# Epigenetic regulators and their impact on therapy in acute myeloid leukemia

Friederike Pastore[1] and Ross L. Levine[2]

[1]Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center; [2]Leukemia Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York City, NY, USA

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## ABSTRACT

Genomic studies of hematologic malignancies have identified a spectrum of recurrent somatic alterations that contribute to acute myeloid leukemia initiation and maintenance, and which confer sensitivities to molecularly targeted therapies. The majority of these genetic events are small, site-specific alterations in DNA sequence. In more than two thirds of patients with *de novo* acute myeloid leukemia mutations epigenetic modifiers are detected. Epigenetic modifiers encompass a large group of proteins that modify DNA at cytosine residues or cause post-translational histone modifications such as methylations or acetylations. Altered functions of these epigenetic modifiers disturb the physiological balance between gene activation and gene repression and contribute to aberrant gene expression regulation found in acute myeloid leukemia. This review provides an overview of the epigenetic modifiers mutated in acute myeloid leukemia, their clinical relevance and how a deeper understanding of their biological function has led to the discovery of new specific targets, some of which are currently tested in mechanism-based clinical trials.

**Correspondence:**

leviner@mskcc.org

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## Introduction

Next-generation whole genome and whole exome sequencing of large AML patient cohorts has broadened our understanding and led to the discovery of new classes of mutations, including in genes involved in epigenetic regulation. At least 70% of patients with *de novo* AML display at least one mutation in an epigenetic modifier.[1] Epigenetic modifiers include proteins that chemically modify DNA or catalyze post-translational modifications on histones. Abnormal epigenetic patterns caused by these mutations can lead to aberrant gene expression in AML. Several novel specific epigenetic therapies are in pre-clinical testing or have recently entered clinical trials.

## Mutations in epigenetic regulators

### *Cytosine modifications*

*DNMT3A*

DNA methyltransferase 3A (DNMT3A) is a highly conserved 130 kDa protein that catalyzes *de novo* methylation of cytosine residues in DNA. Mutations in *DNMT3A* occur in 20-25% of *de novo* AML patients[2-4] and were first identified in 2010. *DNMT3A* mutations often co-occur with *NPM1* mutations and *FLT3*-ITD and confer adverse risk.[4,5] Although mutations can occur in different functional domains, almost 60% of patients display a heterozygous substitution of arginine 882 in the catalytic domain that abrogates methyltransferase activity and DNA binding *in vitro*.[6,7] The R882 mutation in AML patients correlates with global hypomethylation, especially at CpG islands, shores and promoters,[8] although promoter hypermethylation has also been described.[7,9,10] Mutant *Dnmt3A* - predominantly mutant R882 - has been shown to interact with wild-type *Dnmt3A* and *Dnmt3B* in a dominant negative manner inhibiting the wild-type methyltransferase activity of the tetrameric complex.[8,11]

*Dnmt3A* is required for the normal self-renewal capacity of HSCs in adult mice and for maintaining the differentiation potential of serially transplanted HSCs in wild-type recipients.[12] Conditional deletion of *DNMT3A* in murine HSC causes a higher self-renewal capacity and reduced differentiation resulting in an accumulation of HSC in the bone marrow.[10,13] In two studies, patients with *DNMT3A* mutations had higher survival rates when treated with high-dose daunorubicin (90 mg/m$^2$) compared to standard-dose daunorubicin (45 mg/m$^2$)[14,15] although this has not been studied in other, well-annotated clinical trial cohorts.

*TET2*

TET2 is a member of the Ten-Eleven translocation protein family of enzymes that regulate DNA methylation through the α-ketoglutarate and Fe(II)-dependent conversion of 5-methylcytosine (5mC) to 5-hydroxymethyl cytosine (5hmC).[16] *TET2* mutations are most often heterozygous with retained expression of the wild-type allele. They occur in 7-23% of AML patients depending on the cohort, and confer a poor prognosis in CN-AML.[17,18] 5hmC is thought to be critical in DNA demethylation[19] but likely has other roles in regulating epigenetic state and transcriptional output. Biochemical analyses have revealed that TET2 mutations are associated with decreased levels of 5hmC[20-22] and DNA hypermethylation, including at promoters and intragenic regions. *Tet2* loss in murine models and in human cells leads to HSC self-renewal, stem cell and progenitor expansion and a skewing to the myelomonocytic and granulocytic lineages.[23-26]

