL (mixed-lineage leukemia) gene translocations. MLL rearrangements result in the loss of the SET-domain H3K4 methyltransferase on the carboxyl terminal, and the remaining amino-terminal MLL protein is fused to one of more than 60 different partner genes (predominantly, AF4, AF9, AF10, and ENL), which is found to recruit DOT1L as a part of transcription protein complexes and subsequently methylates H3K79 instead of H3K4. Such an aberrant epigenetic modification leads to the overexpression of MLL-target genes such as *HOXA9* and *MEIS1* and causes leukemia. Therefore, DOT1L is proposed as a target for the therapeutic intervention of MLL-rearranged leukemia. Several potent small molecules have thus been developed including SAH-like scaffold[21], iodoethyl-containing scaffold[21], urea or benzoimidazole-containing scaffold[22, 23]. Among them, two inhibitors (EPZ004777[22] and EPZ5676[23]) disclosed by Epizyme bearing urea or benzoimidazole group substituted on the side chain of the aminonucleoside were identified as SAM-competitive DOT1L selective inhibitors with high potencies (IC_{50}=0.4 nmol/L for EPZ004777 and K_i=0.08 nmol/L for EPZ5676, respectively) (Table 1). They dose-dependently inhibited global H3K79 levels in MLL-rearranged leukemia cells, transcript levels of MLL fusion target genes such as *HOXA9* and *MEIS1*, and the proliferation of *MLL*-rearranged leukemia cells, with little or no effect on non-*MLL*-rearranged leukemia cells. However, the poor pharmacokinetic properties (short half-life, *etc*) of these two compounds limited their conventional dosing *in vivo,* and they are used with a subcutaneously implanted mini-osmotic pump instead.

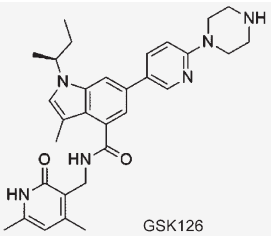
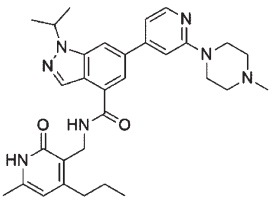
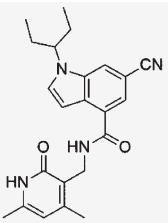
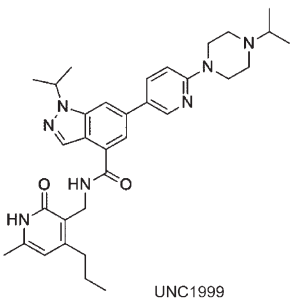
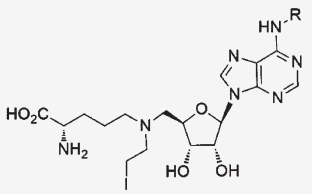
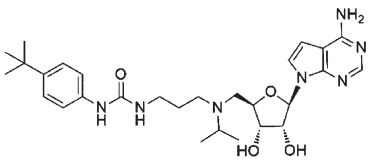
EHMT1/2

Euchromatin histone methyltransferases (EHMTs), including G9a encoded by *EHMT2* and GLP (G9a-like protein) encoded by *EHMT1*, are an evolutionarily conserved protein family, which mono- or di-methylate H3K9 as well as other non-histone substrates such as K373 of the tumor suppressor p53[24, 25]. EHMTs regulate transcriptional repression and activation in the form of a heterodimeric complex in the process of germ cell formation, embryogenesis and cardiac morphogenesis[26, 27]. G9a is highly expressed in a variety of human cancers with a poor prognosis such as leukemia, prostate cancer, lung cancer, and hepatocellular carcinoma[28]. Reduction of G9a expression levels inhibited lung and breast cancer cell proliferation, migration and invasion *in vitro* and suppressed primary tumor growth and metastasis *in vivo*[29]. Renneville[30] *et al* demonstrated in 2015 that knockdown of either *EHMT1* or *EHMT2* could significantly increase the expression of γ-globin genes, and the percentage of cells expressing hemoglobin F (HbF). This discovery may lead to a new therapy for sickle

