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Review

Review

Targeting epigenetic regulations in cancer

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Abstract

Epigenetic regulation of gene expression is a dynamic and reversible process with DNA methylation, histone modifications, and chromatin remodeling. Recently, groundbreaking studies have demonstrated the importance of DNA and chromatin regulatory proteins from different aspects, including stem cell, development, and tumor genesis. Abnormal epigenetic regulation is frequently associated with diseases and drugs targeting DNA methylation and histone acetylation have been approved for cancer therapy. Although the network of epigenetic regulation is more complex than people expect, new potential druggable chromatin-associated proteins are being discovered and tested for clinical application. Here we review the key proteins that mediate epigenetic regulations through DNA methylation, the acetylation and methylation of histones, and the reader proteins that bind to modified histones. We also discuss cancer associations and recent progress of pharmacological development of these proteins.

Key words: epigenetic regulation, anti-cancer drugs, histone modification, DNA methylation, inhibitors

Introduction

Chromatin rolled up with genomic DNA is the basic building blocks of nucleosomes in eukaryotic cells. Nucleosome contains 147 base pairs of DNA, which is wrapped around a histone octamer with two each of histones H2A, H2B, H3, and H4 [1]. In general, there are two major structures in chromatin: heterochromatin and euchromatin. Heterochromatin contains most of inactive genes and is highly condensed. Euchromatin is different; it is more open and contains most of active genes. Gene expression is tightly and spatiotemporally controlled by chromatin structure, and chromatin structure is dynamically regulated by various chromatin modifications, including DNA methylation, histone modifications, nucleosome positioning, and chromatin remodeling [2]. A central dogma of oncology is that cancer is a disease that is initiated and driven by somatic aberrations of the genome. However, the expression or silencing of these genes, which harbor these somatic mutations, is essential for malignant transformation. Recurrent chromosomal translocations, involving transcriptional regulators in several hematopoietic cancers, illustrate the importance of transcriptional dysregulation in cancer development [3]. Therefore, deciphering how the expression or silencing of these genomic alterations culminates into malignant transformation will aid in the identification of novel therapeutic targets. The involvement of DNA methylation in cancer has been discovered for years and DNA methyltransferase (DNMT) inhibitor is the first epigenetic-based drug for cancer therapy. Nucleoside analogs azacitidine (5-azacytidine) and decitabine (5-aza-2′-deoxycytidine) targeting DNA methyltransferase enzymes DNMT1 and DNMT3 are the two approved drugs used for treatments of myelodysplastic syndrome [4]. Shortly after the approval of the two DNA methylation inhibitors, the two histone deacetylase (HDAC) inhibitors suberoylanilide hydroxamic acid (SAHA) and romidepsin were approved for the treatment of refractory cutaneous T-cell lymphoma [5,6]. Current technologies, such as next generation sequencing, have revealed a high frequency of somatic mutations in genes coding for chromatin-associated proteins that are known to regulate DNA methylation, post-translational modifications of histone and chromatin remodeling. *TET2* and *DNMT3A*, which are all involved in regulating DNA methylation patterns, are often found with mutation in leukemia patients [7,8]. This explained the significant response to DNA methylation inhibitors of leukemia patients. Therefore, new technologies will provide a platform for analyzing the complex network of genomic somatic mutations and transcriptional regulation by DNA and chromatin modifications, also will

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guide drug development in the future. In this review, we will discuss the association of DNA methylation, histone modifications, and reader proteins with cancer diagnosis and prognosis as well as their molecular mechanisms in cancer development and their potential for drug targeting.

General Concepts of Epigenetic Regulation

The term 'epigenetics' is the study of alterations caused by modifications in gene expression patterns, which occur during organismal development or during cell proliferation without any change in DNA sequences. Epigenetic regulations include DNA methylation, histone modifications, and RNA interference (RNAi) regulations. DNA methylation and histone modifications play crucial roles in the control of gene activity and nuclear architecture. DNA methylation is the most widely studied in epigenetic area. In normal human, 3%–6% of all cytosines are methylated [1]. On the other aspect, histone acetylation and methylation at specific lysine residues are critical for regulating chromatin structure and gene expression [9,10]. Thus, histone modifications, together with DNA methylation, is involved in regulating transcription and other nuclear processes (**Fig. 1**) [2]. Global alterations of histone modification patterns have the potential to affect the structure and integrity of the genome and to disrupt normal patterns of gene expression, including alterations in DNA methylation, might be causal factors in cancer [3].

Targeting Epigenetic Factors in Cancer

DNA methylation plays a critical role in controlling gene activity and nuclear architecture. In human cells, DNA methylation occurs on cytosines, which precede guanines. These dinucleotide CpGs [11,12] are typically CpG-rich regions, also known as CpG islands, and often appear at the 5′ ends of regulatory regions of many genes. DNA methylation is one of the layers of control of certain tissue-specific genes, such as *BRCA1* [1]. The hypermethylation of specific gene, which is mediated by DNMTs, will cause gene expression repression [13]. DNA methylation often occurs in the context of histone modifications [14].

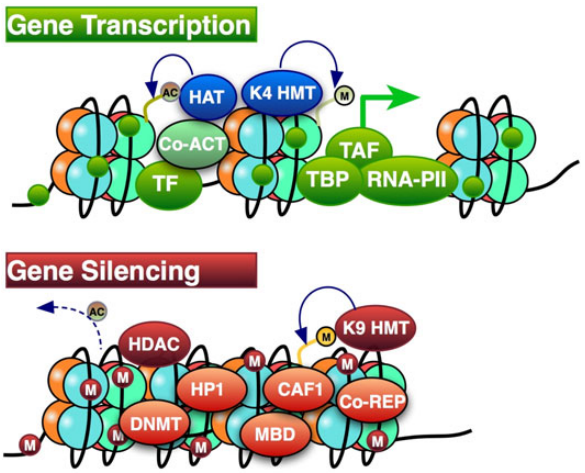

Figure 1. Epigenetic regulation of gene expression Generally, gene expression is controlled in the promoter regions by a combination of DNA methylation and chromatin configuration. In normal cells, active genes are those with open nucleosome spacing around the transcription start site, are unmethylated and associated with acetylated histones. By contrast, gene expression is silenced by condensing chromatin, methylating (at cytosine) DNA, and deacetylating histones. TF, transcription factor; Co-ACT, co-activator; TBP, TATA-binding factor; TAF, TBP-associated factor; RNA-PII, RNA polymerase II. HAT and histone H3 lysine-4 (K4 HMT) are indicated in blue. HDAC and lysine-9 (K9 HMT) are indicated in red. Proteins involved in transcriptional silencing are indicated in red (DNMT, DNA methyltransferase; MBD, methyl-binding domain protein; Co-REP, co-repressor; HP1, heterochromatin protein 1; CAF1, chromatin assembly factor-1).

