Oncogenic Roles and Inhibitors of DNMT1, DNMT3A, and DNMT3B in Acute Myeloid Leukaemia

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ABSTRACT: Epigenetic alteration has been proposed to give rise to numerous classic hallmarks of cancer. Impaired DNA methylation plays a central role in the onset and progression of several types of malignancies, and DNA methylation is mediated by DNA methyltransferases (DNMTs) consisting of DNMT1, DNMT3A, and DNMT3B. DNMTs are frequently implicated in the pathogenesis and aggressiveness of acute myeloid leukaemia (AML) patients. In this review, we describe and discuss the oncogenic roles of DNMT1, DNMT3A, and DNMT3B in AML. The clinical response predictive roles of DNMTs in clinical trials utilising hypomethylating agents (azacitidine and decitabine) in AML patients are presented. Novel hypomethylating agent (guadecitabine) and experimental DNMT inhibitors in AML are also discussed. In summary, hypermethylation of tumour suppressors mediated by DNMT1 or DNMT3B contributes to the progression and severity of AML (except *MLL-AF9* and inv(16)(p13;q22) AML for DNMT3B), while mutation affecting *DNMT3A* represents an early genetic lesion in the pathogenesis of AML. In clinical trials of AML patients, expression of DNMTs is downregulated by hypomethylating agents while the clinical response predictive roles of DNMT biomarkers remain unresolved. Finally, nucleoside hypomethylating agents have continued to show enhanced responses in clinical trials of AML patients, and novel non-nucleoside DNMT inhibitors have demonstrated cytotoxicity against AML cells in pre-clinical settings.

KEYWORDS: acute myeloid leukaemia, DNMT1, DNMT3A, DNMT3B, azacitidine, decitabine, guadecitabine

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Introduction

Epigenetic modifications are inherited changes in DNA that do not change the sequence itself. These include DNA methylation, histone deacetylation, and miRNA regulation.[1] DNA methylation is one of the most extensively studied epigenetic changes and it regulates expression of genes in normal development of mammalian cells. Dysregulation in DNA methylation often plays a vital role in the onset of human diseases, and it has been proposed that epigenetic aberration potentially gives rise to several classic hallmarks of cancer.[2] In particular, promoter hypermethylation of tumour suppressor genes resulting in the silencing of their expression is involved in driving cancer survival, growth, and metastasis.[3,4]

DNA methylation of cytosine bases is prevalent in mammalian and several other eukaryotic genomes. The addition of methyl groups is conducted by DNA methyltransferases (DNMTs) consisting of DNMT1, DNMT3A, and DNMT3B.[5] Methylation occurs at the fifth carbon atom of cytosine bases in specific regions of CpG dinucleotides (Figure 1), and it is formed and preserved by DNMTs.[5,6] Both DNMT3A and DNM3B catalyse de novo DNA methylation, while DNMT1 preserves such patterns of DNA methylation during cell division and preferentially methylates hemimethylated DNA.[6,7]

Structurally, the domains of all DNMTs are distinguished by a variable N-terminal region consisting of regulatory domains

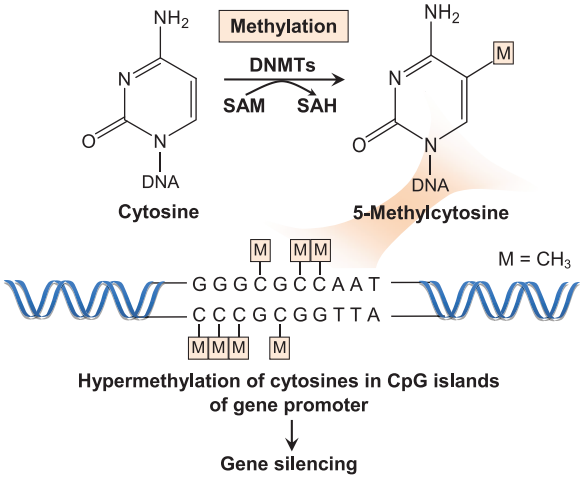

Figure 1. DNMTs catalyse the transfer of a methyl group from the methyl donor S-adenosylmethionine (SAM) to the fifth carbon of the cytosine base in the CpG islands of gene promoter. SAM is converted into S-adenosylhomocysteine (SAH) in the catalytic reaction. Hypermethylation of the cytosines suppresses transcription of the gene.[8,9]

while the C-terminal region contains the methyltransferase domain required for their enzymatic activities.[6] The regulatory domains at N-terminal differ between DNMT1 and DNMT3 whereby the N-terminal region of DNMT1 harbours the DNA methyltransferase-associated protein (DMAP) charged-rich

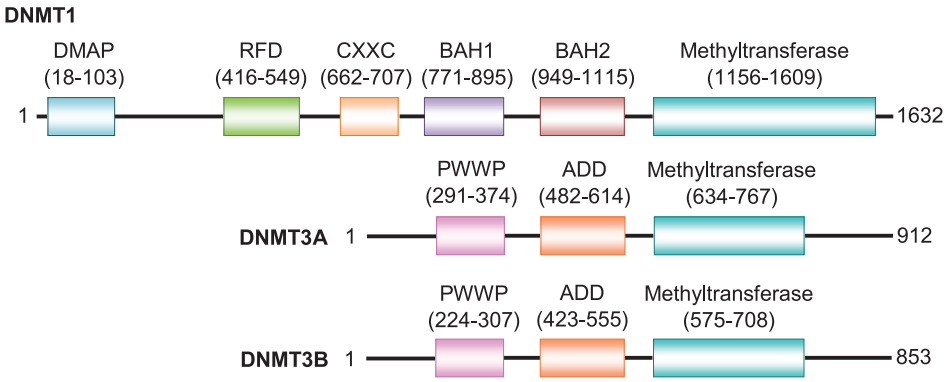

Figure 2. The domains of DNMT1 (RefSeq ID: NP_001124295.1), DNMT3A (RefSeq ID: NP_783328.1), and DNMT3B (RefSeq ID: NP_008823.1) proteins according to Pfam and PROSITE (for ADD domain of DNMT3A or DNMT3B) obtained from Ensembl database (https://www.ensembl.org/index.html). ADD indicates ATRX, DNMT3, DNMT3L zinc finger domain; BAH, bromo-adjacent homology domain; CXXC, CXXC zinc finger domain; DMAP, DNA methyltransferase-associated protein; PWWP, Pro-Trp-Trp-Pro motif domain; RFD, replication foci-targeting domain.

