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The Promise and Failures of Epigenetic Therapies for Cancer Treatment

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Abstract

Genetic mutations and gross structural defects in the DNA sequence permanently alter genetic loci in ways that significantly disrupt gene function. In sharp contrast, genes modified by aberrant epigenetic modifications remain structurally intact and are subject to partial or complete reversal of modifications that restore the original (i.e. non-diseased) state. Such reversibility makes epigenetic modifications ideal targets for therapeutic intervention. The epigenome of cancer cells is extensively modified by specific hypermethylation of the promoters of tumor suppressor genes relative to the extensive hypomethylation of repetitive sequences, overall loss of acetylation, and loss of repressive marks at microsatellite/repeat regions. In this review, we discuss emerging therapies targeting specific epigenetic modifications or epigenetic modifying enzymes either alone or in combination with other treatment regimens. The limitations posed by cancer treatments that elicit unintended epigenetic modifications that result in exacerbation of tumor progression are also discussed. Lastly, a brief discussion of the specificity restrictions posed by epigenetic therapies and ways to address such limitations is presented.

Keywords

Epigenome; Epigenetic; DNA Methyltransferases (DNMTs); Histone Acetyltransferases (HATs); Histone Deacetylases (HDACs); Histone Methyltransferases (HMTs); Histone Demethylases (HDMs)

INTRODUCTION

The epigenome (epi meaning above or beyond) consists of heritable modifications to histones and DNA which are independent of changes to the linear DNA sequence.^{1, 2} These modifications are established by transiently or stably expressed proteins that respond to developmental cues, internal stimuli and environmental factors. These modifications include DNA methylation, histone acetylation, methylation, phosphorylation, ubiquitination, citrullination, sumoylation, and ADP ribosylation. Cues established during development

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allow cells with identical DNA to differentiate into a wide array of cell types, a process which coupled to environmental factors, can lead monozygote twins to establish different epigenetic patterns and consequently, different profiles of gene expression. Such alterations of cellular programming can dictate differences in susceptibility to disease and therefore, represent potential targets for pharmacological intervention.^{3, 4}

Epigenetic modifications are dynamically established by DNA methyltransferases (DNMTs), histone acetyltransferases (HATs), histone methyltransferases (HMTs), kinases, and removed or modified by histone deacetylases (HDACs), histone demethylases (HDMs), ten eleven translocation protein 1-3 (TET1-3) activity, and phosphatases in a highly regulated manner. Such modifications alter the conformation of chromatin by effecting covalent interactions or by acting as special docking sites for reader and effector proteins.⁵ The interaction between chromatin modifying enzymes and reader/effector proteins regulates transcriptional activation or repression leading to varied cellular phenotypes. Epigenetic modifications are not stand alone processes, instead, are subject to considerable crosstalk among the different types of epigenetic marks. Epigenetic marks by themselves, or synergistically with other macromolecular modifications, function to either repress or activate transcription. For example, actively transcribing genes are enriched at the promoters with acetylation (AC), H3 trimethylation at lysine-4 (H3K4me3), and unmethylated CpGs. Further, enhancers are enriched with H3 methylation at lysine-1 (H3K4me1), and H3 acetylation at lysine-27 (H3K27AC), while gene bodies are enriched with H3 dimethylation at lysine-36 (H3K36me2), H3 acetylation at lysine-12 (H3K12AC) and DNA methylation (Figure 1 **Top**).^{6,7,8, 9} On the other hand, the promoters of repressed genes are enriched with DNA methylation at CpG sites, H3 trimethylation at lysine-27 (H3K27me3), H3 trimethylation at lysine-9 (H3K9me3), which can also be present in enhancer and gene bodies, as well as overall loss of acetylation (Figure 1 **Bottom**).^{8, 10, 11, 12} Unlike genetic mutations and gross structural defects that may permanently activate or inactivate genes, all epigenetic modifications identified to date are modifiable, thus conceivably allowing correction of aberrant epigenetic profiles (Figure 1).

Tumor cells undergo massive epigenetic reorganization, with the occurrence of oncogenic phenotypes associated with pronounced CpG-specific hypermethylation in the promoters of tumor suppressor genes, and generalized hypomethylation of the promoters of oncogenes, microsatellite regions and repetitive sequences.^{13, 14} Such changes in the epigenetic landscape contribute to both initiation of tumorigenesis and progression of oncogenic phenotypes. For example, in normal cells, the promoters of tumor suppressor genes are enriched with active transcription marks, such as H3K4me3, H3K9me, H4 acetylation at lysines-5,-8,-12,-16 and 20 (H4 -K5, -K8, -K12, -K16, and -K20 ACs), while satellite regions are enriched with repressive marks, such as H3K27me3, H3K9me2 and H4K16Ac (Figure 2).^{15, 16} In tumor cells, the promoters of tumor suppressor genes lose nearly all acetylation, and acquire repressive marks including H3K27me3 and H3K9me (Figure 2).¹⁷ At repetitive/satellite regions, repressive marks such as DNA methylation (Me), H4K20me3 and H3K27me3 are lost leading to chromosome/microsatellite instability (Figure 2).^{18, 19, 20}

In recent years, therapies targeting epigenetic modifying enzymes (e.g. DNMTs, HATs, HDACs, kinases, HMTs, and HDMs), pathways and accessory proteins have been developed to either block or reverse the aberrant epigenetic modifications (Figure 2). To date, four epigenetic drugs: 5-azacytidine (azacitidine or 5-aza-CR), 5-aza-2'-deoxycytidine (5-aza-CdR or decitabine), Vorinostat (suberoylanilide hydroxamic acid (SAHA)) and Romidepsin have been approved by the US Food and Drug Administration (FDA). Azacitidine and decitabine were approved for the treatment of high risk myelodysplastic syndrome, while vorinostat and romidepsin were approved for Cutaneous T cell Lymphoma

(CTCL). This review highlights some of the latest therapies targeting epigenetic enzymes, pathways and accessory proteins in cancer treatment.

Histone Acetyltransferase (HATs) and HAT Inhibitors (HATIs)

Histone acetylation involves the transfer of an acetyl group from acetyl coenzyme A (acetyl-CoA) to the ϵ -amino group of lysine by HAT.^{21, 22} HATs have been grouped into Type-A and Type-B HATs based on sequence divergence of the HAT domain and intracellular localization. This nomenclature, however, was confusing and a new nomenclature has been adopted as denoted in Table 1.²³ Type-A HATs are nuclear proteins that acetylate histones and other chromatin-associated proteins, while Type-B HATs are both nuclear and cytoplasmic and mainly acetylate *de novo* synthesized histones in the cytoplasm to promote their nuclear localization.^{24, 25} Type-A HATs consist of three families: GNATs, P300/CBP, and MYST. Thus far, histone acetyltransferase-1 (HAT1/KAT1) is the only Type B HAT shown to acetylate H3 at lysines-5 or 12 (K3K5/12).^{22, 26} The different HAT families show little sequence similarity with no homology domain, but most HATs contain a recognizable acetyl-CoA binding domain. Crystal structure analyses of all HATs have provided insight into how these enzymes interact with their substrates²⁷. For example, X-ray crystallography of Type-A HATs uncovered a conserved core domain consisting of three-stranded β -sheets connected to long and parallel α -helices, and this core region supports the conserved interaction of the protein with the acetyl-CoA or related substrates²⁷.

Despite conservation of this core domain, mutational, biochemical and enzymatic analyses showed that the different HAT families employ different mechanism to transfer the acetyl group. The GNAT family employs a one-step bi-bi ternary complex mechanism to transfer the acetyl-CoA. In this mechanism, acetyl-CoA and the substrate bind to form a ternary complex and the glutamate (Glu173 for KAT2A (GCN5) and Glu570 for KAT2B (hPCAF)) at the active site acts as a base and deprotonates the ϵ -amino group of lysine. This allows nucleophilic attack on the carbonyl carbon of acetyl-CoA leading to the formation of a tetrahedral intermediate which dissociates into CoA and the acetylated histone/protein.^{28, 29, 30, 31} The MYST family of HATs is found to either employ the one-step bi-bi ternary mechanism as reported by Berdsen et al. and Yan et al.^{32, 33}, or a two-step ping-pong mechanism in which the substrate and acetyl-CoA binds to each other allowing Cys304 at the active site to attack the acetyl moiety forming an acetylated enzyme intermediate. When the CoA is released from this intermediate, the lysine nucleophile binds the substrate and is deprotonated by Glu338 in the active site resulting in the formation of acetylated histone.³¹ It should be noted, however, that both Yan et al. and Berdsen et al. studied TIP60 (KAT5) in *H. sapiens*, with Yan et al. using a truncated KAT5 and Berdsen et al. using a full length KAT5 in the presence of accessory proteins (NuR4). In addition, substitution of Cys304 to alanine does not affect the catalytic activity of KAT5, suggesting that the GNAT family and MYST families follow similar catalytic mechanisms.³³ On the other hand, the p300/CBP family employs a hit-and-run (i.e. Theorell-Chance) acetyl transfer mechanism. Mutation analysis uncovered Tyr1467 and Trp1436 which are conserved in just the p300/CBP family as the amino acid involved in the acetyl transfer mechanism.³⁴ Trp1436 is believed to either help orient and/or deprotonate the lysine moiety on histone allowing nucleophilic attack on the carbonyl carbon of acetyl CoA leading to the release of CoA. Tyr1467 then donates a proton to CoA such that instead of base catalysis, P300/CBP group follows acid catalysis.^{34, 35}

Based on the catalytic activity of HATs with acetyl CoA, HAT inhibitors (HATIs) are mainly derivatives, conjugates or related compounds of acetyl-CoA and classified into synthetic peptide CoA base bisubstrate HATIs, natural product HATIs or small molecule HATIs (Table 2). The synthetic bisubstrate HATIs were the first to be identified based on the observation that polyamine-CoA conjugates can inhibit HAT activity in cell extracts³⁶.