*WT1*

The zinc finger DNA-binding protein Wilms' tumor 1 (WT1) is a sequence-specific transcription factor. 10-15% of patients with AML harbor mutations of *WT1*.[1,27-29] WT1 mutations predominantly occur as truncating mutations or as nonsense mutations targeted by nonsense-mediated decay and resultant loss of WT1 protein expression.[30] WT1 mutations are negatively correlated with *TET2* and *IDH1/2* mutations[14,31] and to co-occur with *FLT3*-ITD or biallelic *CEBPA* mutations.[32,33] In patients with cytogenetically normal AML, *WT1* mutations are associated with chemo-resistant disease and a lower event-free, 5-year relapse-free and 5-year overall survival rate.[6,29,32,33] DNA methylation analysis revealed similar hypermethylation signatures in *IDH1/2, TET2* and *WT1* mutated patient samples, with a significant overlap between the *TET2* and *WT1* mutant signatures[31] consistent with a converging synergistic effect on DNA methylation. Liquid chromatography-mass spectrometry demonstrated *WT1* mutant AML samples have reduced 5hmC levels, consistent with reduced TET2 enzymatic function. As is observed with shRNA-mediated Tet2 knockdown in murine HSC, shRNA-mediated knockdown of Wt1 reduces 5hmC levels.[31,34] Co-immunoprecipitation studies revealed that Wt1 physically interacts with Tet2 via its zinc-finger domain[31] and can also directly interact with Tet3. The significant overlap of differentially expressed genes in murine HSC with knockdown of Wt1 compared to knockdown of Tet2, and similar phenotype in functional studies, indicate similar effects on hematopoietic differentiation and a similar role in leukemic transformation.

*IDH1 and IDH2*

IDH1 and IDH2 encode NADP-dependent isocitrate dehydrogenases, homodimeric enzymes which normally catalyze the oxidative decarboxylation of isocitrate to alpha-ketoglutarate (α-KG) (synonymous: 2-oxoglutarate (2OG)) within the citric acid cycle. The *IDH1* gene is located on 2q34, the *IDH2* gene is located on 15q26.1. *IDH1/2* mutations are hemizygous missense mutations in single arginine residues within the active site of the enzyme. *IDH1* mutations almost always occur at arginine R132 (alteration predominantly R132H), and *IDH2* mutations occur at the homologous arginine R172 (predominantly R172K) and at arginine R140 (predominantly R140Q).[35,36] Mutations in the *IDH1* gene were initially described in brain tumors.[37] *IDH1* mutations in AML were reported by Mardis *et al.*[38] followed by the discovery of the *IDH2* mutant in AML by Ward *et al.*[35], Gross *et al.*[39] and Marcucci *et al.*[40] *IDH1* and *IDH2* mutations occur with a frequency of 6-7% and 9-11%, respectively, and are mutually exclusive with mutations of *TET2*.[21,36,41,42] *IDH1* mutations show a significant association with mutations in *NPM1* and *MLL*[43]. *IDH1* and *IDH2* mutations are most common in cytogenetically normal AML (CN-AML) with frequencies of 10.4%[36,38,43] and 12.1%-19%, respectively.[40,44] *IDH1/2* mutations have been identified in other myeloid diseases including MDS[45] and MPN.[46] *IDH1* mutations are mainly associated with an inferior outcome, whereas the prognostic relevance of *IDH2* mutations depends on the specific allele and on the choice of anti-leukemic therapy.[47]

All *IDH1* and *IDH2* mutations are novel gain-of-function mutations, leading to a neomorphic enzyme activity catalyzing the reaction from α-KG to 2-hydroxyglutarate (2-HG) and converting NADPH to NADP.[35,39,48] This enzymatic reaction is performed by heterodimers formed by the mutated IDH1 or IDH2 protein together with the wild-type IDH protein. 2-HG enantiomers - R-2-HG and S-2-HG - are physiologically present within cells and tissues at low concentrations. IDH1/2 mutations in AML patients lead to the production and increased serum concentration of R-2-HG, but not of S-2-HG. Thus, R-2-HG has been proposed as a potential biomarker to assess treatment response and to follow minimal residual disease.[39] Experiments in the human erythroleukemic cytokine-dependent (GM-CSF) cell line TF-1, which is able to differentiate in the presence of erythropoietin (EPO),[49] showed that the stable expression of IDH1R132H caused cytokine-independent growth and a block of differentiation.[50] Moreover, R-2-HG itself, in the absence of an IDH1 mutation, was sufficient to induce transformation (cytokine independency and block of differentiation) in this cellular model. This effect was specific to R-2-HG, but not S-2-HG, and could be reversed by withdrawing R-2-HG from the cell medium. Although the mechanisms of action of this oncometabolite R-2-HG in leukemic transformation have not been fully elucidated, many putative functions for R-2-HG have been suggested (for an extensive review please refer to Cairns *et al.*[51]). *IDH1/2* mutations have been shown to competitively inhibit the 2-ketoglutarate-dependent function of *TET2*, resulting in elevated 5-methylcytosine levels. Jumonji C (JmjC) domain-containing histone demethylases (JHDM) are 2-ketoglutarate-dependent dioxygenases leading to a demethylation of mono-/di- and trimethylated lysines on histones in the presence of iron and 2-ketoglutarate.[52] JHDM are competitively inhibited by 2-HG,[53,54] followed by impaired histone demethylation and a block of differentiation.[55] Experiments in mice with either a complete knock-out of Idh1 or knock-in with a heterozygous