domain required for interaction with the transcriptional repressor DMAP1.[10] In addition, DNMT1 contains the replication foci-targeting domain (RFD) that modulates anchoring to the replication fork, the zinc finger CXXC domain that recognises unmethylated CpG, and two bromo-adjacent homology (BAH) domains (ie, BAH1 and BAH2) proposed to act as protein-protein interaction module for the silencing of gene expression.[10] DNMT3A and DNMT3B contain the Pro-Trp-Trp-Pro motif domain (PWWP; a methyl-lysine recognition motif) and the ATRX, DNMT3, DNMT3L (ADD) zinc finger domain (Figure 2). The C-terminal region of all three DNMTs consists of the methyltransferase domain required for their catalytic activities.[6]

Acute myeloid leukaemia (AML) is a clonal disorder of haematopoiesis characterised by the expansion of undifferentiated myeloid precursors, leading to deregulated haematopoiesis and bone marrow failure.[11] AML is the most common type of acute leukaemia with heterogeneous molecular profiles, clinical representations, response to therapies and outcomes. Although intensive chemotherapy and haematopoietic stem cell transplantation (HSCT) have improved the outcomes of AML patients, approximately 50% of younger patients and 80% of patients above the age of 60 years succumb to the disease due to refractory disease, relapse, or treatment-induced mortality.[12] Older AML patients unable to receive intensive chemotherapy due to toxicities have a median survival of just 5-10 months.[13]

AML presents with fewer mutations than majority of other adult cancers and it is a highly heterogeneous disease,[14] implying that other mechanisms such as epigenetics modifications play a role in the pathogenesis and outcomes of the disease. DNA hypermethylation at the CpG islands of tumour suppressors was reported in myelodysplastic syndrome (MDS) and AML nearly two decades ago.[15] Deregulated genome-wide patterns of DNA methylation occur in AML and they are not associated with mutations of known epigenetic regulators.[16]

Apart from DNA methylation, alterations in DNA demethylation also contribute to leukaemogenesis and affect clinical outcomes in AML patients. The DNA demethylase ten-eleven translocation (TET) proteins (including TET1, TET2, and TET3) converts 5-methylcytosine into 5-hydroxymethylcytosine.[17] *TET1* is fused with the *MLL* gene in t(10;11)(q22;q23) AML,[18] while *TET2* mutations occur in 13% to 27% of AML patients with normal or intermediate-risk cytogenetics associated with unfavourable prognosis.[19–21] Moreover, *TET2* mutation status has been shown to predict higher response rate in AML and MDS patients.[22]

Findings in the past two decades demonstrating deregulated DNA methylation in the pathogenesis and aggressiveness of MDS and AML have led to the approval for the clinical use of pyrimidine analogues that inhibit DNMT methylating activities (ie, 5-azacitidine [azacitidine] and 5-aza-2′-deoxycytidine [decitabine]) in both diseases.[23] These agents mimic cytosine and are able to trap DNMTs when incorporated into DNA in S phase of the replication cycle. The proteasome then degrades the trapped DNMTs leading to DNA hypomethylation and re-expression of tumour suppressor genes.[24,25] However, azacitidine is usually administrated for older AML patients who are ineligible for HSCT and with low blasts count (20%-30% bone marrow blasts),[26] while decitabine does not improve complete remission rates compared with supportive care and cytarabine in elderly AML patients.[27] Hence, further understanding of the precise DNMT-mediated oncogenic mechanisms in AML is required to select for specific and potent novel DNMT inhibitors which is currently under intense investigation and discovery.[28–30]

In this review, we describe and discuss the oncogenic properties of DNMT1, DNMT3A, and DNMT3B in AML. We also describe the prognostic and predictive roles of DNMTs in clinical trials of AML patients with hypomethylating agents, as well as novel DNMT inhibitors that have been tested experimentally in AML cells.

DNMT1 in AML

DNMT1 is the most abundantly expressed DNMT in dividing cells and it represents a key therapeutic target in rapidly dividing cancer cells for methylation inhibition and

re-expression of tumour suppressor genes.[31] Several expression and mechanistic studies have shown DNMT1 to be a potential oncoprotein in AML.

DNMT1 protein levels were higher in azacitidine-resistant AML cells (SKM1 azacitidine-sensitive and azacitidine-resistant clones), and reduced expression of anti-DNMT1 miRNAs (ie, targeted *DNMT1* 3′ untranslated region [UTR] for its reduction expression) was associated with azacitidine resistance in AML and high-risk MDS (HRMDS) patients.[32] DNMT1 expression was increased in multi-drug resistant AML cells (HL60/ATRA), and knockdown of a drug resistance-related gene segment, HA117, decreased stem-like signature of the cells and blocked DNMT1 expression.[33]

A key pathogenic mechanism involving DNMT1 in AML is the DNMT1-mediated downregulation of the cyclin-dependent kinase inhibitor *p15* (that encodes p15 protein, a tumour suppressor) expression in the disease. The expression of *p15* is lost in approximately 80% of AML cases, and hypermethylation of its promoter is frequently associated with transformation of the disease to a more aggressive phenotype.[34] *DNMT1* transcripts were found to be upregulated (by 5.3-fold) in bone marrow cells from AML patients compared with bone marrow cells from healthy donors, and *p15* was methylated in 72% of AML patients who had higher levels of DNMT1 expression, indicating the potential of DNMT1 to induce hypermethylation of tumour suppressors in AML.[35]

