

Targeting histone methyltransferase and demethylase in acute myeloid leukemia therapy

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Abstract: Acute myeloid leukemia (AML) is a clonal disorder of myeloid progenitors characterized by the acquisition of chromosomal abnormalities, somatic mutations, and epigenetic changes that determine a consistent degree of biological and clinical heterogeneity. Advances in genomic technologies have increasingly shown the complexity and heterogeneity of genetic and epigenetic alterations in AML. Among the genetic alterations occurring in AML, frequent are the genetic alterations at the level of various genes involved in the epigenetic control of the DNA methylome and histone methylome. In fact, genes involved in DNA demethylation (such as *DNMT3A*, *TET2*, *IDH1*, and *IDH2*) or histone methylation and demethylation (*EZH2*, *MLL*, *DOT1L*) are frequently mutated in primary and secondary AML. Furthermore, some histone demethylases, such as LSD1, are frequently overexpressed in AML. These observations have strongly supported a major role of dysregulated epigenetic regulatory processes in leukemia onset and development. This conclusion was further supported by the observation that mutations in genes encoding epigenetic modifiers, such as *DMT3A*, *ASXL1*, *TET2*, *IDH1*, and *IDH2*, are usually acquired early and are present in the founding leukemic clone. These observations have contributed to development of the idea that targeting epigenetic abnormalities could represent a potentially promising strategy for the development of innovative treatments of AML. In this review, we analyze those proteins and their inhibitors that have already reached the first stages of clinical trials in AML, namely the histone methyltransferase DOT1L, the demethylase LSD1, and the MLL-interacting protein menin.

Keywords: acute myeloid leukemia, epigenetic modifications, methyltransferases, demethylases

Introduction

Epigenetic modification of DNA

In the nucleus of eukaryotic cells, DNA is packaged together with structural proteins (histones) to form a supramolecular complex known as chromatin. Chromatin is arranged in repeating units, known as nucleosomes: each nucleosome is formed by a sequence of DNA wound around eight histone cores. Chromatin may have a condensed conformation, known as heterochromatin, is essentially transcriptionally repressed, or a decondensed conformation known as euchromatin, and is transcriptionally active. The regulation of chromatin conformation is a fundamental biologic process and regulates, at specific sites, accessibility to DNA, a step required to allow gene transcription, replication, recombination, and DNA repair. Epigenetic mechanisms determine heritable changes in gene expression that are due to alterations in the DNA sequence. Various epigenetic mechanisms play an essential role in the control of chromatin conformation, and are mainly represented by posttranslational modifications of histone, adenosine triphosphate (ATP)-dependent nucleosome remodeling, DNA modifications, and replacement of histone with histone variants. Posttranslational modifications of histones

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represent one of the main epigenetic mechanisms to control gene expression.

Histones are highly basic nuclear proteins. They represent spools around which DNA winds, and play an essential role in the control of gene expression.¹⁻³ There are five main families of histones: H1/H5, H2A, H2B, H3, and H4. H1/H5 are known as linker histones, while H2A, H2B, H3, and H4 are known as core histones. The linker histones bind the nucleosome at the entry and exit sites of the DNA, thus maintaining DNA in place. The core histones form dimers, and four dimers form one octameric nucleosome core. Each canonical histone protein – H3, H4, H2A, and H2B – shares a common structural domain consisting of 3 α helices (α 1, α 2, and α 3), separated by two loops (L1 and L2) called the histone fold, facilitating the heterodimerization of H2A with H2B and H3 with H4. These heterodimeric interactions form the dimeric structural unit of nucleosomes. There are variants for the core histones H3, H2AB, and H2B: the histone variant H3.3 differs from the canonical histone H3 in five amino acids required for the genomic localization of H3.3 (usually H3.3 occupies gene promoters), and H2A.Z has considerable amino-acid variation compared to H2A.

Histone proteins undergo many modifications, mainly represented by lysine methylation, arginine methylation, arginine citrullination, lysine acetylation, and serine/threonine/tyrosine phosphorylation. Each nucleosome is composed of a short segment of DNA of about 145–147 base pairs and is engulfed by a histone octamer core, consisting of four dimers of each core histone (H2A, H2B, H3, and H4). The core histone is essentially a globular structure, except for histone tails, composed of about 30 amino acids. These tails are modified by numerous posttranslational modifications, including methylation, phosphorylation, acetylation, ubiquitination, SUMOylation, ADP ribosylation, deamination, and crotonylation. These epigenetic, posttranslation modifications of histone are highly controlled, governed by three categories of enzymatic proteins: “writers” are involved in the addition of chemical groups to histone tails, “erasers” involved in the removal of these chemical groups, and “readers” are proteins that specifically recognize these histone modifications. Notable examples of writers are represented by histone acetyltransferases (HATs) and histone methyltransferases, while erasers are represented by histone deacetylases and lysine demethylases; examples of readers are given by bromodomain-containing proteins, methyl-lysine and methyl-arginine-binding domain-containing proteins and PDH-containing proteins.

Histone acetylation is a dynamic process regulated by two families of enzymes: HATs and histone deacetylases

(HDACs).⁴ HATs catalyze the transfer of an acetyl group to the ϵ -amino group of lysine side chains of the histone protein; these enzymes use acetyl-CoA as a cofactor. This reaction determines the shift of lysine from positively charged to a more neutral condition, reducing the affinity of the histone tail protruding from the nucleosome. As a consequence of these changes, chromatin adopts a more relaxed structure, more suitable for transcriptional activity through the binding of transcription machinery. Particularly, the BET proteins BRD2, BRD3, and BRD4 recognize and bind to the acetylated histone-lysine residues. HDACs act in an opposite way, removing the acetyl groups from histone lysines. HATs comprise many enzymes, including CREBBP (CBP), EP300 (p300), KAT2B (PCAF), KAT5 (Tip60), and KAT6A (Mo2); HDACs include HDAC1–11 and SIRT1–7.

Histone methylation is a very important biologic process through which the expression of many genes is controlled. Histones can be methylated at the level of either lysine or arginine residue.⁵ Lysine residue can be monomethylated, demethylated, and trimethylated. Arginine residues can be monomethylated or demethylated. Methylation is a dynamic process. Methyl units to histone molecules are added by *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase and erased by specific demethylases represented by flavin-dependent LSD1 (also known as KDM1A) and LSD2 (also known as KDM2A) or by the Jmj family of 2-oxoglutarate-dependent demethylase.⁵

Histone-lysine methylation can lead either to transcriptional activation or transcriptional repression. Methylation of histone H3K4, H3K36, and H3K79 is associated with transcriptional activation, while demethylation and trimethylation of H3K9 and H3K27 are associated with transcriptional repression. Methylation at specific lysine residues is catalyzed by specific methyltransferases: H3K4 methylation is catalyzed by the SET domain-containing methyltransferases MLL1 (KMT2A) and MLL2 (KMT2D), H3K79 is methylated by DOT1L, H3K27 methylation is catalyzed by EZH1 and EZH2, H3K36 methylation is performed by SETD2 and WHSC1, H3K9 methylation is induced by MECOM (EVI1) and PRDM16, and H3K9 dimethylation and trimethylation is induced by SUV39H1 and EHMT2. It is important to note that methylation occurring at the level of H3K9 and H3K27 represents the two main mechanisms of gene silencing in mammalian cells. EZH1 and EZH2 make up part of PRC2 in association with two other subunits: EED and SUL12.

It is important to point out that in addition to epigenetic modifications of histone, the epigenetic modification of DNA through methylation represents another key mechanism of gene regulation.⁶ DNA methylation is associated with

transcriptional repression and occurs at the level of the C₅ carbon of cytosine in DNA to form 5-methylcytosine (5-mC), through a reaction catalyzed by three types of DNA methyltransferase: DNMT1, DNMT3A, and DNMT3B. TET enzymes (TET1, TET2, and TET3) promote the oxidation of 5-mC to 5-hydroxymethylcytosine (5-hmC), promoting the demethylation process. IDH1 and IDH2 enzymes catalyze the conversion from isocitrate to α -ketoglutarate (α KG), which is required for the catalytic function of TET enzymes.

Alterations in epigenetic modifiers in acute myeloid leukemia

A considerable number of genetic studies have been performed in acute myeloid leukemia (AML), allowing identification of the most frequent genetic alterations observed in these leukemia types. Comprehensive whole-genome sequencing, exome sequencing, and targeted sequencing studies have shown that mutations at the level of the complex epigenetic gene machinery are frequent in AML. *DNMT3A* is mutated in 20%–25% of adult AML, and frequently involves the substitution of the amino acid arginine at position 882. *DNMT3A* mutation is frequently found in association with *FLT3*^{ITD}, *NPM1*, *IDH1*, and *IDH2* mutations.⁷ *DNMT3A*^{R882H} has dominant negative activity that markedly reduces DNA methylation. DNA hypomethylation is an initiating event in the development of *DNMT3A*-mutated AML and thus represents the cause and not the consequence of leukemia development. Furthermore, CpG-island hypermethylation, a phenomenon frequently observed in *DNMT3A*^{WT} AML, is dramatically reduced in *DNMT3A*-mutated AML.⁸ In line with these observations, conditional knock-in mice develop AML with enlarged hematopoietic stem-cell compartments and involvement of the mTOR pathway. Consequently, *DNMT3A*-mutated leukemic cells are sensitive to the mTOR inhibitor rapamycin.⁹ *DNMT3A* mutations occur during the preleukemic phase of AML pathogenesis, supporting the key role for aberrant DNA methylation and consequent epigenetic reprogramming in malignant transformation of hematopoietic cells. The presence of *DNMT3A* mutations in AML is associated with poor outcome and reduced survival.^{10,11} *DNMT1* is not mutated in AML, but is frequently overexpressed.¹² DNMT1, which methylates hemimethylated DNA, is involved in the differentiation of normal hemopoietic stem cells (HSCs) and maintenance of leukemic SCs through epigenetic silencing of genes that inhibit self-renewal and leukemogenesis.¹³ Recent research has suggested that DNMT1A could represent a therapeutic target for some AML. In fact, DNMT1A expression can be targeted in leukemic cells by inhibitors of FABP4 (upregulated in AML

and stimulates DNMT1A expression in these cells)¹⁴ or by inhibitors of receptor tyrosine kinases.¹⁵ These treatments result in inhibition of tumor growth, induction of cell differentiation, and impairment of leukemic progress in leukemia animal models.^{14,15} Very interestingly, a recent study provided evidence that MUC1-C, a transmembrane oncoprotein aberrantly expressed in leukemic SCs (where it is coexpressed with DNMT1), drives DNMT1 transcription.¹⁶ Targeting MUC1-C with a specific monoclonal antibody, together with the DNMT1 inhibitor decitabine, markedly reduces DNMT1 expression and impairs the survival of AML cells.¹⁶

The *ASXL1* gene encodes a chromatin-binding protein and is mutated in about 3%–5% of AML. The incidence of these mutations is higher in patients with intermediate risk and particularly with high-risk and secondary AML, where it is mutated in about 16% of patients. *ASXL1* mutations, as a single prognostic factor, are associated with a negative outcome.¹⁷ *ASXL1* gene mutations are particularly frequent (20%) in *RUNX1*-mutated AML¹⁸ and are often associated with myelodysplasia-related changes.¹⁹

EZH2, a catalytic component of PRC2, which mediates transcriptional silencing through di- and trimethylation of lysine 27 of histone H3, playing an important role as a histone-lysine *N*-methyltransferase enzyme, is mutated in about 2% of adult AML, where inactivating mutations are usually observed.²⁰ Some AML displays a decrease in EZH2 protein levels due to a posttranslational mechanism triggered by EZH2 phosphorylation induced by CDK1.²¹ EZH2 inactivation is frequently observed at disease relapse, and is associated with *HOX* gene derepression and resistance to multiple drugs.²¹ EZH2 loss is frequently associated with a decrease in H3K27me3 levels.²¹ *EZH2* mutations are more frequent in *RUNX1*-mutated¹⁸ and secondary AML, and are often associated with increased HOXA9 expression.²²