DNA Methylation

DNA methylation and cancer

The methylation of the 5-carbon on cytosine residues (5 mC) in CpG dinucleotides was the first covalent DNA modification identified [15]. DNA methylation of DNA sequences occurs at a variety of locations within the genome, including centromeres, telomeres, inactive X-chromosomes, and repeat sequences [10,16]. In nephroblastoma cells, CpG islands are present in over 70% mammalian promoters. CpG island methylation plays an important role in transcriptional regulation, and it is commonly altered during malignant transformation [10,16]. Hypermethylation of the CpG islands, located within the promoter regions of tumor-suppressor genes, is a major event in the initiation of many cancers. Hypermethylation of CpG-island-containing promoter regions can affect genes regulating the cell cycle, DNA repair, metabolism of carcinogens, cell-to-cell interactions, apoptosis, and angiogenesis, all of which are involved in the development of cancer [11,17]. Hypermethylation occurs at different stages of cancer development and in different cells, and it associates with genetic lesions [18]. Abnormal hypermethylation at gene promoter regions can have as profound effects on cancer as a DNA sequence mutation in the coding-region of one copy of the gene, with further loss of the second copy of the gene (the second hit) initiating cancer development. However, in familial cancer, epigenetic changes do not constitute the initial driver of cancer development, but serve as the second hit. For example, von Hippel-Lindau (VHL) is a key regulator of the hypoxia response pathway, which is vital to tumor survival in low oxygen conditions; in renal cancers, hypermethylation of the promoter region of the *VHL* gene occurs almost exclusively rather than *VHL* gene mutations [19,20]. In addition, in breast and ovarian cancers, promoter hypermethylation is found in the tissue-specific gene *BRCA1*, which is involved in error-free repair of DNA double-strand breaks [21]. The methylation of DNA is facilitated by enzymes mediating maintenance methylation or *de novo* methylation.

DNMT inhibitors

Three DNMT enzymes have been identified, DNMT1, DNMT3A, and DNMT3B. DNMT3A and DNMT3B are responsible for establishing *de novo* methylation patterns, which are then maintained by DNMT1 [22]. The first compounds demonstrated to inhibit DNMT activity and to lead to DNA hypomethylation in cells were the cytidine analogs, 5-azacytidine/vidaza (AZA), and 5-aza-2′-deoxycytidine/dacogen (DAC) [4]. Although early clinical trials using these compounds were designed to test the cytotoxic effects of the inhibitors, the presence of a nitrogen molecule at the 5 position of the azacytidine nucleobase results in an irreversible covalent complex with DNMT1 [23], triggering proteasome-mediated DNMT1 degradation [24]. In the past decade, clinical trials for MDS and acute myeloid leukemia (AML) have employed low doses of AZA and DAC along with treatment schedules, allowing for efficient DNA incorporation with minimal toxicity, and have shown clear clinical benefits. AZA was approved by the U.S. Food and Drug Administration (FDA) in 2004 for the treatment of MDS (Table 1). In a phase III trial, patients treated with AZA showed an improvement in objective clinical response rates

Table 1. DNA methylation inhibitors for cancer treatment

DNMT inhibitor	Compound	Phase	Tumor type	Reference
Vidaza	5-Azacytidine	Approved	MDS	[25]
Decitabine	5-Aza-2′-deoxycytidine	Approved	MDS	[26]
Guadecitabine	SGI-110	Phase II	MDS, CMML, AML	NCT01261312
DHAC	5-6-Dihydro-5-azacytidine	Phase II	Melanoma	[27]

MDS, myelodysplastic syndrome; CMML, chronic myelomonocytic leukemia; AML, acute lymphocytic leukemia.

and a reduction in transfusion requirements relative to best supportive care [28]. DAC was also approved by FDA in 2006 for the treatment of MDS [4], based on objective clinical responses, 16% (15% complete response rate and 1% partial response), and transfusion independence, relative to best supportive care, in a randomized phase III trial [29]. Using preclinical dose and scheduled regimens of DAC for optimal DNA hypomethylation [30], subsequent clinical trials demonstrated significantly improved results, including 39% complete responses [31], and an overall improvement rate of 51% [32]. Similar results were observed in a recent phase II study of DAC in older patients with AML, including a 27% complete response rate [33]. In addition, the clinical benefits of DAC in MDS patients appear to be mechanism-specific and strongly correlate with DNA hypomethylation [26]. However, the metabolic instability of AZA and DAC has prompted the development of modified analogs, including SGI-110. SGI-110 is a dinucleotide that includes a deoxyguanosine (5′-DACpG-3′) and is largely resistant to cytidine deaminase-mediated destabilization [34]. SGI-110 has shown activity in cancer xenograft mouse models, has exhibited DNA hypomethylation activity in primates [35], and is currently being tested in patients with MDS and AML in a phase I clinical trial (**Table 1**).

DNA demethylation and cancer

Alternative to enzymes responsible for DNA methylation, high-resolution genome-wide mapping in pluripotent and differentiated cells confirmed the existence of an enzymatic activity within mammalian cells that either erases or alters this DNA modification [16]. In 2009, two papers describing the presence of 5-hydroxymethylcytosine (5 hmC) offered the first evidence for the recognition and hydroxylation of methylated nucleotides [36,37]. The ten-eleven translocation (TET) family of DNA hydroxylases convert 5-methylcytosine (5 mC) into 5 hmC. Consistent with the notion that 5 hmC is likely to play a role in both transcriptional activation and silencing, the TET proteins also have activating and repressive functions [38]. The TET proteins act as essential intermediates in the process of both active and passive DNA demethylation. Genome-wide mapping of TET1 has demonstrated its strong preference for CpG-rich DNA and its localization to regions enriched in 5 mC and 5 hmC. TET proteins preclude or enhance the binding of several methyl-CpG-binding domain (MBD) proteins, mediating local and global effects by altering the recruitment of chromatin regulators. In a subset of AML patients with chromosomal abnormalities, TET proteins were shown to be involved in these chromosomal rearrangements [39]. In addition, mutations in *TET2* are associated with numerous hematological malignancies [40–42]. Moreover, *Tet2*-deficient mice develop a chronic myelomonocytic leukemia (CMML) phenotype, further suggesting direct involvement of TET proteins in cancer [43,44]. The clinical implications of *TET2* mutations have been largely inconclusive; however, in some subsets of AML patients, *TET2* mutations appear to confer a poor prognosis [45]. Early research of TET2-mediated oncogenesis revealed that patient-associated mutations are largely loss-of-function mutations. Moreover, mutations in *TET2* also appear to confer enhanced self-renewal properties and malignant transformation, suggesting that alterations in this intermediate step in DNA demethylation contributes to cancer development [40]. To date, no TET protein inhibitor has been clinically tested for cancer treatment.