Idh1R132H suggest that the tumor promoting effects of the Idh1 mutations are not caused by the lack of Idh1 protein, but rather by production of the oncometabolite R-2-HG.[51,56] In a conditional knock-in model expressing Idh1R132H from the endogenous locus, Sasaki et al. showed[57] that Idh1R132H expression in all hematopoietic cells (Vav promoter) or in myeloid lineage cells (LysM promoter) resulted in an MDS-like phenotype (appearing within the first year) with expanded stem/progenitor numbers, anemia and extramedullary hematopoiesis. Lowe and colleagues have demonstrated that HSC derived from mice carrying *NRas* or *Flt3*-ITD mutations transduced with a vector containing mutant *Idh2* results in AML. Moreover, *Idh2* mutant-driven AML cells differentiated after inhibition of Brd4 *in vitro*.[58]

### Polycomb group proteins and group interacting proteins

Lineage specific regulation of gene expression in normal hematopoiesis is regulated by histone-modifying enzyme complexes, including polycomb group proteins (PcG) or trithorax group proteins (Trx).

*PRC1 complex*

The PRC1 complex consists of 4 members, CBX, BMI1 and the histone ubiquitin ligases RING1A and RING1B. PRC1 is involved in the maintenance of gene repression by recognizing H3K27me3 via CBX proteins, Histone H2A ubiquitination via RING1A and RING1B and the recruitment of DNA methyltransferases.[59] BMI1 has been associated with increased HSC self-renewal and leukemic reprogramming of myeloid progenitors.[60] Overexpression of BMI-1 and RING1A/RING1B have been detected in myeloid malignancies,61 whereas knock-out of BmiI in MLL-AF9 transformed GMP increased differentiation.

*PRC2 complex*

The canonical PRC2 complex is formed by EZH2, a methyltransferase, catalyzing the di- and trimethylation of the repressive chromatin mark H3K27,[62] SUZ12, EED and RBAP48. Genetic defects in PRC2 components other than EZH2 are not common in myeloid malignancies, and occur more frequently in ALL.[63-65] *EZH2* mutations occur in 1-2% of AML patients, mostly as loss-of-function mutations in contrast to diffuse large cell lymphoma, where they appear as gain-of-function mutations reinforcing H3K27 methylation.[66-68] Mutations in EZH2 are more common in MDS and are associated with cytopenias, but not with disease progression to AML.[69] Conditional Ezh2 deficiency in murine hematopoietic cells leads to diminished generation of pre-B cells and immature B cells in the bone marrow[70] and causes an MPN/MPD phenotype[71] which is accentuated in the setting of concomitant *Tet2* loss.

*Asxl1*

The *ASXL1* gene is 1 of 3 mammalian homologs of the additional sex combs (*Asx*) gene initially identified in Drosophila. It is located on chromosome 20q11.21, contains 13 exons and spans 81 kb.[72] The gene belongs to the group of enhancers of trithorax and polycomb (ETP) genes that are involved in the regulation of *HOX* genes. *ASXL1* has dual functions in the silencing and activation of gene expression. The ASXL1 protein consists of an N-terminal ASX homology (ASXH; amino acids 249-348) region containing 2 putative nuclear receptor coregulator binding (NR box) motifs, the heterochromatin protein-1 (HP1) binding site and the lysine-specific demethylase 1 (LSD1) binding site, 3 other NR box motifs and a C-terminal zinc finger plant homeodomain (PHD; amino acids 1506-1537). ASXL1 is involved in the post-translational modification (PTM) of histones and is a member of a repressive complex containing histone H1.2.[73] Abdel-Wahab *et al.* showed that ASXL1 regulates histone H3K27 methylation through its interaction with the polycomb-repressive complex 2 (PRC2) members EZH2, SUZ12 and EED. ASXL1 loss results in a global loss of H3K27 trimethylation (H3K27me3), although expression levels of PRC2 members remain the same.[74] Furthermore, ASXL1 regulates histone H3K4 and H3K9 methylation by direct interaction of the N-terminal region of ASXL1 with HP1 and LSD1.[75] This leads to an accumulation of methylated H3K9 (repressive histone mark) and unmethylated H3K4 (active histone mark).

