Epigenetics, DNA Methylation, and Chromatin Modifying Drugs

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Abstract

Evidence is emerging that several diseases and behavioral pathologies result from defects in gene function. The best-studied example is cancer, but other diseases such as autoimmune disease, asthma, type 2 diabetes, metabolic disorders, and autism display aberrant gene expression. Gene function may be altered by either a change in the sequence of the DNA or a change in epigenetic programming of a gene in the absence of a sequence change. With epigenetic drugs, it is possible to reverse aberrant gene expression profiles associated with different disease states. Several epigenetic drugs targeting DNA methylation and histone deacetylation enzymes have been tested in clinical trials. Understanding the epigenetic machinery and the differential roles of its components in specific disease states is essential for developing targeted epigenetic therapy.

INTRODUCTION: EPIGENETICS AND HUMAN DISEASE

HDAC: histone deacetylase

HAT: histone acetyltransferase

HDACis: HDAC inhibitors

TSA: trichostatin A

SAHA: suberoylanilide hydroxamic acid

Changes in the normal program of gene expression are the basis for several human diseases. The genome is programmed by the epigenome. The epigenome consists of the chromatin and its modifications, as well as a covalent modification of cytosines residing at the dinucleotide sequence CG in DNA by methylation (1). Recently, a new level of epigenetic regulation by small noncoding RNAs, termed microRNAs, has been discovered (2). A large number of loci in the human genome encode noncoding RNAs, which are processed to short RNAs and target specific genes for silencing. microRNAs regulate gene expression at different levels; they silence chromatin, degrade mRNA, and block translation. microRNAs play an important role in cancer (3) and potentially play an important role in behavioral pathologies, as well (4). Additional forms of noncoding RNA are involved in programming gene expression. For example, the Air RNA regulates IgfIIR gene expression in a manner dependent on the parental origin of the allele (5), and Xist RNA is involved in inactivation of the X chromosome (6). microRNA expression is regulated by epigenetic factors such as DNA methylation and chromatin structure (7).

CHROMATIN MODIFYING DRUGS IN CLINICAL DEVELOPMENT

DNA is wrapped around a protein-based structure termed chromatin. The basic building block of chromatin is the nucleosome, which is formed of an octamer of histone proteins. There are 5 basic forms of histone proteins, H1, H2A, H2B, H3, and H4 (8), as well as other minor variants, which are involved in specific functions such as DNA repair and gene activation (9). The octamer structure of the nucleosome is composed of a H3-H4 tetramer flanked on either side with a H2A-H2B dimer (8). The N-terminal tails of these histones are extensively modified by methylation (10), phosphorylation, acetylation (11), sumoylation (12), and ubiquitination (13). The state of modification of these tails plays an important role in defining the accessibility of the DNA to the transcription machinery. Bidirectional enzymatic machineries modify the chromatin. This offers significant opportunities for developing drugs that can affect the state of chromatin in both directions. The main challenge in using epigenetic modulators for therapy is specificity.

HISTONE DEACETYLASE INHIBITORS AND THEIR ROLE IN CANCER THERAPY

Histone acetylation is a global mark of gene activity. Histone deacetylases (HDACs) remove histones and histone acetyl transferases (HATs) acetylate histones. The most advanced chromatin modification targeted drugs are HDAC inhibitors (HDACis). There is a vast literature demonstrating the involvement of HDACs in suppressing critical genes in cancer (14, 15). HDACis are now being considered as potential therapeutics for mental pathologies, as well (16).

HDAC inhibitors fall into five different structural groups that are currently at varying stages of development. The classic HDACis, trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), are hydroxamate based. They inhibit class 1 and class 2 HDACs. SAHA is the first clinically approved HDACi. A second group includes hydroxamate based HDACis (LBH589, PXD101) that are currently in different stages of clinical development and inhibit class 1 and class 2 HDACs. The third group are aliphatic based and also inhibit class 1 and class 2 HDACs. This group includes sodium butyrate, one of the earliest HDAC inhibitors, as well as the mood stabilizer and antiepileptic valproic acid. The fourth group includes a cyclic peptide based HDACi (FK228) that inhibits class 1 and class 2 HDACs. The fifth group are benzamide based HDACis that inhibit classes 1, 2, and 3 HDACs. An interesting example is MGCD0103, which showed

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isotypic specificity against class 1 HDACs and a broad spectrum of antitumor activity (17). HDAC inhibitors have exhibited anticancer activity in preclinical tumor models and in phase 1 and phase 2 clinical trials [for a review, see (18)], and the first HDACi Vorinostsat (SAHA) was recently approved for clinical use in cutaneous T-cell lymphoma (19). Vorinostat was safe and effective at an oral dose of 400 mg/day with an overall response rate of 30 to 31% in refractory advanced patients with CTCL (19). The HDAC1 isotypic specific MGCD0103 is now being tested in phase 1 and phase 2 clinical trials in solid and hematological tumors and has shown some clinical response (20–22). Several novel HDACis are at different stages of preclinical development.