Histone Modifications

Histones are not merely DNA-packaging proteins, but molecular structures, regulating gene expression. Histones store epigenetic information through post-translational modifications such as lysine acetylation, arginine and lysine methylation, and serine phosphorylation (**Fig. 2**). These modifications affect chromatin structure, and thus, gene transcription and DNA repair. It has been proposed that the sequence and type of histone modifications form a 'histone code' [46]. The functional consequences of the methylation of histones depend on the type of residue lysine (K) or arginine (R) and the specific site that the methylation modifies (e.g. K4, K9, or K20) [47,48]. Methylation of K4 on histone 3 (H3) is closely linked to transcriptional activation [49], whereas methylation of K9 or K27 on H3 and of K20 on H4 is associated with transcriptional repression (**Fig. 2**). Conversely, acetylation of histone lysines is generally associated with transcriptional activation [47,48].

Histone acetylation

The N-acetylation of lysine residues is a major histone modification involved in transcription, chromatin structure, and DNA repair. Histone acetylation is often associated with a more 'open' chromatin conformation. Acetylation is highly dynamic and is regulated by the competing activities of two enzymatic families, the histone lysine acetyltransferases (KATs) and the HDACs. KATs were the first enzymes shown to modify histones. The importance of these findings in cancer was immediately apparent, as one of these enzymes, CBP, was identified by its ability to bind to the malignancy transforming portion of the viral oncoprotein E1A [50]. In addition, numerous examples of recurrent chromosomal translocations [e.g. *MLL-CBP* [51] and *MOZ-TIF2* [52] or coding mutations (e.g. *p300/CBP* [53,54])], involving various KATs, are found in a broad range of solid and hematological malignancies. Although there is only modest structural homology between the different families of KATs, developing specific inhibitors (KAT-I) has proved to be challenging [55]. However, recent progress with derivatives of the naturally occurring KAT-I, such as curcumin, anacardic acid, and garcinol, as well as the synthesis of novel chemical probes, suggests that therapeutically targeting the various KATs with some specificity is likely to be achieved in the near future [55]. However, histones are not the only proteins that can be acetylated. It is also well-established that several non-histone proteins, including many oncogenes and tumor suppressors, such as MYC, p53, and PTEN, are also dynamically acetylated [56]. Therefore, both histone acetylation and non-histone acetylation are likely to be important in cancer development and should be taken into consideration when developing therapeutics [57].

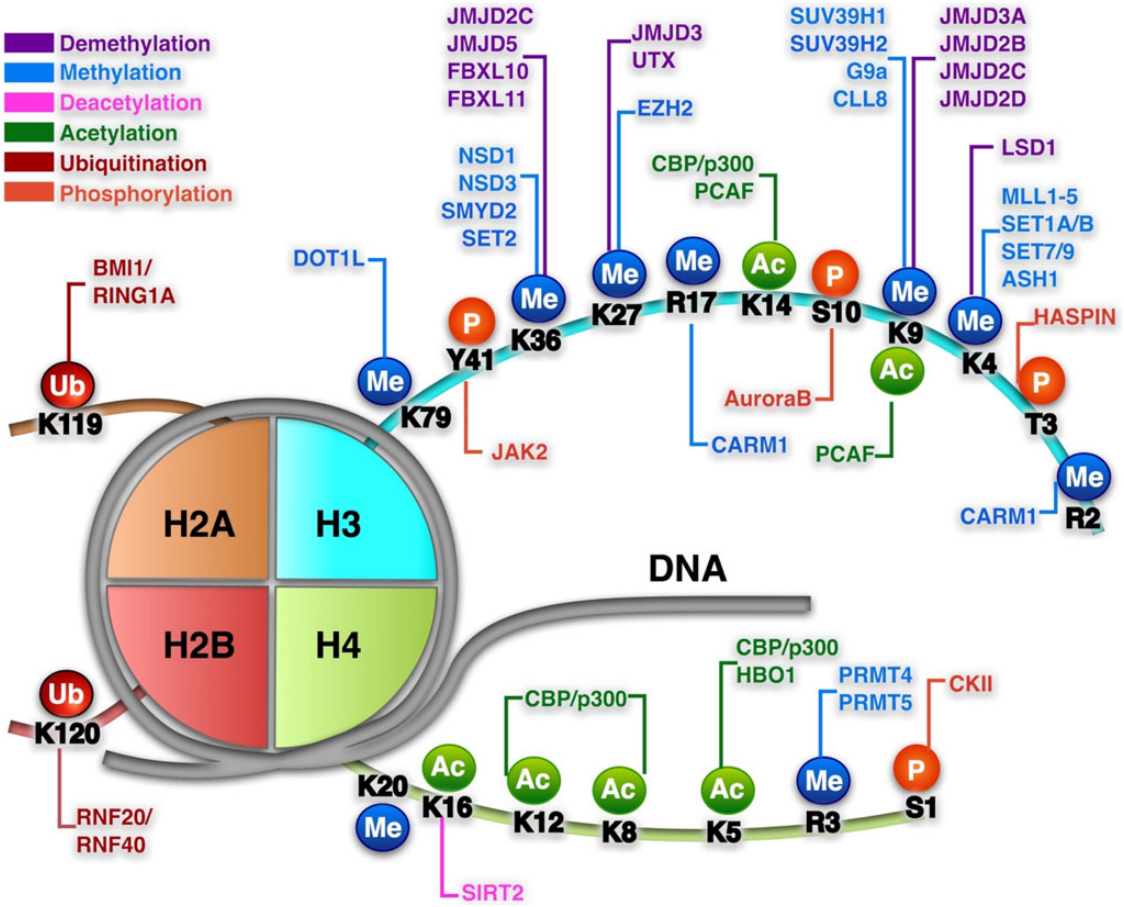

Figure 2. Histone modifying enzymes The histone octamer is assembled from a histone H3:H4 tetramer and two H2A:H2B dimers. The histone tails of all four core histones are subject to a variety of post-translational modifications, including methylation (Me), acetylation (Ac), phosphorylation (P), and ubiquitination (Ub). These modifications are controlled by enzymes such as: histone methyltransferases (HMTs) (dark blue), HDMs (purple), HATs (dark green), HDACs (pink), kinases (orange), and ubiquitin-conjugating enzymes (dark red).