Heterozygous somatic mutations in the ASXL1 gene were described in MDS/MPN/AML by Gelsi-Boyer in 2009.[76] Mutations are present throughout the entire coding region,[77] but are clustered in the 5' end of exon 12. The mutations are most commonly heterozygous frameshift and nonsense mutations, leading to the deletion of the PHD domain.[76] Studies of ASXL1 protein expression in cell lines have indicated a reduced stability of the mutant forms of ASXL1 relative to wild-type and a more rapid degradation of the cDNA of *ASXL1* mutant forms.[78] Interestingly, a truncated ASXL1 protein could not be detected in most of these cases. Hence, leukemogenic *ASXL1* mutations are loss-of-function mutations. *ASXL1* loss results in a similar gene expression pattern as the mixed lineage leukemia-AF9 (MLL-AF9) gene expression signature including an upregulation of the expression of posterior *HOXA* genes.[78] These findings suggest that ASXL1 may function as a tumor suppressor in malignancies of the myeloid lineage by affecting stem or progenitor cell self-renewal and/or differentiation. *ASXL1* mutations have been found in patients with a spectrum of myeloid malignancies including myelodysplastic syndromes (MDS) (13-18.5%), chronic myelomonocytic leukemia (CMML) (43%), myeloproliferative neoplasms (MPN) (2-23%) and AML (9%-18%).[79-81] *ASXL1* mutations have been shown to co-occur with RUNX1 mutations and are inversely correlated with mutations of *NPM1, DNMT3A, FLT3*-ITD and *FLT3*-TKD.[79,81] *ASXL1* mutations are associated with an unfavorable overall survival rate (median overall survival 15.9 months *vs.* 22.3 months; $P$=0.019), and a significantly lower complete response rate to induction chemotherapy (61% *vs.* 79.6%; $P$=0.004) in AML.[82]

*Asxl1* constitutive knock-out mice have partially penetrant perinatal lethality. Surviving knock-out mice exhibit defects in the frequency of differentiation of lymphoid and myeloid progenitors, but not in multipotent progenitors.[83] Myeloerythroid lineage defects in mice with a homozygous deletion of *Asxl1* (*Asxl1*$^{tm1BC}$/*Asxl1*$^{tm1BC}$) are mild.[1,83] A conditional knock-out of Asxl1 in the hematopoietic compartment resulted in a myelodysplastic phenotype[77,78] which was accentuated by concomitant *Tet2* loss.

*JARID2*

The Jumonji at rich interactive domain 2 (JARID2) gene is a PRC2 complex interacting protein, that recruits PRC2 complex to DNA target loci[84] and inhibits the methyltransferase activity of PRC2. *JARID2* mutations (deletions) are

found in progression from chronic myeloid malignancies to acute leukemia.[85]

### Trithorax genes

*MLL1*

*MLL* genes belong to the family of SET domain containing protein lysine methyltransferases that methylate H3K4, a transcriptional activation mark. *MLL* translocations and *MLL* partial tandem duplications occur in AML with frequencies of 5-10% and 5-7%, respectively, and are associated with an inferior prognosis.[86,87-91] Common target genes of MLL fusion proteins are *HOXA* cluster genes[92] and *MEIS1*. Combinations of HOXA9 and MEIS1 as well as of the MLL fusion MLL-AF9 result in leukemic transformation of murine HSC and progenitor cells.[93] Several studies have shown that the H3K79 methyltransferase DOT1L is crucial for the initiation and maintenance of *MLL*-AF9 rearranged AML.[94-96] Menin, a component of the MLL-SET1 like histone methyltransferase complex, is another indispensable protein that interacts with MLL in leukemogenesis. The inhibition of Menin has been shown to induce apoptosis and differentiation in leukemic blasts harboring *MLL* translocations.[97,98]

*Other lysine methyltransferases and demethylases*

Fusions of the histone lysine methyltransferase NSD1 and Nup98 occur in a minority of AML patients, but are associated with a dismal prognosis.[99-101] The methylation induced by NSD1 is physiologically antagonized by demethylases LSD1 and LSD2. Mutations or translocations involving Jumanji C lysine demethylases e.g. JARID1 alter H3K4 methylation. 10% of AML patients with AML M7 harbor *JARID1-NUP98* fusions, and expression of this fusion gene is associated with an aggressive course of disease and an adverse outcome.[102,103]

*Histone acetyltransferases (HAT)*

Histone acetyltransferases alter chromatin compaction in favor of a less compact chromatin by acetylation of lysine residues. Alterations of enzymes belonging to this group e.g. *CBP* (mutations) or *MOZ* (translocations) have been detected in ALL and AML.[104-106]

### Strategies for targeting epigenetic regulators

Multiple studies substantiate that mutations of epigenetic modifiers result in increased self-renewal of murine HSC and HSPC, myeloproliferation and extramedullary hematopoiesis, but do not give rise to AML. Thus, epigenetic modifiers contribute to leukemogenesis, but are alone not capable of causing leukemia. The fact that these mutations can occur in early "pre-leukemic" stages e.g. in patients with preceding MDS, underscores that they are underlying mutations that will be detected in different malignant clones within the same patients at later time points, which makes them a relevant therapeutic target. Epigenetic modifications occur physiologically in the process of differentiation. Thus, these modifications are generally reversible and make them an attractive target (Figure 1 and Figure 2).