About 3%–5% of de novo AML patients display partial tandem duplication of the *MLL* gene, which is characterized by internal tandem duplication of exons 3–9 or 3–11.²³ *MLL*^{PTD} acts as an oncogene by upregulating the expression of *HOX* genes. These AML types display frequent mutations of other epigenetic regulators, such as TET2 (16%), EZH2 (10%), IDH1/2 (31%), and ASXL1 (6%). Furthermore, a typical feature of *MLL*^{PTD} consists of the absence of *NPM1* mutations and frequent *RUNX1* (23%) and *STAG2* mutations (16%).²³ *BCOR* is a component of the variant-group polycomb-repressive complex, mutated in about 4% of karyotype-normal AML. *BCOR* plays an important role in the control of hematopoiesis by inhibiting myeloid-cell proliferation and differentiation and regulates *HOX* gene expression.²⁴

Interestingly, in a recent molecular classification of AML based on the analysis of a large set of samples, one of the largest groups was represented by AML with mutated chromatin, RNA-splicing genes or both, characterized by mutations of genes regulating chromatin (*ASXL1*, *STAG2*, *BCOR*, *MLL*^{PTD}, and *PHF6*), RNA splicing (*SRSF2*, *SF3B1*, *U2AF1*, and *ZRSR2*), or transcription (*RUNX1*).²⁵ As such, no single molecular lesion defines this group. Interestingly, overlapping patterns of mutations are observed among the defining genes of this AML group.²⁵ It is important to note that as stated earlier, this AML subgroup also contains AML types with mutations of the splicing-factor machinery. Interestingly, mutations at the level of these genes interfere with pre-mRNA splicing of genes that are involved in the epigenetic machinery, such as *BCOR*, *MLL2*, and *EZH2*, and through this mechanism affect hematopoiesis.^{26,27} It is important to underline that most patients in the chromatin-spliceosome group were older, with lower white-cell and blast-cell counts and response rates and negative long-term clinical outcome.²⁵ Therefore, it is evident that most of these patients must be classified as high-risk AML.²⁸

About 3% of de novo adult AML harbors a translocation of the *MLL1* gene fused to various partners, including AF4, AF9, ENL, AF10, ELL and AF6; secondary *MLL*-rearranged AML is observed in patients treated with topoisomerase inhibitors.²⁹ These AML types have a negative prognosis, and are thus classified as high-risk AML. The main pathogenic mechanism of these AML types is related to the capacity of the *MLL*-fusion proteins to aberrantly regulate *MLL*-target genes, such as *HOXA* and *MEIS1*, and to modify the genetic program of proliferation and differentiation of *HSC/HPC*.³⁰

TET2 is a protein that acts as an epigenetic modifier to convert methylcytosine to hydroxymethylcytosine. This protein is mutated in about 10%–20% adult AML,³¹ and its frequency is significantly higher in older AML patients.³² *TET2* mutations in AML were not associated with distinct clinical or genetic features, except for *IDH* mutations, which were almost mutually exclusive with *TET2* mutations.³³ At the clinical level, it is unclear whether the presence of *TET2* mutations represents a factor affecting patient outcome. The mutation of such genes as *TET2*, *IDH1*, and *IDH2* causes defective conversion of 5-methylcytosine to 5-hmC, impairing demethylation of DNA. Recent immunocytochemical and biochemical studies have shown that AML with *TET2* mutations shows reduced 5-hmC levels; however, 5-hmC levels were not predictive of survival in AML patients with normal-karyotype AML.^{34,35} Importantly,

TET2 mutations are found also in the white blood cells of otherwise-normal adults with clonal hematopoiesis, a condition related to aging and associated with myeloid-lineage bias and increased risk of development of myelodysplastic syndrome (MDS) or AML.³⁶ These observations have led to a hypothesis that *TET2* mutations represent a preleukemic abnormality required for the initial steps of leukemic transformation, enabling disease progression. In line with this hypothesis, a recent study provided evidence that *TET2* mutations are essential to induce the survival and aberrant self-renewal of leukemic SCs.³⁷ Interestingly, vitamin C, able to enhance 5-hmC in *TET2*-deficient cells, drives DNA hypomethylation, induces the expression of a *TET2*-dependent gene signature, inhibits colony formation of *TET2*-mutated human leukemic cells, and blocks leukemia progression in primary leukemia patient-derived xenografts.³⁷ Finally, vitamin C strongly synergizes with PARP inhibitors to induce the killing of *TET2*-mutated leukemic cells.³⁷

The NADP⁺-dependent isocitrate dehydrogenases (*IDH1* and *IDH2*) are critical metabolic enzymes involved in the interconversion of isocitrate to α KG. Recurrent somatic mutations of *IDH1* occur in 6%–10% of adult AML cases: these mutations affect the arginine residue at position 132 or 170. *IDH2* mutations occur in 8%–12% of adult AML, affecting the arginine residue at position 140 or 172. Leukemia-associated *IDH1/IDH2* mutations confer the neomorphic activity of reducing α KG to the oncometabolite 2-hydroxyglutarate. The accumulation of this oncometabolite inhibits α KG-dependent dioxygenases, including histone demethylases and methylcytosine dioxygenases of the *TET* family.³⁸ The consequent epigenetic deregulation results in DNA and histone hypermethylation, altered gene expression, and blocked cell differentiation.²⁸ The presence of *IDH* mutations does not confer specific properties to leukemic cells, apart from *IDH2*¹⁷² mutations. In fact, *IDH2*¹⁷²-mutant AML represents a subgroup of AML corresponding to about 1% of all AML types²⁵ and displaying gene-expression and DNA-methylation profiles that differ from the profiles for other *IDH* mutations, and display peculiar aberrations in metabolic activity.^{39,40} Specific inhibitors of mutant *IDH1* and *IDH2* enzymes have been developed and introduced into clinical trials. These inhibitors have demonstrated a remarkable single-agent activity in relapsed/refractory AML patients. Patients with relapsing/refractory *IDH2*-mutant AML displayed in 40% of cases a clinical response to treatment with enasidenib (AG221), an *IDH2*-mutant-specific inhibitor. Importantly, 19% of these patients achieved a complete response, with median survival of about 20 months.⁴¹

Interestingly, the responding patients displayed blast-cell leukemic-cell differentiation.⁴¹ Mutations of some genes involved in the epigenetic DNA machinery are frequent in AML, and among these mutations, *TET2*, *ASXL1*, and *DNMT3A* are associated with reduced overall survival.⁴²

In parallel with these observations, other studies have analyzed the occurrence of gene mutations in AML at the clonal level and during the history of disease (ie, at diagnosis and relapse). The results of these analyses have defined the clonal evolution of the AML process. The most relevant results of these studies showed that mutations in genes encoding epigenetic modifiers, such as *DNMT3A*, *TET2*, *ASXL1*, *IDH1*, and *IDH2*, were acquired during the early steps of leukemia development and were present in the founding clone, while other mutations, such as those involving *NPM1*, *FLT3*, and *RAS*, were secondary events occurring at late steps of leukemic development. Importantly, these mutations of epigenetic modifiers are observed also in aging individuals in the context of clonal expansion of hematopoiesis, a preleukemic condition associated with an increased risk of developing leukemia. Furthermore, these mutations usually persist after therapy, lead to clonal expansion during remission, and contribute together with new mutations to disease relapse.^{43,44} The analysis of large sets of older people of different geographical regions without evidence of hematologic malignancies has confirmed a high incidence of clonal hematopoiesis (5%–10% at 70 years; about 20% at 90 years), and has shown that the most commonly mutated genes are *DNMT3A*, *TET2*, and *ASXL1*.^{45,46}

Several recent studies have explored the pattern of epigenetic changes occurring in the various AML types and attempted to classify these leukemias according to this pattern and define possible associations between these epigenetic changes and patient outcome. Large-scale genome-wide DNA-methylation profiling has revealed the existence of distinct DNA-methylation patterns in AML, thus indicating that these leukemias are composed of epigenetically distinct diseases.⁴⁷ Different cytogenetic and molecular AML subtypes were found to display different DNA-methylation profiles.^{47–50} AML characterized by specific translocation events, such as t(8;21)-AML1/ETO, inv(16)-t(16;16)-CBFB-MYH11, t(15;17)-PML-RAR α , and t(v;11q23)-MLL, was characterized by unique DNA-methylation signatures.^{47–50} The group of *NPM1*-mutated AML was heterogeneous for the methylation pattern: four methylation clusters were identified, one hypermethylated and three both hyper- and hypomethylated,⁴⁷ this methylation heterogeneity seemingly reflecting the molecular heterogeneity of this karyotype-normal AML

subgroup and dictated by the co-occurrence of *DNMT3A*, *FLT3*, and *IDH1/IDH2* mutations.^{47,48} *DNMT3A* mutations and particularly *DNMT3A*^{R882} mutations were associated with hypermethylation.⁵¹ *TET2* and *IDH1/IDH2* mutations were associated with a genome-wide hypermethylation signature, particularly pronounced for *IDH1/IDH2*-mutated AMLs.^{51,52}

Various studies have supported a possible role of DNA methylation pattern as a prognostic index for predicting clinical outcome in AML patients. In this context, particularly interesting was a recent study by Lusk et al. These authors performed a multilocus DNA assessment using an xMELP assay and calculated a methylation statistic (M score), showing that: the M score was lower in patients surviving after 2 years compared to that observed in dead patients, and the same applies for complete remission; low-M-score AML patients had better overall survival than high-score patients; and the M score was not associated with established molecular markers, such as *NPM1* and *FLT3*^{ITD} mutations, but was clearly associated with mutations in *DNMT3A* and *IDH1*.⁵³

In this context, Li et al explored epigenetic heterogeneity and possible links among genetic heterogeneity, genetics, and epigenetics in AML.⁵⁴ They observed that the degree of methylation variation, defined as epiallele burden, at the same loci between samples represented a predictor of relapse risk among AML patients. Patients were subdivided according to the level of allelic burden, and those with high allelic burden relapsed more rapidly, compared with those with low epiallele burden. Importantly, the prognostic significance of the level of allelic burden was independent of cytogenetics and white-blood-cell counts at diagnosis. Comparison between AML blasts and normal bone-marrow cells showed that 100% leukemic samples displayed epigenetic allele shifting compared to normal bone marrow. Furthermore, 92% of patients showed epigenetic allele shifting between diagnosis and relapse. Importantly, there was no link between epigenetic burden and mutation burden. Finally, there was no increase in genes that regulate methylation (*DNMT1*, *DNMT3A*, *TET1*, *TET2*, *IDH1*, *IDH2*) in those samples with higher level of epiallele burden.⁵⁴

Constant advancements in the identification of molecular abnormalities of AML has allowed the proposal of new classifications of AML neoplasia, encapsulating information on genetic abnormalities, morphology, immunophenotype, and clinical presentation. The first French–American–British classification defined eight AML subtypes (M0–M7) according to morphological and cytochemical features: M0 (undifferentiated acute myeloblastic leukemia), M1 (acute myeloblastic leukemia with minimal maturation), M2 (acute myeloblastic

leukemia with maturation), M3 (acute promyelocytic leukemia [APL]), M4 (acute myelomonocytic leukemia), M4E (acute myelomonocytic leukemia with eosinophilia), M5 (acute monocytic leukemia), M6 (acute erythroid leukemia), and M7 (acute megakaryoblastic leukemia). In 2008, the World Health Organization (WHO) introduced a new classification based on the integration of molecular and cellular criteria.⁵⁵ Finally, in 2016 a new revised updated version of the WHO classification of myeloid neoplasia was released.⁵⁶

The integration of cytogenetic and molecular criteria allows the stratification of AML into three prognostic subgroups: 1) favorable prognosis for various AML subtypes identified according to the cytogenetic and molecular features t(8;21) with no c-Kit mutations, inv(16), t(15;17), mutated *NPM1* without *FLT3*^{ITD} (karyotype cytogenetically normal [CN]-AML), and mutated biallelic *CEBPA* (CN-AML); 2) intermediate prognosis, including t(8;21) with c-Kit mutation, t(9,11), CN-AML other than those included in the favorable or adverse prognostic group, and cytogenetic abnormalities not included in the favorable or adverse prognostic group; and 3) adverse prognosis, including *TP53* mutation, regardless of cytogenetic profile, CN-AML with *FLT3*^{ITD}; CN-AML with *DNMT3A*, CN-AML with *KTM2A*^{PTD}, inv(3), t(6,9), -5, or del(5q), -7, complex karyotype, and 11q abnormalities other than t(9;11).