In particular, H3-CoA-20 and Lys-CoA specifically inhibit KAT2B (hPCAF) and KAT3B (P300) rather weakly³⁷; however, introduction of a phenyl or methyl group between lysine and CoA improves the inhibition four-fold (Table 2).³⁸ Most of the synthetic bisubstrate HATs work by mimicking the acetyl CoA-lysine intermediate complex in the HAT reactions. Crystal structure information for KAT2A (GCN5) and these HATs shows that KAT2A (GCN5) interacts with the pyrophosphate moiety, the pantothenic moiety and the phosphate group of CoA. The major deficiency for this class of HATs is their high degree of cellular impermeability. Unfortunately, most of the naturally occurring HATs also suffer from a similar problem. For example, anacardic acid, a drug isolated from the shell of cashewnuts, displays permeability restriction in vitro. Nevertheless, garcinol and isogarcinol were both shown to inhibit KAT3B (P300) and KAT2B (PCAF), although they were shown to be toxic (Table 2).^{39, 40} Two derivatives of isogarcinol, LTK14 and LTK15, were shown to selectively inhibit KAT3B (P300), but not KAT2B (PCAF) without cytotoxicity, a finding that shows the great promise of these class of compounds.⁴⁰ The best characterized of the naturally-occurring HATs is Curcumin, which is isolated from the *Curcuma longa* rhizome. Curcumin has shown high efficacy in the prevention and treatment of colorectal, prostate, kidney, lung, ovarian, breast, cervical and liver cancers.³⁹ A derivative of curcumin with bromine substitutions (Table 2) has been shown to inhibit KAT3B with an IC₅₀ value of 5.0 μM.⁴² The last group of HATs includes a number of small molecules designed to overcome challenges with permeability. These include γ -butyrolactone MB-3, quinoline and isothiazolone and their derivatives. Although in their infancy, isothiazolone has been shown to inhibit the enzymatic activity of both KAT2B (PCAF) and KAT3B (P300) leading to reductions in cell proliferation of human ovarian and colon cancer cell lines.⁴¹ γ -butyrolactone MB-3 inhibits KAT2A (GCN5) with K_d calculations showing that the affinity of γ -butyrolactone MB-3 to KAT2A is comparable to the natural substrate H3 lysine.⁴² A derivative of isothiazolones with nitrogen oxide and chlorine substitution at the R2 and R3 position has been shown to effectively inhibit KAT2B. Another derivative of isothiazolones generically called CCT077791 effectively inhibits both KAT2B and KAT3B⁴⁴ (Table 2).

The viability of HATs as drug targets is complicated by the fact that most HATs are part of large multiprotein complexes that help to define the molecular activity of HATs.^{43, 44} In some instances, these complexes have been shown to restrict HAT activity from certain loci⁴⁵, while in others these complexes are assembled to orchestrate the different enzymatic functions required for transcription or alternate cellular functions. For example, the KAT 5 (TIP60) complex not only contains NUR4, but also contains ATM which is a kinase recruited during episodes of DNA damage. As such, KAT5 acetylates ATM which goes on to phosphorylate P53 and CHK2 giving rise to unintended acetylation.⁴⁶ On the basis of these findings, the making of cell permeable HATs is but one step toward making these drugs viable options for cancer treatment.

Histone Deacetylases (HDACs) and HDAC inhibitors (HDACis)

Removal of acetyl groups from histones is catalyzed by a class of enzymes called histone deacetylases (HDACs). In humans, 18 isoenzymes of HDACs have been identified to date, and grouped into four classes based on their homology to yeast HDACs (Table 3). Class I (HDACs 1, 2, 3, and 8) are related to yeast *Rpd3* gene and are mostly located in the nuclei, except HDACs 3 and 8 which can also be cytoplasmic. Class II HDACs (HDACs 4, 5, 6, 7, 9 and 10) are related to yeast *Hda1* gene and primarily located in the cytoplasm, but can also shuttle to the nucleus. Class II HDACs are further divided into two subclasses, IIa (HDAC 4, 5, 7, 9) and IIb (HDAC 6, 10) based on their sequence homology and domain organization. Class III, also known as the sirtuins (sirtuins 1–7), are related to the yeast *Sir2* gene and localized in the cytoplasm, mitochondria and nucleus. Class IV (HDAC 11)

contains a conserved domain that is similar to the catalytic domain of class I and II HDACs. Class I, II and IV share similar structural organization and a common cofactor, Zn^{2+} , while Class III HDACs (sirtuins) are structurally unique and their active site is occupied by the nicotinamide adenine dinucleotide (NAD). Functionally, class II HDACs are regulated by class I HDACs and together they are involved in transcriptional silencing and genomic organization during development. Class III HDACs (sirtuins) are involved in maintenance of acetylation, as well as gene-specific silencing.

HDAC inhibitors (HDACis) have been classified into seven categories based on their chemical structures and mode of inhibition: short chain fatty acids, benzamides, cyclic peptides, electrophilic ketones, hydroxamine-acid-derived compounds, miscellaneous compounds (e.g. Depudecin and MGCD-0103) and sirtuin inhibitors. Class I, II and IV HDACis share a common metal binding domain that serves to block Zn^{2+} chelation at the active site.⁴⁷ Because of the presence of NAD, a different cofactor at the active site of class III HDACs, zinc-dependent HDACis, are ineffective against class III HDACs (sirtuin). Class III HDACis are inhibited by nicotinamide, NAD^+ analogues, indoles, hydroxynaphthaldehyde derivatives, splitomicins, suramins and kinase inhibitors.⁴⁸ Most HDAC inhibitors work by specifically blocking the entry of cofactor into the active site. The mode of action of sirtuin (Class III) inhibitors (i.e indoles, hydroxynaphthaldehyde derivatives, splitomicins, suramins and kinase inhibitors) is not fully understood, and much less is known about their biological consequences.^{49, 50, 51} In fact, sirtuin inhibitors shown to be effective in lower organisms do not work on human subtypes.⁴² Nevertheless, Sirtuin I and 2 specific inhibitors (SEN96 and COMPOUND 6J) are in clinical trials (Table 4)^{52, 53}.

Zinc-dependent HDACis are established anticancer drugs, and two inhibitors (Vorinostat and Romidepsin) have been approved for cancer treatment in the United States (Table 4).⁵⁴ The efficacy of these drugs for cancer treatment has been established. For example, early molecular and cellular biological studies showed that in response to these HDACis, the p21 gene is consistently upregulated in a p53-independent manner leading to G1 cell cycle arrest.⁵⁵ The upregulation of p21 correlates with increased acetylation of histones H3 and H4 near the p21 promoter.⁵⁶ In addition, HDACis such as butyrate and trichostatin A have been shown to stabilize p21 mRNA⁵⁶, to repress cyclins A and D, and to activate p16 and p27 culminating in cell cycle arrest.^{57, 58} In other studies, HDACis have been shown to upregulate the expression of proapoptotic genes (TRAIL, DR5, Bax, Apaf-1, Bmf, Bim and TP2) and/or to downregulate the expression of antiapoptotic genes (i.e., Bcl-2, Mcl1, and XIAP).⁵⁹ In contrast to other epigenetic drugs, a major advantage for HDACis is their potency, as evidenced by their ability to elicit pharmacological effects in the nano/micromolar range.^{60, 61}

HDACis suppress angiogenesis and enhance the host immune system in cancer patients.^{59, 62, 63} In combination therapies, HDACis function synergistically with a host of structurally and functionally diverse chemotherapeutic agents and biologically-active polypeptides. In this manner, HDACis increase target susceptibility and show greater promise. For example, in breast cancer therapy, the effectiveness of topoisomerase II inhibitors can be enhanced by pretreatment with vorinostat.⁶⁴ Further, HDACis have been used in combination with DNA demethylating agents to reactivate silenced genes involved in tumor suppression. For example, three Phase I/II trials combining decitabine with HDAC inhibitors (phenyl butyrate or valproic acid) in patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS) showed both tolerability and promising efficacy.⁶⁵⁻⁶⁷ Of 93 patients treated, 14 showed complete remissions (CR), two showed partial complete remission (pCR), 4 showed partial response (PRs) and 6 showed hematologic improvements (HI) and combined established an overall response rate of 28%.

