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Epigenetics in Acute Myeloid Leukemia

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Abstract

Acute myeloid leukemia (AML) is a disease characterized by uncontrolled proliferation of clonal neoplastic hematopoietic precursor cells. This leads to the disruption of normal hematopoiesis and bone marrow failure. Major breakthroughs in the past have contributed to our understanding of the genetic failures and the changed biology in AML cells that underlie the initiation and progression of the disease. It is now recognized that not only genetic but also epigenetic alterations are similarly important in this process. Since these alterations do not change the DNA sequences and are pharmacologically reversible, they have been regarded as optimal targets for what is now known as epigenetic therapy. In this review, we will discuss our current understanding of normal epigenetic processes, outline our knowledge of epigenetic alterations in AML, and discuss how this information is being used to improve current therapy of this disease.

Acute myeloid leukemia (AML) is characterized by uncontrolled proliferation of clonal neoplastic hematopoietic precursor cells, leading to disruption of normal hematopoiesis and bone marrow failure. Molecular and cytogenetic analyses have been used to define genetic defects that contribute to the initiation and maintenance of this disease. Recurrent chromosomal rearrangements can be detected in more than 50% of AML cases and have been considered one of the most important prognostic factors for the prediction of clinical outcome.[1] However, approximately 45% of AML cases have been shown to harbor normal karyotypes, although in the majority of these cases, one or more defects can be detected at the molecular level.[2–7] The resulting molecular aberrations in these patients have been used to predict clinical outcome and/or, as in the case of internal tandem duplication of *FLT3*, the mutant proteins have been used as therapeutic targets.

In contrast to the aforementioned genetic aberrations that lead to irreversible structural DNA changes, epigenetic alterations result in a loss of gene function but do not modify the DNA coding sequence and can be reversed pharmacologically. In this review, we will outline briefly our current understanding of epigenetic modifications necessary for normal cell functions and describe efforts to unravel alterations that have occurred in these epigenetic patterns and contributed to myeloid leukemogenesis. Furthermore, we will highlight current

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clinical approaches that use this emerging information to develop novel epigenetic strategies.

EPIGENETICS, THE INTERPLAY OF DNA METHYLATION, CHROMATIN, AND NON-CODING RNAs

Once thought to be a simple package for DNA sequences, chromatin is now appreciated as an exceedingly important and complex aspect of normal cell biology. Several decades of research have revealed that modifications of DNA and chromatin proteins play a central role in virtually all functions associated with DNA, including transcriptional control, chromosome stability, DNA repair, and DNA replication. A complex interplay between DNA and chromatin modifications encodes a layer of information that acts in addition to the genetic code. Once established, this “epigenetic” information is preserved during cellular replication and, in certain cases, can even be passed through the germ line and inherited by subsequent generations.[8] Epigenetic information has been shown to be facilitated by three primary factors: DNA methylation, histone modifications, and non-coding RNA.

DNA Methylation

The most well-studied epigenetic mediator is DNA methylation. In mammals, a family of DNA methyltransferase enzymes (DNMTs) catalyzes the addition of a methyl group to the 5’ position of the cytosine ring specifically in CpG dinucleotide sequences. This is the only abundant modification known to occur in mammalian DNA (~70% of CpGs; ~1%–2% of total DNA). The biological importance of DNA methylation is highlighted by the fact that targeted deletion of all DNMT enzymes known to possess methyltransferase activity results in lethality in mice. Deletion of *Dnmt1*, the principal DNMT enzyme that primarily functions to copy DNA methylation to newly synthesized DNA during replication (maintenance methylation), results in embryonic lethality.[9] Deletion of *Dnmt3a* and *Dnmt3b*, DMNTs that have been shown to be involved in both maintenance and de novo methylation activities, also produces a lethal phenotype.[10] In humans, mutations in *DNMT3B* cause ICF syndrome, a disease that is marked by chromomsomal instability, developmental defects, and immunodeficiency.[11] Mutations in the *MECP2* gene that encodes a protein that binds specifically to methylated DNA cause Rett syndrome, which is associated with several neurological defects.[12] In contrast, DNA demethylation is thought to occur passively by failing to maintain methylation during DNA synthesis, although active demethylation also has been observed in specific biological contexts, especially during development,[13] thereby suggesting the possibility that this activity is mediated by enzymatic processes.[14,15]

