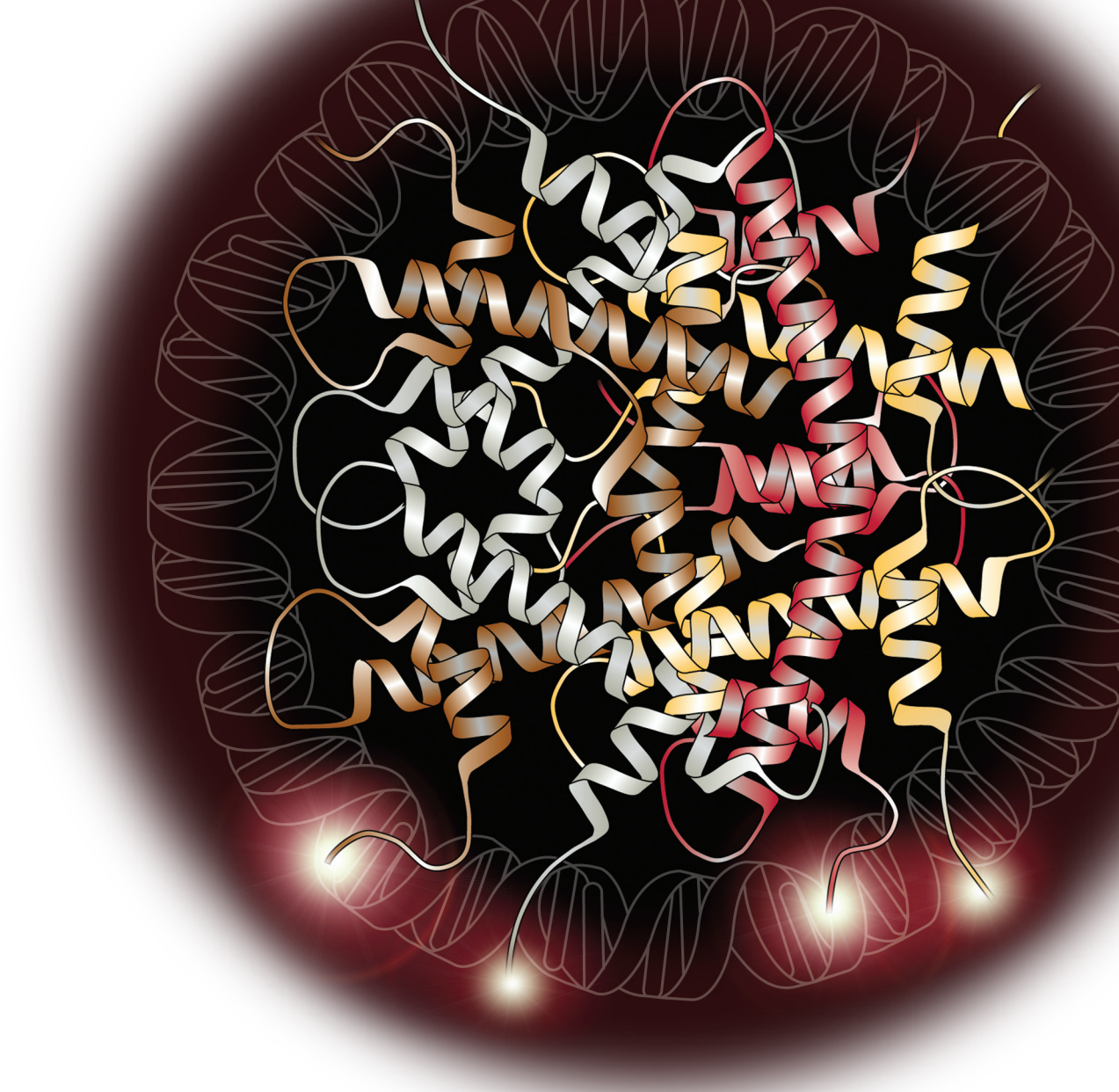

The PROMISE *of* EPIGENETIC THERAPY

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The PROMISE *of* EPIGENETIC THERAPY

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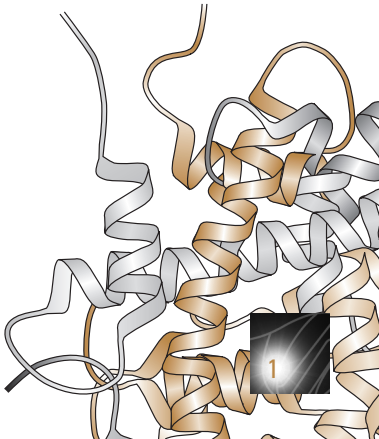

Continuing Medical Education Information

The content of this continuing medical education (CME) activity is derived from a symposium, "The Promise of Epigenetic Therapy," presented at the 48th Annual Meeting of the American Society of Hematology. This activity is offered in two formats: a print monograph and digital real-time symposium presentations. Both versions may be accessed at www.bloodline.net. CME credit may be earned through *one* of these formats.

To earn credit, participants in this activity must read the publication or view and listen to the digital presentations, take the self-assessment quiz, and complete the answer sheet and evaluation form.

Activity release date—August 31, 2007

Expiration date—August 31, 2008

Target Audience

This activity is intended for hematologists and oncologists.

Educational Objectives

At the conclusion of this CME activity, participants should be able to:

- Explain the role of DNA demethylation inhibitors in regulating gene expression in both healthy and cancerous states
- Describe the epigenetic and nonepigenetic mechanisms by which HDAC inhibitors kill hematologic and nonhematologic tumor cells
- Design a treatment strategy that exploits the complementary effects of DNA methylation inhibitors and HDAC inhibitors when used in combination
- Forecast the potential changes in cancer chemotherapy and chemoprevention that translational therapies may make possible

Activity Completion Time

Based upon trials, the estimated time to complete this activity is 2 hours.

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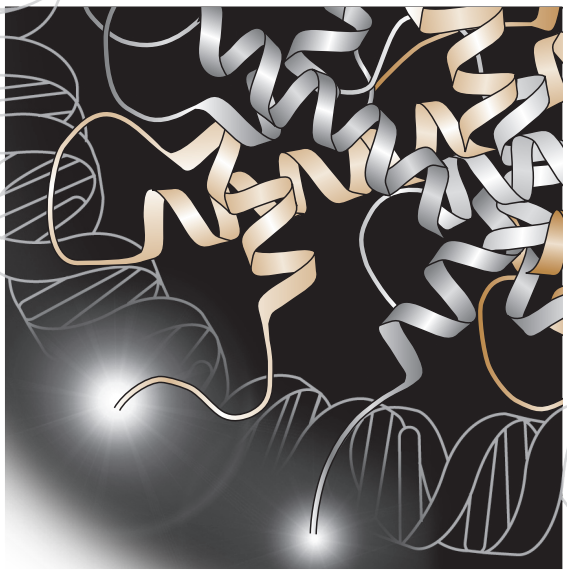
The Promise *of* Epigenetic Therapy



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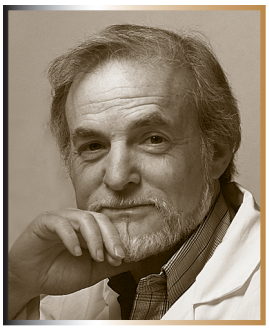
Introduction: The Promise of Epigenetic Therapy

Stephen B. Baylin, MD



Interest in targeting epigenetic changes in cancer as a therapeutic strategy is growing steadily. The term "epigenetic" refers to heritable changes in gene expression patterns that occur without changing the primary base sequence of DNA. Such changes in gene expression patterns form the normal underpinning of development, imprinting, differentiation, and adult cell renewal patterns. In this regard, in essence, epigenetics is the way our DNA is packaged to facilitate its function.

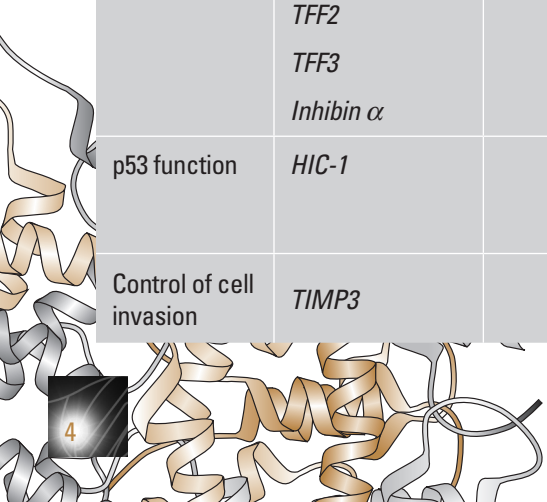
The fundamental unit for packaging DNA is the nucleosome, which consists of a histone octamer around which approximately 150 base pairs of DNA are wrapped. A change in gene expression does not necessarily occur unless there is a modification to DNA packaging, which requires a complex interplay between histone modification, chromatin remodeling, and subsequent methylation of DNA. In a normal, tightly packed genome, DNA methylation may prevent unwanted transcription, for example, to inhibit unchecked copying of repeat sequences, viral sequences, and others. In the cancer genome, however, DNA methylation is often lost or aberrantly increased, resulting in abnormal gene expression. The loss of DNA methylation may result in overexpression of genes associated with loss of chromatin compaction. Because packaging of chromosomes is critical for normal cell mitosis, loss of DNA methylation or chromatin could also contribute to chromosomal and genomic instability.

Table. Mutated and hypermethylated genes in HCT 116 colon cancer cells

Pathway or function	Hypermethylated genes	Genetic changes	Biologic consequences
Wnt pathway	*sFRP1* *sFRP2* *sFRP4* *sFRP5*	Activating *β-catenin* mutation	Aberrant Wnt pathway activation Increased stem/progenitor cell expansion
Mismatch repair	Wild type *MLH1* allele	Mutated *MLH1* allele	Defective mismatch repair
Cell cycle control	Wild type *p16* allele	Mutated *p16* allele	Null state for cyclin D-Rb pathway function
Epithelial differentiation	*GATA4* *GATA5* *TFF1* *TFF2* *TFF3* *Inhibin α*	Mutated *TGF-β* receptor	Failure to properly differentiate
p53 function	*HIC-1*	—	Decreased apoptotic response to DNA damage
Control of cell invasion	*TIMP3*	—	Potential for increased cell invasion

In contrast to the above, there is a growing list of genes within the CpG promoter regions that should normally not be methylated, but which become aberrantly methylated as part of the cancer epigenome. This aberration is associated with compaction of chromatin in an area that should be more open, resulting in a hostile environment for transcription and subsequent silencing of tumor suppressor genes. Much research is now focused on targeting re-expression of such silenced tumor suppressor genes associated with DNA hypermethylation as a cancer prevention/therapy approach.