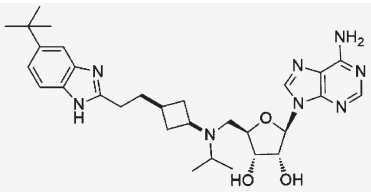
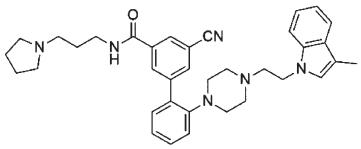
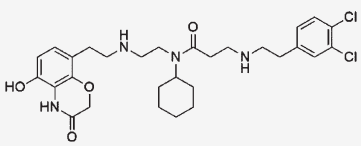
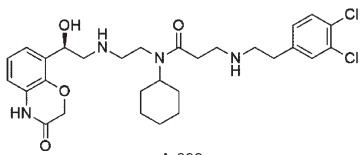
Table 1. The profiles of small molecule inhibitors of histone lysine methyltransferases (HKMTs).

Compound	Target HKMTs	Mechanism	Biochemical potency (IC_{50})	Cellular potency (IC_{50})	*In vivo* activity	Fold selectivity	Status	Reference
 BIX-01294	EHMTs	Substrate competitive	1.9±0.1 μmol/L (EHMT2) 0.7±0.1 μmol/L (EHMT1)	500±43 nmol/L (MDA-MB-231)	No	No inhibition observed (other HMTs)	Preclinical	31, 32, 33
 UNC0638	EHMTs	Substrate competitive	<15 nmol/L (EHMT2) 19±1 nmol/L (EHMT1)	81±9 nmol/L (MDA-MB-231)	No	~500 (other HMTs)	Biological testing	30, 33
 UNC0646	EHMTs	Substrate competitive	0.006 μmol/L (EHMT2)	0.026 μmol/L (MDA-MB-231)	No	No report	Biological testing	34
 UNC0631	EHMTs	Substrate competitive	0.004 μmol/L (EHMT2)	0.025 μmol/L (MDA-MB-231)	No	No report	Biological testing	34
 Cpd 4d	EHMTs	Unknown	1.6 μmol/L (EHMT2) 1.5 μmol/L (EHMT1)	No	No	~100 (other 3 HMTs)	Biological testing	35
 EPZ005687	EZH2	SAM competitive	54±5 nmol/L (EZH2-PRC2)	80±30 nmol/L (OCI-LY19 $EZH2^{WT}$)	No	~50 (EZH1) >500 (other 15 HMTs)	Preclinical	40
 EPZ6438 (tazemetostat)	EZH2	SAM competitive	11±5 nmol/L (EZH2) 2–38 nmol/L (EZH2 mutants)	8 nmol/L (OCI-LY19, $EZH2^{WT}$) 2–90 nmol/L (EZH2 mutants)	Yes	~35 (EZH1) >4500 (other HMTs)	Phase II	51

(To be continued)