A recent study²⁵ based on a very large set of primary adult AML (1,540) patients proposed a detailed molecular classification of these neoplasia, with the identification of 13 subgroups:

- AML with *NPM1* mutations (about 27%), frequently displaying also *DNMT3A*, *FLT3*^{ITD}, *NRAS*, and *TET2* mutations
- AML with mutations in genes encoding chromatin, RNA-splicing genes, or both (about 18%), including *RUNX1*, *MLL*, *SRSF2*, *DNMT3A*, *ASXL1*, and *STAG2* mutations
- AML with *TP53* mutations, chromosome aneuploidy, or both (about 13%), including *TP53* mutations, complex karyotype -5/5q, -7/7q, -12/12p, and +8/8q
- AML with inv(16) or t(16;16), *CBFB-MYH11* (about 9%)
- AML with biallelic *CEBPA* mutations (about 4%)
- AML with t(15;17), PML-RAR α (about 4%)
- AML with t(8;21)(q22;q22), AML1-ETO (about 4%)
- AML with *MLL* fusion genes, t(x;11)(x;q23) (about 3%)
- AML with iv(3) or t(3;3), GATA2, MECOM (about 1%)
- AML with *IDH2*^{R172} mutations (about 1%)
- AML with driver mutations, but no detected class-defining lesions (about 11%), frequently displaying *FLT3*^{ITD} or *DNMT3A* mutations

- AML with no detected driver mutations (4%)
- AML meeting criteria for multiple genomic subgroups (4%).

Adult AML patients with AML usually receive a standard treatment based on 7 days of cytarabine and an anthracycline for 7 days. Using this standard treatment, some groups of AML patients have an approximate “cure” probability (favorable risk), whereas other groups have a survival comprised in a range of 6–18 months. First-generation epigenetic drugs, such as azacitidine and decitabine, currently used for the treatment of MDS, are also used for treating AML patients not eligible for treatment with intensive chemotherapy and with stem-cell transplantation. Under this epigenetic treatment, only 15%–20% of patients display a complete response. More recently, guadecitabine was introduced, a next-generation hypomethylating agent that is not metabolized by cytidine deaminase and the enzyme that degrades decitabine. A recent study showed that >50% of treatment-naïve AML patients >65 years old (77 years mean age) displayed a complete response to treatment with guadecitabine.⁵⁷ Responding patients displayed a median survival >500 days, with an acceptable drug-related toxicity profile. Analysis of the various types of patients enrolled in this study provided evidence about the existence of genetic determinants underlying the response of AML patients to guadecitabine. Patients with *RAS* and *IDH2* mutations had much less chance of getting a complete response to guadecitabine treatment than those without these mutations. In contrast, patients with mutations of other epigenetic regulators, such as *DNMT3A*, *ASXL1*, *EZH2*, *TET2*, *U2AF1*, or *WT1*, had a comparable chance of developing a complete response, as well as those with AML without these mutations.⁵⁸ Furthermore, the frequency of complete responders was higher among patients with naïve AML (56%) than those with relapsed–refractory AML (22%).⁵⁸ Although patients with relapsed–refractory AML displayed a lower rate of complete responses, responder patients displayed long-term survival, and their overall survival was significantly better than that observed for nonresponder patients.⁵⁹ In the whole AML-treated population, 19% of patients survived after 2 years; median overall survival was 6.5 months among nonresponder patients and >29 months in responder patients.⁵⁹ These results are encouraging, and strongly support the use of hypomethylating agents in older AML patients.

AML with *TP53* mutation has a very negative prognosis; these leukemias are usually associated with adverse karyotypes and are more frequent among older AML patients. Welch et al treated 88 AML patients with a 10-day regimen of decitabine and reported high rates of morphological

remission (46%).⁶⁰ Interestingly, they noted higher response rates among AML patients with an unfavorable cytogenetic profile than among those with intermediate or favorable cytogenetic profiles (67% versus 37%) and among patients with *TP53* mutations compared to those without *TP53* mutations (100% versus 41%).

Two recent studies have explored the clinical activity of guadecitabine in high-risk myelodysplasia patients refractory or relapsing after azacitidine and observed only modest response rates.⁶¹ The second study explored a population of untreated high-risk myelodysplasia, and provided evidence that guadecitabine was active in this set of patients (with 28% complete responses), even in patients with adverse biologic features, such as high frequency of complex karyotype, therapy-related disease, and *TP53* mutations.⁶² Targeting epigenetic abnormalities could represent a potentially promising strategy for the development to innovative AML treatments. Here, we analyze some epigenetic modifiers and their inhibitors, focusing on those that have reached the first stages of clinical trials in AML.

LSD1

Structure and function

Histone methylation is a dynamic process. Histone lysine and arginine residues are *N*-methylated at the level of H4; some nonhistone proteins, such as p53, can be also methylated. The effect of methylation on gene transcription is variable in that it can result either in induction or repression of gene transcription, depending on the extent of methylation and the position of the methylated residue. Therefore, examples of methylation typically associated with active gene expression are given by methylation of lysine 4 or 36 of histone H3, while examples of repressive methylation are represented by methylation of lysine 9 or 27 of histone H3. The combination of all the histone-modification events, including methylation, acetylation, phosphorylation, and ubiquitination, determine the final chromatin conformation and the transcriptional activation of a given gene. More than 20 lysine demethylases have been discovered, pertaining to two main gene families: the *KDM1* subfamily containing the LSD enzymes and the *KDM2–KDM7* subfamilies, consisting of JmjC-containing enzymes. The *KDM1* LSDs are mainly represented by LSD1 and LSD2, which are flavin-dependent amine oxidases related to monoamine and polyamine oxidases: these enzymes are dependent on a single electron pair within the lysine for catalysis and consequently can demethylate only mono- and dimethylated lysines. In contrast, JmjC KDMs are Fe²⁺- and 2-oxoglutarate-dependent deoxygenases capable of demethylating mono-, di-, and trimethylated lysines. LSD1 is

specifically involved in the demethylation of monomethylated and dimethylated lysine 4 residues on histone 3. LSD1 can demethylate the lysine residues also of some nonhistone proteins, such as p53 and DNMT1. LSD1 can also methylate H3K9 when it is complexed with the androgen receptor.⁶³ LSD2 specifically demethylates histone H3K4 me1–2 inside areas of its target genes.

The molecular structure of LSD1 and LSD2 proteins has been determined. LSD2 is a homologue of LSD1 based on its sequence, and shares about 30% sequence similarity with LSD1. Several structural/functional domains have been characterized. Some of these domains are present in both LSD1 and LSD2 proteins: an AOL domain, essential for enzymatic activity (the AOL domain has two lobes, one forming a noncovalent FAD-binding site and the other a substrate-binding and -recognition site), and a SWIRM domain, typically observed in chromatin-associated proteins (the SWIRM domain is involved in protein–protein interactions; it is also involved in the association with the androgen receptor). Other domains are specific to LSD1, such as the TOWER domain, while others are specific to LSD2, such as the amino zinc-finger domain, whose function is unclear (Figure 1).⁶⁴ As mentioned, the catalytic activity of both LSD1 and LSD2 enzymes is in the AOL domain and requires the cofactor FAD.

The determination of the three-dimensional structure of LSD1 considerably helped in the understanding of the function of this protein at the molecular level. LSD1 forms a highly symmetric, packed domain structure from which a long helical tower domain protrudes. The SWIRM domain, which contributes to the stability of the protein, packs together with the AOL domain through numerous interactions. The active site cavity present at the level of the AOL domain is spacious and capable of accommodating several residues of the histone-tail substrate.^{65,66}

The chemical demethylation reaction catalyzed by LSD1 is complex. During all the reactions catalyzed by LSD1, at each demethylation cycle a molecule of formaldehyde and H₂O₂ is produced and O₂ is consumed. The chemical reaction involves the initial conversion of methylated lysine to an iminium cation by loss of a hydride anion captured by the oxidized FAD prosthetic groups (Figure 1). The imine cation is then hydrolyzed to produce a carbinol amine, decomposing to formaldehyde, and the demethylated residue. The reduced FAD produced during the initial step of the reaction is reoxidized by O₂ to generate a molecule of H₂O₂ and regenerated oxidized FAD (Figure 1).^{67,68}

The capacity of LSD1 to form molecular complexes with other nuclear proteins and transcriptional factors is an

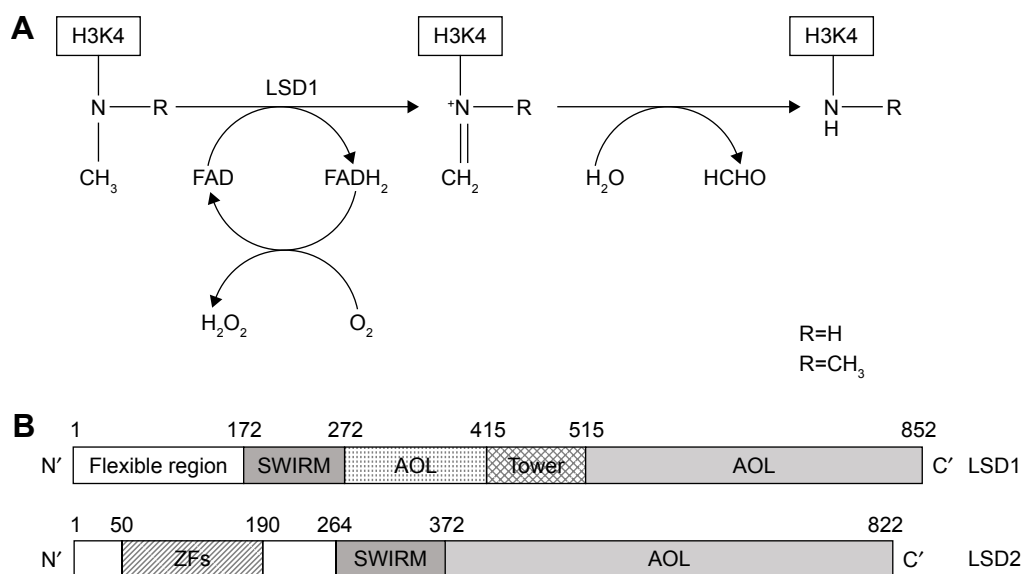


Figure 1 Schematic representation of enzymatic activity and linear structure of human LSD1 and LSD2.

Notes: (A) Enzymatic reaction catalyzed by LSD1. LSD1 catalyzes the demethylation of Lys4 of histone H3 (H3K4) through a flavin-dependent oxidative reaction. LSD1 acts on both di- and monomethylated H3K4. The reaction involves the steps shown from the left to right: first, the histone substrate is bound by the enzyme and the methylated Lys4 side chain is oxidized by the FAD prosthetic group, with consequent reduction of oxygen (O₂) to hydrogen peroxide (H₂O₂); second, the resulting imine intermediate is then hydrolyzed, thus generating the demethylated H3 tail and formaldehyde. (B) Overview of LSD1 and LSD2 structure. Human LSD1 is composed of 852 amino acids with major domains: an N-terminal SWIRM (small α -helical domain), contributing to the steadiness of the molecule; a central protruding Tower domain; and a C-terminal amine oxidase like (AOL) domain. Among these domains, the AOL and SWIRM domains pack together through various interactions (at the level of three-dimensional structure), determining the formation of a spherical structure. At the level of the N-terminal (1–172), there is an N-flexible region. Human LSD2 is composed of 822 amino acids and displays 31% of sequence similarity with LSD1. LSD2 shows three major domains, from the N- to the C-terminal: a ZFs area, a SWIRM, and an AOL domain. It is important to note that LSD2 does not contain a Tower domain, the structure of which plays a crucial role in LSD1 for CoREST binding.

essential property of its activity and the regulation thereof. In this context, it is important to point out that LSD1 was initially discovered as a molecular partner of HDAC2 in HeLa cells.⁶⁹ Subsequently, many other studies have shown that LSD1 participates in the formation of multisubunit complexes involving LSD1, CoREST (also known as RCOR1), HDAC1, HDAC2, ZNF217, PHF21A and HMG20B. This complex is commonly known as the CoREST transcription-repressor complex.^{70,71} The functional implications of all these interactions are not completely defined. However, the LSD1–CoREST interactions are required to protect LSD1 from proteosomal degradation, while the association with HDAC1/HDAC2 is required for both demethylase and HDAC activity.⁷² The molecular protein complex formed by LSD1 was covered in two recent reviews on LSD1.^{73,74}

The *LSD1* gene contains 19 exons, highly conserved among vertebrates. Through a process of alternative RNA splicing, two additional exons, E2a and E8a, can be included into mature mRNA and generate four possible LSD1 isoforms, called LSD1, LSD1-2a, LSD1-8a, and LSD1-2a+8a.⁷⁵ The inclusion of exon E2a can occur in all tissues, while LSD1 transcripts containing E8a are found only in brain and testis.⁷³ While insertion of the 60-nucleotide-long E2a exon does not modify the enzymatic activity of LSD1, insertion

of the 12-nucleotide-long exon E8a could modify LSD1 enzymatic activity (in fact, the LSD1-8a isoform plays a role in mediating H3K9 and H4K20 demethylation).