Histone deacetylation

Inhibitors of HDACs are able to reverse gene repression, which are observed in malignancies. These HDAC inhibitors can induce growth arrest, differentiation, and apoptosis in malignant cells [58,59]. Based on impressive preclinical and clinical data, two pan-HDAC inhibitors, Vorinostat and Romidepsin, have recently been granted FDA approval [60,61] for patients with cutaneous T cell lymphoma (Table 2). Although somatic gene mutations in HDACs do not appear to be prominent in cancer, the activity levels of various HDACs appear to be altered in numerous malignancies (Table 2). Consequently, several novel HDAC inhibitors are currently under investigation for clinical use (Table 2) in a broad range of cancers [58,59]. However, the pleiotropic effects of HDACs continue to pose significant challenges in dissecting the specific effects on histone and non-histone proteins [75]. Recent discoveries make HDACs even more important in different cancers. Mutations in histone acetyl transferase (HAT) genes *CREBBP* and *EP300* were found in tumors [76], indicating decreased tumor acetylation in these patients. So HDAC inhibitors could help re-establish epigenetic controls. HDAC6 inhibitor ricolinostat in phase II trials in multiple myeloma and lymphoma, according to the company's report, has 10 to 20-fold lower potency for HDAC1, HDAC2, and HDAC3 than do the approved pan-HDAC inhibitors. More companies are working on preclinical studies on inhibitors of HDAC1 and HDAC2, of HDAC3, or of HDAC8. HDAC inhibitors reactivate gene expression of cell cycle regulatory proteins to slow down cell growth. However, there were a lot of concerns regarding toxic side effects of HDACs, typically including fatigue, thrombocytopenia, gastrointestinal toxicity, and cardiotoxicity. However, most clinical trials showed that these side effects are manageable [77,78].

Histone methylation

Histones are methylated on the side chains of arginine, lysine, and histidine residues. Lysines may be mono-, di-, or tri-methylated, and arginine residues may be symmetrically or asymmetrically methylated. The best-characterized sites of histone methylation are those that occur on lysine residues, such as H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20 (**Fig. 2**). Specific lysine methylations (H3K4, H3K36, and H3K79) are often associated with active genes in euchromatin ('open chromatin'), whereas others (H3K9, H3K27, and H4K20) are associated with heterochromatin ('closed chromatin') [79]. However, different methylation states, i.e. mono- (me), di- (me2), and tri-methylation (me3), may preferentially localize to different genomic regions. For instance, H3K4me2/3 usually spans the transcriptional start site (TSS) of active genes [80], whereas H3K4me1 is a modification associated with active enhancers [80]. Similarly, monomethylation of H3K9 is typically observed at active genes, and trimethylation of H3K9 is associated with gene repression [79].

Table 2. Histone acetylation inhibitors for cancer treatment

HDAC inhibitor target	Compound	Tumor	Clinical status	Reference
HDAC1, HDAC2, HDAC3, and HDAC6	Vorinostat	Colorectal, breast	Approved	[5,6]
HDAC1, HDAC2, HDAC3, and HDAC8	Romidepsin	T-cell lymphoma	Approved	[5,6]
HDAC1, HDAC2, HDAC3, and HDAC6	Panobinostat	Multiple lymphoma	Approved	FDA.gov
HDAC1, HDAC2, HDAC3, and HDAC6	Belinostat	T-cell lymphoma, pancreatic, MDS	Approved	FDA.gov
HDAC1 and HDAC2	Entinostat	Non-small cell lung cancer, breast	Phase II	[5,62]
HDAC1 and HDAC2	Mocetinostat	Hodgkin's lymphoma	Phase II	[5,63,64]
HDAC1, HDAC3, and HDAC6	Resminostat	Colorectal, liver	Phase II	[65,66]
HDAC (class I and II)	Givinostat	Inflammation, oncology	Phase II	[67]
Pan-HDAC	SB939	Prostate	Phase II	[68,69]
HDACs	CUDC-101	Squamous cell carcinoma	Phase Ib	[70]
HDAC (class I and II)	PCI-24781	B-cell lymphoma	Phase I/II	[71]
HDAC (class I and II)	AR-42	ALL	Phase I	[72]
Pan-HDAC	CG200745	Non-small cell lung cancer	Phase I	[73,74]

MDS, myelodysplastic syndrome; ALL, acute lymphocytic leukemia.

Table 3. Histone methylation/demethylation inhibitors for cancer treatment

Inhibitor	Compound	Phase	Tumor type	Reference
HMT inhibitors				
DOT1L inhibitors	EPZ00477	Preclinical	MLL-rearranged leukemia	[86]
	EPZ-5676	Clinical trials	Hematological malignancies	[86]
	SGC0946	Preclinical	Leukemia	[87]
EZH2 inhibitors	DZNeP	Preclinical	Breast, colon, prostate,	[88]
	GSK126	Preclinical	EZH2 mutant lymphomas	[89]
	GSK343	Preclinical	Epithelial ovarian cancer	[90]
	EPZ005687	Preclinical	EZH2 mutant lymphomas	[91]
	EI1	Preclinical	DLBCL	[92]
	EPZ-6438	Clinical trials	Advanced solid tumors, B-cell lymphoma	[93,94]
	UNC1999	Preclinical	DLBCL	[95]
SUV39H1 inhibitor	Chaetocin	Preclinical	AML	[96,97]
G9A inhibitors	BIX-01294	Preclinical	Neuroblastoma	[98]
	UNC0638	Preclinical	AML, Breast	[97,99]
	BRD4770	Preclinical	Pancreas	[100]
SETDB1 inhibitor	Mithramycin	Preclinical	Non-small cell lung cancer	[101]
HDM inhibitors				
LSD1 inhibitors	Tranylxypromine	Preclinical	AML	[102]
	ORY-1001	Clinical trials	Relapsed or refractory acute leukemia	[103]
JMJC domain-containing inhibitors	Pyridine hydrazone	Preclinical	Enzymatic inhibition	[104]
	GSK-J1	Preclinical	Enzymatic inhibition	[105]
	GSK-J4	Preclinical	H3K27M glioma	[106]

The enzymatic protagonists for lysine methylation contain a conserved Su(var)3-9, Enhancer-of-zeste and Trithorax (SET) domain which possesses methyltransferase activity. The only exception is hDOT1L, the enzyme that methylates H3K79 (**Fig. 2**). In contrast to the KATs, the histone lysine methyltransferases (KMTs) tend to be highly specific enzymes, specifically targeting certain lysine residues.