Modification of chromatin patterns throughout the cancer genome has the potential to create several conditions that result in abnormal gene expression and promote tumorigenesis. The Table lists 14 epigenetic and genetic mutations found in a single cell line of colon cancer. Research into the epigenome of cancer is focusing on how gene silencing occurs and is maintained through DNA methylation and histone amino acid changes that contribute to methylation. Additional exploration into chromatin remodeling proteins also offers a promising therapeutic target. This publication describes current knowledge about drugs that modify methylation pathways or allow re-expression of genes and clinical outcomes for demethylating agents approved for treatment of myelodysplastic syndromes.



Targeting DNA Methylation and Other Gene Silencing Processes

Allen S. Yang, MD, PhD

Targeting aberrant DNA methylation to treat cancer is based on the idea that normal cells have tumor suppressor genes that can be turned "on" or "off" (ie, silenced). Silencing of tumor suppressor genes is usually associated with hypermethylation in the promoter regions of cells, which results in loss of expression and subsequent tumorigenesis. This article explains the known mechanisms of DNA methylation and describes therapeutic approaches to reversing aberrant gene silencing.

Mutation and Gene Silencing

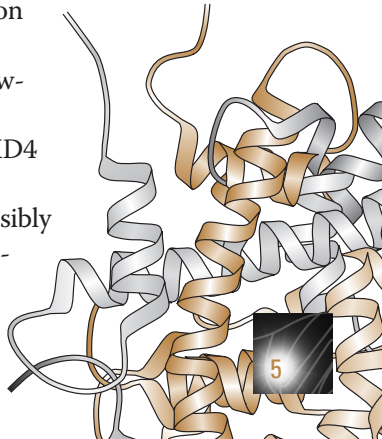
Accumulated gene mutation as a cause for cancer was proposed early in the twentieth century.[1] Knudson refined this theory based on analysis of the occurrence of inherited and sporadic retinoblastoma and subsequently developed the "two-hit" hypothesis of cancer causation.[2] According to this hypothesis, development of cancer is dependent on the occurrence of two mutations, or "hits." One hit may be a pre-existing gene mutation inherited from a parent, such as the hereditary BRCA1 gene mutation associated with increased breast cancer risk. The second hit can be another event such as gene mutation or chromosomal deletion, or it can be epigenetic silencing due to hypermethylation. Figure 1 presents an updated version of Knudson's "two-hit" hypothesis that includes epigenetic mechanisms of gene silencing.[3] Mutation such as chromosomal deletion is irreversible, but hypermethylation leading to gene silencing can be corrected.

DNA Methylation Effects

Although DNA methylation is a single biologic process, it can produce several effects in carcinogenesis. The Table presents six proposed hallmarks or phenotypes of cancer cells and the hypermethylated genes associated with them.[4] In addition to contributing to carcinogenesis, changes in DNA methylation are loosely associated with clinical outcomes, prognosis, and response to chemotherapy in heterogeneous types of tumors. To further characterize the effects of DNA methylation, a study was undertaken to evaluate methylation changes in three genetically homogenous tumors: chronic myelogenous leukemia (CML), acute promyelocytic leukemia (APL), and gastrointestinal stromal tumors (GIST).[5] These tumors are associated with genetic abnormalities—specifically, Philadelphia chromosome translocation or t(9:22) in CML, t(15:17) in APL, and c-kit mutation in GIST. Onset of these genetic abnormalities was the probable initiating event in tumor development.

After gene analysis of the tumors, it appeared that specific gene hypermethylation patterns were unique to each tumor type. In CML, hypermethylation of Abelson murine leukemia viral oncogene homolog 1 (ABL1), which is involved in Philadelphia chromosome translocation, was evident in chronic, accelerated, and blastic phases of the disease. ABL1 hypermethylation was not evident in APL or GIST, however. The presence of ABL1 hypermethylation in early phases of the disease suggests that hypermethylation occurs shortly after translocation or possibly before. Hypermethylation of the tumor suppressor gene ID4 (inhibitor of DNA binding 4) was also evident in CML, although methylation was low during normal and chronic phases but increased during the accelerated and blastic phases. In APL, which is a more aggressive form of leukemia than CML, ID4 methylation patterns were very intense. No hypermethylation of ID4 was found in GIST tumors, however. ID4 hypermethylation was associated with progression of CML and aggressive leukemia and may therefore provide a better target than ABL for methylation inhibitor therapy in leukemias.

Based on these results, it appears that a genetic event such as Philadelphia chromosome translocation initiates the onset of CML. Aberrant methylation may be associated with Philadelphia translocation but not necessarily with the onset of disease. However, methylation of the tumor suppressor gene ID4 begins to increase with disease progression, possibly resulting in more aggressive disease progression. As such, it is a



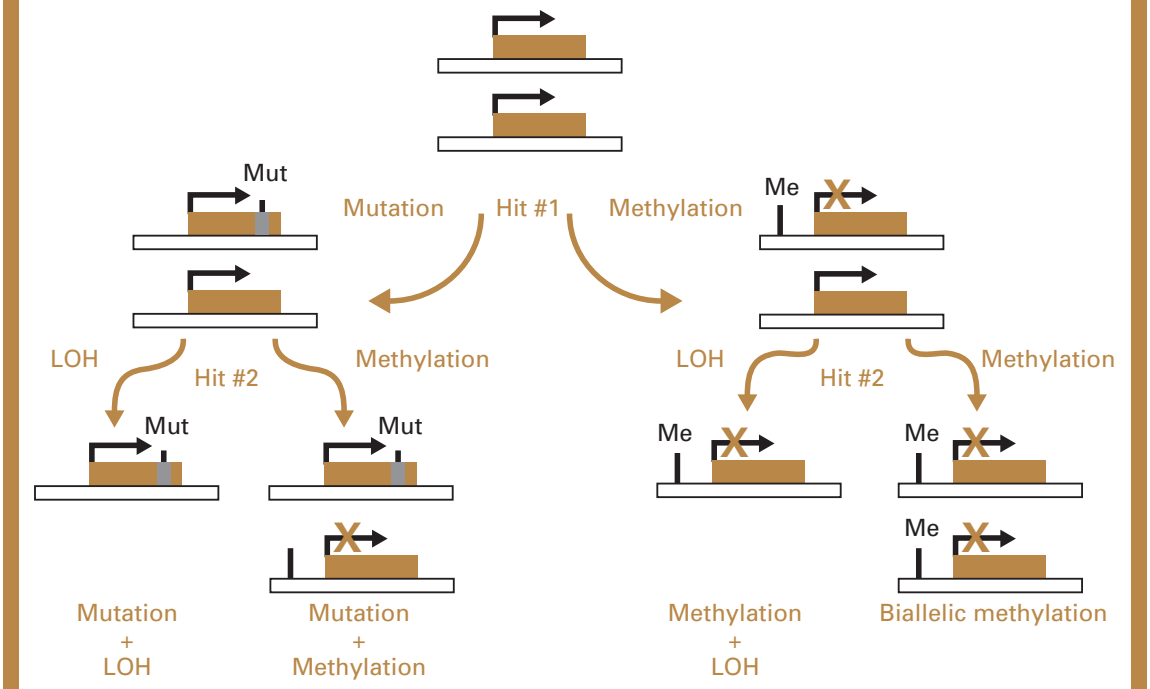


Figure 1. Knudson's "two-hit" hypothesis.

Knudson's hypothesis predicted that both alleles of a tumor suppressor gene would have to be inactivated by germline and/or somatic mutations to cause phenotypic alterations associated with cancer development.[3] The revised version of Knudson's two-hit hypothesis considers the possibility that tumor suppressor gene silencing can result from either genetic (mutation) or epigenetic silencing events. The two functional alleles of a tumor suppressor gene are indicated by the shaded boxes. The first inactivating event affecting one of the two tumor suppressor gene alleles could either be a mutation or transcriptional silencing associated with or caused by hypermethylation of CpG-rich sequences in the promoter region. The silencing event for the second tumor suppressor gene allele—ie, the second "hit"—could be loss of the chromosome containing the tumor suppressor gene allele (ie, loss of heterozygosity) or epigenetic silencing.

LOH = loss of heterozygosity
Reproduced with permission.[3]

potential target for epigenetic therapy. The exact gene or group of genes that are the targets for DNA methylation inhibitor therapy are as yet unknown.

DNA Methylation Inhibitors

The discovery of DNA methylation inhibitors occurred in the late 1970s as a result of research by Jones and Taylor, who were interested in secondary cancers induced by chemotherapy.[6] It is known that pediatric patients with acute lymphoblastic leukemia (ALL) cured with chemotherapy are at increased risk of developing cancer again later in life. Laboratory tests were undertaken to model chemotherapy-induced carcinogenesis. Mouse fibroblast cells were treated in vitro with various chemotherapeutic agents in an attempt to generate cancer. When 5-azacytidine was administered, rather than generating a cancer phenotype, it induced differentiation of fibroblasts into muscle cells; further elegant studies demonstrated that this differentiation was dependent on inhibition of DNA methylation. Linkage of DNA methylation to cellular differentiation was a profound discovery at the time, as the biology of different cellular phenotypes was poorly understood.

Figure 2 presents the mechanism of DNA methylation inhibition by 5-azacytidine. The enzyme DNA methyltransferase forms a covalent bond with cytosine within the DNA. This bond destabilizes the cytosine ring and allows the attachment of a methyl group, resulting in 5-methylcytosine (ie, DNA methylation) and causing subsequent gene silencing. 5-azacytidine is a cytosine analog with a nitrogen atom at the position of DNA methylation. As with cytosine, DNA methyltransferase bonds to 5-azacytidine. However, the nitrogen atom blocks the target site where a methyl group would attempt to attach itself. As a result, the enzyme is covalently trapped, and inhibition of DNA methylation occurs due to irreversible inhibition of the DNA methylation enzyme, DNA methyltransferase.

Jones and Taylor also discovered that there was an optimal dose of 5-azacytidine for inhibition of methylation. They found a correlation between increased concentrations of 5-azacytidine and increased muscle differentiation up to a point, but then muscle differentiation decreased (Figure 3). DNA methylation decreased rapidly upon higher dosing. It was found that lower doses and prolonged exposure to 5-azacytidine was optimal for DNA methylation inhibition and muscle differentiation.[6]

Biology of DNA Methylation Inhibitors

When DNA replicates, its methylation pattern remains on the parent strand but is not replicated on the newly synthesized daughter strand of DNA. Replication or maintenance of the DNA methylation patterns occurs when the DNA methyltransferase enzyme copies them from the parent strand to the daughter. When a methylation inhibitor such as 5-azacytidine or 5-aza-2'deoxycytidine is present, DNA methyltransferase is trapped, as described, and the copying of DNA methylation does not occur. Therefore 5-azacytidine is cell-cycle dependent, and inhibition of DNA methylation is "passive" rather than active. In other words, DNA methylation is not actively removed from the DNA, but passively lost due to failure to copy the DNA methylation pattern after cell division. Thus, decreases in DNA methylation can require several cell divisions and may take a prolonged period of time to occur.