Role of LSD1 in normal hematopoietic differentiation

Gene-knockout studies have contributed greatly to our understanding of the role of LSD1 in the control of normal hematopoiesis. Conditional knockout studies have shown that a deficit of LSD1 expression in the hematopoietic system determines an expansion of HSCs and hematopoietic progenitor cells (HPCs) not associated with a significant increase in marrow cellularity and only a slight increase in white blood cells.⁷⁶ Analysis of bone marrow of LSD1-deficient animals showed an inhibition of granulopoiesis and a stimulation of monocytopenia, thus suggesting that LSD1 plays an important role in lineage choice during granulo/monocytic differentiation.⁷⁶ Analysis of erythroid-cell lineage has shown that LSD1 knockdown results in anemia associated with perturbed terminal erythropoiesis and expansion of early erythroid progenitors.⁷⁰ Other studies have supported the role of LSD1 as an indispensable epigenetic governor of hematopoietic differentiation.⁷⁷ This function is mainly exerted through the capacity of LSD1 to

repress hematopoietic stem and progenitor gene-expression programs during hematopoietic cell differentiation.⁷⁷ Deficient LSD1 expression causes a failure to silence HSC and HPC genes fully, compromising the different steps of the hematopoietic differentiation-cell program.⁷⁷

The activity of LSD1 during hematopoietic differentiation and its regulatory effects on hematopoiesis are regulated by its interaction with CoREST/REST (Rcor) corepressors. Upadhyay et al showed that the three Rcor proteins (Rcor1, Rcor2, and Rcor3) regulate LSD1 activity and cellular differentiation in hematopoietic cells. All the three Rcor proteins interact with LSD1 and the transcription factor Gfi1b: while Rcor1 and Rcor2 facilitate LSD1-mediated nucleosome demethylation, Rcor3 inhibits demethylation.⁷⁸ In line with these observations, Rcor1 and Rcor2 favor differentiation, while Rcor3 inhibits differentiation.⁷⁸ Rcor1 and Rcor3 levels are differentially regulated in erythroid and megakaryocytic cells during terminal stages of maturation.⁷⁸

The capacity of LSD1 to interact with some transcription factors acting as key master regulators of hematopoiesis is essential to explain its effects on hematopoietic differentiation. In hematopoietic cells, LSD1 and Rcor1 associate with Gfi1 and Gfi1b and repress most Gfi1b targets in erythroid cells.⁷⁹ Gfi-transcription factors are master regulators of hematopoietic cell differentiation: Gfi1 is essential for neutrophil differentiation, while Gfi1b is required for the development of erythroid and megakaryocytic lineages.⁸⁰ Furthermore, LSD1–Rcor1 also associates with other transcription factors, such as Scf1/Tal1,^{81,82} Bcl11A,⁸³ and GATA2,⁸⁴ in erythroid cells, inducing repression of their target genes. The excessive and uncoordinated function of all these transcription factors induced by LSD1 deficiency in HPCs determines a derangement of erythroid and megakaryocytic cell differentiation. The interaction between Gfi1 and LSD1 involves the SNAG domain of Gfi1: this domain needs to be methylated at the level of lysine 8 for optimal binding capacity to LSD1.⁸⁵

Finally, recent studies provided evidence that LSD1 plays a relevant functional role at the level of events that determine the initial specification of hematopoietic cell lineage during embryonic development. In vertebrates, the initial appearance of HSCs with long-term repopulation and multilineage-differentiation capacities occurs at the level of the aorta–gonad–mesonephros region. This primitive population of HSCs is generated from a rare subpopulation of endothelial cells, known as hemogenic endothelia and is capable of transdifferentiation through a process of endothelial to hematopoietic transition. This unique transdifferentiation

process requires a molecular orchestration requiring from one side the repression of the endothelial differentiation program and from the other side the induction of the hematopoietic differentiation program. Gfi1 and Gfi1b proteins mark the hemogenic endothelia, and are strictly required for the hemogenic activity of the hemangioblasts: the Gfi proteins, through the recruitment of LSD1 protein, exert their repressive effects on the endothelial differentiation program.⁸⁶ Furthermore, LSD1 activity in the hemangioblast is essential for the inhibition of the endothelial differentiation program through downregulation of the transcription factor Etv2, an essential regulator of vasculogenesis.⁸⁷

Gfi1 and Gfi1B act as transcriptional repressors by recruiting histone-modifying enzymes to promoters and enhancers of target genes, and thus can be considered epigenetic regulators that modify chromatin structure. Several rare hematological diseases are associated with acquired or inheritable mutations in the *GFI1* and *GFI1B* genes; particularly, some patients with severe congenital neutropenia carry mutations in *GFI1* that determine the disruption of the C-terminal zinc-finger domains.⁸⁸ Furthermore, recent studies have suggested a possible role of GFI1 in human leukemias. Hones et al analyzed a large number of AML samples for GFI1 expression, showing that about 10% of these expressed low GFI1 levels.⁸⁹ These leukemia types have a poor outcome and frequently display an adverse cytogenetic FABM0 phenotype, *NRAS* mutations, elevated *EVII* expression, and a leukemic stem-cell signature at the level of the gene-expression profile.⁸³ In experimental mouse models, low GFI1 expression accelerates leukemia development driven by oncofusion proteins, such as *MLLAF9*.⁸⁹ Low GFI1 confers sensitivity of leukemic cells to histone methyltransferase inhibitors, associated with resistance to HDAC inhibitors.⁸⁹ Recently, Volpe et al analyzed AML with normal karyotype for GFI1 expression and observed that those displaying high expression of this transcription factor have frequent *FLT3*^{ITD} and *NPM1* mutations, display an *FLT3*^{ITD} signature, and exhibit high expression of some leukemia-related genes, such as *HOXA9*, *MEIS1*, and *PBX3*.⁹⁰ At variance with the findings of Hönes et al⁸⁹ obtained in the whole AML population, in karyotype-normal AML, high GFI1 expression was associated with a worse outcome.⁹⁰

Very interestingly, mice deleted for Rcor1 were shown to be markedly anemic, showing a block of erythroid precursors at the transition from proerythroblasts to basophilic erythroblasts.⁹¹ Erythroid progenitors purified from Rcor1-null bone marrow cultured in vitro form myeloid colonies, but fail to form erythroid colonies;⁹¹ mutant proerythroblasts

aberrantly express genes typically expressed in myeloid cells and in SCs/PCs.⁹¹ Evaluation of the myelomonocytic lineage of *Rcor1*^{-/-} animals showed absence of mature neutrophils associated with an increase in monocytes, a feature observed also in *LSD1*^{-/-} mice.⁹²

LSD1 in leukemia

LSD1 is not mutated in acute leukemias or in other blood neoplastic disease. However, LSD1 is overexpressed in many hematologic diseases, including AML, acute lymphoblastic leukemia (ALL), myeloproliferative neoplasms, chronic myelomonocytic leukemia, and MDS.⁹³ In AMLs, LSD1 was overexpressed in about 60% of cases.⁹³

Using a model of human MLLAF9 leukemia, Harris et al identified LSD1 as a key regulator of leukemic stem-cell potential, in that this demethylase acts at the level of the genomic loci bound by MLLAF9, favoring the effect of the MLL-fusion protein and thus preventing cell differentiation and apoptosis.⁹⁴ In line with this finding, primary AML cells bearing various MLL rearrangements were shown to be markedly inhibited by small-molecule LSD1 inhibitors.⁹⁴ Interestingly, LSD1 was expressed in MLL-rearranged AML, as well as in other molecular subtypes of AML. In addition to MLL-rearranged AML, AML associated with PML-RARA and RUNX1-RUNX1T1 was shown to be sensitive to the LSD1 inhibitors.⁹⁴ Consistent with these findings, LSD1 was found among the 5% most highly expressed genes in prospectively purified immunophenotypic leukemic SCs from a variety of distinct AML subtypes.⁹⁵

According to these observations, elevated LSD1 expression in AML and in other hematological neoplasia may contribute to leukemia development. To test this hypothesis, Wada et al evaluated the effect of LSD1 overexpression in HSCs and HPCs by generating transgenic mice that overexpress LSD1 in HSCs/HPCs under control of the *Scal* promoter.⁹⁶ First, these authors showed that among acute leukemias LSD1 is expressed at the highest levels in T-cell ALL.⁸⁸ The overexpression of the short LSD1 isoform, which lacks E2a and E8a, induced a marked increase in the self-renewal activity of HSCs via upregulation of *HOXA* genes, but retained multidifferentiation capacities. Transgenic mice overexpressing LSD1 did not develop any hematological neoplasia; however, these mice developed high frequency T-cell ALL after γ -irradiation.⁹⁶

Schenk et al explored the capacity of LSD1 inhibitors to confer to non-APL cells the capacity to differentiate in response to all-*trans* retinoic acid (ATRA). In fact, while APL cells have the remarkable property to differentiate

under treatment with ATRA, non-APL cells fail to undergo differentiation. Schenk et al showed that non-APL cell lines, as well as primary AML cells, undergo granulocytic differentiation when incubated with ATRA and an LSD1 inhibitor.⁹⁷ Analysis of leukemic cells treated with LSD1 inhibitors showed that these drugs did not lead to a large-scale increase of H3K4me across the genome, but increased H3K4me and gene expression at the level of selected genes, particularly some genes involved in myeloid differentiation.⁹⁷

LSD1 inhibitors

The development of specific LSD1 inhibitors has been the objective of many recent studies. These inhibitors represent a fundamental tool to improve our understanding of the role of LSD1 in normal and pathological conditions, and offer a unique strategy to develop preclinical studies to evaluate the therapeutic impact of LSD1 inhibition in animal models of various pathologic conditions and to translate these studies in the clinic.

Some recent papers have analyzed in detail the structure and mechanism of action of the main LSD1 inhibitors that have been developed in the last 10 years.⁹⁵ Here, we analyze the main LSD1 inhibitors and particularly those under clinical development, with emphasis on studies focused on AML treatment (Table 1). According to Niwa and Umehara, LSD1 inhibitors can be subdivided into two main groups: irreversible covalent inhibitors and reversible noncovalent inhibitors.⁹⁸ The irreversible covalent inhibitors can be subdivided into three subgroups: (\pm)-*trans*-2-phenylcyclopropylamine (2-PCPA), *N*-alkylated 2-PCPA derivatives, and inhibitors other than 2-PCPA derivatives.

Studies on the MAO inhibitor tranylcypromine have led to the discovery that this compound exhibited better LSD1-inhibitory activity than the other MAO inhibitors known at the time.⁹⁹ It is important to briefly discuss the properties of Tranylcypromine (TCP), the compound initially investigated for its capacity to inhibit LSD1. TCP was approved as a drug for the treatment of depression. In combination with ATRA, TCP is under exploration in three clinical trials in AML patients: TCP in combination with ATRA in AML patients who cannot undergo chemotherapy (NCT02261779), TCP at four doses (10, 20, 40, and 60 mg) in combination with ATRA in AML and MDS patients (NCT02273102), and TCP in combination with ATRA and the chemotherapy agent cytarabine (DRKS00006055) (Table 1).