In another phase I clinical trial, the efficacy of CI-994 and various other chemotherapeutic agents was examined in 54 patients. The results showed that CI-994 at doses of 4-10mg/m²/day can be safely administered to patients for 7-21 days in a 3-4 week dosing regimen.⁶⁸⁻⁷⁰ In this study, two patients with esophageal and bladder cancers, respectively, showed complete remission (CR) and 5 (3 with non-small lung carcinoma and 2 with colorectal cancer) demonstrated partial remission (PR). On a related note, some leukemias and breast cancers plagued with the expression of fusion proteins (RAR-PML, RAR-PLZF or AML-ETO chimeras) that inhibit differentiation have shown improvement when HDACis are combined with trans-retinoic acid (ATRA).^{67, 71, 72, 73} Another emerging area of combination therapy has been the use of HDACis with tyrosine kinase inhibitors in cancers that overexpress antiapoptotic genes. For example, vorinostat, LBH-589, LAQ-824 and romidepsin have demonstrated synergistic proapoptotic activity in combination with imatinib and other tyrosine kinase inhibitors in the treatment of both imatinib-sensitive as well as imatinib-resistant *bcr-abl* leukemic cells.⁷⁴⁻⁷⁷

DNA Methyltransferases (DNMTs) and DNMT Inhibitors (DNMTis)

DNA methylation involves the enzymatic transfer of a methyl group (CH₃) to carbon-5 of the pyrimidine base, cytosine, by DNA methyltransferases (DNMTs). Five DNA methyltransferases (DNMTs) have been identified in higher eukaryotes: DNMT1, DNMT2, DNMT3L, DNMT3a and DNMT3b but only three (DNMT1, DNMT3a and 3b) are involved in direct DNA methylation. DNMT3L lacks DNA methylation activity⁷⁸, but has been shown to colocalize and to stimulate DNMT3a and DNMT3b during maternal genomic imprinting.⁷⁹ In addition, DNMT3L has been shown to recruit DNMT3a and DNMT3b to H3 histone tails to methylate DNA⁸, and to facilitate H3K36me₃ (a repressive mark).⁸⁰ DNMT2 knockout mice do not display aberrant DNA methylation⁸¹, and instead, show methylation of tRNA outside the nucleus.⁸² DNMT1 is a maintenance methyltransferase that recognizes and methylates hemimethylated DNA during DNA replication⁸³, and can interact with both DNMT3a and DNMT3b to influence transcription. DNMT1 and DNMT3a have both been shown to bind to KMT1A (SUV39H1), a histone methyltransferase, to mediate repression via H3K9me₃⁸⁴, and DNMT1 and DNMT3b were shown to interact with HDACs to repress gene expression.^{85,86} DNMT3a and DNMT3b primarily function as *de novo* methyltransferases and establish DNA methylation during embryogenesis.⁸⁷ DNA methylation provides binding sites for several methyl-binding proteins including Methyl-CpG-binding domain protein 1-3 (MBD1-3) family members and CpG-methylated DNA binding complex (MeCP2) which in turn function to recruit histone modifying enzymes (e.g. HDACs, TETs, kinases, E3 ligases, HMTs).⁸⁸ DNA methylation in most instances (except on gene bodies) results in the formation of heterochromatin leading to transcriptional repression.

Although previously considered irreversible, two seminal studies have shown that 5-methylcytosine (5-mC) can be oxidized to 5-hydroxymethylcytosine (5-hmC) by Ten Eleven Translocation (TET1-3) family of dioxygenases.^{89, 90} The role of 5-hydroxymethylcytosine (5-hmC) conversion is not fully understood since this modification is found in gene bodies, active and repressed promoters, as well as transcriptionally poised genes, suggesting that this might be an intermediate step in the demethylation process.⁹¹ In fact, other derivatives of 5-hydroxymethylcytosine such as 5-formylcytosine and 5-carboxycytosine have been uncovered more recently.⁹² Functionally, demethylation is a process that favors transcriptional activation, as most of the promoters of actively transcribing genes are unmethylated.

Inhibitors of DNA methylation (DNMTis) cause reactivation of silenced genes, inhibition of cell proliferation, apoptosis and enhancement of sensitivity to other cancer drugs. DNMTis are grouped into nucleoside DNMTis and non-nucleoside DNMTis based on their structure

and mode of action (Table 5). Nucleoside DNMTis are analogues or derivatives of the nucleoside cytidine and they include 5-Azacytidine (5-Aza-CR), 5-Aza-2-deoxycytidine (5-Aza-CdR), Zebularine, Cytarabine and 5-Fluoro-2-deoxycytidine (FdCyd). The anticancer activity of these drugs is mediated via two mechanisms: (1) cytotoxicity that stems from the incorporation of these drugs into DNA and/or RNA, and/or (2) reactivation of tumor suppressor genes by passive demethylation of their promoter regions. These drugs do not demethylate DNA per se, but rather with continued replication, cytidines are replaced by the cytidine analogues resulting in serial dilution of methylable cytosines. Furthermore, DNMTs are trapped as covalent adducts with DNA through the incorporated cytidine analogues.

5-Aza-CR and 5-Aza-CdR are taken into the cell through the concentrated nucleoside transporter-1 (hCNT1), or equilibrative nucleoside transporters (hENT1), or the Proton-coupled oligopeptide transporters.⁹³ Once inside the cell, these agents are monophosphorylated by Uridine-cytidine kinase (Urd-Cyd Kinase) and Deoxycytidine kinase (dCyd Kinase), respectively.^{94, 95} This step is followed by sequential phosphorylation by UMP-CMP kinase and Nucleoside-dCP kinase to the active triphosphates: 5-Aza-CTP and 5-Aza-dCTP. 5-Aza-CTP/-dCTP incorporated into the DNA forms covalent adducts between DNMTs and DNA which traps the DNMTs and prevents further methylation.⁹⁶ In other studies, 5-Aza-dCTP was shown to be incorporated into RNA which interferes with ribosomal biogenesis and protein synthesis.^{94, 96} In support of this mechanism, it has been shown that 5-Aza-CR and 5-Aza-CdR hypomethylate the genome through passive dilution of cytidine^{97, 98}. Clinical trials using Aza-CR and sodium phenylbutyrate⁹⁹, Aza-CR and Valproic acid¹⁰⁰, Aza-CR and lenalidomide¹⁰¹ in patients with solid tumors or MDS show greater than 40% efficacy.

Because of the cytotoxicity and instability of 5-Aza-CR and 5-Aza-CdR, DNMTis cannot be continually given to patients. For this reason, zebularine was developed as an alternative. Although this drug works in a manner similar to 5-Aza-CR and 5-Aza-CdR, it is more stable and less toxic than 5-Aza-CR and 5-Aza-CdR DNMTis.^{102, 103} For example, zebularine has been shown to reactivate tumor suppressor genes^{104, 105}, enhance tumor cell response to chemotherapy and radiation sensitivity¹⁰⁶, and exert angiostatic and antimitogenic activities.^{107, 108} This agent is stable for oral administration^{103, 109} thus, providing a significant advantage over other agents (Table 5). In addition, at low doses zebularine can be given to patients continuously without the overt cytotoxicity associated with 5-Aza-CR and 5-Aza-CdR.