Azacitidine and Decitabine

Two methylation inhibitors, azacitidine and decitabine, have generated much interest as cancer therapies. Azacitidine (5-azacytidine) is a ribonucleic acid (RNA) precursor, whereas decitabine (5-aza-2'deoxycytidine) is a DNA precursor. Chemically, azacitidine differs from decitabine in structure only slightly by having a hydroxyl group that is lacking in decitabine. Azacitidine is phosphorylated by the enzyme uridine-cytidine kinase and is then incorporated into RNA. It is incorporated into DNA by conversion to a deoxyribose form by the enzyme ribonucleotide reductase, which converts ribose to deoxyribose. Decitabine is phosphorylated by deoxycytidine kinase and then incorporated into DNA. Both drugs are prodrugs to 5-azadeoxycytidine triphosphate; however, their biochemical differences may allow one to work in a patient when the other does not.

Ribonucleotide reductase, which is necessary for incorporation of azacitidine into DNA, can be inhibited by hydroxyurea. This is important information for clinicians because hydroxyurea is commonly used to control white blood cell counts in patients with leukemia and to manage symptoms of sickle cell disease.[7] Studies of the effects of hydroxyurea on methylation when used alone or with azacitidine or decitabine demonstrate that hydroxyurea alone has no effect on DNA

Table. Hallmarks of cancer and associated hypermethylated genes[4]

Hallmarks	Associated hypermethylated genes
Evading apoptosis	*TMS1* *P73*
Self-sufficiency in growth signals	*P15INK4B* *P16INK4A* *P14ARF*
Insensitivity to antigrowth signals	*P15INK4B*
Tissue invasion and metastasis	*TIMP3* *ECAD*
Limitless replicative potential	*P16INK4A*
Sustained angiogenesis	*THBS1* *THBS2* *VHL*

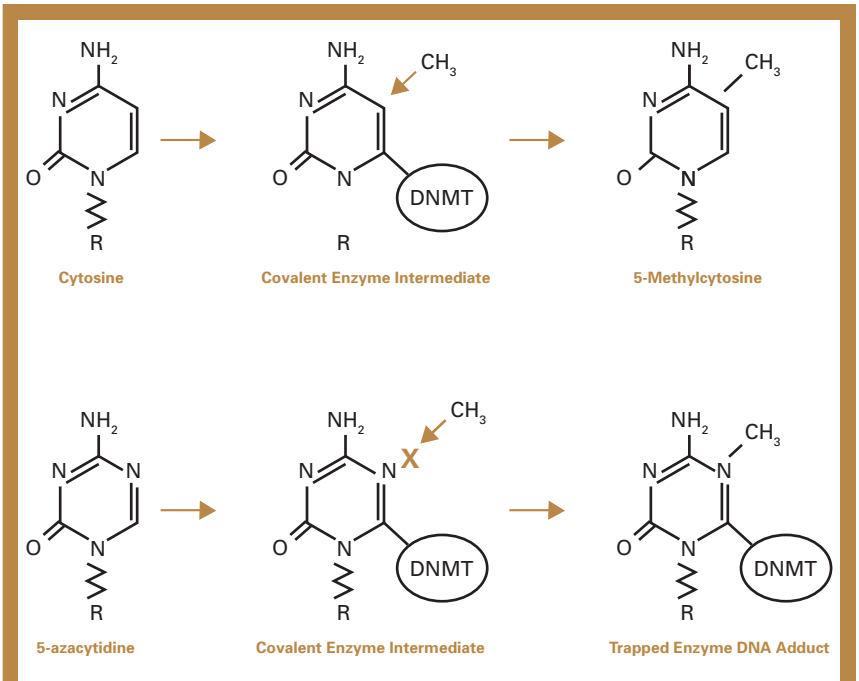


Figure 2. Mechanism of DNA methylation inhibitors.

The enzyme DNA methyltransferase forms a covalent bond with cytosine within the DNA. This bond destabilizes the cytosine ring and allows the attachment of a methyl group, resulting in 5-methylcytosine (ie, DNA methylation) and causing subsequent gene silencing. 5-azacytidine is a cytosine analog with an additional nitrogen atom. As with cytosine, DNA methyltransferase bonds to 5-azacytidine. However, the nitrogen atom interferes with the target site where a methyl group would attempt to attach itself. As a result, the covalent enzyme is trapped, and DNA methylation is inhibited.

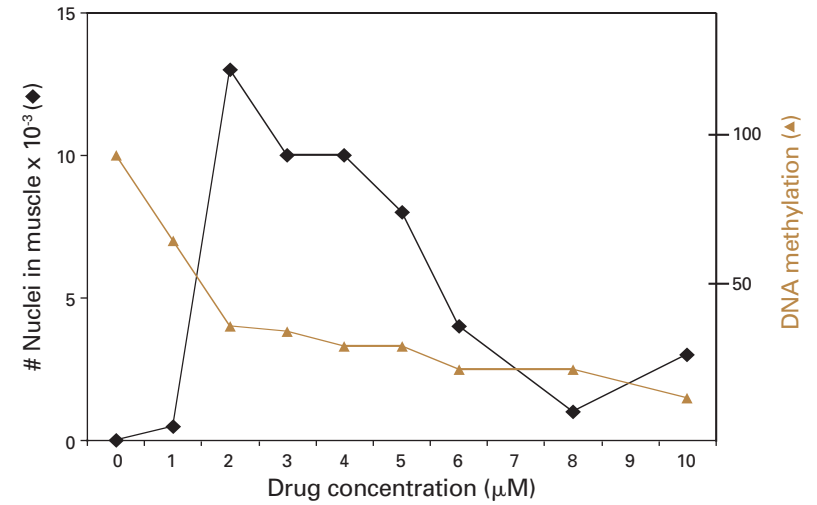


Figure 3. Muscle differentiation induced by 5-azacytidine.[6]

The optimal doses of 5-azacytidine for DNA methylation inhibition and muscle differentiation appeared to coincide.

Reproduced with permission.[6]

methylation. When azacitidine or decitabine is administered with low doses of hydroxyurea, methylation is inhibited. As doses of hydroxyurea increase, however, the ability of azacitidine and decitabine to inhibit methylation decreases.[8] These inhibitory effects on azacitidine and decitabine appear to be related to the ability of hydroxyurea to block ribonucleotide reductase, thereby arresting cell division during the S phase of the cell cycle. That is, hydroxyurea inhibits the ability of azacitidine and decitabine to incorporate into DNA, thereby decreasing their ability to inhibit methylation. This underscores the dependence of these drugs on incorporation into DNA during DNA replication, and the potential for antagonism when they are combined with other agents that inhibit the cell cycle.

Other Factors Associated with Gene Silencing

Reverse transcription-polymerase chain reaction (RT-PCR), used to identify gene expression in people with and without leukemia, reveals that patients with leukemia often lose expression of tumor suppressor genes and this loss of expression is linked to aberrant DNA hypermethylation of the tumor suppressor gene. In some of these patients, it appears that these genes may be silenced before DNA hypermethylation occurs. Therefore, it is correct to say that methylation is *associated* with gene silencing, but it is not the sole cause of it. Rather, methylation should be viewed as a signal or a target that attracts methyl-binding proteins and histone-modifying proteins. Together these elements comprise the field of "epigenetics," which is loosely defined as heritable information that is not coded for in the DNA sequence itself. Histone modifications such as acetylation, methylation, and phosphorylation affect several biologic processes, including gene regulation. This led to increasing clinical interest in combining DNA methylation inhibitors with drugs that can modify histones, such as histone deacetylase inhibitors (see "Modulating Gene Expression by HDAC Inhibition," page 9).

Conclusion

The methylation inhibitors azacitidine and decitabine are already clinically available, but additional drugs are in development or are being tested for new clinical indications. These include zebularine, 5-fluorodeoxycytidine, mitoxantrone, procainamide, MG98, ECGC, and RG108. In addition, there are several agents that work synergistically with methylation inhibitors, including the histone deacetylase inhibitors trichostatin, phenylbutyrate, MG103, and SAHA. Although the mechanisms for some of these drugs are not yet known, epigenetic therapy is a very promising biologic model for cancer therapy. The biology of DNA methylation is well understood, and the tools to study epigenetic changes are available. We should expect to see an increase in the number of epigenetic therapies to become clinically available as a better understanding of the association between cancer and epigenetics is developed.

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Modulating Gene Expression by HDAC Inhibition

Steven Grant, MD

Epigenetic alterations refer to biochemical modifications of chromatin that affect gene expression. The most studied modifications include DNA methylation and changes in the biochemical composition of nucleosome-associated histone tails. DNA methylation is associated with gene silencing, whereas alterations of the histone code are more dynamic and can be associated with either an open or closed chromatin configuration. Aberrant gene silencing in cancer can occur through DNA methylation or through establishment of a repressive histone code. DNA methylation is associated with specific repressive histone codes. Methylation inhibitor agents combined with histone deacetylase (HDAC) inhibitors often exert synergistic effects associated with reactivation of aberrantly silenced genes. HDAC inhibitors that have demonstrated clinical activity against leukemia and myelodysplastic syndromes (MDS) include valproic acid, vorinostat, MGCD0103, and MS-275. The combination of methylation inhibitors and HDAC inhibitors has been shown to produce significant clinical activity in MDS and acute myelogenous leukemia (AML).

HDAC Inhibitor Activities

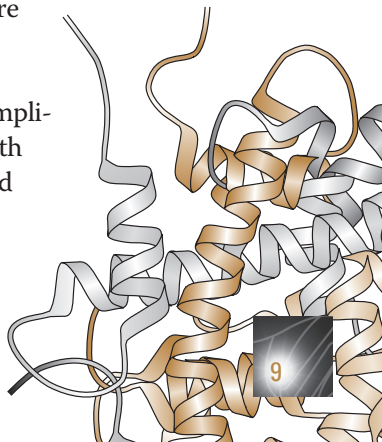
HDAC inhibitors are prototypical epigenetic agents that regulate gene expression through chromatin remodeling. Activities associated with HDAC inhibitors include promotion of histone acetylation, uncoiling of chromatin, and transcription of genes responsible for cellular maturation, growth, and cell death. HDAC inhibitors can trigger cell cycle arrest and reactivate genes responsible for cell death, but also downregulate expression of these genes. In fact, gene array studies have shown that HDAC inhibitors downregulate approximately as many genes as they upregulate, presumably a consequence of the complex interplay that exists between histone acetylation and other epigenetic modifications (eg, histone methylation) in the regulation of gene expression. As their name implies, the actions of HDAC inhibitors are associated with histones, but they promote acetylation of other proteins, as well, and perhaps should be classified more generically as "protein acetylases." The various activities of HDAC inhibitors illustrate their diverse epigenetic and nonepigenetic mechanisms of lethality. Their primary mode of action is uncertain, but preclinical studies demonstrate that HDAC inhibitors potently induce differentiation in some cancer cells, such as leukemia cells, but at higher concentrations can induce apoptosis. Consequently, HDAC inhibitors can either modulate gene expression, induce cytotoxicity, or exert a combination of these actions.