Compound	Target HKMTs	Mechanism	Biochemical potency (IC_{50})	Cellular potency (IC_{50})	*In vivo* activity	Fold selectivity	Status	Reference
 GSK126	EZH2	SAM competitive	9.9 nmol/L (EZH2)	28–861 nmol/L (DLBCL, EZH2 mutants)	Yes	~150 (EZH1) ~1000 (other 20 HMTs)	Phase I	49
 GKS343	EZH2	SAM competitive	4 nmol/L (EZH2)	174±84 nmol/L (HCC1806)	No	~60 (EZH1) ~1000 (other HMTs)	Preclinical	48
 EI1	EZH2	SAM competitive	15±2 nmol/L ($EZH2^{WT}$) 13±3 nmol/L ($EZH2^{Y641F}$)	~370 nmol/L (SU-DHL6, $EZH2^{Y641N}$)	No	~90 (EZH1) >10 000 (other HMTs)	Biological testing	50
 UNC1999	EZH2	SAM competitive	<10 nmol/L (EZH2)	124±11 nmol/L (MCF10A, $EZH2^{WT}$)	Yes	~10 (EZH1) ~10000 (other HMTs)	Preclinical	47
 Cpd 4 R=H Cpd 5 R=CH_3 Cpd 6 R=benzyl	DOT1L	SAM competitive	38 nmol/L (Cpd 4) 120 nmol/L (Cpd 5) 110 nmol/L (Cpd 6)	No	No	>29 (other 4 HMTs)	Biological testing	21
 EPZ004777	DOT1L	SAM competitive	0.4±0.1 nmol/L	0.17–6.47 μmol/L (MLL-rearranged leukemia cell lines)	Yes	>1000 (other HMTs)	Preclinical	22

(To be continued)

Compound	Target HKMTs	Mechanism	Biochemical potency (IC_{50})	Cellular potency (IC_{50})	*In vivo* activity	Fold selectivity	Status	Reference
 EPZ5676	DOT1L	SAM competitive	K_i=0.08±0.03 nmol/L	3.5±0.7 nmol/L (MV4-11)	Yes	>37 000 (other 15 HMTs)	Phase I	23
 LLY-507	SMYD2	Substrate competitive	<15 nmol/L (p53) 31 nmol/L (H4)	0.6 μmol/L (U2OS) 0.6 μmol/L (KYSE-150)	No	>100 (other 25 HMTs and non-HMTs)	Biological testing	56
 AZ505	SMYD2	Substrate competitive	0.12 μmol/L	No	No	>700 (6 other HMTs)	Biological testing	59
 A-893	SMYD2	Substrate competitive	2.8 nmol/L	42% reduction of p53K370me1 (A549)	No	No significant inhibition (>50%) at 1 μmol/L for other 30 HMTs	Biological testing	60

cell disease (SCD) because induction of HbF is a well-validated strategy, and novel epigenetic regulators such as HbF inducers are attractive targets for SCD treatment.

BIX01294, identified through a high-throughput screening, was the first reported[31, 32] selective small molecule inhibitor of G9a and GLP, showing an IC_{50} of 1.9 μmol/L for G9a and 0.7 μmol/L for GLP, separately. The crystal structure of GLP with SAH and BIX01294 demonstrated that BIX01294 did not bind to the SAM binding pocket but was in a substrate groove that was usually occupied by residues H3K4-H3R8 at the N-terminal of the target H3K9. The structure of BIX01294 consists of three parts: quinazoline, piperidine and diazepane subunits. Further structure-activity relationship (SAR) studies were conducted, including the extension of 7-methoxy into the target lysine binding channel and replacement of the benzyl group on the piperidine, represented by UNC0638[30, 33], UNC0646[34] and UNC0631[34]. All three compounds showed high potency both in a fluorescence-based SAH-coupled assay (IC_{50}: 4–19 nmol/L) and in the H3K9me2 In-Cell Western (ICW) assay (IC_{50}: 25–81 nmol/L), with an excellent toxicity to function ratio range of 110–140 in MDA-MB-231 (breast carcinoma) cells compared with BIX01294 (<6). UNC0638 could also dose-dependently increase human γ-globin expression, HbF expression, and mouse embryonic γ-globin gene expression without altering the cellular morphology at a concentration up to 0.25 μmol/L and without affecting cell proliferation and viability up to 0.1 μmol/L in primary adult human erythroid cells *ex vivo*. A combination of UNC0638 with entinostat (HDAC inhibitor) or decitabine (inhibitor of DNA methyltransferase) additively increased the gene expression of γ-globin, suggesting a different γ-globin inducing mechanism of EHMT1/2 inhibition in comparison to entinostat or decitabine[30]. Our group[35] has also reported a group of sinefungin analogues which dose-dependently inhibited EHMT1/2 with an IC_{50} of 1.5–1.6 μmol/L. However, it was not competitive with SAM or substrate, which is indicative of other binding sites in EHMT1/2.