Another cytidine analogue, 5-fluoro-2-deoxycytidine (FdCyd) demethylates DNA in human breast and lung cancer cells (Table 5).¹¹⁰ In the case of FdCyd, the hydrogen atom at carbon 5 (C5), which functions as the methyl acceptor during the methylation reaction, is replaced by a fluorine atom. When FdCyd is incorporated into DNA, the -elimination step in which DNMT transfers the methyl group to the cytidine is inhibited. At the same time, the fluorine atom traps the DNMT to prevent elimination of the FdCyd moiety.^{111, 112} FdCyd is currently in clinical trials for the treatment of breast and other solid tumors.¹¹³ Moreover, FdCyd, in combination with other epigenetic drugs (i.e. tetrahydrouridine and dihydro-5-azacytidine (DHAC), is being evaluated in clinical studies for the treatment of malignant mesothelioma.¹¹⁴

Although nucleoside DNMTis have proven effective for the treatment of cancers, their cytotoxicity remains a significant limitation. To address this shortcoming, non-nucleoside DNMTis such as procaine, L-tryptophan derivatives, RG108, hydralazine, MG98, procainamide, and epigallocatechin-3-gallate (EGCG) are being evaluated. Procaine is a local anesthetic drug that can also function as a DNMTi. For example, procaine has been shown to cause global demethylation and reactivation of tumor suppressor genes in human

breast cancer cells.¹¹⁵ Unlike the nucleoside analogues, procaine competes with DNMTs for binding to CpG rich regions.¹¹⁵ Procainamide and hydralazine are antiarrhythmic drugs that can also function as DNMTis, and both agents have been shown to inhibit DNA methylation through interactions between the nitrogen atom of procainamide and hydralazine with the lys-162 and Arg-240 moities in the catalytic site of DNMTs.¹¹⁶⁻¹¹⁸ In accord with these findings, procainamide has been shown to specifically inhibit DNMT1.¹¹⁹ RG108 is a small molecule inhibitor of DNMTs that inhibits free DNMTs.¹²⁰ This drug works by blocking the catalytic pocket of DNMTs without the formation of covalent adducts that cause cytotoxicity.⁹⁴ Studies have also shown RG108 to cause demethylation and reactivation of tumor suppressor genes without affecting the methylation level of microsatellite regions in lung cancer cells¹²¹, suggesting a specificity level in RG108 that has not been seen with other DNMTis (Table 5).

Histone methyltransferases (HMTs) and HMT inhibitors

Histone methylation occurs in all three basic amino acid residues: lysine, arginine and histidine. S-adenosyl methionine (SAM) donates the methyl residue in all three cases. However, histidine methylation is rare and has not been studied extensively.^{122, 123} Lysine residues are mono-, di-, tri-methylated by a class of enzymes called lysine methyltransferases (KMTs)²³, which were originally categorized into the SET and the DOTL domain HMTs based on the presence or absence of the SET-domain. This unconventional and non-coherent naming of KMTs has now been replaced by a more conventional system based on the type of enzymatic activity, substrate specificity, sequence homology, structural organization of the catalytic domain and order of discovery (Table 6).²³ Thus far, about fifty KMTs have been identified in humans. Arginine residues are methylated by protein arginine methyltransferases (PRMTs) which are categorized into Types-1 and 2 based on whether the methyl group is added to the guanidiny group symmetrically (i.e. dimethyl group added to different nitrogen atoms (Type 2)) or asymmetrically (i.e. dimethyl group added to single nitrogen atom (type 1)). Eleven PRMTs have been identified in humans and classified as Type-1 PRMTs except for PRMTs-5, 7 and -9 (Table 7). In depth analysis of methyl addition to arginine residues has led to further characterization of the types of PRMTs involved, however, this is beyond the scope of this review.

In humans, HMTs generally contain at most four domains: the SET, I-SET, Pre-SET and Post-SET domains. The core SET-domain, which is the catalytic domain, is a sequence of ~130 amino acids named after the drosophila *Su(var)*, *E(z)* and *Trithorax* genes. Crystal structure and topological analyses of the SET-domain showed that it consists of a series of α -sheets folded into three discrete sheets surrounding a threaded-loop which is defined by signature conserved motifs ELxF/YDY and NHS/CxxPN (i.e. x is any amino acid).¹²⁴⁻¹²⁶ All HMTs, except KMT4 (i.e. DOT1L), contain the SET-domain, however, the catalytic domain of KMT4 shares similar structural folds to the SET domain of PRMTs.¹²⁷ The Pre-SET, I-SET, and Post-SET domains vary in nature and sequence among the different HMTs and are present in different combinations with the core SET-domain. These domains act as a scaffold around the SET-domain thereby providing interacting and recognition sites for lysine or arginine substrates and cofactors for general catalysis.^{128,129} Within the context of the ternary structure of HMTs, the I-SET domain is packed immediately after the SET-domain to form the substrate binding groove and part of the cofactor binding pocket. The Pre-SET domain generally provides structural support by interacting with different regions of the SET-domain, while the Post-SET domain forms part of the active site by providing an aromatic residue to pack against the SET-domain, thereby constructing a hydrophobic pore.¹³⁰⁻¹³² This structural arrangement forms two distinct binding pockets for the substrate and cofactor and allows them to meet at the core for catalysis to occur.

The lysines or arginines to be methylated are inserted through the hydrophobic pore into the substrate binding pocket and the catalytic addition of a methyl group(s) using an S_N2 mechanism^{133, 134}. The lone pair of electrons on the nitrogen of lysine or arginine acts as a nucleophile that attacks the electrophilic methyl-sulphonium cation on SAM forming a penta-coordinate carbon transition complex which dissociates into a methylated-lysine/arginine and S-adenosyl-L-homocysteine (SAH). For each round of methylation, the nitrogen atom has to be deprotonated before a methyl group can be added. The mechanism by which the initial or multiple rounds of nitrogen deprotonation occurs is still debated. The general base theory^{135, 136} which regards the conserved tyrosine residues (e.g., Tyr-335 in KMT7, Tyr-283 in DIM-5, Tyr-287 in Rubisco LSMT, Tyr-336 in KMT5A) in the active site as the general bases that deprotonate the nitrogen residue were found to be too far from the active site to participate in general base catalysis. Another deficiency of this theory is that it does not explain repeated deprotonation of nitrogen residues for di- and tri-methylation. Other findings have shown that tyrosine residues function by providing hydrogen bonding sites to the substrate which function to restrain and align the lone pair of electrons to the scissile bond of the sulfonium group.¹³⁷⁻¹⁴¹ Alternatively, the bulk solvent theory explains many of the shortcomings of the general base theory.¹⁴² In this model, HMTs bind a substrate and lose a proton to a bulk solvent (e.g. H₂O) followed by methyl transfer through an S_N2 mechanism. In the case of mono-methylation, the HMTs dissociate from the substrate to complete the methylation cycle. However, for the di- or tri-methylated state, the lysine or arginine residues are sequentially deprotonated by the bulk solvent for the addition of the second and third methyl-groups. Bulk solvent deprotonation was challenged by current findings on the ternary structure of KMT1D and KMT7 which revealed that lysine inserts into the narrow groove at the junction of SET, Pre-SET and Post-SET domain.¹⁴³ In this conformation, the lysine substrate is shielded from the solvent, thus arguing against bulk solvent deprotonation.

Unlike DNA methylation which generally represses transcription (except at gene bodies), or acetylation which generally activates transcription, the functional impact of histone methylation is contextual and can lead to both transcriptional activation and repression. A possible explanation for this conundrum lies on the timing and manner in which the HMTs are recruited to their target destination, the protein composition of HMT complexes, the occurrence of other epigenetic marks, the degree of histone methylation, and the contribution of small non-coding RNAs (sncRNAs) and long non-coding RNAs (lncRNAs). The influence of most of these factors is explained by the fact that histone methylation does not alter the charge on histone tails. Instead, it influences the basicity and affinity of different reader proteins to the methylated site (s). For example, histone methylation reader proteins (i.e. PHD finger domain, WD40 repeats, PWWP domains, chromo- and bromo-domain proteins, royal-family domain) have been shown to recruit specific HMTs to their target sites. In particular, H3K4 methylation by KMT2A (i.e. the mixed lineage leukemia (MLL)) is followed by recruitment of bromo-domain-containing complexes which result in transcriptional activation.¹⁴⁴⁻¹⁴⁶ KMT6 (i.e. EZH2) methylation at H3K27 leads to recruitment of chromo domain-containing polycomb complexes resulting in silencing of homeotic genes and KMT1A (i.e. SUV39h1) trimethylation at H3K9 of the promoters of cell cycle control genes, recruitment of chromo domain-containing heterochromatin protein 1 (HP1) and transcriptional repression.¹⁴⁷⁻¹⁴⁹ Other factors such as tissue-specific expression of HMTs, lncRNAs and sncRNAs are beyond the scope of this review.