The putative mechanism of action of HDACis in cancer is as follows: Blockage of HDACs tilts the balance of acetylation-deactylation reactions toward acetylation. This results in hyperacetylation of histone tails and induction of genes that suppress the cancer phenotype such as tumor suppressor genes and metastasis- and invasion-inhibitory genes. A well-characterized example is the tumor suppressor *P21* that is induced in response to treatment with TSA (23). Another example is *E CADHERIN*, a gene that blocks mesenchymal to epithelial transition and cell invasiveness and is induced by TSA (24). HDACis block multiple biological steps in cancer progression in cultured cancer cells, including cell cycle arrest (25), apoptosis (26), epithelial to mesenchymal transition (27), and invasion (28).

All the known HDACis block one class or several classes of HDACs and thus should have a global effect on gene expression. Nevertheless, comprehensive microarray gene expression experiments reveal that only a fraction of the transcriptome is activated or suppressed with HDAC inhibition (29–32). HDACs and HATs are targeted to specific genes. HDACis will affect only genes that are associated with HDACs and are also targets of HATs. Thus, the specific gene expression response to an HDACi will be determined by the profile of distribution of HDACs and HATs in the genome. Oncogenic pathways target HDACs to specific genes. For example, SNAIL targets HDAC1/2 to the promoter of *E CADHERIN* (33). The relatively specific effects of HDACi on cancer cell growth suggest that critical cancer genes are HDAC bound and HAT targeted.

There are 4 defined phylogenic classes of HDACs. Specificity of HDACis might increase if the isotypes of HDACs involved in cancer are specifically targeted. Evidence points to the involvement of class 1 HDACs, HDAC1 and HDAC3, in several types of cancer (34). A recent example of an isotypic-specific drug is MGCD0103, which has high affinity to HDAC1 and has shown excellent activity in vitro and in vivo (17) in tumor models and is now in clinical trials (21). However, even isotypic-specific inhibitors that target HDAC1 act on an enzyme with multiple genomic targets.

HISTONE DEACETYLASE INHIBITORS AND THEIR ROLE IN MENTAL HEALTH

Chromatin acetylation and memory were shown to be impaired in CBP knockout mice, which suggests a role for acetylation in memory formation (35). The fact that valproic acid, a long established antiepileptic and mood stabilizer, is also a HDAC inhibitor (36) alludes to a possible role for HDACis in treating certain mental conditions such as schizophrenia. Valproic acid has had some effect alleviating psychotic agitation as an adjunct to antipsychotics in schizophrenia (37, 38). HDACis were shown to improve memory and induce dendritic sprouting in a transgenic mouse model of neurodegeneration, which suggests that HDACis might be of use in treating neurodegeneration and memory loss, as well (39). Although biological and behavioral effects of HDACis in the brain are somewhat characterized, their specific gene targets and their function in mental pathologies are not well delineated. Nevertheless, the limited clinical and animal data suggest a potentially important role for HDACis in treatment of mental disorders.

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HMTase: histone methyltransferase

DNMT: DNA methyltransferase

Recent clinical developments are focusing on schizophrenia. Experiments with a novel HDACi from the benzamide class N-(2-aminophenyl)-4-[N-(pyridin-3-yl-methoxycarbonyl)aminomethyl]benzamide derivative (MS-275) in mice resulted in brain region–specific induction of acetylation in the frontal cortex at two genes involved with schizophrenia pathogenesis, *reelin* and *gad(67)* (16). Valproic acid was shown to induce the expression of *reelin*, which was silenced by methionine treatment in mice (40). These studies raise the possibility that treatment of schizophrenics with an HDACi might cause activation of expression of critical genes such as *REELIN*, and could reverse the course of this disease (41). Several clinical trials tested valproate as an adjunctive therapy to antipsychotics in schizophrenia (38, 42, 43). There are indications that valproate might improve violent episodes in a subset of schizophrenia patients (42), and might, in combination with antipsychotics, have an effect on euphoric mania (38) and features of manic symptomatology in bipolar disorders (38). Further clinical trials are needed with valproate and with more potent and selective HDACis to methodically test their therapeutic potential in mental pathologies. Isotypic-specific HDACis might enhance the efficacy and potency of the treatment and reduce its toxicity.