EZH2 is the catalytic component of the PRC2 complex, which is primarily responsible for the methylation of H3K27 (**Fig. 2**). Early gene expression studies implicated *EZH2* over-expression as a progressive event, which conferred a poor prognosis in prostate and breast cancer patients [81], suggesting that *EZH2* was an oncogene. However, targeted resequencing of cancer genomes has recently identified coding mutations within EZH2 in various lymphoid and myeloid neoplasms, suggesting that EZH2 may be both oncogenic and tumor-suppressive. Heterozygous missense mutations resulting in the substitution of tyrosine 641 (Y641) within the SET domain of EZH2 were noted in 22% of patients with diffuse large B-cell lymphoma (DLBCL) [82]. Functional characterization of this mutation demonstrated that it conferred increased catalytic activity and a preference for converting H3K27me1 to H3K27me2/3, further supporting the contention that *EZH2* is an oncogene [83]. In contrast, loss-of-function mutations in the *EZH2* gene have been described in myeloid malignancies [84,85] and in T-cell acute lymphoblastic leukemia (T-ALL) (**Table 3**) and are associated with poor prognosis. Fiskus *et al.* [107] reported that the EZH2 inhibitor, DZNep, induced cell cycle genes p16, p21, and p27 expression, while development gene HOXA9 decreased. In addition, DZNep treatment induced apoptosis in cultured and primary AML cells. Furthermore, combination treatment with DZNep and the pan-histone deacetylase inhibitor, panobinostat, caused more reduced activities of EZH2, induced more apoptosis of AML cells, but not normal $CD34^+$ bone marrow progenitor cells, and significantly improved survival of non-obese diabetic/severe combined immunodeficiency mice with HL-60 leukemia compared with treatment using either single agent alone. These findings indicate that the

combination of DZNep and panobinostat is an effective and relatively selective epigenetic therapy against AML, B-cell lymphomas, and follicular lymphomas [107]. To investigate if tumor growth is dependent on the enzymatic activity of Ezh2, a potent and selective small-molecule inhibitor EI1 was developed. EI1 inhibits the enzymatic activity of Ezh2 through direct binding to the enzyme and competing with the methyl group donor S-adenosyl methionine [92,107]. EI1-treated cells exhibit genome-wide loss of H3K27 methylation and activation of PRC2 target genes. Furthermore, inhibition of Ezh2 by EI1 in diffused large B-cell lymphoma cells, carrying the Y641 mutations, results in decreased proliferation, cell cycle arrest, and apoptosis. These results provide strong validation of Ezh2 as a potential therapeutic target for the treatment of cancer (**Table 3**) [92]. Another inhibitor, GSK126, effectively inhibits the proliferation of EZH2 mutant DLBCL cell lines and markedly inhibits the growth of EZH2 mutant DLBCL xenografts in mice. Together, these data demonstrate that pharmacological inhibition of EZH2 activity may provide a promising treatment for EZH2 mutant lymphoma [89]. In June 2013, Epizyme and Eisai initiated a phase 1/2 clinical trial of EPZ-6438 (E7438) in patients with advanced solid tumors or B-cell lymphomas. In preclinical research report from Epizyme, EPZ-6438 shows killing abilities in EZH2 mutant-bearing lymphoma cell lines, and broadened the activity of EPZ-6438 to all germinal center NHL cell lines. Combining EPZ-6438 with dexamethasone, another corticosteroid, yielded similar synergistic results. In three dose cohorts completed to date: maximum tolerated dose (MTD) not reached; pharmacokinetics (PK) were dose-proportional; pharmacodynamic (PD) evidence of target inhibition was observed; objective responses were seen in two NHL patients [108]. Two NHL patients achieved objective responses: a partial response in a patient with relapsed transformed DLBCL, and an ongoing partial response in a patient with primary refractory mediastinal B-cell lymphoma (PMBCL) [93].

Histone demethylation

The initial notion that histone lysine methylation was a highly stable, non-dynamic modification has now been irrefutably overturned by the identification of two classes of lysine demethylases [109]. Histone demethylase (HDM) proteins have a variety of domains with different functions and these multisubunit complexes confer their target specificity. These functions include binding to the histone, recognizing the correct methylated amino acid substrate, catalyzing the reaction, and binding of cofactors. For example, lysine-specific demethylase 1 (LSD1, KDM1A) belongs to the first class of demethylases that demethylates lysines via an amine oxidation reaction with flavin adenine dinucleotide (FAD) as a cofactor. Another example is the Jumonji demethylases. Jumonji demethylases have a conserved JMJC domain which functions via an oxidative mechanism and free radical attack (involving Fe^{2+} and α-ketoglutarate). In addition, mutations in *UTX*, a member of Jumonji demethylases, are prevalent in a large number of solid and hematological cancers, suggesting that histone demethylation plays an important role in cancer. Small-molecule inhibitors of the two families of HDMs are at various stages of development, and this interest will be spurred on by emerging preclinical data showing the therapeutic potential of compounds, inhibiting LSD1/KDM1A in AML [102,110].

Inhibitors of HDMs

HDMs are considered to be putative drug targets, because several members of this family play a role in diseases and are thought to be druggable proteins. This is assumed to be due to the well-defined active site cavity of HDMs, which is generally a good structural feature for the development of high-affinity and selective small-molecule inhibitors. Although inhibitors of LSD1 and JMJC family proteins have been developed, HDM inhibitors are still in its infancy. Accumulating information from biochemical, structural, and *in vivo* biological characterizations of the known small-molecule inhibitors will further progress drug development (**Table 3**).

LSD1 inhibitors

The recent demonstration that LSD1 is required for the development and maintenance of AML has gained the most attention [102,111]. Specifically, both genetic and pharmacological *in vitro* and *in vivo* data indicate the requirement of LSD1 in sustaining the expression of genes, induced by the MLL-AF9 oncoprotein; and thus, mediating leukemia stem cell maintenance [111]. A plethora of data have suggested that LSD1 could be a potential therapeutic target in cancer, because of its high expression levels in prostate cancer, undifferentiated neuroblastoma, oestrogen-negative breast cancer, bladder cancer, and colorectal cancer [112,113]. In addition, the catalytic domains of the LSD proteins share sequence homology with monoamine oxidase A (MAOA) and monoamine oxidase B (MAOB), in which many inhibitors are available. However, non-selective monoamine oxidase inhibitors may lead to adverse effects and may not be ideal cancer therapeutics (**Table 3**).