Although several non-mutually exclusive roles for DNA methylation have been described, including chromosome stability[16] and genome compartmentalization,[17] the vast majority of the data available strongly support a role in transcriptional regulation. The genomic distribution of CpG dinucleotides and their subsequent methylation is remarkably non-random; relative to the genome at large, CpGs are highly enriched in short sequences called CpG islands (CGIs) commonly found within or very near the 5' regions of genes. CpGs within CGIs are strongly resistant to methylation in normal somatic tissues, whereas most non-5′, non-CGI CpGs are methylated. The reverse may occur in cancer cells. A multitude of studies have demonstrated an inverse correlation between methylation of CGIs and gene expression. DNA hypomethylation is generally permissive for transcription, whereas DNA hypermethylation is associated with gene repression. Indeed, pharmacologically induced DNA demethylation can result in re-expression of hypermethylated genes. Most evidence supports that DNA methylation exerts its repressive effects either by directly blocking transcription factor binding or via a host of DNA binding proteins that specifically interact

with methylated DNA. For example, DNA methylation blocks the binding of CTCF, a key protein that functions to insulate actively transcribed domains from neighboring heterchromatin.[18] Proteins that share a conserved methyl-CpG-binding domain motif, MBD1-4 and MeCP2, along with Kaiso make up a family of proteins that selectively bind to methylated DNA and recruit additional factors with repressive properties.[19]

Histone Modifications

The four core histone proteins (H2A, H2B, H3, and H4) along with 147 bp of DNA comprise the nucleosome, the repeated structural unit of chromatin. The N-terminal histone tails protrude from the core assembly and are subject to a vast array of covalent modifications, including acetylation, methylation, ubiquitination, adenosine triphosphate (ADP)-ribosylation, and sumolation of lysines (K), methylation of arginine (R) residues, and phosphorylation of serines and threonines (for review see Kouzarides[20]). Together, these modifications are thought to comprise the “histone code,” a complex series of modifications that, when integrated, signal for either active euchromatic or silent heterochromatic states. Histone modifications function by, with the exception of methylation, reversing the net electrical charge of the histone tails thus reducing the affinity of inter-nucleosome and/or DNA-histone interactions. Acetylation of histones H3 and H4 by protein complexes that retain histone acetyltransferase (HAT) activity are a hallmark of active transcription. This activity is opposed by histone deacetylases (HDACs) that remove acetyl groups, promoting the formation of heterochromatin. Histone modifications also function by enhancing or decreasing the affinity for co-activator or co-repressor complexes. For example, acetylation promotes the association of several transcriptional activator complexes through the specific interaction of bromo domain-containing proteins. Histone methylation also functions in this manner and can signal for either eu- or heterochromatin though the interaction of several proteins that contain specific chromo-like and PHD domains. Methylation of H3K9 and/or H3K27 by various histone methyltransferases (HMTs), including Suv39h1, Suv39h2, EHMT1, EHMT2/G9a, SETBD1/ESET, and Ezh2 promote the association of heterochromatin protein 1 (HP1), a major component of heterochromatin, and other repressive polycomb group proteins. Methylation of H3K4 signals for euchromatin by recruiting ATP-dependent chromatin remodeling complexes and other factors that activate transcription. Importantly, protein complexes that are recruited to chromatin commonly have enzymatic activities that in addition modify other histone tail residues to promote further the intended chromatin state. Crosstalk between histone modifications forms multiple layers of information that provide stability to the euchromatic or heterochromatic state.

Non-Coding RNAs

Small non–(protein)-coding RNAs have been shown to influence gene expression through a variety of mechanisms in many organisms. RNA-mediated gene repression is mainly achieved through post-transcriptional gene silencing via the expression of an RNA molecule that is complementary (in the anti-sense orientation) to a target transcript. Homology-dependent transcript degradation is mediated by the Argonaut family of proteins that initially process and stabilize anti-sense RNAs, and later facilitate transcript cleavage or transcriptional interference.[21] MicroRNAs (miRNAs) are small RNA molecules (19–23 nucleotides in length) that target specific 3' untranslated regions (UTRs) of genes and cause reduced protein production in a gene-specific manner. Although RNA-mediated post-translational gene silencing is not strictly defined as epigenetic, miRNAs are likely to play an important role in the regulation of genes with important epigenetic functions. In a recent study, DNMT3A and DNMT3B were found to be modulated by miR-29, a miRNA gene that is downregulated in lung cancer.[22] Decreased miR-29 expression results in elevated DNMT3A and -3B, which contributes to aberrant hypermethylation of genes with tumor-suppressor functions.