HDAC INHIBITORS AND THEIR ROLE IN OTHER HEALTH CONDITIONS

HDACis are potential therapeutics for other health conditions. One interesting area is transplantation. A special subset of T cells, regulatory T cells (Tregs), control and maintain transplant tolerance by suppressing immune responsiveness to the transplant. HDACis activate the transcription factor *FOXP3*, which plays a cardinal role in the immunosuppressive function of Treg cells (44). HDAC9 has proven particularly important in negatively regulating FOXP3-dependent suppression (44), thus raising the attractive possibility that isotypic-specific HDAC9 inhibitors might serve as excellent agents for suppressing the antitransplant response in transplantation therapy. In addition, it might be potentially important in suppression of other autoimmune and proinflammatory conditions. Recent preclinical trials with SAHA in the rhesus macaque demonstrated efficacy in primates in induction of Treg function (45).

Other candidates for HDACis are metabolic diseases such as type 2 diabetes. The critical glucose transporter, GLUT4, response to exercise is regulated by HDAC5. Activation of GLUT4 through inhibition of HDAC5 might be an interesting approach to type 2 diabetes (46). Because HDACi treatment of type 2 diabetes is anticipated to be chronic and long-term, it is especially critical to focus on isotypic-specific inhibitors in order to limit systemic toxicity.

HISTONE METHYLTRANSFERASE INHIBITORS

A new area of potential interest is the development of histone methyltransferase (HMTase) inhibitors. H3K9Me2 histone is a hallmark of gene silencing and was shown to mark silenced tumor suppressor genes (47–49). H3K27 methylation, which is targeted by the polycomb group protein and histone methyltransferase EZH2, is another interesting target for inhibition (**Figure 1**). EZH2 associates with DNA methyltransferases (DNMTs) in silencing of tumor suppressor genes (50). HMTase inhibitors could be used therapeutically to activate silenced tumor suppressor genes. Two HMTase inhibitors were recently described. The fungal mycotoxin chaetocin, which belongs to the class of 3–6 epidithio-diketopiperazines (ETPs), specifically inhibits the *Drosophila* HMTase dSU(VAR)3-9 and its human homolog (51). A small-molecule inhibitor of G9a histone methyltransferase was reported last year and was shown to block H3K9Me2 in vitro and in cell culture (52). However, it is not known whether these compounds have anticancer activity or systemic toxicity.

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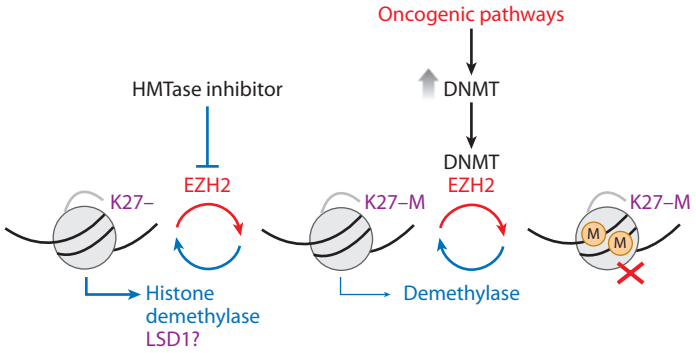

Figure 1

Interaction of histone methyltransferase and DNA methyltransferases (DNMTs) in methylation of tumor suppressor genes. Tumor suppressor genes are marked by EZH2 binding and K27 methylation (K27-M). Increase in DNMT expression as a result of activation of oncogenic pathways such as RAS or RB knockdown increases cellular levels of DNMT1, which is then recruited to EZH2 sites in the genome, resulting in methylation of EZH2-associated DNA (circled M). This illustrates the tight correlation between chromatin and DNA modifications. HMTase inhibitors should cause DNA demethylation as well.

Another interesting group of targets are histone demethylases (53, 54). H3K4Me2 is a hallmark of active genes. Because the state of histone methylation is a balance of methylation and demethylation reactions, inhibition of H3K4 demethylase would result in increased H3K4 histone methylation and activation of genes, including potential tumor suppressor genes. A candidate target is the histone, lysine-specific demethylase 1 (LSD1), that demethylates H3K4Me2. Novel biguanide and bisguanidine polyamine analogues were shown to inhibit LSD1, a homologue of polyamine oxidase, and activate multiple aberrantly silenced genes in colorectal cancer cells (55). Nonselective monoamine oxidase inhibitors such as tranylcypromine, which were used as antidepressive medication in psychiatry, were also found to be LSD1 inhibitors (56). It is possible that LSD1 inhibition is involved in the mechanism of action of antidepressive agents. It is tempting to speculate that selective inhibitors of LSD1 might be effective as antidepressants, as well.

DNA METHYLATION PATTERNS

A major element of epigenetic regulation in vertebrates is the pattern of distribution of a covalent modification of cytosines by methylation in the genome. The primary methylated sequence in vertebrates is composed of only two bases, the di-nucleotide sequence CG (57). Only <80% of the methylatable CG population is methylated. Different CG sites are methylated in different tissues, creating a pattern of methylation that is gene and tissue specific (57). This pattern creates a layer of information that confers upon a genome its specific cell type identity. The DNA methylation pattern is copied by independent enzymatic machinery, the DNMT (58). DNA methylation patterns in vertebrates are distinguished by their tight correlation with chromatin structure. Active regions of the chromatin, which enable gene expression, are associated with hypomethylated DNA, whereas hypermethylated DNA is packaged in inactive chromatin (58).