Histone methylation and associated chromatin regulators play an important role in tumorigenesis. For example, aberrant H3K9 methylation by KMT1C has been associated with chromosomal instability and epithelial to mesenchymal transition, with recurrent translocation and/or mutation of KMT2, KMT3A, and KMT6 found in several cancers.^{150, 151} Loss of KMT1 is associated with decreased viability, genomic instability

and increase tumor risk in mice.¹⁵² In addition, frequent somatic mutation involving the conversion of Tyr to Phe in the active site of KMT6 has been reported in lymphomas.¹³² Tyr to Phe mutation converts KMT6 from a multifunctional mono-, di- and tri-methylase into a dominant tri-methylase.¹⁵³ KMT4 which methylates H3K79 has been shown to be mutated, or its gene translocated or fused to other genes (i.e. AF4, AF5, AF9, ENL and AF10) in many leukemias resulting in ectopic histone methylation.^{154, 155}

The direct involvement of HMTs in tumorigenesis has led to the development of two types of HMT inhibitors (HMTi) namely the SET-domain inhibitors and non-SET-domain inhibitors. The SET-domain inhibitors include analogs of SAM such as SAH, and sinefungin, a natural inhibitor isolated from *Streptomyces*, small molecule inhibitors such as Chaetocin, BIX-01294 and BIX-01294 derivatives (i.e. UNCO224, UNC0631 and UNC0638), and arginine methyltransferase inhibitors 1-9 (i.e. AMI1-9) (Table 8).¹⁵⁶⁻¹⁵⁹ SAH has limited effects against HMTs because its affinity for HMTs is less than, or comparable to SAM. Sinefungin is about 3-10 fold more potent than SAM, but found to be non-selective toward most KMTs and to target DNA methyltransferase or other enzymes that use SAM as a co-factor.¹⁵⁹ Chaetocin, a fungal mycotoxin, was shown to be a competitive inhibitor of SAM and displays specificity toward H3K9-specific KMTs such as KMT1, KMT1A, KMT1C, but its drug-ability is dampened by significant cytotoxicity.^{160,161}

BIX-01294 is a selective inhibitor of a structurally similar KMT1C and KMT1D with IC₅₀ values of 1.7μM and 0.7μM, respectively, over KMT1A and PRMT1 (Table 8).¹⁶²⁻¹⁶⁵ BIX-01294 is made of a central quinazoline ring connected to a seven-membered diazepane and to a benzylated six-membered piperidine. Crystal structure analysis of BIX-01294 in complex with KMT1D and SAM show that BIX-01294 occupies the substrate binding site with the quinazoline moiety interacting with the four aspartate residues on the acidic surface of SET-domain.¹⁶² This interaction blocks the insertion and binding of the substrate to the substrate binding pocket. Because of the selectivity and potency of BIX-01294, several derivatives of this drug have been developed such as UNCO224.^{163,165} In UNCO224, the methoxy group on carbon 5 of the quinazoline ring was replaced by a 7-aminoalkoxy side chain to increase the interaction at the substrate binding site. Accordingly, this switch increases the potency 7-fold with IC₅₀ values of 1.7nM and 1.9nM for KMT1C and KMT1D, and more than 1000-fold selectivity for KMT1C over KMT5A and KMT7 in *in-vitro* assays.¹⁶² Other analogues with cyclic amino groups such as pyrrolidine and piperidine substituted at the 5-methoxy position have been shown to be more potent than UNCO224 because these cyclic side chains pack more efficiently in the substrate binding groove. However, their potency is dampened by their impermeability.^{163,164} To address permeability deficits, other derivatives such as UNC0638 have been developed. In the case of UNC0638, the nitrogen on the piperidine ring was substituted by isopropyl group, the diazepane ring with cyclohexyl group and the methoxy group on carbon-5 of the quinazoline with a *****propyl-pyrrolidin group.¹⁶⁵ In vitro testing of this compound showed increased permeability, a potency with IC₅₀ values of 15nM and 19nM for KMT1C and KMT1D and non-activity against KMT1, KMT6, KMT4, KMT7, KMT5A, HRMT1 and HRDM3.¹⁶⁵ Although still in their infancy, BIX-01294 and its derivatives have shown great potential for the treatment of cancers that involve dysregulation of KMTs.

The first inhibitors of HRMTs are binaphthylureas, symmetrical sulfonated ureas, and their derivatives, named arginine methyltransferase inhibitors 1-9 (i.e. AMI1-9)^{157, 166, 168}. Cell culture studies using RNA binding fusion protein GFP-Np13 as a target showed that AMI-1 decreases methylation of GFP-Np13 by inhibiting PRMT1 with IC₅₀ values of 9μM (Table 8).¹⁶⁴ AMI-1 was shown to be a competitive inhibitor of the arginine substrate and to occupy the substrate binding site of PRMTs.¹⁵⁷ Because of the structural similarity between

AMI-1 and sirtuin inhibitors (i.e. surnamin), AMI-1 has been shown to inhibit sirtuin-1 (SIRT-1) with an IC_{50} of $32\mu M$.¹⁶⁶ To increase specificity, several derivatives have been developed using AMI-1 and AMI-5 as templates.¹⁶⁵ In AMI-6, these investigators substituted bromines at positions 2, 4, 5, and 7 on the xanthene ring resulting in greater specificity for PRMT1 with an IC_{50} of $1.4\mu M$ (Table 8).¹⁵⁷

The so called non-SET domain inhibitors targeting KMT4 are regarded as analogs of SAM in spite of the absence of the SET domain because the protein uses SAM as a cofactor. The first of these drugs, EPZ004777, was synthesized by replacing the methionine amino acid moiety in SAM with urea (Table 8).¹⁶⁸ EPZ004777 was found to be greater than 1000-fold selective for KMT4 over CARM1, PRMT1, PRMT5, PRMT8, KMT1D, KMT6, and KMT7. This agent showed antiproliferative, antidifferentiation and apoptotic activity toward cells harboring MLL fusion.¹⁶⁸ EPZ004777 was also shown to prolong survival in a mouse model of mixed-lineage leukemia.¹⁶⁸ This molecule has poor permeability across the membrane due to the presence of 6 hydrogen bonding sites. Other derivatives have been developed to address this limitation.

The value of focusing on HMTs as viable targets for therapeutic intervention in cancer stems from their high specificity for lysine and arginine methylation.¹⁶⁹ For example, KMTA/1B preferentially trimethylates H3K9 compared to its mono-methylated state and KMT1C has shown preference for the mono-methylated compared to the dimethylated state H3K9.¹⁷⁰ Nevertheless, the specificity of HMTs and their potential as therapeutic targets is dampened by the fact that they also methylate non-histone proteins and can be redundant with regard to the number of HMTs that methylate the same residue. For example, KMT1C and PRMT1 have been shown to specifically methylate H3K9 and H4R3, respectively, but other KMTs (i.e. KMT1, KMT1B, KMT1C) and PRMTs (i.e. PRMT6a, PRMT5, PRMT7) have been shown to methylate the same lysine and arginine residues, respectively.^{172, 173} Recently, AstraZeneca developed a KMT specific drug, AZ505, which specifically inhibits KMT3C.¹⁷⁴ KMT3C has been shown to methylate H3K36 as well as K370 and K860 in p53 and RB, respectively. K370 methylation prevents p53 from binding to the promoters of target genes and methylation at K860 in RB has been shown to create an epitope that selectively recognizes the transcriptional repressor L3MBTL1.¹⁷⁵⁻¹⁷⁸ Binding studies show that AZ505 is a competitive inhibitor of the lysine substrate and shows selectivity toward KMT3C with an IC_{50} value of $0.12\mu M$ compared to greater than $83.3\mu M$ IC_{50} values for KMT4, KMT6, KMT1D, KMT1C and KMT7. KMT1C, which is inhibited by BIX-01294 and its derivatives, has also been shown to dimethylate K373 in p53 leading to inactivation (Table 8).¹⁷⁴ As such, drugs targeting aberrant methylation on histones and non-histone proteins are being developed to combat cancer.

Histone demethylase (HDMs) and HDM inhibitors

Two classes of enzymes are responsible for removing methyl groups from histones: lysine-specific demethylase (KDM) and arginine-specific demethylase (RDM). KDM includes amine oxidase-like (AOL) domain-containing demethylases¹⁷⁹ and Jumonji C (JmjC) domain-containing demethylases.¹⁸⁰ RDM includes peptidyl-arginine deiminase 4 (PAD4) (Table 9).¹⁸¹ PAD4 is the only known RDM and it does not demethylate arginine, instead it converts methylarginine to citrulline.¹⁸¹ Citrullination has been described as a unique epigenetic modification because PAD4 can convert unmethylated arginines to citrulline.¹⁸² This modification implicates citrullination as a mechanism to deplete methylable arginines.

Thus far, KDM1A (LSD1) and KDM1B (LSD2) are the only AOL domain-containing demethylases.^{179, 183} Structurally both enzymes consist of three domains: a common SWIRM (SWI3P, RSC8p and Moira) domain, an AOL domain interrupted by a spacer domain (i.e. the so called tower domain) in KDM1A only, and a CW-type zinc-finger

domain present only in KDM1B. The SWIRM domain is required to stabilize the enzyme and through interactions with the AOL domain form a wide cleft that provides an interface for substrate interaction¹⁸⁴. The tower domain is believed to provide an allosteric interacting surface that directs the specificity of the enzyme.¹⁸⁵ The AOL domain is homologous to the convention FAD-dependent binding domain and consists of two subdomains: the FAD binding and substrate binding subdomains; the interface of the two subdomains form the catalytic site.