Subsequent studies have shown that treatment with receptor tyrosine kinase (RTK) inhibitor, nilotinib, reduced DNMT1 expression resulting in decreased global DNA methylation and upregulation of *p15* expression via promoter hypomethylation in AML cells (MV4-11 and Kasumi-1) and patient blasts.[36] Treatment with nilotinib led to apoptosis of AML leukaemia cell lines, leukaemia regression in mice (C1498 mouse AML cells injected into C57BL/6 mice), and impaired AML patient cell expansion ex vivo and in vivo through reduction of DNMT1. Also, *p15* expression was increased through promoter hypomethylation. Moreover, treatment with harmine (a beta carboline alkaloid derivative of *Peganum harmala*, a type of herb originated from Asia) in AML cells (NB4) supressed their proliferation, decreased *DNMT1* gene expression, and increased *p15* promoter hypomethylation and reactivation.[37]

Interestingly, emerging evidence has shown an association between DNMT1 and lipid metabolism protein in the suppression of *p15* expression in AML. Fatty acid-binding protein 4 (FABP4), a key regulator of lipid metabolism, is upregulated in AML cells and enhances their aggressiveness via DNMT1-dependent DNA methylation. Increased FABP4 expression induced IL-6 expression and STAT3 phosphorylation, causing DNMT1 overexpression and subsequent silencing of *p15* expression while *FABP4* silencing suppressed DNMT1-dependent DNA methylation that restored *p15* expression in AML cells (C1498, MV4-11, and Kasumi-1).[38] Similarly, inhibition of FABP4 by its selective inhibitor BMS309403 resulted in suppressed DNMT1 expression, a decrease in global DNA methylation, and re-expression of *p15* through promoter DNA hypomethylation in AML cells (C1498, MV4-11, and Kasumi-1). Impairment of their growth in vitro, ex vivo (human and mouse AML primary cells), and in vivo (C1498 cells injected into C57BL/6 mice) was also reported.[39]

Synergistic inhibition of DNMT1 and other oncogenic proteins through the use of pharmacological inhibitors has been proposed to treat AML. MUC1C, a transmembrane oncoprotein expressed in AML stem-like cells, induced DNMT1 expression by activating NF-κB p65 pathway that drove *DNMT1* mRNA transcription.[40] Synergistic pharmacological inhibition of MUC1C (with GO-203) and DNMT1 (with decitabine) reduced DNMT1 levels followed by decreased AML cell survival in cell lines (THP-1 and MOML-14) and primary AML cells.[40]

DNMT1 has also been implicated in various other haematological malignancies. The protein is frequently expressed in diffuse large B-cell lymphoma (DLBCL) being highly associated with Ki-67 expression as demonstrated in our multi-centre series of DLBCL cases.[41] We also showed that stable knockdown of DNMT1 in DLBCL cells upregulated expression of genes involved in the activation of cell cycles,[42] similar with the DNMT1-*p15* axis (p15 protein inhibits cell cycle progression) frequently reported in AML. Recent studies have also demonstrated its potentially oncogenic properties in acute lymphoblastic leukaemia,[43] chronic myeloid leukaemia,[44] multiple myeloma,[45] and Burkitt's lymphoma.[46] Collectively, DNMT1 appears to be a promising target for therapeutic modulation in haematological malignancies.

DNMT3A in AML

Mutations affecting the loci of epigenetic regulators are commonplace in AML with DNMT3A being the most commonly mutated epigenetic regulator in the disease. Around 22%-33% of AML cases present with *DNMT3A* mutations.[14,47–49]

One of the vital findings pertaining to DNMT3A in AML is the identification of *DNMT3A* missense mutations that affect the encoding of arginine R882 (codon CGC), causing loss of methylation activity of DNMT3A.[50] R882 mutation occurred in 13.2% (n = 37/281) of AML patients, and other types of mutations encompassing *DNMT3A* were found in 25 AML patients (8.9%).[51] These mutations were significantly more frequent in patients with an intermediate-risk cytogenetic profile but absent in all patients with a favourable-risk cytogenetic profile ($P < .001$), and *DNMT3A* mutations conferred significantly shorter survival ($P < .001$). In a study of AML patients younger than 60 years of age, a similar proportion (14%, n = 58/415) of patients were found to harbour missense mutations of R882 and R882 mutations were significantly associated with inferior overall survival (OS; $P = .018$) and relapse-free survival (RFS; $P = .029$).[52]

Mutated *DNMT3A* is considered an early genetic lesion in the pathogenesis of leukaemia and *DNMT3A* mutation alone might be insufficient to cause malignancies as observed in asymptomatic older carriers,[53–55] suggesting that additional genetic perturbation is required to transform into leukaemia cells. In line with this, functional studies in *Dnmt3a* knockout mice (C57BL/6-CD45.2 background) with *Flt3* internal tandem duplication (*Flt3-ITD*) overexpression have shown the roles of *DNMT3A* mutation as a reservoir for the clonal expansion of HSCs until the acquirement of the additional genetic lesion *Flt3-ITD*, causing transformation into AML cells.[56]

An independent study also showed that mice (C57BL/6-CD45.2 background) with *Flt3-ITD* and inducible deletion of *Dnmt3a* developed a rapidly lethal, penetrant, and transplantable AML.[57] The authors reported that single-cell assays identified clonogenic subpopulations expressing genes sensitive to methylation and responsive to Dnmt3a levels, and concluded that *Dnmt3a* haploinsufficiency transformed *Flt3-ITD* myeloproliferative neoplasms into AML by regulating methylation-sensitive gene expression. Likewise, a recent analysis of the mutational landscape of 85 AML patients with partial tandem duplication of MLL (*MLL-PTD*) suggested that *DNMT3A* (among others including *IDH2, TET2*, or *U2AF1*) mutations are clonal with early onset, and *MLL-PTD* likely occurs after these initial mutations while proliferative mutations involving *FLT3* or *RAS*, that usually appear later, are largely subclonal.[58]