Mechanisms of Silencing of Gene Expression by DNA Methylation

DNA methylation is a highly effective mechanism for silencing of gene expression in vertebrates and plants. DNA methylation silences gene expression either by interfering with the binding of

LSD1: lysine-specific demethylase 1

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transcription factors (59, 60), or by attracting methylated DNA-binding proteins (MBDs) such as MeCP2 (61). MeCP2 recruits other proteins such as SIN3A and histone modifying enzymes, which leads to formation of a closed chromatin configuration and silencing of gene expression (61). Several methylated DNA-binding proteins such as MeCP2, MBD1, MBD2, and MBD3 suppress gene expression by a similar mechanism (62–64). MBD3 does not bind directly methylated DNA, but it associates with the NurD complex that contains MBD2 as the methylated DNA-binding factor (65). Certain MBDs have other enzymatic activities. MBD4 is a thymidine glycosylase (66), and MBD2 was suggested to bear demethylase activity (67–71), although this is highly contested.

MBD: methylated DNA binding domain

SAM: S-adenosyl-L-methionine

DNA Methyltransferases

The DNA methylation reaction is catalyzed by DNMT (58). Methylation of DNA occurs immediately after replication by a transfer of a methyl moiety from the donor S-adenosyl-L-methionine (SAM, or AdoMet) in a reaction catalyzed by DNMT. Three distinct phylogenic DNA methyltransferases were identified in mammals. DNMT1 shows preference for hemimethylated DNA in vitro, which is consistent with its role as a maintenance DNMT, whereas DNMT3a and DNMT3b methylate unmethylated and methylated DNA at an equal rate, which is consistent with a de novo DNMT role (72). It is clear, however, that this classic distinction between de novo and maintenance DNMT doesn't always apply. Both classes of enzymes participate in both de novo and maintenance methylation, and DNA methylation is a targeted process.

Is DNA Methylation a Reversible Reaction?

The most controversial issue in the DNA methylation field is the question of whether the DNA methylation reaction is reversible (73). Dynamic reversibility is essential for life-long responsiveness of the DNA methylation pattern to drugs. There is a long list of data from both cell culture and early mouse development supporting the hypotheses that active methylation occurs in embryonal and somatic cells, and that a dynamic, reversible DNA methylation pattern is involved in memory in the brain (74), as well as in an estrogen induced gene (75).

Several enzymatic activities were proposed to cause DNA demethylation. A G/T mismatch repair glycosylase functions as a 5-methylcytosine DNA glycosylase, recognizes methyl cytosines, and cleaves the bond between the sugar and the base. The abasic site is then repaired and replaced with a nonmethylated cytosine resulting in demethylation (76). An additional protein with similar activity was recently identified, the methylated DNA binding protein 4 (MBD4) (77). MBD2b (a shorter isoform of MBD2) was shown to directly remove the methyl group from methylated cytosine in methylated CpGs (78), but this was contested by several groups (63). GADD45A, a damage response protein, was proposed to trigger active DNA demethylation through a repair-mediated process (79). However, this was also contested by a later study (80). More recently, it was proposed that the DNA methyltransferase DNMT3A acts as a demethylase, possibly through a mechanism that involves deamination (81).

BILATERAL RELATIONSHIP BETWEEN CHROMATIN STRUCTURE AND DNA METHYLATION

Correlation Between Chromatin and DNA Methylation States

The two components of the epigenome, DNA methylation and chromatin, are tightly correlated (**Figures 1** and **2**). More than three decades ago, Cedar & Razin showed that inactive chromatin is

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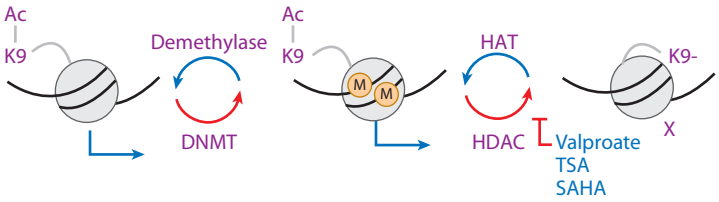

Figure 2

Interrelation between chromatin modifying drugs and DNA methylation. Histone deacetylase inhibitors (HDACis) cause acetylation of target genes, which facilitates demethylation. This could serve as a way to demethylate genes in the brain, where 5-aza-cytidine (5-azaC), a replication-dependent DNA methylation inhibitor, would not be functional. Acetylation (Ac); methylcytidine (M).

enriched with hypermethylated DNA and that active chromatin is associated with hypomethylated DNA (58). These correlations were confirmed by detailed analyses of specific genes, as well as genome-wide ChIP-on-chip analyses. The relationship between chromatin and DNA methylation is bilateral (82).