Non-coding RNAs also have been shown to influence gene expression by inducing changes in chromatin conformation.[23] While these processes have been well described in plants and some lower organisms, less is known about this function in mammals. Interestingly, X-chromosome inactivation in mammals is dependent on the coating of the repressed chromosome with RNA from the *XIST* gene in mammals, preceding changes in DNA methylation and histone modifications.[24] As the activity of DNMTs and histone modifying enzymes do not appear to be contingent on the underlying sequence, RNA-based mediation of epigenetic phenomena would assist to explain the locus-specific nature of DNA methylation and histone modifications.

DNA Methylation and Histones: An Integrated View of Epigenetic Transcriptional Regulation

Recent findings have highlighted a strong interdependence between DNA methylation and histone modifications. A first line of evidence came from the discovery that DNMT1 interacts with HDAC1[25] and HDAC2,[26] demonstrating a functional relationship between histone acetylation and DNA methylation. Experimental induction of DNA demethylation leads to histone acetylation, and demethylation can be caused by the HDAC inhibitor, trichostatin A.[27] the histone methyltransferase that methylates H3K9, Suv39h, associates with DNMT1[28] and the H3K27 methylase, Ezh2, associates with all three catalytically active DNMTs.[29] Furthermore, several ATP-dependent chromatin remodeling complexes that interact with chromatin in a histone modification-dependent manner influence DNA methylation, including *Suv39h*,[30] ATRX,[31] and *Lsh*.[32] *Lsh* associates with *Dnmt3a* and *Dnmt3b*, demonstrating how this functional relationship might occur.[33] A major question that remains to be answered is which of these epigenetic marks drives the establishment and maintenance of chromatin states. Two recent studies found that H3K4 methylation blocks the developmental acquisition of DNA methylation, indicative of a predominant role of histones in determining chromatin state and acquisition of other epigenetic marks.[34,35] Other evidence shows that upon promoter activation, DNA demethylation precedes H3K4 methylation.[36] As DNA methylation is the only epigenetic modification to be covalently associated with the DNA itself, CpG methylation status is in the unique position to provide long-term stability of gene expression states. Histone sliding and eviction occurs frequently in transcribed regions.[37] DNA methylation may enable a lasting “chromatin memory” that endures the prolonged cellular lifespan of mammalian cells and persists through activities such as transcription and mitosis.

Tissue-Specificity and Development of Genome-Wide Epigenetic Patterns

The importance of epigenetic information is underscored by the fact that genome-wide patterns are unique between virtually all healthy cell types and tissues from within an individual.[38,39] Commonly these patterns are altered during disease.[40] Normal cellular differentiation and tissue development coincide with developmental acquisition of genome-wide patterns of DNA methylation. It is theorized that epigenetic factors are involved in the control of cellular totipotency, differentiation, and lineage selection. In support of this hypothesis, embryonic stem cells have distinct patterns of histone modifications that simultaneously signal for active and repressive chromatin, a precursor state to the choice of cell fate.[41] Following cell lineage selection, tissue-specific hypermethylation of 5′ regions of genes generally correlates with gene repression in the particular tissue.[39,42] However, as only a small proportion of genes demonstrate tissue specificity in DNA methylation patterns, the roles of other epigenetic factors in this process await evaluation on a genome-wide scale. Interestingly, DNA methylation differences between tissues are vastly more common in non-5′ regions.[38] These changes may be involved in the regulation of non-coding RNAs or modulation of regional chromatin structure via interactions with the nuclear matrix. In

support of this, MeCP2 binds CpGs in AT-rich sequence contexts[43] and associates with matrix proteins.[44]