Tranylcypromine (TCP) was the first developed LSD inhibitor [102]. Subsequently, ORY-1001, developed by the biotech company, Oryzon Genomics [102], is even more specific and 1000-fold more potent than TCP [111]. It reduces leukemogenesis and stem cell colony formation, and induces differentiation of AML cell lines at subnanomolar concentrations [102]. Moreover, ORY-1001 leads to a time- and dose-dependent increase in H3K4me2 levels at LSD1 target genes, and to increased differentiation and differentiation markers (e.g. CD11b), followed by cellular apoptosis in AML cell lines. Inhibition of LSD1 activity is not associated with a global increase in H3K4me2 levels, but is associated with increased H3K4me2 levels at MLL-AF9-bound genes and genes involved in differentiation [102,111]. Taken together, these studies provide a proof of concept that LSD1 is a therapeutic target in leukemia. However, apart from the TCP derivatives, no truly promising drug candidates that selectively target LSD1 have so far been published. Future development of LSD1-specific inhibitors may lead to novel therapeutics, targeting not only leukemia, but also other cancers.

Inhibitors of JMJC demethylases

Inhibitors of JMJC demethylases have been considered to be lead structures for drug development. Most of the reported inhibitors are metal chelators that bind competitively with the 2-oxoglutarate cofactor [114,115]. A high-throughput screening effort with an *in vitro* enzymatic assay identified 8-hydroxyquinolines as pan-specific low micromolar JMJC demethylase inhibitors (**Table 3**) [116]. A derivative of this inhibitor was recently prepared and used to block herpes simplex virus (HSV) reactivation in a mouse-derived explant model [117]. JMJD3 is associated with inflammatory responses and reprograming [118,119]. Interestingly, recent studies have shown a relationship between up-regulated JMJD3 and cancer progression. JMJD3 is up-regulated in prostate cancer with higher expression levels in metastatic prostate cancer [120]. Therefore, JMJD3 may be a promising therapeutic target for cancer treatment.

One of the most potential leading compounds reported thus far is GSK-J1 [105], which is a JMJD3 inhibitor. This compound has an IC_{50} in the submicromolar range and is remarkably selective for the JMJD subfamily (UTX, JMJD1, and JMJD2) [105]. GSK-J1 binds competitively to the 2-oxoglutarate cofactor and has the functionality

to chelate the Fe^{2+} within the active site. This chelating interaction is likely to significantly contribute to the affinity of the inhibitor. The observation of the flexibility of the metal position within the active site is an important finding [105]. When GSK-J1 and GSK-J4 are bound, the active Fe^{2+} is repositioned and one of the interactions with a residue of the facial triad is made through an intermediate water molecule. As chelation may be critical for the binding of the inhibitor deep into the catalytic site, this flexibility of the metal position may allow for a wider set of inhibitor scaffolds, and thus, increase the likelihood of finding a more selective inhibitor [121].

In 2012, Schwartzentruber *et al.* [122] found mutations in histone H3.3 (H3K27M) in pediatric glioblastoma. H3K27M inhibits the enzyme activity of PRC2 through interaction with the EZH2 subunit [123]. JMJD3 inhibitor GSKJ4, which was found to increase H3K27 methylation by inhibiting K27 demethylase, is a potential drug for H3K27M mutation glioblastoma [106]. GSKJ4 treatment of K27M-expressing cells is dose-dependent for inhibition of cellular viability. GSKJ4 also completely inhibited the clonal growth of all K27M-expressing cells but had no effect on the clonal growth of the cells expressing wild-type H3.3 [106].

Histone phosphorylation and ubiquitylation

Besides methylation and acetylation, phosphorylation and ubiquitylation also affect the functions of histone. Aurora-B is a chromosome passenger protein required for phosphorylation of histone H3S10 and cytokinesis [124]. Three Aurora-kinase inhibitors have recently been described, ZM447439, Hesperadin, and VX-680. All three inhibitors induce similar phenotypes in cell-based assays and, interestingly, VX-680 shows antitumor activity in rodent xenograft models. However, Aurora-B might be an anticancer drug target not for H3K9 phosphorylation but simply because inhibition of Aurora-B rapidly results in a catastrophic mitosis, which leads to cell death [125,126].

Another kinase for histone phosphorylation (H3Y41p) is Janus-associated kinase 2 (JAK2) which is one member of a family of cytoplasmic tyrosine kinases. The JAK enzymes are required for signaling by cytokine and growth factor receptors that lack intrinsic kinase activity [127,128]. Discovery of an activating tyrosine kinase mutation known as JAK2V617F in myeloproliferative neoplasms (MPNs), including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), has generated a great deal of interest in targeting [128]. A phase I-II clinical trial showed that JAK inhibitor INCB018424 is associated with marked and durable clinical benefits in patients with myelofibrosis. With a 15-mg twice-daily starting dose, 52% patients had a rapid objective response lasting for 12 months or more [128]. Other JAK2 inhibitors Lestaurtinib and Pacritinib (SB1518) are under clinical trials for myelogenous leukemia (AML) [129], relapsed lymphoma, and advanced myeloid malignancies [130].

Histone ubiquitylation, which is mediated by polycomb-repressive complex 1 (PRC1), also plays an important role in gene silencing in human. PRT4165 is reported as a potent inhibitor for PRC1-mediated H2A ubiquitylation *in vivo* and *in vitro*. *In vitro* E3 ubiquitin ligase activity assays showed that PRT4165 inhibits both RNF2 and RING 1A, which accounts for the E3 ubiquitin ligase activity in PRC1 complexes. So PRT4165 is considered as a potential target for cancer therapy [131].