Implications of the Interrelationship of Chromatin and DNA Methylation on the Use of Chromatin Modifying Drugs

The interrelation between chromatin state and DNA methylation suggests that there is a crosstalk between drugs targeting chromatin and those targeting DNA methylation. This could be utilized therapeutically. For example, because HDACis not only affect histone acetylation but also facilitate replication-independent DNA demethylation (70), they could be utilized to induce demethylation in post mitotic nondividing tissues such as brain or heart (**Figure 2**). Catalytic inhibitors of DNMT1 such as 5-aza-cytidine (5-azaC) or Zebularine need to be incorporated into DNA; only then do they inhibit DNMT during passage of the replication fork. Indeed, valproate, the antiepileptic drug that is also an HDACi, induces replication-independent demethylation (69, 83) in cell culture and demethylation of the *reelin* gene in mouse brain in vivo (84).

HISTONE MODIFYING ENZYMES AND RECRUITMENT OF DNA METHYLTRANSFERASES AND DEMETHYLASES TO SPECIFIC GENES

Specific DNA methylation patterns could be directed by chromatin modification. It is now well established that histone modification enzymes interact with DNA methylating enzymes and recruit DNA methylation activity to specific targets. A growing list of histone modifying enzymes such as HDAC1 and HDAC2 have been shown to interact with DNMT1, DNMT3a, the histone methyltransferases SUV3-9, EZH2, and PRC2/3, a member of the multi-protein polycomb complex that methylates H3 histone at the K27 residue (85–88), as well as the heterochromatin protein HP1, which binds H3-K9 methylated histones (89). The methylated DNA binding protein MeCP2 interacts with the HMT SUV3-9 (87).

One of the most important links between chromatin and DNA methylation is the association of EZH2 HMTase, methylation of H3-histones at K27 residues, and DNA methylation of tumor suppressor genes (**Figure 2**). A survey of CG islands methylated in lung cancer revealed that they were also PcG EZH2 targets (90). Thus, sites bound by EZH2 are poised to become methylated, but in normal cells the level of DNMT1 keeps the EZH2 targets unmethylated. In the process of tumorigenesis, DNMT1 levels are induced by activation of several oncogenic pathways and

5-azaC: 5-aza-cytidine

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silencing of tumor suppressor pathways (91–95). It is therefore anticipated that EZH2 inhibitors will trigger selective loss of methylation of tumor suppressor genes.

Similar to DNA methylation, demethylation is targeted to genes by chromatin modification changes. Transcription factors recruit HATs to specific genes. This triggers gene-specific acetylation and recruitment of RNApolII to the gene, which is followed by demethylation (96). There are examples in the literature indicating that transcription factors such as *NF-κB* (97) and NGFI-A (98) are required for replication-independent active demethylation.

In summary, DNA methylation pattern and chromatin structure are found in a dynamic balance. This balance is required to maintain the homeostasis of epigenetic information. A change in either of these parameters would trigger a change in the DNA methylation state.

DNA METHYLATION PHARMACOLOGY

DNA methylation could be modified pharmacologically. By modification of DNA methylation, it would be possible to alter gene expression programs, including those for pathological gene expression. There are potentially multiple diseases that are candidates for DNA methylation therapy. The critical issues are: understanding the aberrations in methylation involved in the disease, the complexity of the DNA methylation machinery, and the multiple interactions of the DNA methylation machinery with other cellular machineries.

Aberrations of DNA Methylation Patterns and DNA Methylation Machinery in Cancer

Cancer was the first disease for which DNA methylation was proposed as a therapeutic target (99). The first DNA methylation inhibitor 5-azaC (or 5AC) and its deoxy analog 5-deoxycytidine (5-azaCdR or DAC) (100) were recently approved by the FDA for treatment of myelodysplastic syndromes (MDS) (101) (see **Figure 3**). Three types of aberration in the DNA methylation machinery occur in cancer: hypermethylation of tumor suppressor genes, aberrant expression

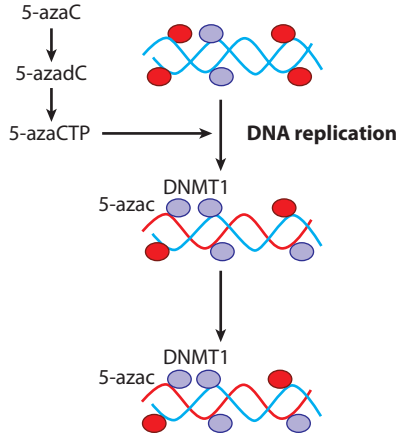

Figure 3

Mechanism of action of 5-aza-cytidine (5-azaC) and 5-azadC. 5-azadC is a prodrug, which needs to be phosphorylated by cellular cytidine kinases to the triphosphate nucleotide 5-azadCTP. 5-azadCTP is incorporated into DNA and traps the DNA methyltransferase (DNMT) in the progressing fork, resulting in passive demethylation of the nascent strand. Methylated CGs (*red circles*); unmethylated CGs (*purple circles*).