KDM1A and KDM1B specifically catalyze the removal of mono/di- methyl groups from H3K4; however they have been shown to change substrate specificity when in complex with different accessory proteins. For example, KDM1A in complex with androgen receptor is able to demethylate H3K9.¹⁸⁶ In contrast, KDM1A when in complex with silencer corepressor of RE1 silencing transcription factor (CoREST) can demethylate histones within nucleosome substrates.^{187,187} The catalytic removal of methyl groups is initiated by the formation of an imine intermediate through oxidative protonation of the lone pair of electrons on the nitrogen atom.¹⁷⁹ The iminium intermediate is then hydrolyzed to a carbinolamine intermediate that degrades spontaneously into formaldehyde and demethylated H3K4. The reduced cofactor, FADH₂, is reoxidized by O₂ to FAD and H₂O₂. Because the initial step requires a lone pair of electrons on the nitrogen atom for protonation, KDM1 family members cannot demethylate tri-methylated residues.

About 30 members of the Jumonji C (JmjC) domain-containing demethylases have been discovered in humans and divided into 7-groups based on phylogenetic and structural analysis. Structurally they consist of a minimum of a core Jmj domain which has been shown to be sufficient to perform the catalytic demethylation.¹⁸⁰ The additional domains are believed to enhance demethylation by facilitating substrate and/or protein-protein interactions. The catalytic demethylation of JmjC domain containing enzymes is different from the KDM family members in that a lone pair of electrons is not needed to initiate catalysis; hence they can demethylate all three states of methylation.¹⁸⁸ Rather, the catalysis depends on the two cofactors: Fe²⁺ and α -ketoglutarate.

In the unbound state, the Fe²⁺ at the active site is coordinated by three molecules of water, two histidine residues and one glutamate residue.¹⁸⁹ It is believed that in the initial step of the demethylation, molecular dioxygen and α -ketoglutarate coordinate to Fe²⁺ by displacing the water molecules, and this is followed by a single electron transfer from Fe²⁺ to the dioxygen creating reactive peroxide radical. The peroxide radical attacks the C2 bond of α -ketoglutarate creating a bond between α -ketoglutarate and Fe³⁺. In the next step, α -ketoglutarate decarboxylates into succinate and CO₂ with the generation of Fe³⁺-OXO radical in the catalytic core. The highly reactive hydroxyl radical group then activates the C-H bond in the substrate methyl-lysine and transfers oxygen to the methyl group forming hydroxymethyllysine which is an unstable hemiaminal. The hemiaminal readily decomposes into formaldehyde and a lysine residue lacking a methyl group.^{180, 188} The coordination of the three water molecules to the Fe²⁺ regenerates the initial catalytic species which can undergo a second and third round of demethylation.¹⁹⁰⁻¹⁹²

Dysregulation of demethylases have been reported in several cancers. For example, KDM1A overexpression is a predictive biomarker of prostate cancer¹⁹³ and JMD2C and KDM1A have been identified as coactivators of the androgen receptor through demethylation of the repressive mark H3K9 leading to expression of androgen dependent genes.^{184,193} Overexpression of the H3K9 methylation mark is associated with higher risk of relapse in prostate cancer. In addition, the microenvironment of tumors is depleted of oxygen which leads to the activation of hypoxia inducible factor (HIF) which in turn targets the JMJD1 and JMJD2 family of demethylases.¹⁹⁴⁻¹⁹⁷ Also, the overexpression of KDM5A has been

implicated in drug resistant cancers.¹⁹⁸ Therefore, inhibitors have been developed for both classes of demethylases and their potency toward the different HDMs tested.

Three different classes of inhibitors have been developed to specifically target KDM1A: Mono Amine Oxidase Inhibitors, Substrate-based Inhibitors and Bisguanidine analogues (Table 10). The monoamine oxidase inhibitors were originally designed to target Mono Amine Oxidase A & B (MAOA & MAOB) in the treatment of depression and neurological disorders.¹⁹⁹⁻²⁰¹ While the MAO activity removes the amine groups of neurotransmitters in an FAD dependent manner, sequence and structural analyses showed that the MOA domain is similar to the AOL domain of KDM1As. As such, tranylcypromine (PCPA), while itself not effective against KDM1A ($K_i = 357\mu\text{M}$), served as the lead to design potent inhibitors of KDM1A.²⁰² A homoserine derivative of PCPA have been shown to be 100-fold more potent than PCPA and is selective against MAOA & MAOB (Table 10). Methylation analysis showed recovery of H3K4 dimethylation at a concentration of $6-67\mu\text{M}$ in HEK293 cells.²⁰³ Another derivative, S2101 increased dimethylation at H3K4 at concentrations of $1\mu\text{M}$ with a K_i value of $0.6\mu\text{M}$ against KDM1A.²⁰⁴ Crystal structure analyses determined that these drugs inhibit the activity of KDM1A by forming a covalent adduct with the FAD cofactor.²⁰²

Substrate base inhibitors are designed from the last 21 amino acid peptide sequence of the histone-3 tail with the addition of chemical subgroups at lysine-4 (K4) of the peptide sequence.²⁰⁵ The most potent of this class of drugs is a compound in which a dimethyl-hydrazine group was substituted at the K4 position.²⁰² Compared to the lead substrate (methionine substitution at K4), this compound displays an inhibition rate in the nanomolar range ($K_i = 4.4\text{nM}$) making it the most potent inhibitor of KDM1A identified thus far.²⁰² It is believed that substrate based inhibitors act through suicide inactivation by permanently trapping the enzyme thereby preventing it from acting on another substrate. Substrate based inhibitors are promising in the sense that they are structurally similar to the natural substrates and with smart design can remain inert until acted on by the KDM1A enzyme.

Bisguanidine analogues were developed based on the observation that KDM1A shares considerable homology with FAD-dependent polyamine oxidases and that biguanidines can inhibit polyamines oxidases.²⁰⁶⁻²⁰⁸ Thus far, two analogues (PG-1150 and PG-11144) have shown more than 50% inhibition of KDM1A at a concentration of $1\mu\text{M}$ and a noncompetitive inhibition profile of purified KDM1A enzymes at a concentration of $2.5\mu\text{M}$.^{206, 209} Exposure of HCT116 human colon carcinoma cells to these compounds significantly increased mono-/dimethylation at H3K4 followed by reexpression of previously silenced genes.^{206, 209} An interesting finding in these studies is that the bisguanidine analogues exhibit noncompetitive inhibition despite the structural similarity between the bisguanidine analogues and the natural substrate.

Thus far, three classes of inhibitors have been identified for JmjC HDMs: N-oxalylglycine analogues, HDAC inhibitors and pyridine carboxylate derivatives. N-oxalylglycine analogues were originally designed to target Fe^{2+} and α -ketoglutarate dependent oxygenases such as Hypoxia Inducible Factor (HIF), prolyl hydroxylase PHD2 ($K_i = 8\mu\text{M}$) and Asparaginyl hydroxylase factor-inhibiting –HIF ($K_i = 1.2\text{mM}$).²⁰⁹⁻²¹¹ N-oxalyl glycine, the lead compound in this group, was tested against JMJD2E and showed an IC_{50} of $78\mu\text{M}$ which decreased to $28\mu\text{M}$ with a 30 minute preincubation.²¹² Other derivatives with substitution of the benzyl or ethylphenyl group at the N-oxalylated D-amino acid, namely, N-Oxalyl-D-phenylalanine and N-oxalyl-D-homophenylalanine, showed IC_{50} values of 320 and $100\mu\text{M}$, respectively.²¹² The most effective in this group is the N-oxalyltyrosine derivative which showed an IC_{50} of $5.4\mu\text{M}$ against JMJD2E (Table 10).²¹³

HDACs inhibitors (e.g. SAHA or Vorinostat) are defined in part by a hydroxamic acid group and a lipophilic stretch that allows them to coordinate to Zn^{2+} in the cofactor binding pocket of HDACs. Since the catalytic mechanism employed by JmjC involves coordination to Fe^{2+} , it was thought that HDAC inhibitors such as SAHA could also inhibit JmjC HDMs. In addition, some of the JmjC HDMs such as JMJD2 family members (A-E) contain a zinc binding site.²¹⁴ As such, HDAC inhibitors such as trichostatin A (TSA), SAHA and butyric acid were tested against JMJD3E.²¹² Butyric acid was ineffective against JMJD2E, and TSA could not be assayed because it inhibited formaldehyde dehydrogenase (FDH) which was used in the assay to oxidize the product of demethylation, formaldehyde to formate.²¹² SAHA on the other hand was moderately effective against JMJD2E with an IC_{50} value of $540\mu\text{M}$ which significantly decreased to $14\mu\text{M}$ with a 30 minute preincubation.²¹² In comparison, SAHA has been shown to inhibit HDAC1 with an IC_{50} value of 48nM which is more than 1000-fold more effective.²¹⁵ Nevertheless, the advantage of JmjC HDAC specific inhibitors such as SAHA and TSA is that these drugs are already in the market and enzyme kinetic studies have shown mixed competitive inhibition for SAHA against JMJD2E, suggesting that the way SAHA inhibits JmjC HDMs is different from HDACs.