Three classes of HDACs have been identified. Class I HDACs are homologues of yeast RPD3 and are primarily located in the nucleus. Class II HDACs are homologues of yeast HDA1 and are found in both the cell nucleus and cytoplasm. Class III HDACs, which are structurally distinct from classes I and II, are homologues of yeast and mouse SIR2 and are found in both the nucleus and cytoplasm. They are involved in oxidative response, longevity, and caloric distribution.[1] More recently, a fourth class of HDACs, containing HDAC11, has been proposed.

There are four broad categories of HDAC inhibitors (Table 1). They vary in mode of action and specificity for different classes of HDACs. At this point, it is unclear whether more specific HDAC inhibitors directed against one or more HDACs would be beneficial.

Effects on Chromatin Structure

Nucleosomes contain positively charged histone tails. Lysine residues on histone tails are subject to acetylation, which results in a more open chromatin structure and usually enhances gene expression. In contrast, deacetylation of histones causes chromatin structure to become more compact, which usually inhibits gene expression. When an HDAC inhibitor is present, deacetylation is inhibited, resulting in more open chromatin structure and generally enhanced gene expression. This explanation is greatly simplified; HDACs interact with many proteins associated with gene expression, including DNA methyltransferases, histone methyltransferases,



and coregulatory and corepressor complexes. In essence, HDAC inhibitors interfere with these protein interactions and presumably block or reverse the effects of HDAC repression of gene expression and differentiation.

Table 1. Four classes of HDAC inhibitors

Class I	Class II	Class III	Class IV
Hydroxamic acids:	**Short-chain fatty acids:**	**Synthetic benzamide derivatives:**	**Cyclic tetrapeptides:**
SAHA	Na butyrate	MS-275	Depsipeptide
Pyroxamide	AN-9	CI-994	Trapoxin
TSA	Phenylbutyrate		Apicidin
Oxamflatin	Phenylacetate		CHAPs
CHAPs	Valproic acid		
LAQ824			
LBH589			
BL1521			

SAHA = suberoylanilide hydroxamic acid; CHAP = cyclic hydroxamic acid-containing peptide.

Mechanisms of Cell Death

The mechanisms by which HDAC inhibitors cause tumor cell death are still under investigation. Mechanisms that have been identified include epigenetic processes such as induction of chromatin decondensation and induction of genes responsible for differentiation and cell death. HDAC inhibitors may also work through indirect epigenetic processes such as acetylation of proteins that affect cell death and differentiation. This mechanism can be considered indirectly epigenetic, or nonepigenetic, because it is the acetylation process that regulates cellular functions. The ultimate effect of HDAC inhibitors depends on a complex interplay between both epigenetic and indirect epigenetic actions. Table 2 lists several mechanisms of HDAC inhibitor lethality.

Nonhistone protein targets of HDAC inhibitors control several functions that have direct or indirect effects on gene expression. HDAC inhibitors can modify the function of signaling proteins, oncoproteins such as BCL-6, the tumor suppressor p53, and transcription factors E2F-1 and NF-κB, which have several secondary effects on gene expression. They also modify the function of chaperone proteins such as Hsp^{90}, which leads to alterations in the expression of other signaling proteins including mitogen-activated protein kinase (MAPK). HDAC inhibitors also acetylate structural proteins, such as α-tubulin, and DNA repair proteins, such as Ku70.

Epigenetic Mechanisms of Lethality

Several potential mechanisms of epigenetic-induced cell death are under study. This section summarizes clinical data and theories of lethality associated with HDAC inhibitors.

Reactive Oxygen Species

There is significant interest in induction of oxidative stress, or reactive oxygen species (ROS) generation, as a mechanism of lethality of HDAC inhibitors. Suberoylanilide hydroxamic acid (SAHA; vorinostat), an HDAC inhibitor approved for the treatment of cutaneous T-cell lymphoma, was studied in vitro to determine the mechanism by which it induces cell death.[2] Generation of ROS was central to vorinostat-mediated cell death, but this process was inhibited by the antioxidants pyrrolidine dithiocarbamate and N-acetylcysteine and by the electron chain uncoupler carbonyl cyanide m-chlorophenoxylhydrazone (CCCP). This study demonstrated that generation of ROS does not merely correlate with cell death but contributes to cell death induced by vorinostat.

Another in vitro study evaluated the HDAC inhibitor MS-275 in leukemia cells.[3] MS-275 led to marked acetylation of histones H3 and H4, but the effects on survival were dramatically different. There was a marked discordance between histone acetylation, differentiation induction, and cell death. The correlation between induction of ROS and cell death was high, however. For example, a concentration of 5 μM MS-275 induced a dramatic increase in ROS generation as early as 2 hours after drug exposure and persisted for 24 hours. Addition of the free radical scavenger L-NAC blocked ROS, which resulted in inhibition of cell death by MS-275. This study suggests that MS-275 is a potent inducer of ROS in leukemia cells, an event that is important in MS-275–mediated mitochondrial damage and cell death. Furthermore, while histone acetylation may be necessary for HDAC inhibitor actions, it is insufficient to completely explain the lethality of HDAC inhibitors.

Thioredoxin

Other research has focused on HDAC inhibitor upregulation and downregulation of genes. An in vitro study of vorinostat evaluated in prostate, bladder, and breast cancer cells determined that it increases expression of the protein 1/thioredoxin-binding protein-2, and this reciprocally downregulates thioredoxin gene expression.[4] Further study of vorinostat and MS-275 found that both HDAC inhibitors caused upregulation of thioredoxin protein in normal cells but not in transformed cells.[5] This increase in thioredoxin in normal cells was associated with lack of ROS generation. Downregulation of thioredoxin in transformed cells correlated with greater sensitivity to the HDAC inhibitor and increased ROS. Differential gene expression of normal cells vs transformed cells might therefore contribute to potential selectivity of HDAC inhibitors.

E2F

E2F is a transcription factor that can be regulated and acetylated by HDAC inhibitors. Effects of HDAC inhibitors on the oncogenic Rb-E2F1 pathway, which is frequently deregulated in human cancers, was investigated.[6] Cancer cells with elevated E2F1 activity were shown to be highly susceptible to the HDAC inhibitors vorinostat or trichostatin A (TSA), which led to a dramatic increase in expression of the pro-apoptotic protein Bim. This activity is an example of a cell death mechanism that may be either directly or indirectly epigenetic.

Death Receptors: TRAIL

Two Italian studies reported that HDAC inhibitors induce expression of a tumor necrosis factor (TNF) death receptor, TNF apoptosis-inducing ligand (TRAIL), in AML cells and blasts.[7,8] Increased death receptor expression was associated with enhanced sensitivity to TRAIL-induced lethality in leukemic but not normal cells. Although this specific death receptor pathway was linked to cell death, other reports suggest that lethality may be either dependent on or independent of death receptors. A study that exposed U937 leukemia cells to TRAIL and HDAC inhibitors produced significant lethality, but the cells did not exhibit upregulation of death receptors.[9] Cell death was attributed to the simultaneous activation of intrinsic pathways due to the HDAC inhibitors and extrinsic pathways due to TRAIL. Thus, induction of death receptors is not always necessary for significant lethality to occur in cells exposed to both TRAIL and HDAC inhibitors.

CDK Inhibitor p21

The cyclin-dependent kinase (CDK) inhibitor p21 appears to critically affect cell response, particularly the response of hematopoietic cells to HDAC inhibitors. p21 is a cell cycle inhibitor that is important for cell cycle arrest in leukemia cells during differentiation. It is also involved in DNA synthesis and opposes apoptosis. p21 binds to and inhibits procaspase-3 and stress-related kinases. It is universally induced by HDAC inhibitors and is a critical regulator of HDAC inhibitor lethality. Exposure of U937 leukemia cells to the HDAC inhibitor SAHA results in robust induction of p21, significant increase in lethality, and a reciprocal decrease in cell differentiation.

Flavopiridol is the first CDK inhibitor to be used clinically. It inhibits several types of CDKs and is also a pan-CDK inhibitor. It is a potent inhibitor of the CDK9/cyclin T complex (PTEF-b) and is therefore a potent transcriptional repressor, which leads to downregulation of short-life proteins, including p21. When flavopiridol is

Table 2. Determinants of HDAC inhibitor-mediated lethality

Reactive oxygen species (ROS) generation
Bid activation
Downregulation of antiapoptotic genes (Bcl-x_L, XIAP)
Upregulation of proapoptotic genes (Bax, Bak)
Induction of death receptors (DR4, DR5), Fas, TRAIL
Proteasome inhibition
Induction of $p21^{CIP1}$
Interference with Hsp^{90} function
Disruption of G_2 and mitotic checkpoints
Activation of stress-related kinase (JNK)
Inactivation of cytoprotective pathways (Raf/MEK/ERK, Akt, Bcr/Abl)
NF-κB activation/acetylation
Ceramide generation

combined with vorinostat, it acts as a transcriptional repressor, blocking the induction of p21. The combination of flavopiridol and SAHA produces a dramatic induction of apoptosis.

Nonepigenetic Mechanisms of Lethality

Cell Cycle Disruption

Disruption of the cell cycle is one explanation for the selectivity of HDAC inhibitors in causing death to cancer cells but not normal cells.[10] HDAC inhibitors cause cell cycle arrest in G1 and in some cases G2, thereby adversely affecting mitosis. The effects of HDAC inhibitors in causing cell cycle disruption appear to be dependent on concentration and the genetic background of the cell.

Ku70

Another area of interest is the association between the Ku70 polypeptide and Bax, a member of the BCL2 family of proteins. Bax triggers mitochondrial injury. In a study of HDAC inhibitor activity in neuroblastoma cells, it was found that Ku70 binds to Bax in an acetylation-sensitive manner. After exposure to an HDAC inhibitor, acetylated Ku70 releases Bax, allowing it to trigger mitochondrial depolarization.[11] Acetylation of Ku70, release of Bax, and induction of mitochondrial injury appear to represent another nonepigenetic mechanism of lethality of HDAC inhibitors.

NF-κB

The transcription factor NF-κB is emerging as an important determinant of HDAC inhibitor lethality. In a study involving leukemia cells, exposure of cells to MS-275 or vorinostat led to activation of NF-κB and hyperacetylation and nuclear translocation of RelA, a protein member of the NF-κB family.[12] RelA acetylation antagonizes HDAC inhibitor induction of cell death. However, coadministration of an IKK inhibitor blocked RelA acetylation and translocation, trapping it in cell cytoplasm. The administration of an IKK with an HDAC inhibitor led to a dramatic increase in lethality. These findings suggest that blocking activation of NF-κB markedly enhances HDAC inhibitor lethality.