In conjunction with the aforementioned studies, several reports have also proposed the $DNMT3A^{mut}$/*Flt3-ITD* axis as a promising therapeutic target for AML patients. AML patients with *FLT3-ITD* displayed inferior outcomes in AML patients after allogeneic HSCT due to higher risk of early relapse.[59,60] Co-occurrence of *DNMT3A* mutations with *FLT3-ITD* comprised about 13% of AML patients (n = 210/1571),[61] and in adult AML patients (n = 128), patients with *DNMT3A* R882 mutations positive for *FLT3-ITD* ($DNMT3A^{mut}$/$FLT3\text{-}ITD^{pos}$) had the worst OS ($P = .025$) and RFS ($P = .011$) compared with three other groups ($DNMT3A^{wt}$/$FLT3\text{-}ITD^{neg}$, $DNMT3A^{wt}$/$FLT3\text{-}ITD^{pos}$, $DNMT3A^{mut}$/$FLT3\text{-}ITD^{neg}$).[62] In normal-karyotype (NK)-AML patients after allogeneic HSCT, patients with $FLT3\text{-}ITD^{pos}$ and $DNMT3A$ $R882^{mut}$ had significantly worse survival compared with patients harbouring $FLT3\text{-}ITD^{neg}$/*DNMT3A* $R882^{wt}$, $FLT3\text{-}ITD^{neg}$/*DNMT3A* $R882^{mut}$, and $FLT3\text{-}ITD^{pos}$/*DNMT3A* $R882^{mut}$ (all $P < .05$).[63]

There also appears to be less conventional findings pertaining to *DNMT3A* mutations in AML patients. The frequency of R882 mutation was much lower in Chinese patients where R882 mutations occurred in 6.6% (n = 12/182) of AML patients and in 7.8% (n = 4/51) of patients with MDS.[64] None were found in patients with chronic myeloid leukaemia or myeloproliferative neoplasms in the same study. In AML patients with *DNMT3A* mutations (R882H/R882C), mutated *DNMT3A* transcript levels were higher in bone marrow than in the blood after induction and consolidation therapies but did not have association with endpoints remission duration and OS, although its levels were persistently high in remission.[65] Interestingly, the molecular landscape of paediatric AML derived from nearly 1000 participants in the Children's Oncology Group (COG) AML trials demonstrated that no mutations affecting the protein-coding regions of DNMT3A were found in paediatric AML, although these mutations were frequent in adults, implying that mutations in *DNMT3A* that promote leukaemogenesis occur in many apparently healthy adults but are rare in children.[66]

Other recent findings include the following: (1) *DNMT3A* was mutated in approximately 19% of AML cases with *RUNX1* mutations that negatively impacted OS of the patients ($P = .001$)[67]; (2) methylation profiling studies on TCGA AML patients (n = 194) identified two distinct subgroups with profound hypomethylation signatures termed as DMP.1 and DMP.2. Mutations affecting *DNMT3A* and *FLT3* were significantly enriched in the DMP.1 cases ($P < .001$).[68] The DMP.1 group had the worst survival compared with DMP-negative and DMP.2 group, and immune response genes were enriched in the DMP.1 group, suggesting a link between *DNMT3A* mutations and altered immune response in AML.

Apart from the therapeutic potential, assessment of *DNMT3A* mutations might represent a diagnostic tool for AML. A recent study of women who were healthy at study baseline but were eventually diagnosed with AML (n = 212), somatic mutations of *DNMT3A* (among mutations of other genes) in peripheral blood DNA were found to predict an increased risk of developing AML years before diagnosis.[69] Thus, screening peripheral blood for *DNMT3A* mutations in asymptomatic AML patients warrants expanded future research.

DNMT3B in AML

Unlike DNMT3A, DNMT3B mutation is a rare event in AML. Nonetheless, multiple lines of evidence have demonstrated that its expression in AML is associated with worse clinical outcomes:

1. Overexpression of *DNMT3B* was associated with inferior event-free survival (EFS; $P = .006$) and a trend towards worse OS ($P = .056$) in a panel of de novo AML patients (n = 191)[70].
2. In an integrated analysis of the methylome and transcriptome of 151 paediatric AML patients, increased DNMT3B expression and reduced methylation were associated with poorer clinical outcomes ($P \leqslant 10^{-5}$; $q \leqslant 0.002$), and higher DNMT3B expression was associated with worse minimal residual disease (MRD), higher rate of relapse or resistant disease, worse EFS, and higher genome-wide methylation burden (GWMB) in both the training and validation cohort (all $P < .03$ in both cohorts).[71] Higher GWMB was also associated with worse MRD and EFS (all $P < .05$ in both cohorts), implying that DNMT3B-induced GWMB may play a role in the aggressiveness of paediatric AML.

3. Older adults (⩾60 years old) with primary, cytogenetically normal AML (n = 210) who expressed high *DNMT3B* transcript levels had significantly fewer complete remissions ($P = .002$), inferior disease-free survival (DFS; $P = .02$), and OS ($P < .001$), and all three characteristics remained significant ($P < .05$) in multivariable analyses.[72] High *DNMT3B* levels in these patients were associated with gene expression profiles implicated in differentiation, proliferation, and survival pathways, but surprisingly high *DNMT3B* levels were not associated with DNA methylation changes, consistent with other reports demonstrating that *DNMT3B* expression did not influence the DNA methylation levels of leukaemic blasts derived from cytogenetically normal AML patients.[73] These suggest that older AML patients with high DNMT3B expression might not respond to hypomethylation agents, and that DNMT3B might exert its effects through an additional, methylation-independent mechanism in older AML patients.