The pyridine carboxylate derivatives were originally developed to inhibit collagen prolyl hydroxylases (e.g. collagen prolyl-4-hydroxylases) with Fe^{2+} as a cofactor. The most effective of these inhibitors are pyridine-2,4-dicarboxylic acid (2,4-PDCA) with an IC_{50} of $1.4\mu\text{M}$ for JMJD2E²¹², 8-hydroxyquinoline-5-carboxylic acid (8-HQ-5-COOH) with an IC_{50} of $2.0\mu\text{M}$ for JMJD2A²¹⁶ and internal hydroxamic acid with an IC_{50} of $2\mu\text{M}$ for JMJD2A and $1.0\mu\text{M}$ for JMJD2C.²¹⁷ To be effective, JmjC inhibitors should be nonspecific against other $\text{Fe}^{2+}/2^-$ -ketoglutarate dependent oxygenases (i.e. HIF asparaginyl hydroxylase, factor-inhibiting HIF (FIH), and the human HIF prolyl hydroxylase domain 2 (PHD2) whose activities have been shown to control a wide array of genes. In this context, small molecules that target different regions of these JmjC enzymes will be the most promising candidates to remedy the problem of specificity.

Closing Remarks and Perspectives

Despite the promise of epigenetic therapies, in most cases available therapies lack specificity. Epigenetic drugs targeting DNA methylation or DNMTs show considerable cytotoxicity because these drugs cause global demethylation by passive demethylation or trapping of DNMTs. Such unintended consequences have limited the use of these drugs for prolonged periods of time. Because HDACs and HATs are part of macromolecular protein complexes, targeting them can also lead to unintended consequences. The lack of specificity is in keeping with the fact that epigenetic modifications are not stand alone processes, with synergistic interactions between and within marks increasing complexity of regulatory control. The inhibitors designed to target HMTs and HDMs mainly target cofactors and/or cofactor binding sites, leading to a considerable degree of non-specificity in light of the vast array of enzymes using the same co-factors to catalyze multiple processes. Thus, a more comprehensive structural analysis is needed to identify unique domain(s) and residues critical to the catalytic mechanisms used by these enzymes. Increased understanding of the histone code and the macromolecular protein complexes involved in epigenetic regulation will help refine the targeting of epigenetic therapies. This will involve the design of so called smart drugs that will target only specific epigenetic modifications, either alone or in combination with other marks. Thus, unless our understanding of the epigenome continues to improve and the key regulators of epigenetic control are identified, therapies targeting epigenetic modifications within the human genome will remain of limited value and restricted for widespread dissemination in the clinical setting.

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Figure 1.

Dynamic epigenetic regulation in normal cells showing crossed talk between epigenetic modifications resulting in transcriptional activation or repression. This figure depicts different epigenetic modifications under normal conditions for both expressed and repressed genetic targets. Histone Acetyltransferases (HATs), Histone Methyltransferase (HMTs), Ubiquitin ligases (E3), Ten Eleven Translocation (TET) DNA hydroxylase (convert 5mC to 5HmC) and Kinases add transcriptionally active epigenetic marks (e.g. AC, H3K4me3, H3K4me1, H3K27Ac, H3K36me3, H3K12Ac and 5HmC) to promoters, enhancers and gene bodies which promote the formation of euchromatin and transcriptional activation (**Top**). DNA Methyltransferase (DNMT), Histone Methyltransferase (HMT), Ubiquitin ligase (E3), Histone Deacetyltransferase (HDAC) put repressive epigenetic marks (H3K27me3, H3K9me3 and DNA methylation) to promoters, enhancers and gene bodies promoting the formation of heterochromatin and transcriptional repression (**Bottom**).

Figure 2.

Simplified version of the dynamic plasticity of epigenetic modifications in normal and tumor cells. In normal cells, gene rich regions are dynamically enriched with active transcription marks (i.e. H4 acetylation at lysine-5,-8,-12 and -16 (H4-(K5,8,12,16)Ac), H4 acetylation at lysine-9 (H4K9Ac), H3 trimethylation at lysine-4 (H3K4me3), 5-hydroxymethylcytosine (5-HmC)) to allow the transcription of tumor suppressor genes, while repeat regions are enriched with repressive marks (H4 acetylation at lysine 16 (H4K16Ac), H4 trimethylation at lysine-20 (H3K20me3), H3 trimethylation at lysine-27(H3K27me3), H3 dimethylation at lysine-9(H3K9me2) and DNA methylation (Me)) that lead to heterochromatin formation. In tumor cells, this dynamic is reversed leading to increased repressive marks at gene rich region and active marks at repeat regions. The dotted red arrow denotes the reversal of these epigenetic modifications by epigenetic drugs to the original state or to an alternate intermediate state. The red X denotes blockage of aberrant epigenetic modification by epigenetic drugs.

Table 1

Histone Acetyltransferases: Classes, Nomenclature and Substrate Specificity

NOMENCLATURE OF HUMAN HATs		SUBSTRATE SPECIFICITY
OLD	NEW	
TYPE A		
GNAT FAMILY • hGCN5 • hPCAF	• KAT2A • KAT2B	H2B, H3-K9/14/23/27, H4-K8/16 H3-K14, H4-K8
P300/CBP FAMILY • CBP • P300	• KAT3A • KAT3B	H2A-K5, H2B-K12/15/20, H3-K14/18/23, H4-K5/8/12 H2A-K5, H2B-K12/15/20, H3-K14/18/23, H4-K5/8
MYST FAMILY • TAF1 • TIP60/FLIP • MOZ/MYST3 • MORF/MYST4 • HMOF/MYST1 • HBO1/MYST2 • ELP3	• KAT4 • KAT5 • KAT6A • KAT6B • KAT7 • KAT8 • KAT9	H3 > H4 H2A-K5, H3-K14, H4-K5/8/12 H3, H4 H3-14 H4 (-5, -8, -12) > H3 H4-16 H3
TYPE B		
• HAT1	• KAT1	• H4-K5/-12

Table 2

Specificity and Activity of Histone Acetyltransferase Inhibitors

HATi COMPOUND	NAMES	HAT SPECIFICITY	ACTIVITY(IC ₅₀)	REF
SYNTHETIC HATis				
	Lys-CoA	KAT3B (P300)	3.2μM	37
	Lys-CoA-CH ₃	KAT2B (PCAF)	0.8μM	37
	Ph-Lys-CoA	KAT3B (P300/)	0.7μM	38
NATURAL HATis				
	Anacardic Acid	KAT3B (P300) KAT2B (PCAF)	8.5μM 5.0μM	39
	Garcinol (Toxic)	KAT3B (P300) KAT2B (PCAF)	7.0μM 5.0μM	40, 41
	Isogarcinol (Toxic) LTK14 (R = CH ₃ / -C(CH ₃) ₂) (Non-Toxic)	KAT3B (P300) KAT2B (PCAF) KAT3B (P300)	7.0μM 5.0μM 5.0-7.0μM	41
	Curcumin (R = OCH ₃)	KAT3B (P300)	25μM	40, 42
	Compound-11 R=Br	KAT3B (P300)	5.0μM	42
SMALL MOLECULE HATis				
	-Butyrolactone (R = nC ₃ H ₇)	KAT2A (hGCN5)	100μM	43

HATi COMPOUND	NAMES	HAT SPECIFICITY	ACTIVITY(IC ₅₀)	REF
	Isothiazolones (R1 = H, R2 = NO ₂ , R3 = Cl, R ₄₋₆ = H)	KAT2B (PCAF)	1.1μM	44
	CCT077791	KAT3B (P300) KAT2B (PCAF)	3.0μM 7.3μM	44

Red arrows or circles denote sites of side chain substitution

Table 3

Histone Deacetylases: Classes, Yeast Homologs, Cofactors and Localization

HDAC CLASSES	HOMOLOGY TO YEAST DEACEYTLASE DOMAIN	CO-FACTOR	LOCALIZATION
I • HDAC 1 & 2 • HDAC 3, & 8	<i>Rpd3</i> 81, 82 % 76, 65 %	Zn ²⁺ Zn ²⁺	Nucleus Nucleus/Cytoplasm
IIa • HDAC 4, 5,7 & 9	<i>Hda1</i> 52, 53, 51, 51 %	Zn ²⁺	Nucleus/Cytoplasm
IIb • HDAC 6 & 10	<i>Hda1</i> 61 & 65 %	Zn ²⁺	Nucleus/Cytoplasm
III • SIRT 1, & 2 • SIRT 3 • SIRT 4 & 5 • SIRT 6 & 7	<i>Sir2</i>	NAD ⁺ NAD ⁺ NAD ⁺ NAD ⁺	Nucleus/Cytoplasm Mitochondria/Nucleus Mitochondria Nucleus
IV • HDAC 11	<i>Rpd3</i> & <i>Hda1</i> 42 & 41%	Zn ²⁺	Nucleus

Table 4

Histone Deacetylase Inhibitors: Specificity and Current Status.