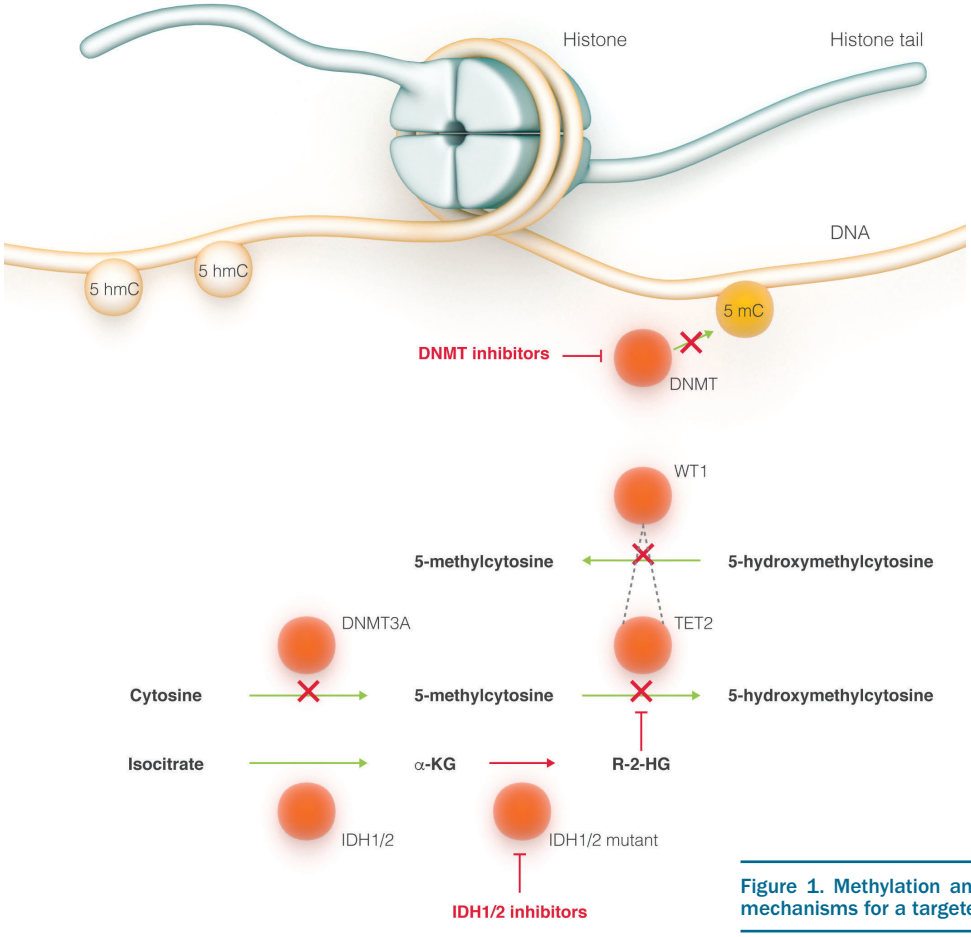


**Figure 1. Methylation and hydroxymethylation of DNA and mechanisms for a targeted therapy.**

*DNMT methyltransferase inhibitors*

The nucleoside analogs azacytidine (AZA) and 5-aza-2-deoxycytidine [decitabine (DAC)] integrate into normal DNA during S phase, inhibit DNA methyltransferases by forming bonds with DNMTs and cause their degradation.[107,108] Although both agents can induce robust global methylation changes (hypomethylation) *in vitro* and *in vivo*, overall response rates are low at 30-50%.[109]

*Histone deacetylase inhibitors*

The balance between histone acetylation and deacetylation plays a critical role in the regulation of gene expression. Histone acetylation induced by histone acetyl transferases (HATs) is associated with gene transcription, whereas histone hypoacetylation induced by histone deacetylases (HDACs) confers gene silencing.[110] 18 human HDACs can be assigned to four different classes of HDACs. HDACs have also been shown to deacetylate proteins involved in cell cycle control, differentiation and aopotosis.[110,111] The potential of HDAC inhibitors as monotherapy in the treatment of MDS and AML has been assessed in a number of clinical trials. Although HDAC inhibition showed anti-leukemic activity *in vivo*, therapeutic outcome with single agent HDAC therapy remained poor with response rates of up to 17% for vorinostat,[112-114] 13% for the oral inhibitor MGCD0103115 and less for other inhibitors.[116,117] Combinations of HDAC inhibitors with hypomethylating agents have been assessed in clinical studies and showed higher response rates and synergistic anti-leukemic effects.[118-120] Combinations of HDAC inhibitor panobinostat with JQ1 inhibitors, EZH2 inhibitors or LSD1 inhibitors showed synergistically lethal effects on AML cells.[121-123] A recently presented phase 1 study, in which a short course of panobinostat was given in addition to the classical "7+3" induction chemotherapy in 22 patients >60 years with *de novo* AML or high-risk MDS, showed a CR/CRi rate of 40% and a median survival rate of 16 months in responders in this high-risk cohort.[124]