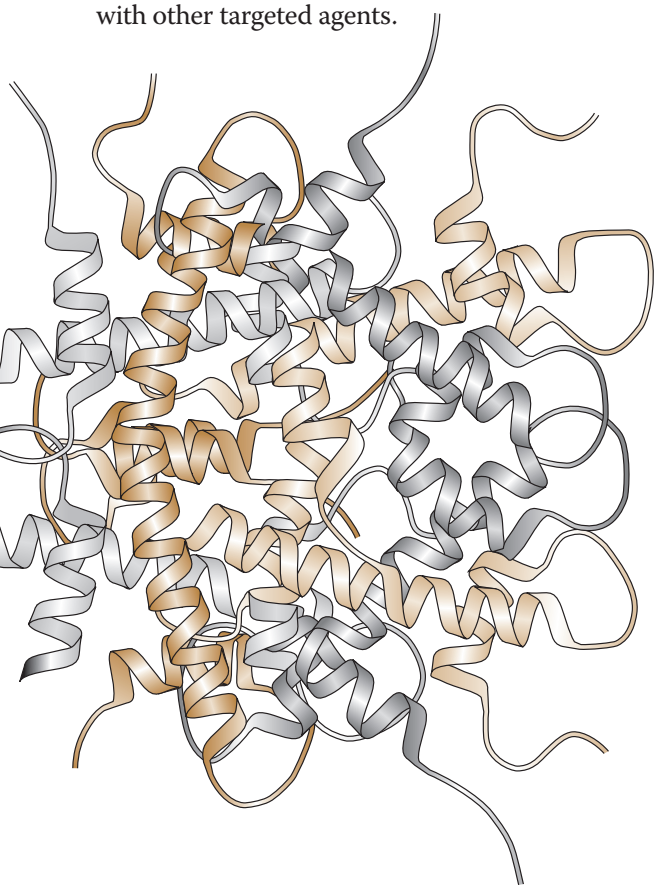
Another example of nonepigenetic changes associated with NF-κB exposure is modification of the expression of the antioxidant enzyme superoxide dismutase (SOD), which represents a target of NF-κB and is induced by HDAC inhibitors. In IκBα "super-repressor" cells in which NF-κB is disabled, SOD is no longer induced by HDAC inhibitors, and lethality is increased. In addition, TBAP, a SOD mimetic, blocks enhanced HDAC inhibitor lethality in IκBα mutant cells by diminishing oxidative damage. In essence, HDAC inhibitors have two potentially opposing and mutually exclusive effects: they induce differentiation, but they also induce oxidative damage. It is possible that one way in which HDAC inhibitors permit differentiation of leukemia cells is by preventing cell death through acetylation of NF-κB and subsequent induction of antioxidant enzymes that oppose cell death through stress-related pathways. However, if acetylation is blocked, HDAC inhibitors induce oxidative injury and apoptosis. Therefore, agents that interrupt the NF-κB pathway enhance HDAC inhibitor-mediated lethality, presumably through a nonepigenetic, oxidative damage-related mechanism.

Proteasome Inhibitors

Proteasome inhibitors, such as bortezomib, used with HDAC inhibitors exert synergistic effects in several types of malignant hematopoietic cells. By blocking the action of proteasomes, these agents cause accumulation of proapoptotic proteins within the cell. HDAC inhibitors, particularly those that inhibit Class IIB HDACs, exacerbate protein accumulation by acetylating heat-shock protein 90 (Hsp90), a chaperone molecule, thereby disrupting the disposal of unwanted proteins. The combined activity of proteasome inhibitors and HDAC inhibitors leads to a dramatic increase in cell death and can lead to endoplasmic reticulum (ER) stress. Proteasome inhibitors also prevent degradation of I-κBα and inhibit the NF-κB pathway, which can also lead to ER stress. Antioxidants can substantially reduce the lethality of combined proteasome inhibitors and HDAC inhibitors, suggesting that ROS generation might be a final pathway by which cell death occurs.

Conclusion

HDAC inhibitor activity may stem from alterations in gene expression such as up- or downregulation. Lethality may be due to epigenetic or nonepigenetic effects, the latter including protein acetylation, effects on transcription factors, and acetylation of chaperone proteins. These epigenetic and nonepigenetic mechanisms are not mutually exclusive but in fact collaborate to induce cell death. In other words, genetic changes can result from acetylation of both histones as well as other proteins implicated in the regulation of gene expression. Conversely, the response of a cell to nonepigenetic actions of HDAC inhibitors are likely to be influenced by alterations in gene expression induced by these agents. Further understanding of these mechanisms will be essential for attempts to optimize the use of HDAC inhibitors in hematologic and other malignancies, particularly in combination with other targeted agents.



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Using Combination Therapy to Improve Control: DNA Methylation and HDAC Inhibition

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Inhibitors of DNA methylation or histone deacetylation (HDAC) are the two classes of epigenetic therapy currently used for the treatment of myeloid malignancies. As single agents, drugs in each class produce significant effects in leukemia and lymphomas. In combination, DNA methylation inhibitors and HDAC inhibitors have synergistic effects that have been clinically shown to produce significant activity against acute myelogenous leukemia (AML) and myelodysplastic syndromes (MDS).

This article summarizes clinical data describing DNA methylation inhibitors and HDAC inhibitors and their effects on the natural history of myeloid diseases such as MDS. Clinical overviews for the methylation inhibitors azacitidine and decitabine and the HDAC inhibitors vorinostat, MGCD0103, and valproic acid are included. Results from studies of combination therapy with both classes of agents are also reviewed.

Azacitidine

Much of what is known about the clinical effects of azacitidine, a DNA methylation inhibitor, is based on results from the Cancer and Leukemia Group B (CALGB) 9221 study.[1] This was a randomized, controlled study including 191 patients with MDS. The study objective was to compare azacitidine 75 mg/m^2/d administered for 7 days every 28 days with standard supportive care. Patients in both treatment arms received transfusions and antibiotics as required. Patients in the supportive care arm whose disease worsened were allowed to cross over to the azacitidine treatment group. Results are presented in Table 1.

Table 1. CALGB 9221 response[1]

	Supportive care N = 92	Azacitidine N = 99	Crossover N = 49
CR	0 (0%)	7 (7%)[a]	5 (10%)
PR	0 (0%)	15 (16%)[b]	2 (4%)
Improved	5 (5%)	38 (37%)[b]	16 (36%)
Total	5 (5%)	60 (60%)[b]	23 (47%)

[a]$P < 0.01$; [b]$P < 0.001$; CR = complete response; PR = partial response.
Reproduced with permission.[1]

The crossover design limited statistical power to determine survival rates, but it did provide information about the effects of azacitidine after delaying therapy. Response rates were still favorable even after a delay in starting azacitidine therapy. Transformation to AML occurred in 15% of patients who received azacitidine and in 38% of patients who received supportive care. The median duration of survival was 20 months for patients in the azacitidine group and 14 months for patients who received supportive care, 53% of whom were crossed over to azacitidine therapy.

An additional survival analysis was performed to eliminate the confounding effect caused by including crossover patients in the overall survival analysis. Survival results for the three subgroups—azacitidine, supportive care, and crossover patients—were compared from a 6-month landmark date. The landmark analysis included supportive care patients who never crossed over or who crossed over after 6 months, supportive care patients who crossed over before 6 months, and patients who initially received azacitidine. Median durations of survival after the 6-month landmark date were 11, 14, and 18 months for the three groups, respectively (Figure 1). Patients who died prior to the 6-month date (N = 36) were not included in the landmark analysis. Results for patients initially treated with azacitidine were statistically significantly better than results for patients who received supportive care or who crossed over late or who never crossed over ($P = 0.03$). Patients who crossed over early had improved survival compared with other supportive care patients, but the difference was not statistically significant ($P = 0.1$). Results from the landmark analysis support the view that azacitidine alters the natural history of MDS.

Quality of life was another end point for the CALGB study.[2] Patients were interviewed by

telephone at baseline and at days 50, 106, and 182. Fatigue, dyspnea, physical functioning, psychological distress, and "positive effect" were improved for patients who received azacitidine compared with patients who received supportive care. The most common treatment-related toxicity from azacitidine was myelosuppression (Table 2). Adverse events were generally transient, with patients recovering in time for the next treatment cycle.

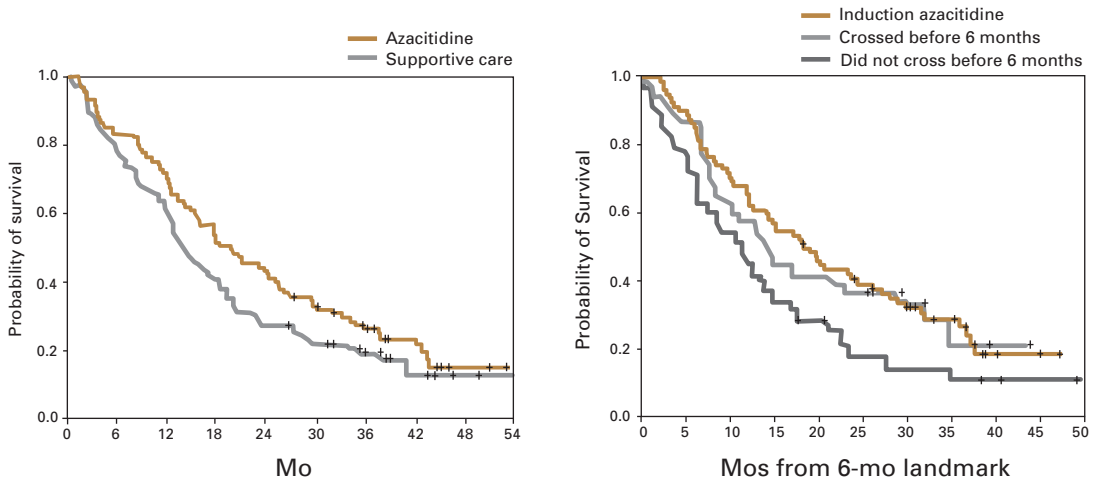

Figure 1. Landmark analysis: CALGB 9221 survival.[1]

Survival results for patients who received azacitidine, supportive care, or azacitidine after failure of supportive care (crossover patients) were compared from a 6-month landmark date. Median durations of survival after the 6-month landmark date were 11, 14, and 18 months for the three groups, respectively. Results for patients initially treated with azacitidine were statistically significantly better than results for patients who received supportive care or who crossed over late or who never crossed over ($P = 0.03$). Patients who crossed over early had improved survival compared with other supportive care patients, but the difference was not statistically significant ($P = 0.1$).