The 2-PCPA scaffold represents the basic structure used for the development of the large majority of irreversible LSD1 inhibitors.¹⁰⁰ The introduction of various chemical

Table I Main LSD1 inhibitors introduced into a plan of clinical development

Trial identifier	Title	Disease	Phase	Epigenetic drug	End points
EUDRACT 2013-002447-29	A Phase I study of human pharmacokinetics and safety of ORY1001, an LSD1 inhibitor, in relapsed or refractory acute leukemia (AL)	Acute myeloid leukemia	I/II	ORY1001 (RG6016) (Oryzon Genomics Barcelona, Spain)	Safety, tolerability, pharmacokinetics, pharmacodynamics, effects on cell differentiation, clinical response in acute myeloid leukemia subtypes
NCT02913443	A dose-finding and -expansion study of RO7051790 administered orally in patients with relapsed, extensive-stage disease small-cell lung cancer (ED SCLC)	Small-cell lung cancer	I	ORY1001 (RG6016) (Oryzon)	Safety, tolerability, recommended dose for Phase II, overall survival
NCT02034123	Investigation of GSK2879552 in subjects with relapsed/refractory small-cell lung carcinoma	Small-cell lung cancer	I	GSK2879552 (GlaxoSmithKline Philadelphia, PA, USA)	Safety, tolerability, pharmacokinetics, pharmacodynamics, recommended dose for Phase II, clinical activity
NCT02177812	A Phase I dose-escalation study of GSK2879552 in subjects with relapsed/refractory acute myeloid leukemia (AML)	Acute myeloid leukemia	I	GSK2879552 (GlaxoSmithKline)	Safety, tolerability, pharmacodynamics, pharmacokinetics, recommended dose for Phase II, clinical activity
NCT02712905	An open-label, dose-escalation/dose-expansion safety study of INCB059872 in subjects with advanced malignancies	Advanced cancer	I/II	INCB059872 (Incyte, Palo Alto, CA, USA)	Safety, tolerability, tumor-response rates, pharmacokinetics
NCT03132324	A study to evaluate safety, pharmacokinetic, and biological activity of INCB059872 in subjects with sickle-cell disease	Sickle-cell disease	I	INCB059872 (Incyte)	Safety, tolerability, pharmacodynamic parameters (fetal hemoglobin levels)
NCT03136185	IMG7289 in patients with myelofibrosis	Primary myelofibrosis postpolycythemia and post-essential thrombocythemia myelofibrosis	I	IMG7289 (Imago Biosciences, San Francisco, CA, USA)	Safety, tolerability, pharmacokinetic studies
NCT02842827	IMG7289, with and without ATRA, in patients with myelofibrosis	Acute myeloid leukemia myelodysplastic syndromes	I	IMG7289 (Imago Biosciences)	Safety, tolerability, pharmacokinetic, and pharmacodynamic effects of IMG7289 with or without ATRA
NCT02875223	A safety and efficacy study of CC90011 in subjects with relapsed and/or refractory solid tumors and non-Hodgkin's lymphomas	Non-Hodgkin's lymphoma neoplasms	I	CC90011 (Celgene, Summit, NJ, USA)	Safety, tolerability, pharmacokinetics, clinical response

Abbreviation: ATRA, all-*trans* retinoic acid.

modifications to this basic structure has led to the identification of many new compounds, some exhibiting higher LSD1-inhibitory activity and a reduced MAO-inhibitory activity.⁹⁸ Particularly, chemical substitutions on the phenyl ring of tranylcypromine have considerably improved the potency and selectivity of these LSD1 inhibitors.⁹⁹

Most LSD1 inhibitors under clinical development pertain to the family of *N*-alkylated 2-PCPA derivatives. This category of compounds was discovered by Oryzon Genomics (Barcelona, Spain), showing that LSD1 inhibition is markedly improved by the development of *N*-alkylated 2-PCPA derivatives, exhibiting LSD1-inhibitory potency in

the nanomolar range. One of these compounds, ORY1001, displayed potent LSD1-inhibitory activity (IC_{50} of about 18 nM) and specificity (>1,000-fold selectivity over MAOs and LSD2).⁹⁷ A cellular assay on the THP1 cell line (MLL – AF9 cells) displayed pharmacologic activity of ORY1001 (as evaluated by methylation and cell differentiation assay) at subnanomolar concentrations.¹⁰⁰ Leukemic cells with MLL translocations were particularly sensitive to the inhibitory effects of this compound. Daily oral doses <20 µg/kg have shown a potent antileukemic effect in mice transplanted with MV (4;11) cells.¹⁰⁰ Finally, preclinical pharmacologic and toxicologic studies showed

good oral bioavailability and a good safety profile.¹⁰¹ Given this favorable pharmacologic profile, ORY1001 entered a plan of clinical development screening to investigate its safety profile and possible antitumor effects in patients with non-small-cell lung cancer and AML (Table 1).

A preliminary report on the ongoing Phase I/II clinical study on AML patients (EUDRACT 2013-002447-29) was presented at the last meeting of the American Society of Hematology in December 2016. Patients with refractory/relapsed AML were enrolled in this study, and the dose-escalation section allowed definition of a recommended dose of ORY1001 for the expanded phase, of 140 $\mu\text{g}/\text{m}^2/\text{day}$.¹⁰² At this dose, the drug was relatively well tolerated, and the most frequent adverse events were asthenia, febrile neutropenia, constipation, and peripheral edema.¹⁰² Importantly, ORY1001 was evaluated in an extension cohort of 14 AML patients with relapsing AML subtypes predicted to be more sensitive according to preclinical studies (ie, ten AML MLL/translocated and four acute erythroleukemia/M6). Objective responses were seen in 36% and blast-cell differentiation in blood observed in 64% of these patients.¹⁰² These observations further support additional studies exploring the antileukemic effects of ORY1001.

After the end of this Phase I study in AML, further development of ORY1001 will be carried out by Roche (Basel, Switzerland) and the compound name will be RG6016. RG6016 will be additionally evaluated in AML patients and in small-cell lung cancer patients in the context of a Phase I study (NCT02913443). Another LSD1 inhibitor, ORY2001, generated by Oryzon is under evaluation in a Phase I clinical study in neurodegenerative disease (Alzheimer's disease, Parkinson's disease, and Huntington's disease). This study was preceded by one initiated in early 2016 to determine the safety, tolerability, and kinetics of ORY2001 in healthy volunteers.

Other *N*-alkylated compounds, RN1 and RN7, are currently being evaluated in preclinical models. Both those compounds displayed a selective and potent LSD1 inhibitory capacity (IC_{50} 10–30 nM); an important property of these compounds is in their brain penetration capacity, eliciting inhibitory effects on long-term, but not short-term memory formation.¹⁰³ RN1 was evaluated in preclinical models for its capacity to act as an agent stimulating fetal hemoglobin synthesis in adult erythroid cells and for its antileukemic properties. Concerning the first point, RN1 was evaluated in a mouse model of sickle-cell disease, showing that drug administration induced fetal hemoglobin synthesis in erythroid cells, reduced disease pathology,¹⁰⁴ and reduced oxidative-induced damage to erythroid cells.¹⁰⁵ Preclinical

studies in baboons support the efficacy (evaluated by fetal hemoglobin increase) and safety of long-term administration of RN1 in baboons.^{106,107} These observations support further development of the LSD1 inhibitor RN1 in the treatment of sickle-cell disease.^{106,107}

Other studies on RN1 were focused on evaluating the antileukemic effects of this inhibitor. Studies performed on leukemic cell lines showed that leukemic cells with MLL translocations or bearing RUNX1–RUNX1T1 (AML1–ETO) translocations are particularly sensitive to cell death, cell differentiation, and inhibition of cell proliferation in vitro and in vivo induced by RN1.¹⁰⁶ The in vitro assay indicated that RN1 is a potent LSD1 inhibitor with an IC_{50} assayed on inhibition of cell proliferation corresponding to 1–5 nM.¹⁰⁸ These observations suggest that both MLL-rearranged and RUNX1-rearranged AML may converge on similar downstream oncogenic pathways that are impacted by LSD1 inhibition.¹⁰⁸

In 2015, the Cancer Epigenetic Department of GlaxoSmithKline (Philadelphia, PA, USA) performed a screening on a library of 2.5 million compounds, and discovered a series of small molecules with LSD1-inhibitory activity that led to the development of three potent LSD1 inhibitors: GSK2879552, GSKLSD1 and GSK2699537. All three molecules are *N*-alkylated cyclopropylamine derivatives and act as potent irreversible LSD1 inhibitors. The pharmacokinetic and pharmacodynamic properties of GSK2879552 indicated that this compound was suitable for clinical development. GSK2879552 treatment of a large panel of tumor cell lines indicated that AML and small-cell lung cancer cells were uniquely sensitive.¹⁰⁹ The antitumor effect against small-cell lung cancer was characterized in detail, providing evidence that the sensitivity of these tumor cells to GSK2879552 correlated with DNA hypomethylation of a signature set of probes.¹⁰⁹ GSK2879552 is under investigation in the context of a Phase I study in a subset of cancer patients with relapsed/refractory small-cell lung cancer (NCT02034123) (Table 1).

Incyte (Palo Alto, CA, USA) recently developed a new LSD1 inhibitor, INCB059872, displaying properties suitable for clinical development. This inhibitor was potent and selective for LSD1 and was orally bioavailable. Initial pharmacodynamic studies confirmed the capacity of this inhibitor, like other LSD1 inhibitors, to induce apoptosis and differentiation of leukemic cells displaying MLL rearrangements.¹⁰⁸ Studies in xenograft mice transplanted with human MLLAF9 leukemic cells showed prolonged in vivo effects of INCB059872, allowing its administration with an alternate-day regimen.¹¹⁰ Preclinical studies with INCB059872 showed a synergistic interaction with ATRA¹¹¹ and with a BET inhibitor¹¹² in inducing cell differentiation and inhibiting

the growth of non-APL AML cells. In another set of studies, preliminary evidence was provided on possible synergy between INCB059872 and various signal-transduction inhibitors (such as PIM-kinase inhibitors, JAK1/2 inhibitors, or PI3K δ -selective inhibitor) in some AML models.¹¹³ Other studies were focused on exploring the possible antitumor effects of INCB059872 in some solid tumors. Interestingly, preclinical studies showed significant antitumor effects of this LSD1 inhibitor alone or in combination with chemotherapy in Ewing sarcoma cell lines.¹¹³ In vivo studies in mice xenografted with human Ewing sarcoma cell lines confirmed their sensitivity to the antitumor effects of INCB059872.¹¹⁴ Interestingly, using patient-derived xenograft models, evidence was provided that tumors with EWS–FLI translocations were particularly inhibited in their growth by the LSD1 inhibitor.¹¹⁵ Finally, other preclinical studies confirmed the observations made with ORY1001 and showed clear sensitivity of small-cell lung cancer to LSD1 inhibitors.¹¹⁶ On the basis of these observations, Incyte has launched two clinical trials based on the administration of INCB059872: a Phase I/II study of dose escalation/dose expansion in patients with advanced cancer (NCT02712905), and a Phase I dose-escalation study in sickle-cell disease (NCT03132324) (Table 1).