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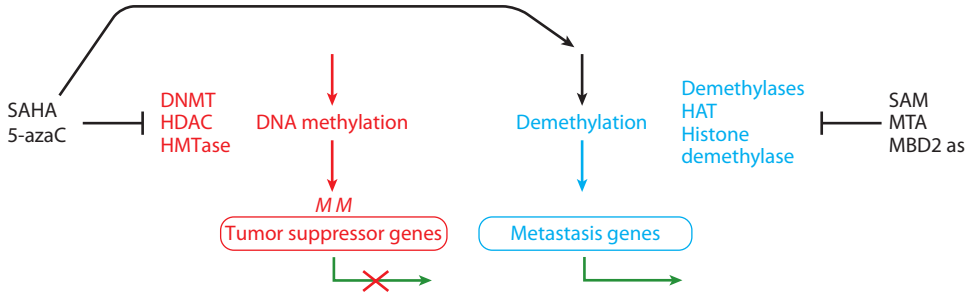

Figure 4

Epigenetic changes in cancer involve both methylation of tumor suppressor genes and demethylation of prometastatic genes. The process of gene activation involves participation of histone acetyl transferases (HATs), histone demethylases, and possibly DNA demethylases. The process of gene inactivation involves participation of DNA methyltransferases (DNMTs), histone deacetylases (HDACs), and histone methyltransferases (HMTases). Tumor suppressors could be activated by either HDACi or DNMT inhibitors, however, it is possible that the DNA demethylation would activate prometastatic genes. Several agents were shown to suppress prometastatic genes in culture and in vivo: S-adenosyl-L-methionine (SAM), 5′-methylthioadenosine (MTA), and a methylated DNA binding domain 2 (MBD2) antisense oligonucleotide.

of DNMT1 and other DNMTs, and hypomethylation of unique genes and repetitive sequences (102–104). It is important to consider all three when designing therapeutic strategies targeting DNA methylation (**Figure 4**).

Mechanism of Action of DNMT1 Inhibitors

The expression of DNMT1 is tightly regulated with the state of cell growth by transcriptional and posttranscriptional mechanisms (105, 106). Several oncogenic pathways lead to overexpression of DNMT1 through transcriptional and posttranscriptional control of DNMT1 (91–93, 107). Deregulation of the proper cell-cycle coordinated expression of *DNMT1* causes cellular transformation (107). Overexpression of *DNMT1* in nontransformed cells leads to cellular transformation (108), whereas knockout of *dnmt1* protects mice from colorectal cancer (109). Taken together, these data support the idea that inhibition of DNMT1 should be a reasonable strategy for anticancer therapeutics. The anticancer effects of DNMT1 inhibition were demonstrated pharmacologically using antisense oligonucleotide inhibitors (110, 111), and genetically using *dnmt1-/-* mice (109).

In order to properly design and utilize therapeutic strategies that involve DNMT1 inhibition, it is essential to understand why DNMT1 transforms cells and why DNMT1 inhibition blocks tumor growth. DNMT1 is an enzyme that methylates DNA. The commonly accepted and attractively straightforward model is that DNMT1 inhibition causes loss of methylation during DNA synthesis and, as a result, aberrantly methylated tumor suppressor genes are activated, arresting tumor growth. However, knockdown of DNMT1 by siRNA or antisense oligonucleotides blocks the growth of cancer cells by mechanisms independent of DNA methylation through induction of tumor suppressor genes (112). This triggers a DNA damage response and inhibition of DNA replication (113). It is plausible, therefore, that DNMT1 transforms cells by a mechanism independent of DNA methylation and that targeting the DNA methylation–independent functions of DNMT1 will have a strong anticancer growth effect (**Figure 4**).

DNA Methylation Inhibitors and DNMT1 Modulators

The three most commonly used catalytic inhibitors of DNMTs are the nucleoside analogs 5-azaC, 5-azaCdR, and Zebularine. The mechanism of action of these inhibitors is somewhat unique. They

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are first phosphorylated to the triphosphate nucleotide and incorporated into DNA during DNA synthesis (**Figure 3**). DNMT1 forms a covalent bond with the carbon at position 6 of the cytosine, as well as at the 5-aza-cytosine ring in DNA. Under normal conditions, the enzyme transfers the methyl group from SAM to the 5′ carbon position of the cytosine ring. This enables the release of the enzyme from its covalent bond with cytosine. When a 5′-aza-cytosine ring replaces cytosine in the DNA, the methyl transfer does not take place and the DNMT is trapped on the DNA (114). The replication fork progresses in the absence of DNMT, resulting in passive loss of DNA methylation in the nascent strand but not the template. Zebularine is a nucleoside analog that, unlike 5-azaC, is chemically stable and orally bioavailable. Zebularine was originally identified as a cytidine deaminase inhibitor (115). Its mechanism of action is predicted to be similar to that of 5-azaC. This compound exhibits DNA demethylation activity and shows reduced potency and toxicity in comparison to 5-azaC.