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Time to Response

Clinicians who use induction therapy for AML and high-risk MDS generally expect to see some level of response after the first treatment. But clinical data suggest that it can take 4 to 6 treatment cycles with azacitidine to see maximum effects in MDS. A study evaluating MDS response using International Working Group criteria found the median number of cycles from first azacitidine treatment to any response was three cycles.[3] Most responders (90%) achieved a response by cycle 6. Among responders, 75% responded by cycle 4 while the remainder responded during a period that extended as late as cycle 17.[4]

Effects in High-Risk Patients

Patients with refractory anemia with excess blasts (RAEB) or RAEB in transformation who are 65 years of age or older have poor prognoses and few treatment options other than supportive care. A subgroup analysis of the 191 patients in the CALGB 9221 study was performed to evaluate the effects of azacitidine in patients meeting these criteria.[5] An intent-to-treat design was used based on randomization to azacitidine treatment (N = 31) or supportive care (N = 37). Efficacy end points included overall survival, time to AML transformation, and time to death or AML transformation. Patients who received azacitidine had statistically significantly better outcomes for all three end points compared with patients who received supportive care.

When the CALGB 9221 study was initiated, patient cytogenetics were not routinely gathered, so the International Prognostic Scoring System (IPSS) was not used to assess high-risk patients. An alternative prognostic model was used to identify high-risk MDS patients with an expected survival time of ≤1.2 years, the equivalent of an IPSS Intermediate-2, or high-risk, classification.[6] All 70 patients in this subgroup were followed until death. Again, high-risk patients who received azacitidine had significantly different overall survival rates compared with supportive care patients ($P = 0.03$). Survival rates for the azacitidine and supportive care groups, respectively, were 63% and 37% at 1 year and 35% and 13% at 2 years. Duration of time to AML transformation and time to death or AML transformation were also statistically significantly longer for patients in the azacitidine group compared with patients who received supportive care.

Decitabine

In clinical trials, the DNA methylation inhibitor decitabine also produces significantly higher response rates among patients with MDS

Table 2. CALGB 9221 treatment-related toxicity (N = 191)[1]

Adverse event	Percent
Leukopenia	43
Granulocytopenia	58
Thrombocytopenia	52
Infection	20
Nausea and vomiting	4
Death	≤1

compared with supportive care alone. In a phase 3 randomized study, 170 patients with MDS received either decitabine (N = 89) or supportive care (N = 81).[7] Patients treated with decitabine achieved a significantly higher overall response rate (17%), including a 9% complete response rate and an 8% partial response rate, compared with no response (0%) among patients receiving supportive care ($P < 0.001$). Correct dosing of decitabine is crucial, however, due to the potential for toxicity. Decitabine exerts methylation inhibition very effectively at low doses, but higher doses are associated with cytotoxicity.[8] A phase 1 study evaluated the effectiveness of a low-dose extended regimen of decitabine for treatment of relapsed and refractory leukemias.[9] Patients received decitabine in doses of 5, 10, 15, or 20 mg/m^2 intravenously over a 1-hour period daily for 5 days per week for 2 weeks. These doses were approximately 5- to 30-fold lower than the maximum tolerated dose (MTD). Two groups also received 15 mg/m^2 daily for 15 or 20 days, respectively. All regimens were well tolerated and produced clinical response with the best response rate evident among patients who received 15 mg/m^2 for 10 days. Fewer clinical responses were observed with escalated or prolonged dosages. A low-dose regimen administered over a long duration is recommended for patients receiving decitabine.

Vorinostat

The HDAC inhibitor vorinostat, also known as suberoylanilide hydroxamic acid (SAHA), was evaluated in a phase 1 dosing study.[10] A total of 41 patients with relapsed or refractory chronic acute leukemia or MDS were enrolled. The initial dosing schedule was oral vorinostat 100 mg three times daily. Dosing was increased by 50 mg until a maximum dose of 300 mg was reached, which is above the MTD. Vorinostat 300 mg was administered for 14 days every 21 days. Gastrointestinal toxicity occurred, so the dose was decreased to 200 mg twice daily for 14 days. Overall, nine patients (21%) had objective evidence of response as follows: one complete response, two complete responses with no recovery of platelet counts, one partial response, and five complete marrow responses (blasts <5%). All responses were observed in patients with AML, and five (41%) of these responses were observed at an oral dose of 200 mg three times daily. Histone acetylation was observed in all patients at all dose levels. In this study, the MTD of oral vorinostat was either 200 mg three times daily or 200 mg twice daily for 14 days every 21 days in patients with leukemia.

MGCD0103

The HDAC inhibitor MGCD0103 is the first agent in this class to demonstrate selectivity by targeting Class I HDACs. A phase 1 open-label escalation study of MGCD0103 was undertaken to determine the MTD and to characterize pharmacokinetic and pharmacodynamic characteristics.[11] MGCD0103 was administered orally three times weekly in patients with relapsed or refractory leukemia or MDS, or in older untreated patients. Doses included 20, 40, 60, or 80 mg/m^2. A total of 22 patients were enrolled. MGCD0103 was well tolerated at doses below the MTD of 80 mg/m^2. Pharmacokinetics revealed HDAC inhibition at all doses, but responses were dose-dependent. Complete marrow response was observed in three patients. Oral doses of MGCD0103 produced mild clinical responses in patients with advanced leukemia or MDS who were refractory to other treatment options.

Valproic Acid

Valproic acid, generally used as an anticonvulsant and mood stabilizer, is being studied both alone and in combination for its HDAC inhibitor effects in malignant diseases.[12] The in vitro effects of valproic acid used in combination with decitabine were evaluated using leukemic cell lines.[13] Figure 2 presents the effects of each agent and the combination on apoptosis. Valproic acid induced global histone acetylation, an effect that was enhanced by decitabine. The combination of valproic acid and decitabine produced synergistic effects on growth inhibition, apoptosis, and reactivation of methylated genes.

Based on these results, a phase 1/2 study was designed to evaluate the effects of a combination regimen of azacitidine, valproic acid, and all-

trans retinoic acid (ATRA) for the treatment of leukemia.[14] Patients with high-risk MDS (≥10% blasts), relapsed or refractory AML, and patients older than 60 years of age were included in the study. The treatment regimen included a fixed dose of azacitidine 75 mg/m^2/d administered for 7 days, oral ATRA 45 mg/m^2/d for 5 days starting on day 3 of the azacitidine schedule, and escalating doses of valproic acid administered orally once daily for 7 days concomitantly with azacitidine. The initial dose of valproic acid was 50 mg/kg, followed by dose escalations to 62.5 and 75 mg/kg once daily. Treatment cycles were 21 days long. The phase 2 response end point was ≥30% with stopping rules.

A total of 53 patients (median age 69 years) were enrolled. Most patients (92%) had AML, and the remainder had high-risk MDS. The median number of prior treatments was two, although some patients received up to six treatments prior to enrollment. During phase I, the MTD of valproic acid was established at 50 mg/kg. Dose-limiting toxicity symptoms included neurotoxicity, confusion, and somnolence. The total response rate was 42%, and the overall response for patients receiving the MTD of 50 mg/kg valproic acid was 47%. Figure 3 presents overall survival rates. The median survival duration of patients who responded to treatment was more than 55 weeks (N = 15). Two patients with advanced refractory disease died. Patients who responded tended to have higher blood levels of valproic acid than nonresponders.

Is Combination Therapy Better Than A Single Agent?

There is inadequate clinical data to definitively demonstrate the superiority of combination therapy over single agents. Additional studies are needed, but a summary of results from the CALGB azacitidine studies revealed complete response rates of no more than 12%,[15] compared with a complete response rate of 25% among patients who received combination therapy with azacitidine, valproic acid 50 mg/kg, and ATRA.[14] In addition, the median time to response for this combination was one course of treatment, compared with three or more for azacitidine alone. The combination of decitabine and valproic acid also produced a 22% objective response in patients with leukemia in a phase 1/2 study.[16] The primary concern with valproic acid is its toxicity profile and the high doses required to produce a response. If valproic acid were replaced by other HDAC inhibitors used in combination with DNA methylation inhibitors, clinical response may be synergistically enhanced with better toxicity profiles.

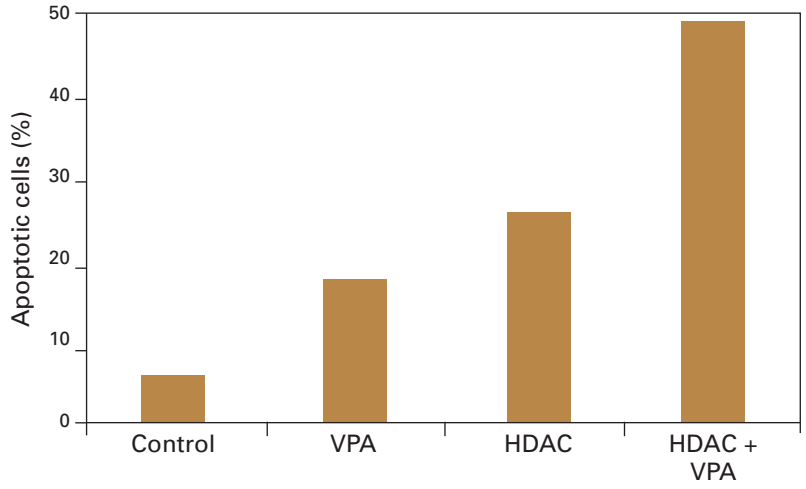

Figure 2. Synergistic effects of valproic acid and decitabine.[13]

Effects on apoptosis for valproic acid, decitabine, and the combination of the two agents are presented. Combination therapy produced synergistic effects, including increased apoptosis of leukemic cells.

HDAC = histone deacetylase; VPA = valproic acid.

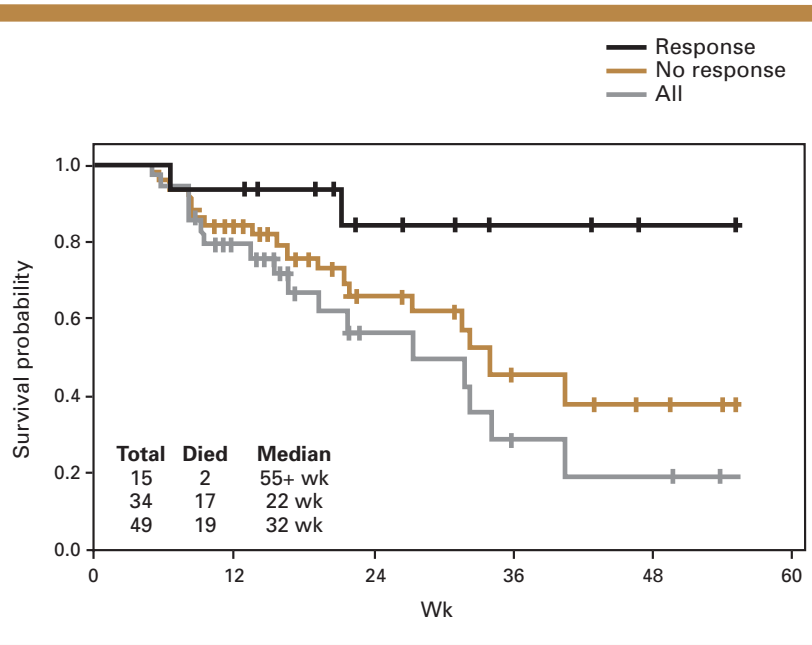

Figure 3. Overall survival.[14]

The median overall duration of survival for patients who responded to combination therapy with azacitidine, valproic acid, and all-trans retinoic acid was at least 55 weeks (N = 15) . The total response rate among these patients was 47%. Two patients with advanced refractory disease died.