HDAC COMPOUND	NAMES	HDAC SPECIFICITY	STATUS	REF
	Vorinostat	HDAC1, HDAC2, HDAC3, HDAC6	APPROVED	54
	Romidepsin	HDAC1, HDAC2, HDAC3, HDAC8	APPROVED	54
	Panobinostat	HDAC1, HDAC2, HDAC3, HDAC6	PHASE II	53
	Valporic acid	CLASS I & IIa	PHASE I	65-67
	Phenyl butyrate	CLASS I & IIa	PHASE I/II	65-67
	SEN196	SIRT1	PHASE II	52
	COMPOUND 6J (R= -C ₄ H ₈)	SIRT-II	IC ₅₀ = 1.0 μM	54

Red circles denotes site of side chain substitution.

Table 5

DNA Methyl Transferase Inhibitors: Mode of inhibition and Current Status.

DNMTi COMPOUND	NAMES	DNMT SPECIFICITY	STATUS	REF
NUCLEOSIDE ANOLOGUES				
	Azacitine (5-Aza-CR)	DNA DNMT ADUCTs	APPROVED	96
	Decitabine (5-Aza-CdR)	DNA/RNA DNMT ADUCTs	APPROVED	96
	Zebularine	DNA/RNA DNMT ADUCTs	Preclinical	102-109
	5-Fluoro- deoxycytidine (FdCyd/FDAC)	DNA/RNA DNMT ADUCTs	Phase I/II	111-114
NON-NUCLEOSIDE ANOLOGUES				
	Procaine	DNMT1	Phase I	115
	Procanamide	DNMT1	Phase I	119
	Hydralazine	DNMT1, DNMT3A & 3B	Phase II/III	116-118
	RG108	DNMT1	IC ₅₀ = 1000μM	121

Red circles denote unique atoms or side groups relative to the parent compound.

Table 6

Histone Lysine Methyl Transferases: Nomenclature and Substrate Specificity.

NOMENCLATURE HMTs		SUBSTRATE SPECIFICITY
NEW	OLD	
KMT1A	SUV39H1	H3K9 (P53 at K373 by KMT1C)
KMT1B	SUV39H2	
KMT1C	G9a	
KMT1D	GLP	
KMT1E	ESET/SETDB1	
KMT1F	CLL8	
KMT (2A-2F)	MLL (1-5)	H3K36
KMT2G	hSET1A	
KMT2H	ASH	
KMT3A	SET2	H3K36
KMT3B	NSD1	
KMT3C	SYMD2	H3K36 P53 (K370), RB (K860)
KMT5A	PRESET7/8	H4K20
KMT5B	SUV4-20H1	
KMT5C	SUV4-20H1	
KMT6	EZH2	H3K27
KMT7	SET7/9	H3K4/P53
KMT8A	RIZ1	H3K9
KMT8B	PRDM9	H3K4me3
KMT8C	PRDM6	H3K20 (+KMT1C)
KMT8D	PRDM8	H3K9
KMT8E	PRDM3	
KMT8F	PRDM16	
KMT4	DOT1L	H3K79

Table 7

Histone Arginine Methyltransferases: Classification and Substrate Specificity.

HPRMTs	OTHER NAMES	TYPE	SUBSTRATE SPECIFICITY
HRMT1	ANM1, HCP1 IR1B4, HRMT1L2	I	H4R3, Ewing sarcoma (EWS) gene
HRMT2	MGC11137 HRMT1L1	I	H4
HRMT3	HRMT1L3	I	Ribosomal Protein S2 (RPS2)
HRMT4	CARM1	I	H3R17 (H3K18ac required), p160, NF- b.
HRMT5	JBP1, SKB1, IBP72 SKB1hs, HRMT1L5	II	H2A, H3, H4
HRMT6	FLJ10559 HRMT1L6	I	H3R2, H4R3, H2A-R3 DNA polymerase B (R83, R152)
HRMT7	FLJ10640 KIAA1933	II	H2A, H4R3
HRMT8 (Brain specific)	HRMT1L4	I	Ewing sarcoma (EWS) gene
HRMT9	FBXO11, VIT1 UBR6, FLJ12673 MGC44383	II	Not characterized
HRMT10	LOC90826 FLJ46629	Unknown	Not characterized
HRMT11	FBX10, FBXO10 FLJ41992 MGC149840	Unknown	Not characterized

Table 8

Histone Methyl Transferase Inhibitors: Specificity and Activity.

HMTi COMPOUND	NAMES	HMT SPECIFICITY	ACTIVITY IC ₅₀	REF
HISTONE METHYLTRANSFERASE INHIBITORS (HMTis)				
	SAH	Non-Specific	-	154
	Sinefungin	Non-Specific	-	157
	Chaetocin	KMT1 KMT1A KMT1C	-	157,158
	BIX01294	KMT1C KMT1D	1.7nM 0.7nM	159
	UNC-0624	KMT1C KMT1D	1.7nM 1.9nM	160
	UNC-0638	KMT1C KMT1D	15nM 19nM	160
	AMI-1	Non-Specific	9μM	165
	AMI-6	PRMT1	1.4μM	165
	EPZ00477	KMT4	0.4nM	166
	AZ505	KMT3C	0.12μM	172

Red circles denote position where side groups (R) are substituted.

Table 9

Histone Demethylases: Nomenclature and Substrate Specificity.

NOMENCLATURE HDMs		SUBSTRATE
NEW	OLD	
KDM1A	LSD1/AOF2	H3K4me2/me1; H3K9me2/me (AR)
KDM1B	LSD2/AOF1	H3K4me2/me1
KDM2A	FBXL11A, JHDM1A	H3K36me2/me1
KDM2B	FBXL10B, JHDM1B	H3K36me2/me1; H3K4me3
KDM3A	JMJB1A, JHDM2A	H3K9me3/me1
KDM3B	JMJB1B, JHDM2B	
KDM4A	JMJB2A, JHDM3A	H3K9me3/me2 H3K36me3/me2
KDM4B	JMJD2B	
KDM4C	JMJD2C, GASC1	
KDM4D	JMJD2D	H3K9me3/me2/me1; H3K36me3/me2
KDM4E	JMJD2E	H3K9me3/me2
KDM5A	JARID1A, RBP2	H3K4me3/me2
KDM5B	JARID1B, PLU1	
KDM5C	JARID1C, SMCX	
KDM5D	JARID1D, SMCY	
KDM6A	UTX, MGC141941	H3K27me3/me2
KDM6B	JMJD3, KIAA1111	H3K27me3/me2; H3K9me2/me1 H4K20me1
KDM7	KIAA1718	H3K4me2/me1 H3K27m2/me1
KDM8	JMJD5/ FLJ13798	H3K36me2

Table 10

Histone Demethylase Inhibitors: Specificity and Activity.

HDMis COMPOUND	NAMES	HDM SPECIFICITY	ACTIVITY IC ₅₀	REF
HISTONE DEMETHYLASE INHIBITORS (HDMis)				
	TRANLYCYPROMINE (PCPA) (R = H)	KMD1A	Ki = 357μM Non-Specific	200
	Compound 2	KMD1A	1.9μM	201
	S2101 (R = H)	KMD1A	0.6μM	203
	Compound 22 (R = -CH ₂ CH ₂ NHNH ₂)	KMD1A	4.4nM	203
	PG11140 (Cis-Form) PG11150 (Trans-Form)	KMD1A KMD1A	1.0μM 5.0μM	202
	N-OXALYLGLYCINE (R = H)	JMJD2E	78μM 28μM (30mins)	215
	N-OXALYLTYROSINE	JMJD2E	5.4μM	215
	SAHA	JMJD2E	520μM 14μM (30mins)	213
	Pyridine-2,4-dicarboxylic acid (3,4-PDCA)	JMJD2E	1.4μM	210

HDMis COMPOUND	NAMES	HDM SPECIFICITY	ACTIVITY IC ₅₀	REF
	Hydroxyquinoline Carboxylic acid (8-HQ-5-COOH)	JMJD2A	2.0μM	214
	HYDROXAMINE	JMJD2C JMJD2A	1.0μM 3.0μM	215

Red circles denote position where side groups (R) are substituted or where conformation stereoisomer originate (i.e. PG11144 and PG11150).