Observations showing that malignant cells maintain aberrant epigenetic marks, particularly DNA methylation, compared to their normal counterparts has dominated the field of epigenetics. Global hypomethylation is commonly observed in cancerous cells with, paradoxically, locus-specific hypermethylation.[45] Mechanisms of tumorigenesis based on hypermethylation and silencing of tumor-suppressor genes have been well-described; whereas the role of global hypomethylation is less clear as very few loci outside of CGIs, which are normally unmethylated, have been investigated. Most studies have invoked a strategy of investigating the DNA methylation status of candidate loci, based on their biological function and/or involvement in other types of cancer. Non-biased, genome-wide approaches have determined that epigenetic changes in cancer occur at relatively high frequency and display tumor type-specific patterns.[46] It is now appreciated that epigenetic changes in cancer are generally more abundant in comparison to genetic changes.[47] As genome-wide scanning approaches become more comprehensive, critical information about the patterns of epigenetic aberrations in cancer will become clearer.

EPIGENETIC ALTERATIONS IN AML

While major efforts have led to a better understanding of molecular defects encoded in the DNA sequences of malignant AML cells, our understanding of how epigenetic alterations contribute to myeloid leukemogenesis remains to be fully elucidated. Aberrant epigenetics in AML were first reported in 1987 with the description of altered DNA methylation in the 5′ regulatory region of the calcitonin gene in primary leukemia samples.[48] Subsequently, Pfeifer et al reported that overall levels of 5-methylcytosine do not significantly change in newly diagnosed acute leukemias[49] while they decrease at relapse, suggesting that global loss of methylation (hypomethylation) is not part of the initiating steps of leukemogeneis, but rather it constitute an additional “hit” leading to therapy resistance.[49] Follow-up studies confirmed global hypomethylation of the leukemia genome and identified repetitive sequences as the targets for DNA hypomethylation.[50]

Subsequent studies in malignant blasts focused mainly on hypermethylation of genes that are unmethylated and expressed in normal non-malignant cells. It became clear that hypermethylation correlates with gene silencing and similar to a genetic mutation, inactivate gene transcription. The list of genes hypermethylated in AMLs includes tumor-suppressor genes with well-established functions in cell cycle control, apoptosis, or DNA repair.[51–59]

In a first attempt to estimate the extent of hypermethylation in AML, Melki et al used bisulfite genomic sequencing to study hypermethylation in the promoter regions of eight cancer-related genes in 20 AML patients.[60] Nineteen of the 20 patients showed methylation in at least one gene, and 15 had aberrant methylation in two or more promoters. Based on this study, the authors concluded that AML might be characterized by a general deregulation of CpG island methylation. In a similar study analyzing the DNA methylation status of 14 gene promoter–associated CpG islands in AML,[61] the authors described concordance of DNA methylation events, and the existence of a methylator phenotype was suggested. With the development of a novel genome scan for aberrant DNA methylation, an assay called restriction landmark genomic scanning (RLGS), it became possible to evaluate the DNA methylation status of thousands of gene promoters within a single assay. Surprising results out of these studies from genome scans in AML cells were that the levels of global promoter methylation could reach up to 8.3% of all CGIs.[62] Based on an estimated 29,000 CGIs in the human genome,[63] this number would translate into more than 2,000 aberrantly methylated CGIs or the silencing of over 2000 genes. Interestingly, the patterns of DNA methylation

were non-random, which suggests an underlying mechanism that leads to specific methylation of target genes or a selection process leading to the enrichment of cells with specific subset genes that have been inactivated by DNA methylation. Furthermore, comparing DNA methylation profiles from different tumor types to those patterns observed in AML revealed that each tumor type has a characteristic pattern and identified tumor type-specific DNA methylation events,[46] again supporting the argument that DNA methylation events could develop into potential diagnostic and prognostic biomarkers.