## IDH inhibitors

Several small molecule targeting mutant *IDH1/2* enzymes have been developed and tested in pre-clinical models and are currently being evaluated in clinical trials. The IDH1-R132H inhibitor AGI-5198 was first described in the context of glioma cells harboring *IDH1* mutations and shown to reduce 2-ketoglutarate levels, demethylation of H3K9me3 and induce differentiation.[125] Treatment of *IDH1*-mutated AML progenitor cells with HMS-101, another IDH1 inhibitor, resulted in a decrease of 2 HG levels and block of colony formation.[126] First results of the clinical phase I trial of the orally administered IDH1 inhibitor, AG-120, in 66 patients with relapsed/primary refractory AML harboring an *IDH1* mutation (NCT02074839) demonstrate an overall response rate of 36% with a CR rate of 18% and a median response duration of 5.6 months.[127] The IDH1 inhibitor IDH305, and the IDH1 and IDH2 mutant inhibitor AG-881 are currently being tested in patients with *Idh* mutant malignancies in trials which are currently recruiting (NCT02381886, NCT02492737). The IDH2-R140Q inhibitor AGI-6780 binds allosterically to the IDH2R140Q dimeric interface and causes differentiation of the erythroleukemic cell line TF-1 and primary human AML blasts.[128] The IDH2 inhibitor AG-221 decreased 2-HG levels by >90%, induced differentiation and prolonged survival in a dose-

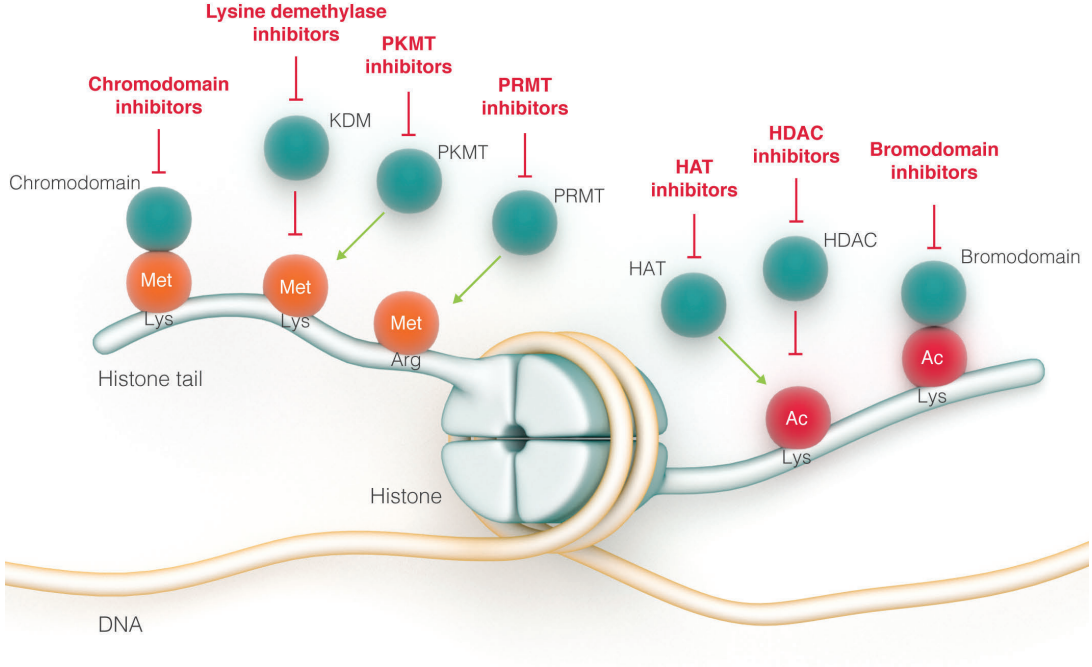


**Figure 2. Posttranslational methylation and acetylation of histones and mechanisms for a targeted therapy.** Arg: Arginine; HAT: histone acetyltransferase; HDAC: histone deacetylase; KDM: Histone lysine demethylase; Lys: lysine; PKMT: protein lysine methyltransferase; PRMT: protein arginine methyltransferase.