Because both 5-azaC and Zebularine need to be incorporated into DNA to trap DNMT, they might have additional nonspecific toxicities that are a result of the trapping of DNMT1 onto DNA, and perhaps the trapping of other DNA binding proteins, as well (116). Non-nucleoside-based inhibitors of DNMT1 that inhibit DNMT catalytic activity without incorporation into DNA are therefore of much interest. Such a compound was described, but its efficacy and potency in whole animals and humans are unclear (117).

Other commonly used drugs were shown to bring about demethylation. For example, procainamide, a widely used antiarrythmic drug, inhibits DNMT activity and promotes hypomethylation (118, 119). Recently, analogues of procainamide were synthesized and one lead was reported to inhibit DNMT1 and to cause global hypomethylation (120). Hydralazine, an antidiuretic, induces hypomethylation (118). Valproic acid, a widely used antiepileptic and mood stabilizer, was shown to cause demethylation (69, 83). These data raise the concern that other heavily used drugs affect the DNA methylation pattern and thus can promote the expression of disease-promoting genes (121). Future drug safety tests should include measures of DNA demethylation (121).

Clinical Trials with DNA Methylation Inhibitors

Several clinical trials have been launched with a nucleoside-analog pan DNMT inhibitor 5-azaC and its deoxy analog 5-deoxycytidine (DAC). Responses with tolerable adverse effects were reported in clinical trials in hematological malignancies, especially in myelodysplastic syndrome (MDS) (122). However, there was no significant success reported in solid tumors (123). The weak response of solid tumors might result from pharmacokinetic issues such as delivery problems, as well as dosing and scheduling. Different strategies for combining 5-azaC with other chemotherapeutic agents or chromatin modifiers such as HDACis are now being tested and might be effective in solid tumors (124).

Basic questions regarding the mechanism of action of 5-azaC need to be answered. Although the basic hypothesis is that 5-azaC causes demethylation and reexpression of silenced tumor suppressor genes, this has not yet been proven in the clinic. 5-azaC might be acting through methylation-independent mechanisms, through induction of damage response pathways, or via toxicity associated with incorporation into DNA to induce tumor suppressor genes. It is unclear whether its clinical activity is a result of DNA methylation-independent activities mediated through 5-azaC binding of DNMT1 and other DNMTs, nor is it clear what specific DNMT isoforms are responsible for the anticancer activity of 5-azaC. Identifying the critical DNMT isotype involved would guide the development of isotypic-specific DNMT inhibitors. Understanding why 5-azaC is effective in hematological cancers is critical for developing second-generation potent

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and less toxic DNA methylation inhibitors. These unresolved issues also have implications for the dosing and scheduling of 5-azaC. Under the supposition that 5-azaC causes demethylation at low doses, whereas it is mainly toxic at high doses, researchers in recent trials have focused on doses of 5-azaC that are well below the maximum tolerated dose (MTD) (122). These trials showed better responses than previous trials, but there was no immediate correlation between the response past a given threshold and the extent of demethylation. The response was not correlated with the presence of a hypermethylated *P15* prior to treatment, which served as a readout for the state of tumor suppressor gene methylation (122). In contrast to the hypothesis of low-dose 5-azaC for anticancer activity, a recent animal study showed that 5-azaC dose intensification increased 5-azaC antineoplastic activity (125). The issue of scheduling and dosing is a critical issue that needs to be resolved in animal testing and further clinical trials.

The only isotypic-specific DNMT1 inhibitor tested in clinical trials is MG98, a second-generation antisense oligonucleotide that specifically targets DNMT1 mRNA (126). The mechanism of action of this class of inhibitors is different from catalytic inhibitors of DNMT1. This agent eliminates the expression of DNMT1 protein entirely and thus targets all functional activities of DNMT1, including methylation-independent activities. Knockdown of DNMT1 results in inhibition of DNA replication (127), triggering of damage response (113), and induction of tumor suppressor genes (112). The immediate blockage of replication by DNMT1 knockdown dramatically limits the demethylation induced by DNMT1 inhibition, thus avoiding the potential deleterious impact of global demethylation (113). Knockdown of DNMT1 is devoid of the adverse effect of global hypomethylation. Other isotypic-specific DNMT inhibitors might exhibit different therapeutic effects in different conditions. They might enrich the arsenal and diversity of epigenetic drugs. The main issue with antisense oligonucleotides is delivery to solid tumors. Recently, the clinical trials of this class of drugs were stopped because of lack of objective response in the last phase II trials in metastatic renal cancer (128). Nevertheless, this strategy, as well as therapeutic siRNAs, carries great promise. Searching for agents that knock down DNMT1 rather than inhibit its catalytic activity is an alternative path to DNMT inhibitors that is worth pursuing.