Conclusion

Azacitidine has a significant effect on the natural history of MDS and is improving survival outcomes for patients. HDAC inhibitors have also shown efficacy in MDS and AML and might provide effective single-agent therapy for patients with lower-risk MDS. The combination of methylation inhibitors and HDAC inhibitors appears to provide superior results compared with single agents, however. Studies of various combination therapies have produced better response rates with fewer treatment cycles. The trend for future studies is to evaluate more powerful HDAC inhibitors with lower toxicity, used in combination with methylation inhibitors that are already successful, such as azacitidine and decitabine. Combination therapy offers great promise for the treatment of MDS and AML.

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Future Applications and Directions for Translational Therapies

Steven D. Gore, MD

The future of epigenetic therapies is promising. Along with approved agents, many investigational drugs appear to provide epigenetic modulation that could make additional therapeutic options available, especially when used in combination.

The foundation for selecting drug combinations lies in understanding convergent or complementary molecular mechanisms that can provide therapeutic synergy. Combinations may be selected for clinical research based on in vitro evidence of therapeutic synergy, or on empiric clinical characteristics such as absence of overlapping or synergistic toxicity. Positive clinical effects such as increased response and prolonged survival are the primary justifications for using combinations, even if the mechanisms are incompletely understood.

DNA Methylation Status and Clinical Response

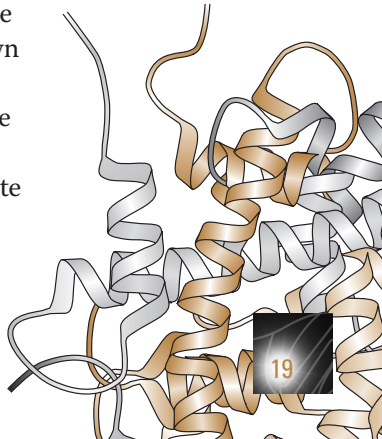
The clinical effects of DNA methylation inhibitors, when used to treat patients with myeloid leukemias, are readily apparent, but the biologic basis for the efficacy is still under investigation. Investigators rationalize the use of DNA methylation inhibitors in these patients because p15 and other genes are frequently hypermethylated in these diseases. To help explain the biologic effects of methylation inhibitors, a study of patients with high-risk MDS was conducted to determine the p15 methylation status of bone marrow mononuclear cells during treatment with decitabine.[1] A methylation-sensitive primer extension assay (Ms-SNuPE) was used to detect methylation. After at least one course of decitabine, a decrease in p15 methylation occurred in 9 of 12 patients and was associated with clinical response, although changes in methylation status did not appear to be required for patients to achieve clinical response.

Another phase 1 study of low-dose, prolonged decitabine dosing effects on relapsed or refractory leukemia used a less sensitive COBRA assay to detect p15 methylation status in peripheral blood.[2] There was no difference in baseline p15 methylation status between clinical responders and nonresponders nor in change of p15 methylation status following treatment, raising questions about whether clinical activity is due to epigenetic mechanisms. The clinical and pharmacodynamic results of different dosing schedules of decitabine were investigated in a randomized study undertaken to further explore optimal dosing schedules of this DNA methyltransferase inhibitor.[3] Adults with advanced MDS or chronic myelomonocytic leukemia (CMML) were assigned to one of three dosing regimens. A sensitive reverse transcription-polymerase chain reaction (RT-PCR) assay was used to determine p15 status.

Responding patients appeared to have higher levels of p15 expression at baseline than nonresponders. After treatment, p15 expression continued to increase among responders compared with nonresponders, but the difference was not significant. Nevertheless, increased p15 expression appeared to be a potential marker of clinical response.

The impetus for combining DNA methylation inhibitors with histone deacetylase (HDAC) inhibitors is based on in vitro data demonstrating re-expression of methylated genes using the sequential application of a methylation inhibitor followed by a HDAC inhibitor. The HDAC inhibitor trichostatin A (TSA) did not reactivate hypermethylated genes when used alone in an in vitro study.[4] Following treatment with decitabine, however, TSA produced robust re-expression of methylated genes such as p15.

The first clinical study of sequential DNA methyltransferase and HDAC inhibitors evaluated the use of azacitidine followed by the HDAC inhibitor sodium phenylbutyrate to treat patients with MDS and AML.[5] Low doses of azacitidine were administered for long durations, followed by a week of sodium phenylbutyrate initiated on the last day of azacitidine dosing. Results are shown in Table 1. Most clinical responses were complete or partial, suggesting enhancement of complete and partial responses compared with historic experience with



azacitidine alone. Changing the dosing schedule of azacitidine or adding an HDAC inhibitor may have improved the quality of response.

Examination of methylation status among responders in this study revealed extensive but incomplete reversal of p15 methylation after the first cycle of azacitidine therapy.[5] There has been ongoing controversy about whether DNA methylation inhibitors demethylate malignant cells or destroy them, allowing replacement with nonmethylated normal cells. The presence of heterogeneous methylation during the first treatment cycle and some remethylation after 7 days of sodium phenylbutyrate therapy suggest that the cells present during cycle one are still malignant. In this study, reversal of methylation appeared to correlate with clinical response. Six patients who responded clinically demonstrated reversal of methylation for genes p15 and CDH-1, the second most commonly methylated gene in myeloid malignancies. In contrast, there was no methylation reversal among nonresponders.

Table 1. Sequential azacitidine and sodium phenylbutyrate[a5]

Azacitidine dose	Patients (N)	Dose-limiting toxicity	Response
75 mg/m^2 x 5 days	6	1	2
50 mg/m^2 x 5 days	6	0	0
50 mg/m^2 x 10 days	8	0	4 (CR/PR)
50 mg/m^2 x 14 days	3	2	2
25 mg/m^2 x 14 days	6	0	3 (1 CR)

[a]sodium phenylbutyrate 376 mg/kg/d IVCI for 7 days beginning on the last day of azacitidine dosing

CR = complete response; PR = partial response.

The pharmacokinetic profile of azacitidine was analyzed after administration of the first dose.[5] A trend toward higher azacitidine area under the curve (AUC) was associated with methylation reversal, suggesting that finding ways to increase the AUC might increase treatment response. In addition, the largest increase of histone acetylation occurred after administration of azacitidine (Figure). This effect on histone acetylation from a methylation inhibitor is not completely understood, but it raised the question of whether there are off-target treatment effects that lead to histone acetylation in response to methylation inhibition. This phenomenon underscores the need to consider off-target effects of therapy, however, and to better understand the molecular pathways involved in clinical response.

Potential Therapeutic Combinations

Intervention targets and drugs under investigation for the treatment of MDS are listed in Table 2. Many of these agents have been studied individually, but the most promising results are likely to emerge from combinations of these agents. This section provides a summary of clinical data about potentially beneficial combinations that are still under study.

MS-275

MS-275 is a benzamide derivative HDAC inhibitor. It is administered orally and has a long half-life of approximately 45 hours. In a phase 1 trial, 39 adults with advanced acute leukemias were treated with MS-275 4 to 8 mg/m^2 once weekly for 2 weeks, repeated every 4 weeks.[6] After 13 patients were treated, the dose was increased to 8 to 10 mg/m^2 administered once weekly for 4 weeks and repeated every 6 weeks. Treatment with MS-275 induced a profound increase in histone acetylation that persisted for several weeks after the drug was administered. The pharmacokinetic and pharmacodynamic effects of MS-275 suggested that it would be useful when combined with a methylation inhibitor, so a phase 1 dose-finding study with azacitidine was undertaken.[7]

This combination is now being studied in a randomized, phase 2 study in the US Intergroup (ECOG1905) to further evaluate the effects of MS-275 used with azacitidine compared with azacitidine therapy alone. Additional evaluation of DNA damage that occurs during treatment will be performed to determine whether clinical response is associated with epigenetic effects or DNA damage.

Several other studies of combination therapies using a methylation inhibitor and HDAC inhibitor are under way, including:

- decitabine plus FK228
- decitabine plus vorinostat
- azacitidine plus vorinostat
- azacitidine plus MGCD0103

Nonepigenetic Effects of HDAC Inhibitors

In addition to their potential epigenetic effects, HDAC inhibitors use other mechanisms unrelated to epigenetic alterations that may be useful in targeting malignant cells.

These agents are associated with induction of reactive oxygen species and oxidative DNA damage; modulation of the TNF apoptosis-inducing ligand (TRAIL) pathway, including TRAIL receptors and death receptors 4 and 5; inhibition of NF-κB activation; and acetylation of Hsp^{90} and disruption of its chaperone function.

These mechanisms may be used in conjunction with other agents to cause cell death (see "Modulating Gene Expression by HDAC Inhibition," page 9).

All-Trans Retinoic Acid

Methylation and silencing of retinoic acid receptor-β (RARβ) in idiopathic myelofibrosis[8] and of retinol-binding proteins in many primary leukemias[9] suggest that altering retinoid pathways with DNA methylation inhibitors may produce clinical effects. A study of combination therapy with azacitidine, valproic acid, and all-trans retinoic acid (ATRA) in leukemia resulted in significant clinical activity.[10] A pilot study of valproic acid and ATRA (with no methylation inhibitor) used to treat refractory and high-risk AML patients also produced phenotypic changes of AML blasts.[11] ATRA was used to activate gene transcription and differentiation in leukemia cells. When used in combination with a methylation inhibitor or HDAC inhibitor, ATRA might improve clinical response. Additional study is needed.

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) and its receptors regulate angiogenesis, thereby affecting tumor growth and dissemination. Increased VEGF concentrations are associated with lower complete response rates to chemotherapy in patients with MDS.[12] In lung cancer, the p16INK4A tumor suppressor gene downregulates VEGF. Decitabine used in one study affected the methylation status of p16 genes, which subsequently affected VEGF expression.[13] Combination therapy with VEGF might provide a pathway for regulating angiogenesis and tumor progression.