Myeloperoxidase (MPO), a microbicidal protein measured in leukaemia blasts by cytochemistry, is a biomarker for the diagnosis of AML and its expression is associated with better prognosis in AML patients. *DNMT3B* was found to have a significantly inverse relationship with MPO levels in $CD34^+$ AML cells ($P = .0283$) without significant association with common mutations in AML (*FLT3-ITD, CEBPA*, or *NPM1* mutations).[74] This suggests that *MPO* gene transcription might be repressed through promoter methylation by DNMT3B, although this is subject to further proof by functional studies.

Furthermore, in a *DNMT3B* genotyping study on de novo AML patients (n = 317) and healthy control subjects (n = 406) of Chinese Han populations, the GG genotype of rs1569686 was most significantly associated with increased risk for AML (OR: 5.76; 95% confidence interval (CI): 2.60-12.73; $P < .01$), compared with the TT genotype, in contrast with the CC genotype of rs2424908 shown to have reduced AML risk (OR: 0.57; 95% CI: 0.36-0.91; $P = .01$) compared with the TT genotype, suggesting that *DNMT3B* gene polymorphisms could play roles in AML leukaemogenesis and as potential markers for AML.[75] Moreover, DNMT3B overexpression leading to DNA hypermethylation has also been reported in T-cell acute lymphoblastic leukaemia and Burkitt's lymphoma,[76] illustrating the oncogenic role of DNMT3B through hypermethylation of tumour suppressor genes in haematological malignancies.

Despite of the association of DNMT3B expression with worse prognosis in AML patients, recent studies have also shown its potential tumour suppressive roles in two subtypes of AML, ie, *MLL-AF9* AML, and inv(16)(p13;q22) AML.

In *MLL-AF9* AML, one of most frequent *MLL* rearrangements in MLL-rearranged leukaemia, deletion of *Dnmt3b* in mice model increased their progression through enhanced stemness and cell cycle progression accompanied by upregulation of oncogenic gene sets, indicating a tumour suppressive role of Dnmt3b in *MLL-AF9* AML.[77] Moreover, the authors also showed that *Dnmt3a/3b* double-KO (DKO) AML cells had accelerated leukaemic development compared with control AML cells or deletion of either *Dnmt3a* or *Dnmt3b* gene, suggesting the synergistic involvement of DNMT3A and DNMT3B in suppressing *MLL-AF9* leukaemia progression. These observations were in line with findings by an independent group in which Dnmt3b overexpression (inducible *Dnmt3b*-knock-in mice) slowed leukaemia development and deregulated leukaemia stem cell (LSC) function, and murine *MLL-AF9* cells with *Dnmt3b*-knock-in demonstrated leukaemia development with prolonged survival compared with *MLL-AF9* alone.[78] These studies appear to tally with previous observations of the Dnmt3b tumour suppressive roles in lymphomas whereby $Dnmt3b^{-/-}$ mice with MYC-induced lymphomas demonstrated accelerated lymphomagenesis.[79]

The inv(16)(p13;q22) is one of the most common recurring chromosomal rearrangements in AML, and the transcriptional coactivator *MN1*, an oncogene in inv(16) AML, is known to be overexpressed in inv(16) AML.[80] Recently, *DNMT3B* expression was found to be lower in inv(16) vs non-inv(16) paediatric AML patients, and knockdown of *DNMT3B* expression in AML cells (HL-60) led to decreased re-methylation efficiency of *MN1* exon-1 locus that subsequently drove *MN1* overexpresion in AML cells.[81] This implies the involvement of DNMT3B in the leukaemogenesis of inv(16) AML and suggests a tumour suppressive role akin to those observed in *MLL-AF9* AML.

miRNAs Implicated in the Regulation of DNMTs Expression

One of the pivotal findings in the regulation of DNMTs expression is through *miR-29b*-mediated suppression. Overexpression of *miR-29b* in AML cells (MV4-11 and Kasumi-1) reduced the expression of DNMT1, DNMT3A, and DNMT3B at both mRNA and protein levels, resulting in reduced global DNA methylation and re-expression of *p15* through promoter DNA hypomethylation.[82] The authors demonstrated that *miR-29b* directly targeted *DNMT3A* and *DNMT3B* in their 3′ UTRs, while *miR-29b* downregulated *DNMT1* indirectly by targeting *SP1*, a transactivator of *DNMT1* expression.

Treatment of AML cell lines (Kasumi-1 and NB4) and leukemic blasts from primary AML patients with a potent histone deacetylase 1 (HDAC1) inhibitor AR-42 resulted in downregulation of *miR-29b* targets including *SP1, DNMT1, DNMT3A*, and *DNMT3B*.[83] Combination of AR-42 and decitabine yielded higher anti-leukemic activity both in vitro (Kasumi-1, NB4, and murine FDC-P1-KIT^{mut}) and in vivo (FDC-P1-KIT^{mut} cells injected into NOD/SCID mice).[83] These findings suggest that *miR-29b* oligonucleotides might be effective hypomethylating therapeutic compounds by supressing DNMTs expression. Moreover, the levels of *miR-29b*, in addition to DNMTs, have since been evaluated as a potential predictive factor in decitabine clinical trials of AML patients as described in the next section.