dependent manner in an AML IDH2R140Q xenograft model.[129] Preliminary results of its first-in-human phase I/II dose escalation study (NCT01915498) in patients with advanced myeloid malignancies showed CR and PR rates of 18% and 15%, respectively, in 128 relapsed/refractory AML patients, irrespective of the number of prior treatment regimens. The median duration of response was 6 months. This clinical benefit is achieved by a differentiation of the malignant clone despite the persistence of the mutant IDH2 VAF.[130]

### EZH2 inhibitors

EZH2 inhibitors have proven efficacy in lymphoma and are currently being explored in these diseases in clinical trials. Of note, GSK126, a potent, highly selective EZH2 inhibitor decreased global H3K27me3 levels, and reduced proliferation of *EZH2* mutant DLBCL cell lines and of *EZH2* mutant DLBCL mouse xenograft models.[131] Although *EZH2* mutations occurring in AML are loss-of-function mutations, in contrast to lymphoma where the majority confers a gain-of-function, an intact EZH2 and PRC2 complex is required for aberrant self-renewal in *MLL*-rearranged AML.[132,133] Deletion of EZH2 in *MLL*-rearranged mouse models impaired growth and progression of AML.[134] Likewise, knockdown of EZH2 in HL-60 cells promoted AML differentiation and reduced clonogeneic potential.[135] Recent data have shown that EZH2 inhibitors e.g. 3-deazaneplanocin (DZNep) induced apoptosis in leukemic *MLL*-rearranged cells and reduced the frequency of leukemia initiating cells (LICs).[136,137] UNC1999, a dual EZH1 and EZH2 inhibitor is a promising new oral target in *MLL*-rearranged leukemia.[138]

### Bromodomain inhibitors

BRDT, BRD2, BRD3, and BRD4 belong to the family of bromodomain and extraterminal (BET) human bromodomain proteins. They facilitate transcriptional activation by binding acetylated chromatin.

### BRD4 inhibitors

JQ1 is a cell-permeable small molecule that binds competitively to the acetyl-lysine recognition motif of BRD4.[139] JQ1 has been demonstrated to have anti-leukemic activity *in vitro* and *in vivo* in diverse AML models.[140,141] In *FLT3*-mutated AML, $CD34^+$ human blast progenitor cells apoptosis was enforced by the combination of JQ1 with the FLT3 tyrosine kinase inhibitor ponatinib or AC220.[121] Also, combinations of JQ1 with the HDAC inhibitor panobinostat increased apoptosis in human AML blast progenitor cells.[122] A more stable and soluble derivate of JQ1 for clinical application, JQ2 (TEN-010), is currently being tested in a phase I study in patients with AML and MDS (NCT02308761). The BRD4 inhibitor, GSK525762, has entered early clinical trials in patients with relapsed refractory hematological malignancies (NCT01943851). The new BRD4 inhibitors in late pre-clinical trials - EP11313 and EP11336 - have demonstrated a favorable pharmacologic profile compared to GSK52762, and led to a growth inhibition of c-myc deregulated AML cell lines, which was more pronounced when combined with ATRA.[142]

### BRD2/3/4 inhibitors

OTX015 is another Bromodomain inhibitor, that specifically prevents BRD2, 3 and 4 from binding to acetylated histones which leads to the suppression of super-enhancer driven oncogenes. OTX015 has proven antiproliferative efficacy in pre-clinical studies in lymphoid cell lines and mouse models.[143] A dose-finding phase I trial of oral OXT015 (NCT01713582) in 41 patients with relapsed/refractory acute leukemia (37 AML, 1 high-risk MDS, 3 ALL), showed 2 complete remissions and 1 CR with incomplete count recovery. The main dose-dependent side effects were diarrhea and thrombocytopenia.[144] The BRD inhibitor CPI-0610 in combination with MDM2 inhibition has shown efficacy in *in vitro* and murine *in vivo* experiments in eradicating p53 wild-type AML and sparing normal hematopoiesis.[145] A phase 1 study of CPI-0610 in patients with pretreated AML, MDS and MPS is currently recruiting patients (NCT02158858).

### LSD1 inhibitors

The histone H3K4/K9 demethylase LSD1 can regulate gene activation and repression in epigenetic regulation and is a key effector of the differentiation block in *MLL*-rearranged leukemia.