Signaling Antagonists

In T cells, inhibition of DNA methylation increases DNA methyltransferase transcription.[14] This increase appears to be associated with signaling through the rasmitogen-activated protein kinase (ras-MAPK) pathway. Possible treatment approaches based on signaling pathways include using agents to prevent remethylation, such as the farnesyl transferase inhibitors tipifarnib and lonafarnib, and the MAPK inhibitor SCIO-469.

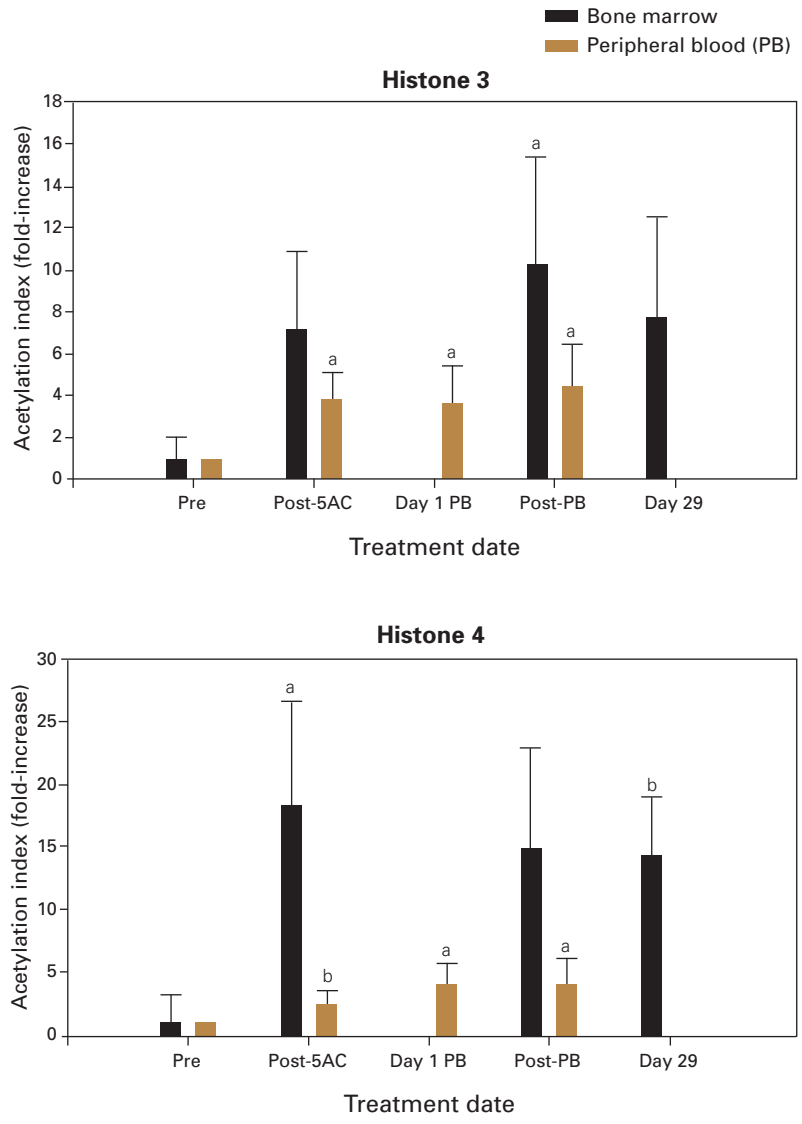


Figure. Histone acetylation after azacitidine (5AC) administration.[5]

Azacitidine, a methylation inhibitor, produced histone acetylation of H3 and H4. The reason for this effect is not understood and suggests potential "off-target" effects of agents used to treat myeloid diseases.

[a] $P < 0.05$, indicates mean > 1
[b] $P < 0.025$, mean fold increase > 1
Reproduced with permission.[5]

Arsenic Trioxide

Arsenic trioxide (ATO) is under study for the treatment of myeloid diseases including MDS. Although the mechanism by which it exerts clinical effects is not understood, ATO appears to produce hematologic improvements in some patients. A phase 2 multicenter study evaluated the safety and efficacy of ATO in two cohorts of patients with MDS, stratified by risk.[15] For lower-risk MDS patients, hematologic improvement was the primary end point. For higher-risk MDS patients, additional end points included complete or partial remission. Among patients who received at least two cycles of treatment, hematologic improvements occurred in 39% of lower-risk patients and in 9% of higher-risk patients. One higher-risk patient achieved complete remission. The median duration of hematologic improvement was 6.8 months.

Table 2. MDS therapeutic targets: mechanisms and drugs

Target mechanisms	Target drugs
Transcription	HDAC inhibitors
Signaling	Lenalidomide
Angiogenesis	Bevacizumab/PTK787
Cytokine milieu	Etanercept/infliximab
Apoptosis	SCIO469
–Increasing	Tipifarnib/lonafarnib
–Decreasing	Arsenic trioxide
Immune modulation	Retinoids/vitamin D
	TLK199

An earlier small study of ATO and ascorbic acid in elderly patients with acute promyelocytic leukemia (APL) produced a reduction in bone marrow blasts from >40% to <5% in 3 patients.[16] These results suggest a possible therapeutic option for elderly patients that is less toxic than chemotherapy.

Lenalidomide

Lenalidomide has been shown to produce hematologic activity in patients with MDS. In one study of 43 patients with transfusion-dependent or symptomatic anemia who had not responded to prior erythropoietin therapy, lenalidomide produced a response in 24 patients (56%), and 20 had sustained independence from transfusion.[17] Lenalidomide appears to be effective in patients with low-risk MDS who are unlikely to benefit from conventional therapy. The combined use of lenalidomide and azacitidine warrants study to determine the possibility of synergistically improved efficacy.

Conclusion

Selecting doses and combinations of therapeutic agents to develop depends on therapeutic objectives. Is the goal to affect a pathway to terminal differentiation, leading to clonal extinction? Is it to kill all malignant cells? Choices of combinations and dosing regimens are based on intermediate and ultimate end points. Being able to vary dosing schedules to produce different biologic effects offers a promising option. In addition, several new therapeutic agents and combinations are being studied, revealing additional mechanisms and potential pathways for treatment. The complexities of some agents can make the task of determining which agent or combination of agents to use somewhat daunting. But the primary objectives in selecting a therapeutic approach are to produce normal hematopoiesis and to improve survival. Studies are adding to our understanding of how to achieve these goals. Agents that are known to produce clinical effect will increasingly be combined with agents that have the potential to enhance outcomes. It is likely that combination regimens will provide the most promising results.

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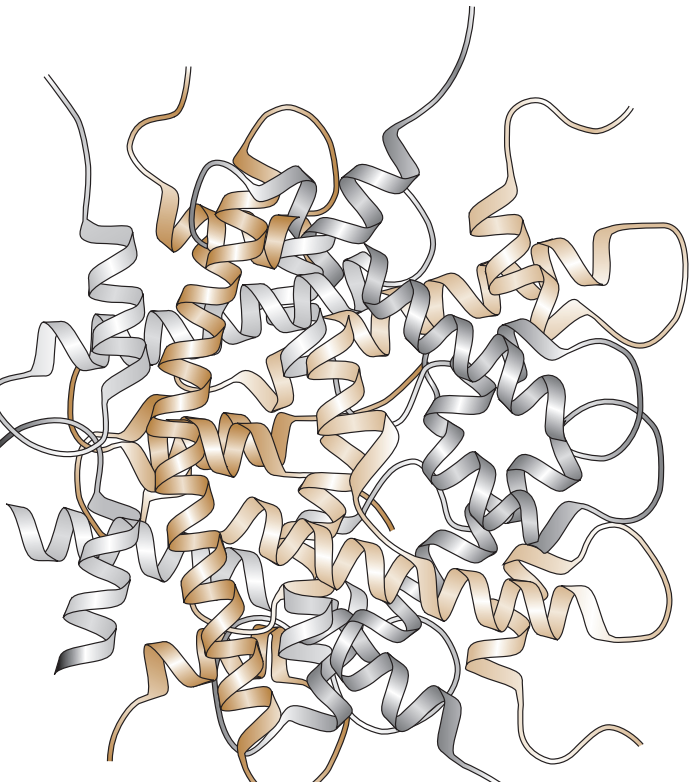


Self-Assessment Quiz

1. **Based on Knudson's "two-hit" hypothesis of cancer causation, a "hit" can be:**
   a. an inherited gene mutation.
   b. epigenetic silencing.
   c. loss of heterozygosity.
   d. all of the above.

2. **Which agent is incorporated into DNA?**
   a. Decitabine (5-aza-2'deoxycytidine)
   b. Azacitadine (5-azacytidine)
   c. Hydroxyurea
   d. 5-aza-2'deoxycytidine triphosphate

3. **Which activities are associated with HDAC inhibitors?**
   a. Histone acetylation
   b. Uncoiling of chromatin
   c. Transcription of genes
   d. All of the above

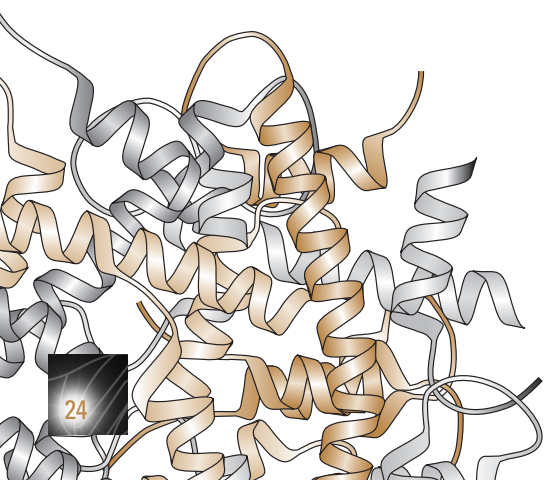
4. **How many classes of HDACs have been identified?**
   a. One
   b. Two
   c. Three
   d. Four

5. **A study evaluating MDS response using International Working Group criteria found that the median number of cycles from first azacitidine treatment to any response was:**
   a. 1 cycle.
   b. 3 cycles.
   c. 6 cycles.
   d. 17 cycles.

6. **The median survival duration of patients who responded to treatment with combination azacitidine, valproic acid, and all-trans retinoic acid (ATRA) was:**
   a. 55+ weeks.
   b. 34 weeks.
   c. 22 weeks.
   d. 17 weeks.

7. **Clinical data support which approach to combination therapy with methylation inhibitors and HDAC inhibitors?**
   a. HDAC inhibitor followed by a methylation inhibitor
   b. Methylation inhibitor followed by an HDAC inhibitor
   c. Concurrent use of both classes of agents
   d. Neither approach produces clinical effect

8. **Clinical data support the use of lenalidomide for which patient population?**
   a. Any patient with myeloid leukemia
   b. Lenalidomide has not produced useful clinical effects
   c. Elderly never-treated patients
   d. Patients with low-risk MDS who are unlikely to benefit from conventional therapy



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