EZH2 and PRC2

The polycomb repressive complex 2 (PRC2), which is responsible for mono-, di-, and tri-methylation of H3K27, is composed of EED (embryonic ectoderm development), SUZ12, RbAp48 and an enzymatic subunit of either EZH2 (enhancer of zeste homologue 2, also known as KMT6 or KMT6A) or its homolog EZH1 (enhancer of zeste homologue 1, also known as KMT6B), which shares 96% sequence identity in the SET

domains with EZH2[36]. EZH2 overexpression is implicated in several tumor types correlated with poor prognosis such as prostate cancer, breast cancer, myeloma, hepatocellular carcinoma and gastric cancer[37]. Mutations of the Y641 residue within the catalytic SET domain of EZH2 occur in 22% of diffuse large B-cell lymphomas (DLBCLs) and follicular lymphomas (FL)[38, 39], and 8%–24% of non-Hodgkin lymphomas (NHL)[40]. Homology modeling[41, 42] and the solved *apo*-structure of the EZH2 SET domain[43, 44] have disclosed Y641 as a key amino acid for substrate specificity. Mutations of Y641 into other smaller amino acids, such as phenylalanine (F), serine (S), histidine (H) and cysteine (C) resulted in changes in pocket dimensions, as well as hydrogen bonding, which allowed the free rotation of H3K27me2 and subsequent tri-methylation to H3K27me3 (a transcriptionally repressive mark) and led to repression of key tumor suppressor genes[45, 46]. Therefore, EZH2 and PRC2 have been proposed as attractive drug targets for cancer therapy, which has inspired several pharmaceutical companies to launch high-throughput screening campaigns to discover inhibitors of EZH2.

Several selective EZH2/1 inhibitors have been reported, which could be divided into 3 series according to their different structures: pyridone-indazole scaffold (EPZ005687, UNC1999, and GSK343), pyridone-indole scaffold (GSK126 and EI1), and pyridone-phenyl scaffold (EPZ006088 and EPZ6438). EPZ005687 was the first small molecule reported by Epizyme[40] as a selective inhibitor of EZH2, with approximately 50-fold potent selectivity for EZH1 and more than 150-fold selectivity for 15 other HMTs. Docking results for EPZ005687 from the homology model of EZH2 built using GLP as a template indicated key hydrogen bonds (the central amide and N688, pyridone and H689) and hydrophobic interactions of the pyridone-indazole core with EZH2, excluding the morpholinomethyl group exposed to the solvent. Further structural modifications surrounding the morpholinomethyl moiety led to UNC1999[47] (N-isopropyl piperazine instead), with a desirable physicochemical property (clogP=3.1) that increased its oral bioavailability. It is worth noting that UNC1999 is a SAM-competitive, dual inhibitor of EZH2 and EZH1 (selectivity: ~10-fold), giving it an advantage in killing cancer cells such as MLL-rearranged acute leukemia, where H3K27 is methylated by both PRC2-EZH2 and PRC2-EZH1. GSK343[48], with an n-propyl group substituted on the 4-pyridone core, selectively inhibited EZH2 with an IC_{50} of 4 nmol/L. However, the high clearance in rat pharmacokinetics studies limited its use *in vivo*.

The representatives of a pyridone-indole scaffold such as GSK126 and EI1, which were reported almost at the same time as EPZ005687, were identified as selective and SAM-competitive inhibitors of EZH2. Different from EPZ005687, GSK126 showed *in vivo* activity, *ie*, subcutaneous dosing in nude mice with xenografts of KARPAS-422 and Pfeiffer cells (mutant DLBCL cell lines) dose-dependently decreased the level of global H3K27me3[49]. The structure of EI1 was reported to be similar to GSK126, with a cyano instead of a pyridine-piperazine group. It inhibited the proliferation of EZH2 mutant DLBCL cell lines with no effect on wild types[50].