Growing evidence has shown the potential mechanisms involving DNMT3B-mediated hypermethylation in the promotion of leukaemogenesis and aggressiveness of AML, particularly involving miRNAs.[82,84,85] *miR-375* has been demonstrated to be a tumour suppressive miRNA in various cancers; its expression conferred better OS (P=.007) and DFS (P=.015) in AML patients (n=102), and DNA hypermethylation of the promoter of precursor-*miR-375* (pre-*miR-375*) caused lower *miR-375* expression in AML.[85] In the same study, it was reported that overexpression of *miR-375* suppressed proliferation and colony formation of AML cells and reduced tumour size with prolonged survival in leukaemia xenograft mouse model (HL-60 cells in nude mice). More importantly, HOXB3 (a homeobox protein) induced DNMT3B expression to bind pre-*miR-375* promoter that led to increased DNA hypermethylation of pre-*miR-375*, causing lower expression of *miR-375* in AML cells (HL-60 and THP1), and the authors proposed a novel *miR-375*-HOXB3-CDCA3/DNMT3B regulatory circuitry in AML.[85]

Predictive Roles of DNMTs in AML Clinical Trials

In clinical trials of AML patients with and without hypomethylating agents that have assessed changes in DNMTs, the clinical response predictive role of DNMT markers remains unresolved. In a phase I study of decitabine alone or in combination with valproic acid, DNMT1 protein was detected in eight of 14 (57.1%) AML patients. DNMT1 was significantly (P=.02) decreased by decitabine treatment in the patients but the depletion was not associated with clinical response.[86] In another phase I trial of decitabine plus bortezomib, bone marrow samples from five patients showed that *DNMT1, DNMT3A*, and *DNMT3B* were downregulated while *miR-29b* and estrogen receptor (*ESR*) were upregulated post-treatment (day 26) but none of the genes reached statistical significance.[87]

In terms of decitabine treatment alone, a phase II study of untreated older (⩾60 years old) AML patients receiving low-dose decitabine showed that responders (n=14) had a significantly higher pre-treatment level of *miR-29b* vs non-responders (n=9) (P=.02).[88] In the same study, a trend for lower *DNMT3A* (P=.06) was observed in responders vs non-responders, and no significant differences in the levels of *DNMT1, DNMT3B*, or *ESR1*. The COG conducted a trial on eight young adults or children with refractory/relapsed (R/R) AML to receive low-dose decitabine alone.[89] Three bone marrow samples were available in the study for expression analysis that showed increased *miR-29b* expression associated with a decrease in *DNMT1* expression post-treatment. Furthermore, a phase I/II trial of adult R/R AML patients (n=122) receiving guadecitabine showed that global DNA demethylation was strongly associated with clinical response, and *DNMT3B* (but not *DNMT1* and *DNMT3A*) expression contributed >5% in predicting clinical response. *FLT3-ITD* and *NPM1* mutations did not predict response in this cohort of AML patients.[90]

Although DNMT1-mediated suppression of *p15* is common in AML, the expression of *p15* in clinical trials also does not always correlate with response or prognosis. In a phase I/II study of 5-azacitidine in combination with valproic acid and *all-trans* retinoic acid, *p15* mRNA expression was significantly upregulated after 7 days of treatment (P=.02) but it was not associated with clinical response.[91] Likewise, in a separate phase I study, no association was observed between *p15* methylation status at baseline or after therapy and response to decitabine in R/R AML patients.[92] Conversely, *p15* mRNA levels were increased in five of six (83.3%) assessable AML patients treated with decitabine, and global DNA hypomethylation occurred in 7 of 12 (58.3%) patients.[86] Changes in DNMT1, DNMT3A, or DNMT3B expression and response predictive roles of each biomarker in clinical trials of AML patients treated with DNMT inhibitors are summarised in Table 1.

Novel DNMT Inhibitors and Combination Therapies in Clinical Trials of AML

Complete tumour responses are infrequent in AML and MDS patients treated with azacitidine or decitabine (a deoxy derivative of azacitidine). This is thought to be due to degradation of both agents by cytidine-deaminase (CDA) present in the liver, spleen, and intestinal epithelium, resulting in their short plasma half-life.[93–95] Guadecitabine (SGI-110), a dinucleotide of decitabine, demonstrates improved stability in plasma due to CDA resistance,[96,97] and it is under active assessment in several AML clinical trials. In a multicenter phase I study of R/R AML (n=74) and MDS patients (n=19), the dosage 60 mg/m^2 daily in a 5-day schedule and 28-day cycle administered subcutaneously (60 mg/m^2/d SC 5-day/28) was shown to be well-tolerated in both patient populations.[98]

The clinical trial was followed by a phase II study of elderly (⩾65 years) treatment-naïve AML patients (n=107) to receive guadecitabine at different doses and treatment schedules.[99] Over 50% of the patients achieved a composite complete response at all doses and schedules of guadecitabine administration, and 60 mg/m^2/d SC 5-day/28 maintained as the recommended regimen. Similar findings were reported by another multicenter phase II study of R/R AML and MDS (n=55) where eight (14.3%) patients responded to guadecitabine (60 mg/m^2/d SC 5-day/28) with prolonged survival of 17.9 months vs median survival of 7.1 months.[100] In the study, patients with no or few somatic mutations (mutated *DNMT3A* was present in 20% of the study cohort) was the only factor that significantly predicted clinical response (P=.035), and high rate of demethylation in blood was significantly associated with longer survival (P=.03).

Ongoing clinical trials involving DNMT inhibitors (azacitidine, decitabine, and guadecitabine) in AML patients are summarised in Table 2. In particular, a multicenter phase III randomised clinical trial of guadecitabine vs treatment of choice in 404 previously treated AML patients is currently underway (ClinicalTrials.gov ID: NCT02920008). Interestingly, combination of decitabine with talazoparib, a novel inhibitor recently approved by the Federal Drug Administration (FDA) in October 2018 for the treatment of *BRCA*$^+$ breast cancer, is also under investigation by a phase I/II study in untreated AML and R/R AML patients (n=171; ID: NCT02878785) (Table 2).