Targeting Epigenetic Readers

In epigenetic regulation, enzymes, such as HMTs, are considered as 'writers', enzymes which remove these modifications are considered 'erasers', and proteins which specifically recognize and target these modifications are considered as 'readers'. These 'readers' include malignant brain tumor (MBT), bromodomain and extraterminal (BET) proteins, and plant homeodomain (PHD) finger proteins. Many chromatin binding proteins including several catalytic enzymes act as 'chromatin readers', possessing specialized domains that bind to distinct nucleosomal covalent modifications in response to upstream signaling cascades [132]. Histone lysine residues are capable of harboring at least eight different covalent modifications, including acetylation, methylation, ubiquitylation, and sumoylation. Further complexity exists, because each lysine residue may be unmethylated, monomethylated, dimethylated, or trimethylated. Critical residues within the binding pocket of chromatin reader domains confer a preference for specific modification states, whereas residues outside the binding pocket determine histone-sequence specificity (**Fig. 3**). This modular combination allows proteins with similar binding domains to dock at different modified residues, or at the same amino acid, displaying a different modification state. For instance, proteins with a similar PHD finger domain can bind to different methylated histones. PHD fingers of the proteins BHC80 and DNMT3L preferentially bind to unmethylated lysine residues [134,135], whereas the PHD finger of ING2 binds most avidly to dimethylated and trimethylated lysines (**Fig. 3**) [136]. Additional lysine residue modifications, such as acetylation, may further provide docking sites for other proteins containing acetyl–lysine binding domains, such as bromodomains [137]. Finally, to add to the complexity, many chromatin regulators have more than one type of reader domain, and their chromatin binding can be further influenced by neighboring histone modifications, the so-called multivalent engagement-of-histone modifications [138].

BET protein inhibitors

Mutations that abrogate the chromatin-reading capacity of many epigenetic regulators play an influential role in a variety of diseases, including cancer [139]. Using these chromatin reader modules as therapeutic targets may offer a unique opportunity to tailor therapies to specific diseases. Recently, small molecules have been developed that specifically and avidly inhibit the tandem bromodomains of BET family of proteins [140]. Bromodomains are highly conserved motifs present in a number of proteins [137]. More than 40 different human proteins contain a bromodomain, and some have multiple bromodomains. Although the acetyl–lysine binding pocket for all bromodomains is hydrophobic, considerable variations exist in the electrostatic interactions at the pocket opening among bromodomain families. This variation determines the specificity of individual bromodomains and provides an opportunity to develop specific small molecules that target against certain families of bromodomains, including the BET proteins [141].

The BET family has four members, including bromodomain-containing proteins 2, 3, and 4 (BRD2, BRD3, and BRD4) whose expressions are ubiquitous, and BRDT whose expression is confined to germ cells. Early studies characterizing the BET proteins showed that BRD4 is associated with the mediator complex [142], an important multicomponent protein complex facilitating the initiation of transcription [143]. BRD4 is also associated with the active form of positive transcription elongation factor b (P-TEFb), a complex that regulates RNA polymerase II activity at the onset of transcriptional elongation [144]. Underscoring the physiological importance of the BET proteins in cellular homeostasis is the observation that the experimental knockout of either *Brd2* or *Brd4* in mice results in early

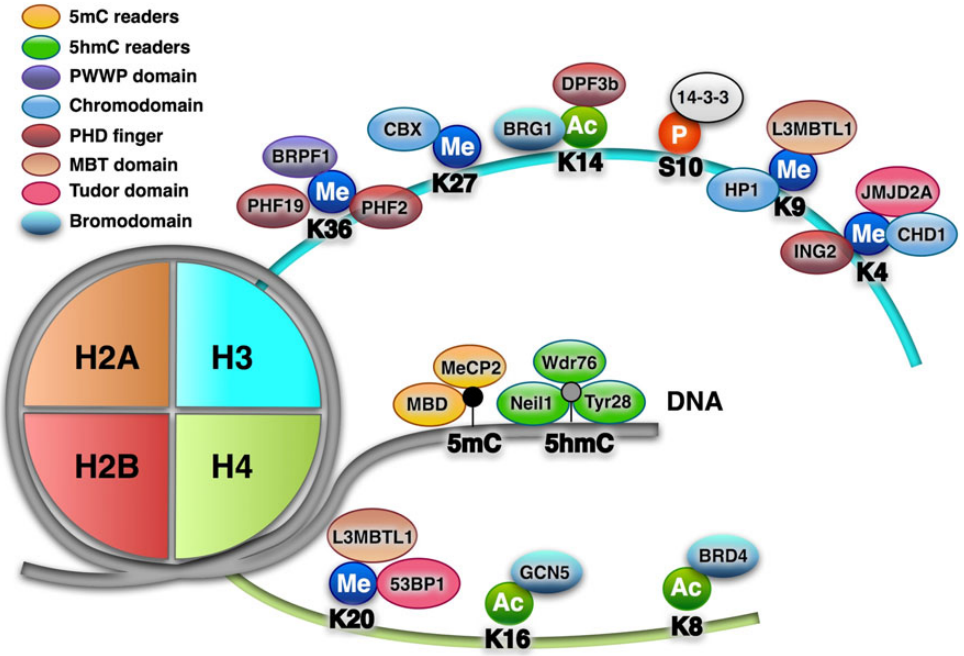

Figure 3. Epigenetic readers Epigenetic reader domains consist of specialized protein–protein interaction motifs, which recognize and discriminate between various post-translational modifications. These domains include: PWWP domains (purple), Chromodomains (light blue), PHD fingers (dark red), MBT domains (light red), Tudor domains (pink), Bromodomains (blue), and Methylation binding (MBT) domains (orange). 5-methylcytosine (5mC) readers (yellow) and 5-hydroxymethylcytosine (5 hmC) readers (green) [133].

Table 4. Epigenetic reader inhibitors for cancer treatment

Reader domain	Compound	Phase	Tumor type	Reference
Bromodomain inhibitors				
BET inhibitors	JQ1	Preclinical	MLL-rearranged leukemia	[147]
	dBET-1	Preclinical	Breast cancer, leukemia	[148]
	I-BET151	Preclinical	Hematological malignancies	[149,150]
	I-BET762	Clinical trials	Leukemia	NCT01943851, NCT10587703
BAZ2B inhibitor	GSK2801	Preclinical	Breast, colon, prostate	SGC website
Chromodomain inhibitors				
L3MBTL1 inhibitor	UNC669	Preclinical	Epithelial ovarian cancer	[151]
L3MBTL3 inhibitor	UNC1215	Preclinical	EZH2 mutant lymphomas	[152]

MLL, myeloid/lymphoid leukemia; NCT01943851 and NCT10587703, from https://clinicaltrials.gov; SGC website, http://www.thesgc.org/chemical-probes/GSK2801.

embryonic lethality [145,146]. In addition, RNAi screening strategies have identified an important role for BRD4 in AML. These findings have provided the impetus for investigating the potential of BET bromodomain inhibitors as novel anticancer agents (Table 4).