Currently it is unclear what the mechanisms are that lead to aberrant DNA methylation in a leukemia genome. Several reports have described the active recruitment of DNMTs to target sites by onco-fusion proteins.[64,65] A well-characterized example is provided by Di Croce et al. for the onco-fusion protein PML-RAR, generated by the translocation of chromosome 15 (location of the *PML* gene) and chromosome 17 (location of the retinoic receptor alpha) in acute promyelocytic leukemia.[64] In this study it was shown that PML-RAR onco-fusion protein has the ability to recruit DNA methyltransferases to target promoters containing a retinoic acid response element. This recruitment leads to DNA methylation of target promoters (eg, RARβ2) and subsequent gene silencing. This epigenetic silencing involves additional recruitment of polycomb repressive complex 2[66] and indirect interaction through an HDAC3-mediated complex with MBD1.[67] However, this mechanism alone does not explain the complexity of epigenetic silencing seen in primary leukemic cells since it was found that PML-RAR translocations are rarely associated with RARβ2 promoter methylation.[68] A similar mechanism has been proposed also for t(8;21) AML, where the AML1-ETO fusion protein was shown to recruit DNMT1, in addition to HDACs, on the promoter regions of target genes.[64,65] Recently, other studies have provided evidence for epigenetic control of the gene expression patterns of precursor or stem cells.[69] A large proportion of aberrantly methylated genes show repressive histone H3K27 methylation in addition to an active mark H3K4 methylation in embryonic stem cells.[70–72] Another possibility would be a random targeting of promoter sequences followed by a selection process supporting the outgrowth or survival of cells containing a specific DNA methylation pattern in addition to other genetic aberrations.[73]

The importance of chromatin changes associate with alterations in DNA methylation patterns in leukemia is exemplified by alterations including the mixed lineage leukemia (*MLL*) gene. *MLL* is a frequent partner for recurrent translocations in acute leukemias, possesses a DNA binding domain, and is a H3K4 methyltransferase. In normal cells, *MLL* positively regulates gene expression of several genes, including *HOX* genes, whereas the fusion proteins including the MLL portion have lost the H3K4 methyltransferase activity, resulting in deregulation of target genes and transformation into leukemic stem cells.[74] In contrast, the partial duplication of *MLL*-PTD, occurring mostly in AML with a normal karyotype,[75] unlike the MLL chimeric fusion proteins, results in an enlongated protein that retains all functional domains of the MLL wild-type protein, including the C-terminal SET domain that confers histone methyltransferase activity.[76] Nevertheless, the presence of this chimeric protein results in aberrant expression of the *HOX* gene and silencing of the concurrently present *MLL* wild-type allele via epigenetic alterations.[77]

EPIGENETIC THERAPIES IN AML

Pharmacologic reversal of aberrant epigenetic changes that silence genes important in hematopoiesis can restore normal bone marrow function and lead to clinical disease response in AML. We review here clinical results with three different epigenetic-targeted therapeutic approaches: (1) hypomethylation with azanucleosides, (2) histone deacetylation with HDAC inhibitors, and (3) dual targeting of aberrant methylation and deacetylation with combinations of these agents.

Clinical Studies With Hypomethylating Agents

Although there are no drugs in this category specifically approved for AML, currently there are two hypomethylating agents approved by the US Food and Drug Administration (FDA) for treatment of myelodysplastic syndrome (MDS): 5-azacytidine (Vidaza, AZA; Pharmion Corp) and 5-aza-2'-deoxycytidine (decitabine, Dacogen; MGI Pharma/SuperGen). Both of these azanucleosides have activity in myeloid neoplasms, although the optimal doses and schedules of these agents in AML are still under investigation. Introduced decades ago as cytotoxic agents, these compounds seem to act via demethylation/differentiation rather than cytotoxicity, when used at doses far lower than the maximum tolerated dose (MTD). These compounds exert their hypomethylating activity by competing with the endogenous pool of deoxynucleosides for incorporation into newly synthesized DNA. Once incorporated into DNA, azanucleosides covalently bind and sequester DNMTs, leading to loss of promoter hypermethylation and re-expression of silenced genes (Figure 1).