Talazoparib inhibits the activities of poly (ADP-ribose) polymerase (PARP) that subsequently induces DNA damage and cell death of *BRCA*$^+$ breast cancer cells through synthetic lethality (ie, simultaneous disruption of multiple genes).[108–110] PARP-BRCA represents the first synthetic lethality approach that has successfully translated into the clinic,[111] and similar synthetic lethality interaction is thought to occur for PARP-DNMT. The PARP inhibitor olaparib has been shown to synergise with decitabine whereby treatment with both inhibitors caused synthetic lethality with increased apoptosis and DNA damage in a panel of AML cell lines (HL60, K562, Mv4-11, KG1a, and PL21).[112]

Likewise, treatment of AML cell lines (KBM3/Bu2506 and MOLM14) with combinations of decitabine (DNMT inhibitor), niraparib (PARP inhibitor), and romidepsin or panobinostat (both HDAC inhibitors) suppressed their proliferation through trapping of PARP1 and DNMT1 to chromatin, leading to increased double-strand DNA breaks and cell death.[113] These findings support the ongoing or future clinical trials of PARP-DNMT inhibitors combination in AML patients.

Experimental DNMT Inhibitors Targeting AML

Approved nucleoside analogues for AML treatment (azacitidine and decitabine) are relatively non-specific with low chemical stability, confer significant toxicities, and require incorporation into DNA to exert their effects as covalent inhibitors.[114] This intensifies the identification and characterisation of novel, non-nucleoside DNMT inhibitors with various chemical scaffolds, and multiple of such inhibitors have recently been assessed in AML cells.

A recent structure-based virtual screening in combination with biological assays identified two compounds, termed as compounds 40 and 40_3, that inhibited DNMT3A activities with IC_{50} of 46.5 and 41 μM, respectively.[29] The authors demonstrated that both compounds were more cytotoxic against AML cell lines (Kasumi-1, KG-1, MV4-11, and THP-1) than the cervical cancer HeLa cells, and both compounds showed similar growth inhibitory effects. Compounds 40 and 40_3 at 50 μM concentration conferred over 50% inhibition in MV4-11 AML cells.

NSC-319745 is an inhibitor of DNMT1 originally identified through docking-based virtual screening and enzymatic assays, although it contains relatively low potency against DNMT1.[115] Recently, an independent research group synthesised hydroxamic acid derivatives of NSC-319745, resulting in compounds potent against DNMTs and HDACs.[116] One of the inhibitors termed as compound 15a showed higher DNMT1 inhibitory potency than NSC-319745 (% inhibition at 100 μM: 69.88 ± 1.97) as well as inhibiting HDAC1 (IC_{50}: 57 nM) and HDAC6 (IC_{50}: 17 nM). Compound 15a was cytotoxic against AML (U937; IC_{50}: 1.06 ± 0.09 μM) and chronic myeloid leukaemia (CML) (K562; IC_{50}: 2.85 ± 0.12 μM) cells, and induced expression of *p16* through its CpG islands demethylation and histones acetylation. The compound also demonstrated inhibition, albeit lower than DNMT1, against DNMT3A (% inhibition at 100 μM: 23.39 ± 4.12) and DNMT3B (% inhibition at 100 μM: 48.53 ± 2.38).[116]

Harmine, a type of β-carboline alkaloid originally isolated from the seeds of the herbal plant *Peganum harmala*, decreased *DNMT1* expression and re-activated *p15* expression through promoter hypomethylation. In AML cells (NB4), the compound significantly suppressed cell proliferation ($P < .05$) in a dose- and time-dependent manner and arrested the cells in G_0/G_1 phase.[37] In addition, an analogue of procaine (a non-nucleoside DNMT inhibitor) termed as derivative 3b was capable of inducing the demethylation of chromosomal satellite repeats in HL60 AML cell line, although it was not cytotoxic against the cells.[117]

Conclusions

DNMT1 is rarely mutated in AML and its high expression levels in AML imply the requirement of its increased functions, through overexpression, for the survival of AML cells. DNMT1 frequently targets the promoter region of *p15* for expression downregulation. Hence, assessment of increased *p15* levels serves as a recommended parameter to measure the impaired activity of DNMT1 in AML, and this is applicable in future research for the identification and characterisation of novel inhibitors specific against DNMT1 which has been a subject of active investigations in recent years.[28,118,119]

A wide body of reports have demonstrated the high frequency of *DNMT3A* mutations occurring in AML. Mutations of *DNMT3A* locus is regarded as one of the prerequisite early genetic lesions in the pathogenesis of AML before the acquisition of additional mutations such as *FLT3-ITD* that transform a pre-malignant clone into AML, while R882 mutations confer loss-of-function of the encoded protein. *DNMT3A*-specific therapeutic modalities might require genetic editing for the restoration of wild-type DNMT3A protein expression.

The roles of DNMT3B in AML might be subtype specific as it appears to be oncogenic in AML xenograft mouse models as well as cell lines. Paradoxically, it may also play a tumour suppressive role in *MLL-AF9* AML, and inv(16)(p13;q22) AML. Its oncogenic properties have been frequently shown to be associated with HOX genes, particularly the oncoprotein HOXB3 in AML as well as breast cancer,[120] and DNMT3B expression is positively associated with those of HOX family genes in AML.[71] Disruption of the expression or functions of DNMT3B as a potential treatment for AML patients might thus require assessment for the expression levels of HOX genes.

The expression of DNMTs was not predictive of response in most clinical trials of hypomethylating agents in AML. However, there are limitations to the interpretation of predictive values whereby most clinical trials had limited number of samples for expression studies pre- and post-treatment. Furthermore, different patient populations (young or older AML patients) or regimens (hypomethylation monotherapy or combination treatments) might contribute to differences in their DNMTs expression or prediction of response results. Nonetheless, majority of the studies reported downregulation

Table 1. Changes in DNMT1, DNMT3A, or DNMT3B expression in AML patients treated with DNMT inhibitors in clinical trials.