High LSD1 expression blocks differentiation and is associated with a poor prognosis in AML. LSD1 can be targeted by tranylcypromine analogs or downregulated by RNA interference which induces differentiation of *MLL*-rearranged leukemic cells.[146] The combination of ATRA and LSD1 inhibition by tranylcypromine in cell lines and primary AML samples showed a more potent anti-leukemic effect than either drug alone.[147] The selective tranylcypromine derivative LSD1 inhibitors ORY-1001 developed by Oryzon Genomics (EudraCT number 2013-002447-29) and GSK2879552 developed by GlaxoSmithKline (NCT02177812) have entered early phase clinical trials in patients with relapsed and refractory acute leukemia. A phase I clinical study of ATRA and tranylcypromine for adult patients with AML and MDS (NCT02273102), and a phase I/II trial of ATRA and tranylcypromine in patients with relapsed or refractory AML and no intensive treatment possibility (NCT02261779) are currently recruiting. Co-treatment of the LSD1 inhibitor 2509 and panobinostat showed synergistic lethality of primary AML blasts and prolonged survival in xenograft AML mouse models compared to either agent alone.[123]

IMG-98 is a novel LSD1 inhibitor that irreversibly binds to LSD1's essential cofactor FAD and thereby leads to its inactivated enzyme form. Exposure of AML cell lines to IMG-98 has been shown to promote differentiation and growth inhibition of AML blasts, especially in combination with ATRA. The first clinical trials with an optimized drug closely related to IMG-98 are expected to start in early 2016.[148]

### DOT1L inhibitors

DOT1L inhibitors selectively impair DOT1L-mediated H3K79 methylation and inhibit the expression of leukemogenic genes. Several DOT1L inhibitors have been successfully tested in *MLL*-rearranged AML (*MLL-AF6* and *MLL-AF9*) cells in xenograft models, where they have impeded proliferation and caused cell cycle arrest in cells expressing the MLL fusion.[149-153] The DOT1L inhibitor Pinometostat (EPZ-5676) was able to cause sustained complete remission in a xenograft model. The first results from its phase I clinical trial for, thus far, 49 patients with advanced hematological malignancies, including relapsed/refractory *MLL*-rearranged AML (NCT01684150), revealed an overall response in 6 patients (2 of whom

achieved a CR) with an acceptable safety profile. Pinometostat plasma concentrations in these patients correlated with the inhibition of global H3K79 methylation in PBMC and reductions in methylation of MLL target genes.[154] Data from Chen *et al.* demonstrated that DOT1L inhibition favors an open chromatin state by the inhibition of chromatin localization of the repressive SIRT1/H3K9 methyltransferase SUV39H1 complex.[155] Of note, a combination therapy of a DOT1L inhibitor and SIRT activators have demonstrated enhanced antiproliferative activity against *MLL*-rearranged cell lines.[155]

### JmjC-containing demethylases

H3K27me3 negatively regulates gene transcription by promoting a compact chromatin structure.[156,157] Mutations in members or associated proteins of the PRC2 complex such as *EZH2, SUZ12, EED* and *ASXL1* result in a loss of H3K27me3, providing a rationale for a therapy that inhibits demethylation on H3K27.[64,67,78] Demethylation by JmjC containing demethylases is α-KG-dependent and can be inhibited by small molecules. GSK-1 - or its corresponding ethyl ester prodrug GSK-4 - is a selective H3K27 demethylase inhibitor that inhibits JMJD3 and UTX and impairs TNF-α production by human primary macrophages in an H3K27-dependent manner.[158,159]

## Summary

In recent years our knowledge of the mutational landscape of AML has deeply improved and the relevance of mutations involving epigenetic modifiers has been highlighted. Epigenetic modifiers represent a new class of mutations that affect global chromatin state and DNA methylation, and control large numbers of genes and pathways by inducing alterations in DNA methylation or DNA hydroxymethylation and histone post-translational modifications. *In vitro* and *in vivo* models have delineated that mutations in epigenetic regulators are not by themselves sufficient to initiate AML, but can induce the expansion of a stem/progenitor clone that is susceptible to other mutations. In fact, mutations in epigenetic modifiers often co-occur with classical mutations in signaling effectors and transcription factors, but also with other epigenetic mutations. Furthermore mutations of epigenetic modifiers have not only been found in AML, but occur also in MPN and MDS, and even in subjects with clonal hematopoiesis in the absence of a myeloid neoplasia.

The number of different epigenetic mutations that are potentially targetable for a specific personalized therapy currently outnumber the available inhibitors. However, some newly developed compounds demonstrate target inhibition and are currently been investigated in clinical trials with promising clinical efficacy. There is growing evidence that the inhibition of a specific epigenetic modifier may not kill the malignant clone, but in many cases rather leads to the differentiation of leukemia cells. Consequently, these agents will require a longer time to display their full therapeutic effects as compared to chemotherapy. A plausible clinical setting for these kinds of inhibitors could therefore be as an "add on" to conventional post-remission therapy. Pre-clinical studies have suggested that combination therapies of two or more epigenetic drugs, or a combination of an epigenetic drug combined with a kinase inhibitor, may have additional synergistic effects. Critical ongoing efforts include further accurate pre-clinical models to elucidate how mutations in epigenetic modifiers interact with other AML disease alleles, and clinical studies to assess the efficacy of epigenetic therapies alone or in combination with other anti-leukemic agents.

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