AZA was approved for use in MDS patients based on a Cancer and Leukemia Group B (CALGB) trial.[78,79] Treatment with low-dose AZA (75 mg/m^2 day subcutaneously for 7 days each month) resulted in a significantly better response, longer median time to AML progression, and decreased probability of leukemic transformation compared with best supportive care. The AZA arm had a complete remission (CR) rate of only 7%, but the majority of patients had evidence of clinical benefit and improved quality of life. Interestingly, most responders showed clinical effects beginning in the third or fourth month, suggesting that repetitive exposure of malignant cells to intermittent, low-dose AZA may affect only a fraction of cells during each cycle and that clinical response may thus not be apparent initially. Preliminary results of a new phase III, multicenter, randomized study of AZA versus conventional care regimens (several permitted) in higher risk MDS patients demonstrated a significant survival advantage for patients receiving AZA for a median of nine cycles at the same dose as in the CALGB study. Two-year overall survival (OS) was significantly higher for the AZA group (51% *v* 26%, $P < .0001$) with a median OS also superior for the AZA group (24.4 months *v* 15 months). There was no increase in early mortality detected between in the AZA group versus best supportive care.[80] Low-dose AZA as a single-agent also has been explored in AML; a CR rate of 13% was reported at the annual meeting of the American Society of Hematology (ASH) in 2007.[81]

Decitabine also was recently approved by the FDA for the treatment of MDS on the basis of favorable clinical response for poor-risk patients compared with best supportive care. The schedule of decitabine administered was 15 mg/m^2 intravenously over 3 hours, every 8 hours, for nine doses, every 6 weeks with a significantly higher response rate for decitabine (17%, including a 9% CR rate) versus best supportive care ($P < .001$).[82] However, even lower doses of decitabine have shown promising results, with a remarkable 39% CR rate in a single-center report when administered at 20 mg/m^2 intravenously daily for 5 days, every 28 days.[83]

Single-agent studies with decitabine in AML also have shown activity. In a dose-finding study of 5–20 mg/m^2/d over 1 hour for 5 days a week × 2 weeks (10 doses) in a range of hematologic malignancies (n = 50, including 44 with AML/MDS), the response rate of for 15 mg/m^2/d was 11/17 (65%), including six CRs.[84] Among the patients with AML, 14% (5/37) had a CR. Dose-dependent global demethylation of DNA by decitabine at 5–20 mg/m^2/d was associated with a clinical response in AML patients.[85] Recently, Cashen et al reported a phase II study of single-agent decitabine in older, untreated AML patients who were unable to receive conventional induction therapy.[86] Decitabine was given in doses of 20 mg/m^2 over 1 hour, for 5 consecutive days every 4 weeks, resulting in a 26% response rate (seven of 27 patients). At The Ohio State University, a more intensive, 10-day schedule of inductions with decitabine, ie, 20 mg/m^2 over 1 hour, for 10 consecutive days every 4

weeks is currently being tested, based on promising clinical and pharmacodynamic results from a phase I study (discussed later in this review). Further studies with decitabine in a novel role, maintenance therapy for AML in first CR after completion of intensive induction and consolidation, are also being pursued by different groups.

Clinical Studies With HDAC Inhibitors

The potential for therapy targeting aberrant HDAC activity in AML was demonstrated a decade ago.[87] Several groups have demonstrated that the fusion protein PML-RAR aberrantly recruits HDACs, thereby inducing chromatin hypoacetylation and gene silencing. Warrell et al initially reported that in a patient with refractory acute promyelocytic leukemia (APL) and clinically resistant to all-*trans* retinoic acid (ATRA), PB administered intravenously twice daily in combination with ATRA led to eventual complete morphologic, cytogenetic, and molecular remission along with a time-dependent increase in histone acetylation.[87,88] A platform for further development of PB as a regulator of transcription via HDAC inhibition was subsequently established by Gore et al. This group demonstrated feasibility and achievement of pharmacologically relevant PB plasma levels with a prolonged infusion of PB in patients with MDS and AML, although few hematologic responses were observed.[89]

Since these initial studies, clinical responses with HDAC inhibitors as single agents in subsets of AML other than t(15;17) have been noted, but overall results have been disappointing. AML activity of depsipeptide in several phase I/II single-agent studies was limited to blast count reductions.[90] In other clinical trials, oral administration of suberoylanilide hydroxamic acid (SAHA) has been investigated in a phase I study of patients with MDS or refractory leukemia.[91] Of 34 AML/MDS patients on the study, only two achieved CR and two incomplete CR (CRi, failure to recover normal neutrophils and/or platelet counts without evidence of leukemia). Several groups have studied valproic acid with or without ATRA in AML[92] with rare clinical responses. Gojo et al recently reported results of a phase I study of MS-275 administered orally (weekly, duration of treatment varied) to 38 AML/MDS patients.[93] Although no objective responses were observed, correlative studies confirmed histone H3/H4 acetylation in most patients by week 2 or 3 of therapy.