TREATMENT	PHASE AND ID	PATIENTS	REGIMEN	TYPE OF SAMPLES[a]	CHANGES IN EXPRESSION	PREDICTIVE OF RESPONSE
Decitabine or decitabine + valproic acid (VA)[86]	Phase I ID: NCT 00079378	Adult relapsed AML (n = 13) and older (>60 y/o) untreated AML (n = 12)	Decitabine alone for OBD (days 1-10; n = 14) or decitabine for 10 days + VA for MTD (days 5-21)	Unselected bone marrow mononuclear cells (n = 14)	Post-treatment: ↑*p15, ER, ER* promoter demethylation; ↓DNMT1 protein, global hypomethylation	*ER* expression
Decitabine + bortezomib[87]	Phase I ID: NCT 00703300	Untreated poor-risk older (⩾65 y/o) AML patients (n = 19)	Decitabine (days 1-10) with cycles repeated every 28 days + bortezomib (days 5 and 8, or days 5, 8, 12, and 15)	Serial bone marrow samples (n = 5)	Post-treatment: ↑*miR-29b, ESR;* ↓*DNMT1, DNMT3A, DNMT3B*	N/A
Decitabine[89]	N/A	Young adults or children R/R AML (n = 8)	Decitabine (days 1-10) at ~4-week intervals. Subsequent courses shortened to 5 days in responders	Sequential bone marrow samples (n = 3)	Post-treatment: ↑*miR-29b;* ↓*DNMT1*	N/A
Decitabine[88]	Phase II ID: NC T00492401	Untreated older (⩾60 y/o) AML patients (n = 53)	Low-dose decitabine (days 1-10)	Unselected diagnostic bone marrow samples (n = 23)	Responders: ↑*miR-29b*, a trend for lower *DNMT3A*. No differences in *DNMT1, DNMT3B*, or *ESR1*	*miR-29b* expression
Guadecitabine[90]	Phase I/II ID: NCT 01261312	Adult R/R AML (n = 122)	Guadecitabine for 5 (daily ×5) or 10 days (daily ×10) up to 4 cycles followed by daily ×5 cycles. All regimens dosed with a 28-day treatment cycle	Type of samples not stated (n = 122)	N/A	Global DNA demethylation; *DNMT3B* contributed >5%

For the primary outcomes (eg, response rate, survival) of the actual clinical trial, readers are directed to the individual reference for each study.
Abbreviaitons: MTD, maximum-tolerated dose; N/A, not available; OBD, optimal biological dose.
[a]Denotes the type of samples used to examine the changes in the levels of DNMTs and other genes, and the number of available samples.

Table 2. Ongoing clinical trials of DNMT inhibitors in AML patients.

TREATMENT	PHASE AND ID	PATIENTS	PRIMARY OUTCOME MEASURES	REGIMEN	PRIMARY COMPLETION
Guadecitabine vs treatment of choice (TC)[101]	Phase III ID: NCT 02920008	Previously treated AML (n=404)	Overall survival (OS)	Guadecitabine or TC in a 1:1 ratio. Guadecitabine is given in 28-day cycles. TC includes either high- or low-intensity regimen, or best supportive care.	September 2019
Guadecitabine[102]	Phase I ID: NCT 02293993	Relapsed or not eligible for chemotherapy AML (n=21)	Dose-limiting toxicity (DLT) when administered subcutaneously	Patients divided into four cohorts each receiving different dose once daily for 5 consecutive days followed by a 23-day non-dosing period, or once daily for 10 days followed by a 16-day non-dosing period	December 2019
Guadecitabine[103]	Phase II ID: NCT 03603964	AML and MDS who participated in a previous guadecitabine clinical study (n=250)	Incidence of adverse events (AEs)	Guadecitabine at the same dose received in the last cycle of their prior study or at a different dose. Treatment is continued as long as the patient continues to benefit based on investigator's judgement	July 2020
Guadecitabine[104]	Phase II ID: NCT 03454984	Previously treated AML and MDS after allogeneic stem cell transplantation (n=40)	Disease-free survival (DFS)	Guadecitabine for 5 days in a total of 10 cycles (cycle=28 days)	September 2020
Decitabine+talazoparib[105]	Phase I/II ID: NCT 02878785	Untreated AML and relapsed/refractory AML (n=171)	Dose finding and efficacy	Phase I: decitabine daily for 5 days every 28 days. Talazoparib orally daily days 1-28; Phase II: decitabine for 5 days every 28 days. Talazoparib orally daily days 1-28	December 2020
Azacitidine or decitabine+chemotherapy[106]	Phase II ID: NCT 03164057	Untreated AML (n=200)	Safety, change in genome-wide methylation and its association with event-free survival (EFS)	Azacitidine or decitabine for 5 days prior to induction chemotherapy followed by intensification chemotherapy. Objective is to evaluate safety and efficacy of epigenetic priming prior to chemotherapy	June 2025
Guadecitabine vs guadecitabine+idarubicin[107]	Phase II ID: NCT 02096055	Untreated elderly AML (n=44)	Complete remission (CR) and toxicity	Group A: guadecitabine for 5 days; Group B: guadecitabine plus idarubicin for 5 days	April 2026

of DNMTs expression accompanied with increased expression of their suppressor *mir-29b* post-treatment (Table 1).

In conclusion, increased hypermethylation of tumour suppressor genes in AML is attributable to the aberrant activities of DNMT proteins whose expression is primarily regulated by *miR-29b*. Expression of DNMTs is downregulated in clinical trials of AML patients treated with hypomethylating agents targeting DNMTs. Recent clinical trials have shown the novel hypomethylating drug guadecitabine conferring enhanced response in AML patients. Multiple phase II and III clinical trials are currently underway to test the efficacy of azacitidine, decitabine, or guadecitabine in combination with other agents (eg, PARP inhibitor or chemotherapy regimens) for AML patients. Collectively, these imply that nucleoside-based agents might still be the mainstay for AML hypomethylation therapy in the upcoming years. Novel non-nucleoside DNMT inhibitors have demonstrated cytotoxicity in pre-clinical experimental settings against AML cells, and their translation into the clinic remains to be elucidated.

Author Contributions

KKW conceived and designed the manuscript, conducted literature searches, prepared the figures, and wrote the manuscript. CHL and TMG critically revised and edited the manuscript.

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