Recently, new LSD1 inhibitors pertaining to this chemical subtype have been reported. These compounds are promising for their potency, specificity, and pharmacologic properties. Takeda (Osaka, Japan) reported a new LSD1 inhibitor, T3775440, a tranlycypromine derivate characterized by high LSD1-inhibitory activity (EC_{50} 2 nM). A screening of the effects of this compound against a large panel of leukemic cell lines showed a sensitivity of erythroid and megakaryocytic leukemic cell lines. The growth-inhibitory effect observed on these cells seems to be related to a trans-differentiating effect consisting of the induction of monocyte differentiation.¹¹⁷ In vivo experiments in normal mice showed that T3775440, as well as other LSD1 inhibitors, exerted an inhibitory effect on erythroid and megakaryocytic progenitors, resulting in thrombocytopenia.¹¹⁷ This inhibitor effect on erythromegakaryopoiesis could be related to the capacity of T3775440 to disrupt Gfi1b-containing transcriptional complexes.¹¹¹ These observations support the hypothesis that LSD1 inhibitors could be evaluated for the treatment of M6 and M7 AML. In line with this observation, as mentioned, M6 AML patients were shown to be frequent responders to treatment with the ORY1001 LSD1 inhibitor.¹⁰²

Ogasawara et al developed novel LSD1 inhibitors, NCD25 and NCD38, which consist of two moieties: a tranlycypromine-based moiety with LSD1-inhibiting activity and a lysine that is designed to recognize, with high affinity, an LSD1

enzymatic pocket, thus allowing selective inhibitory activity restricted to LSD1 and not extended to other MAO enzymes.¹¹⁸ These two LSD1 inhibitors were recently characterized for their anticancer properties. Sugino et al explored the antileukemic activity of NCD25 and NCD38.¹¹⁹ NCD25 and NCD38 inhibited LSD1 with EC_{50} of about 500 nM. Through the study of a panel of sensitive leukemic cell lines and gene-expression studies in LSD1 inhibitor-treated cells, the conclusion was reached that these inhibitors derepress superenhancers of hematopoietic regulators (such as Gfi1, ERG, and CEBPA) that are abnormally silenced by LSD1 and via this mechanism inhibit leukemic programs and promote cell differentiation.¹¹⁹ Furthermore, NCD38 showed a marked inhibitory effect on colony formation by primary leukemic cells derived from MLL-rearranged AML, erythroleukemia, and MDS.¹¹⁹ These findings provide a rationale for clinical trials of LSD1 inhibitors in MDSs.¹¹⁹ Interestingly, NCD38 and NCL1, another lysine-specific LSD1 inhibitor,¹²⁰ were shown to inhibit glioma SCs.¹²¹

The use of noncovalent reversible LSD1 inhibitors could provide some advantages compared to irreversible inhibitors, particularly as regards a safer metabolic profile. Only a limited number of noncovalent LSD1 inhibitors have been developed, and some are promising in preclinical assay in terms of development of antileukemic drugs.¹²² Several compounds with these properties and submicromolar potency have been developed. Among these compounds, particularly interesting is the inhibitor SP2509, containing a benzohydrazide scaffold, and with a K_i on LSD1 activity of about 30 nM.¹²³

The effect of SP2509 on leukemic cell lines was tested, providing evidence about effects comparable to those induced by irreversible LSD1 inhibitors (ie, inhibition of proliferation, induction of apoptosis and cell differentiation).¹²⁴ This compound was tested also on primary AML cells, providing evidence that NPM1 and MLL-rearranged leukemic cells are particularly sensitive to the effects of SP2509.¹²⁴ Interestingly, cotreatment with an HDAC inhibitor (panobinostat) and SP2509 was synergistically inhibitory for AML blasts.¹²⁴

Another reversible LSD1 inhibitor, JL1037, was recently identified through computer-aided drug-design technology.¹²⁵ This compound displayed a reversible and selective LSD1-inhibitory activity with IC_{50} of 100 nM and antileukemic effects like those observed using other LSD1 inhibitors.¹²⁵ Exploration of the inhibitory activity of JL1037 on leukemic cells revealed induction of apoptotic and autophagic responses.¹²⁵

From high-throughput screening, a new series of compounds was identified, named 4*H*-thieno[3,2-*b*]pyrrole-5-carboxamide, with inhibitory activity against LSD1.¹²⁶

Structure-guided optimization of this chemical series led to the isolation of some compounds (46, 49, and 50) displaying selective inhibitory activity for LSD1 and pharmacological effects comparable to those observed for other LSD1 inhibitors.¹²⁶

A high throughput screen and subsequent in silico screening led to the development of (4-cyanophenyl) glycine derivatives as potent reversible LSD1 inhibitors.¹²⁷ The scaffold-hopping method was used to develop structurally new LSD1 inhibitors: a series of 4-(pyrrolidin-3-yl) benzonitrile derivatives that act as compounds mimicking the GSK690 LSD1 inhibitor, with improved selectivity over the *HERG* ion channel and no activity against related MOA enzymes.¹²⁸ Among these inhibitors, particularly promising is the compound 21G.¹²⁸

Recently, the development of tranlycypromine derivatives targeting both LSD1 and HDAC was reported.¹²⁹ Particularly, compound 7 displayed potent inhibitory activity against HDAC1 and HDAC2, with IC_{50} of 15 nM and 13 nM, respectively, as well as potent inhibition against LSD1, with IC_{50} of 1.20 μ M.¹²³ At the biochemical level, this compound increased cellular H3K4 and H3K9 methylation, as well as acetylation.¹²⁹

DOT1L

Structure and function

One of the most important and studied histone-modification pathways is represented by lysine methylation, occurring as mono-, di-, or tri methylation. The biologic consequences of histone-lysine methylation are variable in that they can result in both repression or derepression of gene expression.

There are two distinct classes of histone-lysine KMTs: a first class of proteins is characterized by the presence in its sequence of an evolutionary conserved SET catalytic methyltransferase domain; a second class lacks an SET domain and is represented by a single member – DOT1/DOT1L.

DOT1 was originally identified in yeast as a gene that disrupts telomeric silencing when overexpressed. In mammals, this protein is called DOT1L. DOT1L is the only known KMT able to methylate H3K79: this methylation is associated with active transcription and occurs on the nucleosome core, not on the histone tail.¹³⁰ No demethylase has been identified to remove H3K79 methylation, thus indicating that DOT1L is the key determinant for H3K79 methylation. In addition to its role in DNA methylation, DOT1L is also involved in DNA-repair mechanisms and cell-cycle regulation.¹²⁴ DOT1L is a unique enzyme in the KMT superfamily for its structure. Although DOT1L is a lysine methyltransferase, sequence characteristics indicate similarity to the arginine methyltransferase family.¹³¹

The three-dimensional structure of DOT1L reveals an elongated molecule with two domains different from those observed in SET-containing proteins and like those observed in non-SET domain-containing methyltransferases;¹³² the SAM-binding site of DOT1L presented at the level of the C-terminal α/β domain (Figure 2).¹³² The DOT1L protein is a dynamic enzyme able to have different conformational states: particularly, following SAM binding, the protein displays a more closed conformation.¹³² Study of the protein conformation, when DOT1L binds various types of competitive SAM inhibitors, indicates that loops in SAM binding and near this binding site adopt different conformations. Though not

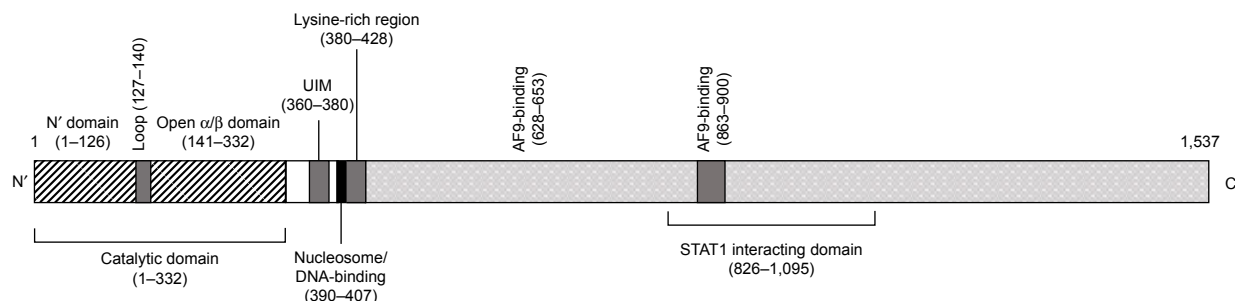


Figure 2 Schematic representation of the DOT1L protein.

Notes: Some physiologically relevant domains are indicated. A large catalytic region (1–332) contains three domains: N' (1–126), loop (127–140), and an open α/β domain. The N-terminal domain consists of five α -helices and two β -strand hairpins. Loop 127–140 is a protein-flexible loop. The open α/β domain (amino-acid residues 141–332) contains a seven-strand central β -sheet and five α -helices. Part of the flexible loop region and the open α/β domain contributes to the formation of an S-adenosyl-L-methionine (SAM)-binding pocket. A ubiquitin-interaction motif (UIM) is located at amino-acid residues 360–380 and involved in ubiquitin H2B and DOT1L-Bat 3 interactions, both required to facilitate efficient H3K79 dimethylation and trimethylation. A lysine-rich region (380–428) is required for nucleosome binding and interacts with the ubiquitin H2B: deletion of this region resulted in reduced demethylation and trimethylation. Within the lysine-rich region, there is a nucleosome/DNA-binding motif (390–407). Two AF9-binding regions have been mapped: the 628–653 binding region contains one binding site for AF9; the 863–900 binding region contains two AF9-binding sites. The DOT1L_{865–874}-binding domain is strictly required for the binding of the AF9-ENL complex through the C-terminal region of ENL. The 580–1,183 region contains an STT1-binding site.

mutated, DOT1L plays an important role in leukemogenesis. In fact, MLL fusion proteins interact directly or indirectly with DOT1L and result in inappropriate recruitment of this KMT at the level of the gene targets of MLL fusion proteins, such as *HOXA* and *MEIS1*, resulting in their transcriptional activation.^{133,134}

Recent studies have in part clarified the mechanism through which DOT1L modulates transcriptional activity and contributes to the deregulated gene expression observed in MLL-rearranged leukemias. The capacity of DOT1L to interact with other proteins to form multiprotein complexes is fundamental for its physiological function of transcriptional regulator and for its relevant contribution to the pathogenesis of MLL-rearranged leukemias. DOT1L has been found to be involved in the formation of complexes with an ENL family protein and an AF10 family protein, such as AF10 or AF9. The association of DOT1L with these proteins increases the methyltransferase activity of DOT1L to produce highly methylated H3K79 me2/3 markers.¹³⁵

Many of these proteins complexing with DOT1L are also partners of MLL in the formation of MLL-fusion proteins. In MLL-rearranged leukemias, these partners interact with DOT1L, and through this interaction drive the DOT1L complex at the level of MLL-fusion target genes, such as *HOXA* and *MEIS1*, thus inducing their high H3K79 methylation and consequently their high expression. This is the basic mechanism through which DOT1L contributes in a relevant way to MLL-fusion leukemia, enabling a high expression of MLL target genes. The AF9 and AF10 proteins act as readers of histone modifications.^{136,137}

AF9 interacts with DOT1L, forming a stable complex with its C-terminus (Figure 2). AF9 interacts with H3K9ac, which is enriched in transcriptionally active genes.¹²⁶ Most AF9-bound genes are enriched for both H3K9 ac and H3K79 me3.¹³⁷ Importantly, AF9 knockdown leads to decreased H3K79 methylation on target genes.¹³⁷ AF10 determines higher H3K79 methylation states, and thus enhances the methyltransferase activity of DOT1L.¹³⁵ AF10 interacts with unmodified H3K27, but not methylated H3K27, thus explaining why H3K27 methylation does not often co-occur with H3K79 methylation on the genetic region.¹³⁶

Another important element in the understanding of the mechanism through which DOT1L exerts its leukemogenic function derives from a recent study showing that DOT1L prevents SIRT1-mediated gene silencing at the level of gene regions recognized by MLL-fusion proteins. Particularly, DOT1L recruited at the level of MLL-fusion target loci prevents SIRT1 from deacetylating H3K9.¹³⁸

When DOT1L is inactivated by chemical inhibitors, SIRT1 reacquires the capacity to bind to these genetic loci and promotes deacetylation of H3K9, which leads to H3K9 methylation and consequent downregulation of MLL-fusion target-gene expression.¹³⁸ Finally, SIRT1 activators synergize with DOT1L inhibitors and accelerate downregulation of MLL-fusion target genes.¹³⁸ The mechanisms through which DOT1L contributes to MLL-driven leukemogenesis are very complex, and this is in large part related to the capacity of MLL-fusion proteins to form large complexes interacting with numerous other nuclear proteins. More than 70 genes have been shown to fuse with MLL. In most cases, MLL is fused with a component of the AF4-ENL-P-TEFb (AEP) complex or the DOT1L-AF10-ENL complex. These complexes involve a member of the ENL family, such as ENL and AF9. The ENL proteins contain a YEATS domain, recognizing H3K9/18/27 Ac. The AEP complex contains AF4 protein families, which provide various interaction platforms for cofactors involved in initiation and elongation of transcription. The mechanism through which the MLL-AF10-ENL complex transforms HSCs operates through the DOT1L-interaction domain,¹³³ while the MLL-ENL-AF9 involves the ANCI-homology domain, which interacts with both DOT1L and AF4.¹³⁹

A recent study provided evidence that MLL-F10-DOT1L acts through a more complex mechanism that does not need the simple recruitment of DOT1L to the target promoters, but involves also the capacity of DOT1L through its ENL-binding motifs to recruit AF4.¹³² According to these observations, it was suggested that the simultaneous inhibition of the MLL fusion-AF4 complex and DOT1L may provide more effective treatment of MLL-rearranged leukemia.¹⁴⁰