Combination of Hypomethylating Agents and HDAC Inhibitors in the Clinic

Given the interplay of the different epigenetic silencing mechanisms, dual targeting of aberrant DNA methylation and histone deacetylation has been pursued in AML. Several combination studies of a hypomethylating agent with a HDAC inhibitor have been reported. Gore et al studied AZA and PB in AML/MDS and observed a 14% CR rate in a group of 29 treated patients.[94] Increased RNA expression associated with decreased promoter methylation of the *P15* gene was observed in three of four responders, thereby providing the first definitive evidence in patients of a cause-effect relationship among administration of AZA, DNA hypomethylation, gene re-expression, and clinical response in patients with AML/MDS. Subsequently, Garcia-Manero et al reported a study of decitabine and valproic acid in AML/MDS, with a 19% CR rate seen in 54 patients.[95] Similar results were found with combination of AZA with valproic acid.[96] We recently reported a 16% CR rate in 25 patients enrolled in a study of decitabine with or without valproic acid in AML, administered at a different dose and schedule than those in the study previously reported.[97] Interestingly, in our study, all of the CRs occurred in previously untreated patients older than 60 years and with complex karyotypes. Mechanistically, we demonstrated that re-expression of the *estrogen receptor 1* (*ESR1)* gene, commonly silenced in AML/MDS, was significantly associated with clinical response (CR/CRi). Quantitative methylation studies showed that drug-induced hypomethylation within a unique region of the *ESR1* promoter

was the primary mechanism for re-expression of the gene. However, we did not find any benefit for increased (synergistic or additive) gene re-expression following the addition of tolerable doses of valproic acid to decitabine. Recent preliminary reports also have demonstrated feasibility of other novel combinations of epigenetic therapies including decitabine plus SAHA[98] and AZA plus MGCD0103.[99] A unifying and promising theme across several of the combination studies discussed above is markedly higher response rates in previously untreated, elderly patients who may not be candidates for intensive induction therapy. Altogether, these studies have shown the feasibility of combining different epigenetic targeting therapies and provide promise that further improvements on efficacy can be achieved. Whether combination of newer hypomethylating agents or HDAC inhibitors or treatment restricted to specific subsets of AML in which aberrant epigenetics plays a predominant leukemogenic role will lead to better clinical results remains an important question for further research.[100]

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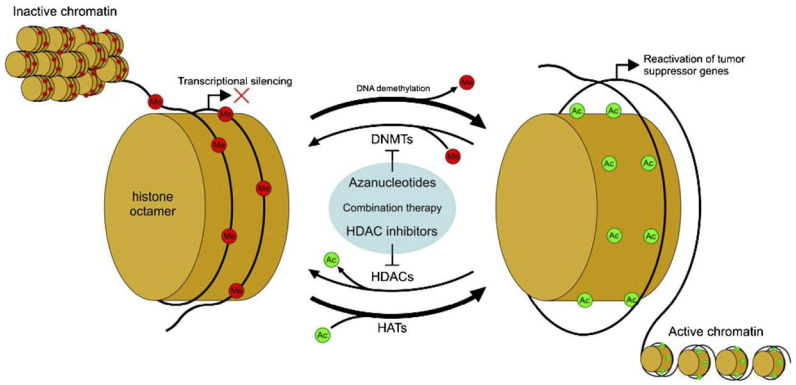

Figure 1.

Mechanism of reactivation of silenced genes in cancer using azanucleosides and HDAC (histone deacetylase) inhibitors. Aberrant silencing of tumor-suppressor genes is maintained by factors that control chromatin states. DNMT (DNA methyltransferase) and HDAC enzymes cooperatively work to methylate DNA and deacetylate histones, respectively. Treatment with azanucleosides reduces DNMT activity and thus promotes net DNA demethylation (occurring during DNA replication or by active processes), while HDAC inhibitors promote a net gain of histone acetylation. In combination, these two therapies work synergistically to promote and maintain active chromatin states. HAT, histone acetyltransferase.