Chinnaiyan and colleagues [153] have found that the BET family of chromatin readers might be one such target. Treatment of a panel of prostate cancer cell lines with the small-molecule BET inhibitor, JQ1, revealed that cells with activated androgen receptor (AR) signaling were sensitive to JQ1-induced apoptosis and cell cycle arrest. JQ1 also globally reduces AR target gene transcription in AR-positive cells, suggesting that BET family proteins are involved in AR-mediated transcriptional programs. One of the proposed major targets for JQ1 is MYC, and MYC levels and MYC-associated gene transcription were reduced by JQ1. However, these authors found that loss of MYC did not completely phenocopy the effects of JQ1 treatment, nor did MYC expression rescue JQ1-mediated effects on cell growth, indicating that other factors also play a role in these cells [153]. A new competitive antagonist of BET bromodomains, dBET1, was developed recently. dBET1 induced highly selective cereblon-dependent BET protein degradation, which is stronger than JQ1 *in vitro* and *in vivo*, and delayed leukemia progression in mice [148].

MBT protein inhibitors

The methyl-lysine reader modules are principally made up of the 'Royal family', including MBT domain, Tudor domain, and Chromodomain proteins [154]. The Tudor domains (named for the Tudor gene in drosophila) have 60 amino acids arranged in 4–5 antiparallel β-sheets to form a barrel-like structure [155,156]. Of the 30 mammalian Tudor containing proteins identified to date, several have been associated with cancer. For example, Tumor domain-containing protein 1 (TDRD1) is over-expressed in ERG-rearranged prostate cancer [157]. L3MBTL3 is a member of MBT proteins, and its loss

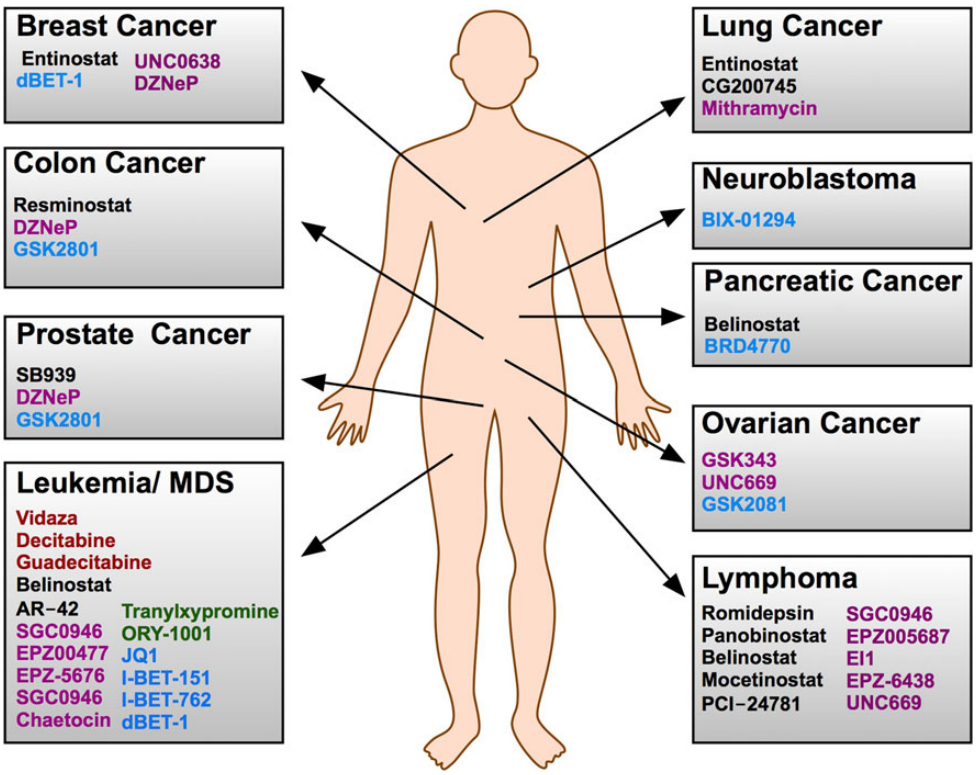

Figure 4. FDA-approved and on-clinical-trial epigenetic drugs for cancer therapy The first generation of FDA-approved epigenetics-based drugs has firmly established that epigenetic modulation is a viable treatment option for a growing list of diseases. So far, epigenetic drugs (especially for DNMT and HDAC inhibitors) have been approved mainly for the treatment of blood cancers, in particular myelodysplastic syndromes (MDS), leukemia, and lymphoma. Recently, HMT and HDM inhibitors start clinical trials for various cancers, including breast cancer, colon center, lung cancer, prostate cancer, ovarian cancer, and neuroblastoma. Epigenetic drugs for cancer treatment including: DNMT inhibitors (red), HDAC inhibitors (black), HMT inhibitors (purple), HDM inhibitors (green), and epigenetic reader inhibitors (blue).

contributes to the development of $20q^-$ hematopoietic malignancies by inducing replicative stress, DNA damage, and genomic instability [158]. UNC1215 is an inhibitor of L3MBTL1, which also contributes to hematopoietic malignancies. Structurally, the compound behaves as an aryl aniline lysine mimetic, binds to the aromatic cage common to the methyl-lysine readers, and has good *in vitro* potency and cellular activity [152]. Another inhibitor, UNC1679, has much higher affinity (IC_{50} = 160 nM) and selectivity for L3MBTL3, when compared with the related L3MBTL1 protein (IC_{50} > 10 nM) [159] (Table 4).

Conclusion and Future Directions

Great progress has been made in developing second-generation small-molecule inhibitors of epigenetic proteins (**Fig. 4**). Several DNMT and HDAC inhibitors have been FDA-approved and inhibitors of LSD, DOT1L, BET, and EZH2 are now entering clinical trials for multiple tumor targets (**Fig. 4**). In addition, progress is being made to develop tools for the identification of epigenetic drivers of cancer, such as next generation sequencing, that will further facilitate epigenetic-targeted drug discovery. The discovery of somatic mutations in epigenetic proteins in cancer reinforces the idea of an epigenetic driver in these malignancies. However, many epigenetic enzymes may also drive cancer progression via non-epigenetic mechanisms. Mutations in some non-chromatin proteins may also be indirectly linked to well-recognized epigenetic proteins (e.g. TET family proteins) [7,8]. In addition, the presence or absence of the components of SWI/ SNF, a nucleosome remodeling complex, may contribute to drug sensitivity, as demonstrated by the pronounced effect of EZH2 inhibitors on SMARCB1 deficient rhabdhoid tumors [160]. These druggable factors offer the possibility of developing orthogonal and synergistic interventional strategies. Identification of patient-specific molecular signatures and markers along with further optimization of epigenetic regulator drugs will be a big challenge for the existing and future clinical trials. These efforts may lead to a personalized medicine approach and more stratified patient populations.

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