Interestingly, recent studies have shown that DOT1L may represent a therapeutic target for the treatment of *DNMT3A*-mutant AML. *DNMT3A* is mutated in about 20% of AML and usually associated with a negative prognosis. In 60% of cases, *DNMT3A* mutations involve the arginine at amino-acid position 882 and determine a marked loss of cellular DNA methyltransferase activity. Ablation of DNMT3A in HSCs determines a pronounced expansion of HSCs and a progressive block in cell differentiation. HSCs deficient in DNMT3A overexpress DOT1L and have increased H3K79 methylation, particularly at the level of DNA regions corresponding to genes highly dysregulated in leukemia.¹³⁵ These observations have suggested that DOT1L could represent a target in *DNMT3A*-mutated AML. In line with this hypothesis, DOT1L inhibitors blocked the growth of *DNMT3A*-mutated cell lines and primary AML cells and induced cell differentiation.¹⁴¹

DOT1L and hematopoiesis

Gene-knockout studies in mice have supported a role for DOT1L in the control of normal hematopoiesis. DOT1L^{-/-} mice developed more slowly and died on embryonic days 10.5–13.5, exhibiting pronounced anemia.¹⁴⁰ Analysis of the hematopoietic tissue of DOT1L-deficient mice displayed a selective defect at the level of erythroid but not myeloid progenitors: erythroid progenitors failed to develop normally, displayed delayed progression through the cell cycle, accumulated in G₀/G₁, and exhibited a great tendency toward apoptosis, even in the presence of erythroid growth factors.¹⁴² GATA2 expression was greatly decreased in erythroid cells of DOT1L-deficient mice, while PU1 levels were increased.¹⁴²

Subsequently, two additional studies demonstrated that DOT1L plays an important role in maintaining normal adult hematopoiesis, using conditional *DOT1L*-knockout mice, coupled with serial bone-transplant assays.^{140,141} Knockout of *DOT1L* in bone marrow causes pancytopenia and depletes HSCs and various progenitor cell (PC) populations, thus indicating an essential role for DOT1L in maintaining HSCs and HPCs.^{143,144}

Daigle et al explored the effect of a potent DOT1L inhibitor (EPZ004777) on in vivo murine hematopoiesis, showing that the administration of this inhibitor at tolerable pharmacodynamically active doses elicited only slight effects consisting of a moderate and unexplained increase of neutrophils, monocytes, and lymphocytes not accompanied by any significant change at the level of frequency of bone-marrow colony-forming cells. HSCs and granulomonocytic progenitors were not affected by the drug.¹⁴⁵ Differences observed in knockout vs drug-inhibition studies are seemingly related to differences in DOT1L silencing achieved by these different procedures.

DOT1L inhibitors

Given the important role of DOT1L in MLL-rearranged leukemia, many recent studies have investigated the development and characterization of DOT1L inhibitors and explored their potential role in leukemia therapy (Table 2). Three compounds were initially reported – EPZ004777, EPZ5676, and SGC0946 – and selected for their specific inhibitory activity against DOT1L.^{145–147}

All these three compounds act as competitive inhibitors of SAM, the cofactor required for the methyltransferase activity of DOT1L. More recently, new DOT1L inhibitors have been identified. Wang et al reported the identification of a new class of DOT1L inhibitors, characterized by a scaffold of [1,2,4]-triazolo-[3,4-*b*] [1,3,4]-thiadiazole. The most

active of these compounds, compound 6 is a selective DOT1L inhibitor with an IC₅₀ of 8.3 μM.¹⁴⁸

The DOT1L inhibitor EPZ004777 is characterized by a considerable inhibitory potency (IC₅₀ 400 pM). This inhibitor was designed to mimic the SAM enzyme cofactor. EPZ was competitive with SAM and not with the peptide substrate. EPZ represents the standard DOT1L inhibitors with several typical pharmacological effects, consisting of global reduction of H3K79 me2 levels, inhibition of the expression of *HOXA9* and *MEIS1*, and inhibition of the growth in vitro and in vivo of MLL-rearranged leukemic cells.¹⁴⁵ Taking advantage of the crystal structure of the DOT1L–EPZ004777 complex, SG0946 inhibitors with increased in vitro and in vivo cellular potencies were developed.¹⁴⁵ SG0946 contains a bromo-substitution at position 7 compared to EPZ004777 and was more potent in chemical and cellular assays.¹⁴⁷ A major advancement in the field was represented by the discovery of the EPZ5676 DOT1L inhibitor by Daigle et al in 2013.¹⁴⁶ This inhibitor binds the DOT1L enzyme at the level of the cofactor-binding site with an affinity of 0.03 nM and reduces H3K79 me2 levels in MV411 cells with an IC₅₀ of 3 nM.¹⁴⁷ Preclinical studies have shown that the continuous infusion of EPZ5676 was able to induce complete tumor regression in immunocompromised mice xenografted with MLL-rearranged MV411 cells.¹⁴⁶ The efficacy of EPZ5676 in preclinical models has triggered the clinical development of this inhibitor as an anticancer drug. In clinical studies, the inhibitor is administered by continuous intravenous infusion. Alternatively, EPZ5676 can be administered by subcutaneous bolus formulation, but this mode of administration has been used only in preclinical studies.¹⁴⁹ Interestingly, in MLL-rearranged AML cells, EPZ5676 synergizes with standard antileukemic drugs and the hypomethylating agent azacitidine, eg, AraC and daunorubicin, to inhibit the growth of leukemic cells.¹⁵⁰

The metabolism and disposition of EPZ5676 has been explored in preclinical animals (rats and dogs) and humans, showing that fecal excretion of the intact drug and its metabolites is responsible for the majority of drug-related elimination, with low renal excretion.¹⁵¹ Despite the pharmacokinetic improvements in EPZ5676 compared to other LSD1 inhibitors, this compound still shows low oral bioavailability.

A first Phase I study (completed) was designed to evaluate safety profile, pharmacokinetics, and pharmacodynamics and obtain preliminary indications on clinical activity in adult patients with relapsed/refractory acute leukemia (either myeloid or lymphoid) (Table 2).¹⁵² A total of 49 patients were treated (either in the dose-escalation or expansion phase), and six patients displayed objective response, including

Table 2 Main properties of DOT1L inhibitors

Name	Clinical structure	Potency of inhibitor activities	Major biological effects	Preclinical and clinical studies	References
EPZ004777	Aminonucleoside, SAM-competitive	DOT1L K_i 0.5 nM MV411 proliferation, IC_{50} 151 nM H3K79 IC_{50} 84 nM	Inhibition of cellular H3K79 methylation; inhibition of proliferation of MLL-rearranged leukemia cells; inhibition of <i>HOXA</i> and <i>MEIS1</i> gene expression	Poor pharmacokinetic properties	Daigle et al ¹⁴⁵
EPZ-5676	Aminonucleoside SAM-competitive	DOT1L K_i 0.08 nM MV411 proliferation, IC_{50} 3–15 nM H3K79 IC_{50} 3–7 nM	Inhibition of cellular H3K79 methylation; inhibition of proliferation of MLL-rearranged leukemia cells in vitro and in vivo; inhibition of <i>HOXA</i> and <i>MEIS1</i> gene expression	In vivo pharmacokinetics showed moderate–high clearance and low oral bioavailability; drug administered by continuous intravenous infusion in phase I clinical trials	Daigle et al ¹⁴⁶
SGC0946	Aminonucleoside (brominated analog of EPZ2004777), SAM-competitive	DOT1L K_i 0.3 nM H3K79ma2 IC_{50} 8 nM	Decrease of viability of MLL-AF9-transformed cells		Yu et al ¹⁴⁷
CN-SAH Compound 12 and 13	5-Adenosyl-L-homocysteine SAM-competitive, but it does not bind to the SAM site, but to a site near to the SAM site	DOT1L K_i 13 nM DOT1L K_i 0.4–1.4 nM MV411 proliferation, IC_{50} 85–128 nM H3K79 IC_{50} 16–23 nM	Inhibition of DOT1L activity Inhibition of cellular H3K79 methylation; inhibition of proliferation of MLL-rearranged leukemia cells; inhibition of <i>HOXA</i> gene expression	None Good oral bioavailability; high blood clearance, high volume of distribution, moderate half-life	Spurr et al ¹⁹¹ Chen et al ¹⁹²
Compound 7	Structure not nucleoside-related	DOT1L K_i <0.1 nM MV4-11 proliferation, IC_{50} 5 nM H3K79 IC_{50} 3 nM	Inhibition of cellular H3K79 methylation; inhibition of proliferation of MLL-rearranged leukemia cells in vitro; inhibition of <i>HOXA</i> gene expression; more potent than EPZ5676 in comparative assays	None	Möbitz et al ¹⁹³
Compound 6	Scaffold of [1,2,4]-triazolo-[3,4-b][1,3,4]thiadiazole	DOT1L K_i 8 nM MV411 proliferation, 22 mM H3K79 IC_{50} 10 nM	Inhibition of proliferation of MLL-rearranged leukemia cells in vitro	None	Wang et al ¹⁴⁸

Abbreviations: SAM, S-adenosyl-L-methionine; MLL, mixed-lineage leukemia.

one morphologic complete remission, one cytogenetic complete response, one partial response, and three resolutions of leukemia cutis.¹⁵² Drug administration is usually well tolerated, and about 15% of treated patients display adverse events.¹⁵² In another Phase I study, pinometostat was evaluated in a cohort of pediatric patients with relapsed or refractory MLL-rearranged acute leukemia.¹⁴⁷ No objective responses were observed in these patients, and only transient reductions in blast-cell counts were observed in 40% of treated patients.¹⁵³

Menin

Menin structure and function

Menin is a 67 kDa protein encoded by the *MEN1* gene localized on chromosome 11q13.¹⁵⁴ Menin is a nuclear protein, ubiquitously expressed.¹⁵⁵ Menin interacts directly with the N-terminal region of MLL;^{156–158} this N-terminal MLL

region is retained in all MLL-fusion proteins and plays a key role in the binding of MLL and MLL-fusion proteins to target genes, such as *HOXA9*.^{156,157,159,160} The capacity of MLL-fusion proteins to bind menin is strictly required for their leukemogenic activity: in fact, mutations at the level of the N-terminal region of MLL-fusion proteins block their capacity to induce leukemia and to upregulate *HOXA* gene expression.¹⁵⁴ These observations have indicated that the menin–MLL interaction represents a potentially important target in the treatment of MLL-rearranged AML. Analysis of the structure of the menin protein is essential for a better understanding of its function. Human menin is composed of 615 amino acids and it is a globular protein. The linear structure of the protein shows four structured regions that form the structured core and represent regions conserved during evolution, interspaced by three loop regions. In contrast to the

structured regions, the loop regions and C-terminal region are variable among species and contain sites of posttranslational modification of the protein, such as phosphorylation and SUMOylation (Figure 3).¹⁶¹ Structural studies have shown that menin is an α -helical protein with three folds based on three tetratricopeptide repeats:¹⁶² these repeats form a large central cavity that represents the binding site for the protein–protein interactions with MLL and JunD8127. This central cavity is a relatively rigid structure that does not change conformation upon binding of protein ligand.

As stated, an essential function of menin is dependent upon its capacity to interact with the N-terminal region of MLL: at the level of this region of the MLL molecule, two menin-binding motifs have been discovered: high-affinity MBM1, encompassing residues 6–13, and low-affinity MBM2, encompassing residues 24–40 (Figure 3).¹⁵⁸ The MBM1 pocket on menin represents a major site for targeting by small-molecule inhibitors.