Another pyridone-phenyl scaffold represented by EPZ006088[51] and EPZ6438[51] was reported by Epizyme in 2016. Modeling of EPZ005687 indicated that the amide group joining pyridone was out of plane owing to the forces of the indazole group, which presents a surrogate pyridone-phenyl core with a methyl group adjacent to the amide providing the possibility of steric-directing effects both on the amide and aniline. EPZ6438 (tazemetostat) was thus discovered with THP substituted on the aniline core. EPZ6438 dose-dependently inhibited H3K27Me3 levels and cell proliferation in human lymphoma cell lines including $EZH2^{WT}$ and EZH2 mutants (A682 and Y646). Further *in vivo* assays in mice showed low clearance and desirable bioavailability, which led to clinical trials in patients with lymphoma (DLBCL and FL) and INI1-deficient solid tumors[51].

SMYD2

SMYD2, a SET and MYND domain containing HKMT, methylates H2B, H3, H4[52, 53], and other non-histone substrates, including retinoblastoma tumor suppressor RbK860 and RbK810[54] as well as tumor suppressors p53K370[55] and HSP90[56]. SMYD2 is highly expressed in 75% of esophageal squamous cell carcinomas (ESCCs)[57] and 95% of pediatric acute lymphoblastic leukemias[58] with poor survival. All of these characteristics point to its potential as a drug target to treat cancers. Several small molecule inhibitors have been developed to investigate the roles of SMYD2 in carcinoma and other biological processes. LLY-507[56] was disclosed as a selective, substrate-competitive inhibitor for SMYD2 with more than 100-fold selectivity over other HMTs and non-HMTs, including its homolog SMYD3. Cell-based assays showed that LLY-507 dose-dependently inhibited the expression of p53K370me1 in U2OS and KYSE-150 cells (ESCC cell lines) and the proliferation of ESCC, HCC and breast cancer cells. However, no obvious changes were observed in the global histone methylation levels using mass spectrometry-based proteomics, suggesting that SMYD2 is mainly present in the cytoplasm. Two other inhibitors, AZ505 and A-893, have also been reported[59, 60]. AZ505 and A-893 have similar structures except for one hydroxy group translocating from the benzoxazinone in AZ505 to the linker in A-893. Both of these inhibitors were shown to be selective, substrate-competitive SMYD2 inhibitors. Cell-based assays indicated that these two compounds reduced the expression levels of p53K370me1 in human A549 lung carcinoma cells. The co-crystal structure of SMYD2 with AZ505/A-893 suggested that two hydrophobic pockets are present: a primary pocket occupied by the cyclohexyl group and a secondary pocket occupied by the dichlorophenyl moiety, with the benzoxazinone group occupying the lysine-binding channel. An additional hydrogen bond between the benzylic alcohol and Y240 was formed in the co-crystal structure of SMYD2 with A-893, which contributes to the increased potency of A-893 compared to AZ505 (>80-fold).

Conclusion

The human histone methyltransferases (HMTs) are a large class of enzymes (>50), many of which are associated with cell differentiation, gene regulation and DNA recombination. Overexpression of HMTs has been linked to a variety of human malignancies. Owing to their common characteristics in chemical catalytic reactions, HMTs are likely to be tractable as targets for drug discovery. At this point, several potent small molecules have been discovered and show inhibitory activities *in vitro* and *in vivo* on various cancer cells. Such inhibitors have been used either as chemical probes to study enzyme functions or as candidates for drug development. Although histone methylation has been studied extensively, investigation of non-histone methylation is still in its infancy, which calls for further systematic research.

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