Menin acts as an oncogenic cofactor of MLL-fusion proteins in leukemia. This function is related to the capacity of menin to interact with the N-terminus of MLL, an MLL region retained in all MLL-fusion proteins. Through this mechanism, menin drives MLL and MLL-fusion proteins to target genes.^{156,157,159,160} The essential role of menin in supporting the oncogenic activity of MLL-fusion proteins is directly shown by the observation that loss of menin binding by MLL-fusion proteins abolishes their capacity to upregulate *HOX* gene expression and to induce leukemia in vivo in mice.¹⁶¹ Furthermore, expression of a dominant-negative inhibitor composed of an amino-terminal MLL sequence acts as a blocking agent of the oncogenic potential of MLL-fusion proteins.¹⁶² Finally, small-molecule inhibitors able to bind to menin and disrupt the binding of these molecules to MLL inhibit the proliferation of MLL-fusion-bearing leukemic cells and induce their differentiation.¹⁶³

The requirement of menin for leukemogenic activity of MLL-fusion proteins is strongly supported by various experimental studies, and represents a strong rationale to develop small-molecule inhibitors to target the menin–MLL interactions. The analysis of a large set of different MLL-fusion proteins clearly supported the capacity of a potent menin inhibitor, MI2-2, to induce growth arrest, differentiation, and downregulation of MLL-fusion target genes in leukemia cells transformed with various MLL fusions.¹⁶⁴

Importantly, this menin inhibitor elicited its inhibitory activity on AML cells displaying different types of MLL-fusion genes. As mentioned, MLL-rearranged AML is sensitive to DOT1L inhibitors. However, DOT1L inhibitors act slowly on these leukemia cells, and their effect on many MLL-rearranged cell lines is limited. To bypass this important limitation, it was discovered that a combined inhibition of both DOT1L and menin resulted in strong, complementary inhibition of MLL-rearranged leukemic cells, as supported by induction of cell killing and differentiation.¹⁶⁵ At the gene-expression level, the combination of the two drugs elicited a more marked inhibitory effect on MLL-fusion and *MYC* target genes.¹⁶⁵

Analysis of *HOXA* and *HOXB* cluster-gene expression in AML allows identification of four AML subgroups:

1. a subgroup not expressing *HOXA* or *HOXB*, represented by AML, characterized by PML–RAR α and AML1–ETO fusion transcripts
2. a subgroup expressing only *HOXA*, represented by AML, characterized by MLL-fusion genes and by frequent complex karyotypes
3. a subgroup expressing only *HOXB*, represented by AML, frequently characterized by CBF β –MYH11 rearrangements
4. a subgroup expressing both *HOXA* and *HOXB*, represented by AML, frequently characterized by *NPM1* mutations in most cases and more rarely by MLL-PTD.¹⁶⁶

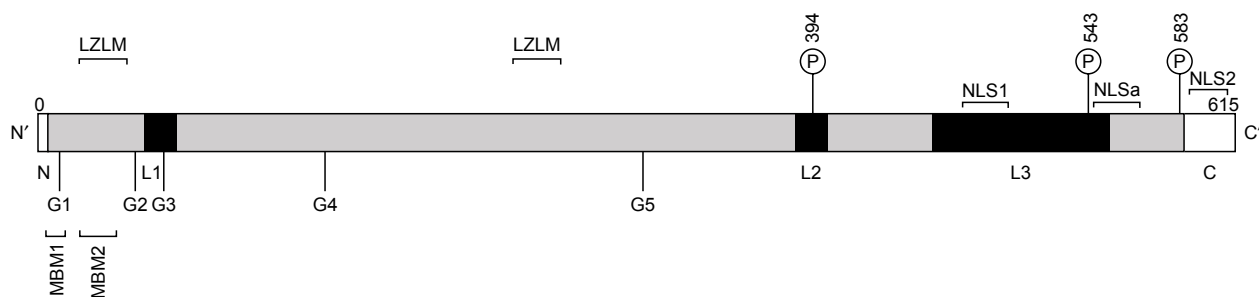


Figure 3 Linear structure of human menin.

Notes: Schematics of a sequence of human menin composed of 615 amino acids. Elements of the folded core of the protein are in gray. These elements contribute to the formation of a central cavity. Three loops, L1–L3, are shown in black. Three phosphorylation sites, located at Ser 394, Ser 543, and Ser 583, are reported and indicated by the symbol P. Two leucine zipper-like motifs (LZLMs) are indicated. Five GTPase motifs (G1–G5) are present in the protein. Two nuclear localization signals (NLS1 and NLS2) and an accessory NLS (NLSa) are reported. The protein possesses two Menin-binding motifs: a high-affinity MBM1 and a low-affinity MBM2.

NPM1-mutated AML represents the largest group of HOX-expressing AML. A recent study provided evidence that pharmacologic inhibition of menin and DOT1L inhibited HOX and FLT3 expression and promoted differentiation of *NPM1*-mutated AML.¹⁶⁷ Menin is involved also in the genesis of other tumors in addition to leukemias. Therefore, mutations of the menin gene are responsible for the development of a peculiar syndrome called multiple endocrine neoplasia type 1 syndrome, an autosomal-dominant disorder in which variable combinations of endocrine and nonendocrine tumors may occur, in the context of varied phenotypic and clinical features.¹⁶⁸ The large majority of menin gene mutations observed in these patients are inactivating and private mutations, without any preferential distribution among selected regions of the menin gene.¹⁶⁸

Enhanced expression of the menin–MLL complex plays an important tumorigenic role in other neoplasia such as Ewing's sarcoma¹⁶⁹ and prostate cancer.¹⁷⁰ In Ewing's sarcoma, expression of both menin and MLL is increased and determines enhanced HOXD expression: inhibition of MLL–menin interaction in these tumor cells decreases their tumorigenicity and inhibits HOXD expression.¹⁶⁷ In prostate cancer cells, the overexpressed MLL–menin complex acts as a coactivator of androgen-receptor signaling and contributes to tumorigenicity. Importantly a menin inhibitor blocks androgen-receptor signaling and reduces growth of castration-resistant tumors *in vivo*.¹⁷⁰

Role of MLL and menin in hematopoiesis

The study of specific knockout mice has contributed to elucidation of the role of MLL and menin 1 in normal hematopoiesis. A basic observation derived from these studies was that while normal hematopoiesis was markedly impaired in the absence of MLL, less pronounced and superimposable defects were observed in menin-deficient mice. During embryonic life, MLL expression is strictly required for the development of both primitive and definitive hematopoiesis.^{171–173} Furthermore, MLL is required also for adult hematopoiesis, particularly for HSC self-renewal and HPC proliferation.^{174,175}

Menin 1 knockout results in mouse lethality during mid-gestation for multiple developmental defects. The possible consequence of a loss of menin 1 expression for hematopoiesis was investigated using a conditional knockout strategy. These studies showed no overt defects of hematopoiesis in menin 1-deficient mice: these mice displayed only a modest decrease in white blood-cell counts, associated

with decreased clonogenic capacity of HPCs in standard methylcellulose assays¹⁷⁶ and reduced repopulating capacity of HSCs in competitive transplantation assays.¹⁷⁷

The analysis of regulation of hematopoiesis in mice deficient in the interaction between MLL1 and menin 1 came to the conclusion that MLL1 and its cofactor menin 1 act independently in regulating hematopoiesis at the level of HSCs.¹⁷⁸ Both MLL1 and menin 1 play an important role in the control of B-lymphocytic cell differentiation and act largely through independent mechanisms (interestingly, some genes, such as *HOXA9* and *MEIS1*, are regulated by both proteins, but other sets of genes are specifically regulated by either MLL1 or menin 1).¹⁷⁸ These findings support the view that these proteins are largely independent for their effects on the control of normal hematopoiesis and the disruption of menin 1–MLL interactions may represent a safe strategy to try to target selectively the aberrant gene expression deriving from the interaction between MLL-fusion proteins and menin 1.¹⁷⁸

Developmentally induced MLL1 loss showed that deficient mice exhibit phenotypically normal fetal hematopoiesis, but rarely survive past 3 weeks of age. Surviving animals were anemic, thrombocytopenic, and exhibited a reduction in HSC number.¹⁷⁹ The role of histone methyltransferase activity in MLL1^{-/-} phenotypes was investigated using the *MLL1* gene deleted from the SET domain, corresponding to the catalytic site, and provided evidence that histone methyltransferase activity is dispensable for the effects of MLL on hematopoiesis.¹⁸⁰

The role of MLL1 in HSC biology was better delineated in additional studies. Artinger et al identified the transcriptional network of MLL in the hematopoietic system and showed that it extends beyond *HOX* genes and involves also a number of other transcriptional regulators, such as MECOM, Prdm16, Pbx1, Eya1, and others involved in HSC maintenance and proliferation.¹⁸¹ Furthermore, the MLL-transcription-dependent network contains genes that are both dependent and independent of menin.¹⁷² The maintenance of MLL activity on gene transcription and HSC function requires the capacity of this protein to recruit an H4k16 acetyltransferase; in fact, H4k16 deacetylase inhibitors are sufficient to restore MLL1-dependent gene expression in MLL1^{-/-} cells.¹⁸¹

Menin 1 inhibitors

The study and understanding of the molecular basis of MLL1–menin 1 interactions have been of fundamental importance in the development of specific small molecule

inhibitors. The interaction between these two proteins is dictated by the binding of MLL to a large hydrophobic cavity present in the menin 1 molecule through two binding motifs: a high-affinity motif encompassing MLL residues 6–13 (MBM1), and a low-affinity motif encompassing MLL residues 24–40 (MBM2) (Figure 3).¹⁸² The MBM1 pocket is of fundamental importance for the interaction between MLL and menin 1, and represents a major site for targeting by small-molecule inhibitors. The mutation of F9 to alanine in MBM1 has a very marked impact on MLL–menin interaction.¹⁸² The role of MBM2 in MLL–menin interaction is less defined. The MBM2 motif carries a positive charge due to the presence of four arginine residues, and is seemingly involved in the mediation of electrostatic interaction with the central cavity of menin 1, which is negatively charged.¹⁸³

In line with these observations, inhibitors able to target MBM1 are capable of blocking MLL–menin interaction and suitable for preclinical and clinical studies. The first small-molecule inhibitors of the MLL–menin interaction were identified using a technology based on fluorescence polarization-based high-throughput screening.¹⁷⁷ Among the various inhibitors identified using this approach, the most potent was the MI2 inhibitor, with an IC_{50} of 446 nM, able to block the interaction between MLL and menin and to inhibit the leukemogenic potential of MLL-fusion proteins.¹⁸⁴

Analysis of the crystal structure of human menin complexed with MI2 allowed the design and the production of a more powerful inhibitor, Mi2-2, with a clear improvement in IC_{50} of about tenfold.¹⁷⁹ The introduction of a cyanoindole ring connected to a thienopyrimidine core allowed the definition of a new chemical core structure suitable for the development of new specific and more potent menin inhibitors.¹⁸⁵ This strategy led to the synthesis and characterization of two new menin inhibitors: MI463, with an IC_{50} of about 15 nM, and MI503, with an IC_{50} of about 15 nM.¹⁷⁹ Both these compounds exhibit a suitable pharmacological profile based on standard preclinical studies.¹⁷⁷ He et al identified another class of hydroxy- and aminomethyl piperidine compounds capable of mimicking the three hydrophobic interactions between MLL and menin: the most notable of these compounds, MIV6, displayed an IC_{50} of about 56 nM.¹⁸⁶

Other studies were based on completely different approaches. Zhou et al designed some macrocyclic peptidomimetic inhibitors, such as MCP1, able to bind the menin pocket as like the native MBM1 MLL motif: two of these compounds exhibited high IC_{50} in the 5–20 nM range but their high molecular weight hampered their further preclinical development.¹⁸⁷ Finally, the screening of existing drugs

allowed the identification of two drugs, the aminoglycosidic antibiotic neomycin and tobramycin, two low-affinity inhibitors with IC_{50} in the 20–60 μ M range.¹⁸⁸

More recently, Xu et al, using pharmacophore-based and structure-based approaches, developed a new screening strategy to discover new inhibitors targeting the MLL–menin interface. M123, one of these compounds, displayed potent inhibitor activity with an IC_{50} of about 5 nM and the capacity to inhibit the growth of MLL-rearranged leukemia cells.¹⁸⁹ Borkin et al recently reported the synthesis and structure-based optimization of a new thienopyrimidine class of compounds able to block the interaction between MLL and menin.¹⁹⁰ This approach led to the identification of a compound (MI136) that showed marked inhibitory activity in vitro and in vivo and seems suitable for preclinical studies. This compound displays a favorable pharmacokinetic profile.¹⁹⁰

Conclusion

Studies carried out in the last few years have provided strong and clear evidence that multiple dysregulations of epigenetic regulatory mechanisms have a central role in leukemia onset and progression. Many epigenetic modifications are suitable for pharmacological interventions. Histone methylation is a very important process in the context of epigenetic processes of control of gene expression, and the study of these processes in leukemia has led to the identification of lysine methyltransferases and demethylases as promising targets for therapeutic interventions. In this review, we have analyzed those enzymes (LSD1 and DOT1L) or molecules (menin) and their inhibitors that have already reached the stage of initial clinical development in leukemia therapy. Although the first indications with these inhibitors are promising, future studies will clarify the real impact of these inhibitors in the treatment of leukemia and other cancers.

Disclosure

The authors report no conflicts of interest in this work.

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