Demethylation in Cancer and Other Diseases: Possible Adverse Effects of Inhibitors of DNA Methylation

One of the main adverse effects of catalytic inhibitors of DNA methylation enzymes is global hypomethylation. There are several lines of data to suggest that this is an undesired effect that might promote cancer metastasis and other disease states such as lupus and autoimmune disease. A recent study suggests that hypomethylation is systemic in certain cancers and could be detected even in cycling lymphocytes in bladder cancer patients (129). Demethylation activates metastatic genes such as *HEPARANASE* (130) and *uPA* and plays an important role in metastasis (131). Screens for hypomethylated genes in different cancers revealed several genes that were characteristically unmethylated in different types of cancer (132, 133). In addition to activation of gene expression through promoter demethylation, hypomethylation causes genomic instability (134) and unleashes the expression of repetitive sequences disrupting gene expression programming (135). Although knockout of *dnmt1* protected mice from colorectal cancer, *dnmt1* hypomorph alleles promoted thymic lymphomas in mice (136).

These data have important implications for DNA methylation therapy. Catalytic inhibitors of DNMTs that cause global hypomethylation, such as 5-azaC, and are now used in anticancer therapy might increase the propensity of cancer cells to metastasize. We have recently shown that treatment of non-invasive breast cancer cells with 5-azaC induces demethylation and expression of

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prometastatic genes, and stimulates invasiveness (137). It is important to carefully examine whether a similar increase in metastases might occur in current clinical trials and to be cognizant of this possibility in new treatments with 5-azaC. If 5-azaC is found to stimulate metastasis in humans, this should prompt an effort to develop other classes of DNMT inhibitors, either isotypic-specific inhibitors that do not induce metastatic genes, or agents that knock down the DNMT1 protein or its interaction with the replication fork, as discussed above. DNMT inhibition is a powerful strategy to block deregulated growth of cancer cells and is worth pursuing, but it is necessary to accomplish this while avoiding the adverse effects of global hypomethylation. In this respect, it is critical to carefully examine and map the changes in gene expression profile in response to knockdown of each of the different DNMT1 isotypes.

Blocking Demethylation as a Therapeutic Strategy

An interesting therapeutic implication of the global hypomethylation observed in cancer is that inhibitors of global hypomethylation might serve as cancer therapeutics (138). It is clear, however, that we need a better understanding of the processes leading to demethylation in cancer and that this is an important field of research that requires additional input.

Two different approaches were used to block demethylation in cancer. The first approach involved treatment with the methyl donor SAM. The common sense rationale behind using SAM is that SAM is a methyl donor of all DNMT reactions and increasing cellular levels of SAM would enhance the activity of DNMT, but this obviously holds true only if the cellular concentration of SAM is well below the Km for the different DNMTs. It is not clear that this is the case. Another proposed explanation is that increased SAM concentrations change the SAM:SAH ratio. SAH is a potent inhibitor of DNMT, therefore, by increasing SAM we reduce inhibition of DNMT and increase the rate of methylation (139). A third hypothesis is that SAM inhibits demethylation, thus tilting the equilibrium of the DNA methylation reaction toward methylation. SAM was shown to inhibit demethylase activity in vitro and in cells (68). SAM is highly unstable and it is not clear whether its in vivo activities are caused by SAM or by SAM metabolites such as 5′-methylthioadenosine (MTA) (140). MTA was recently shown to affect histone methylation as a HMTase inhibitor (141).

Notwithstanding the mechanism through which SAM induces genomic methylation, SAM was previously shown to be chemoprotective in a liver cancer model in rodents (140). In vitro treatment of human breast and prostate cancer cell lines with SAM resulted in inhibition of invasion in vitro, and metastasis and tumor growth when the cells were transplanted into nude mice in vivo (131, 142). These results call for an effort to develop SAM analogues with improved pharmacokinetics.

Another important line of investigation involves identifying proteins responsible for demethylation of metastatic genes in cancer and targeting them for inhibition. The MBD2 controversy focused on in vitro activity of MBD2 following in vitro translation of the recombinant protein (143). However, follow up data showed that transient coexpression of MBD2 and methylated promoters resulted in demethylation and activation of gene expression (144) and knockdown of MBD2-inhibited replication-independent active demethylation induced by valproate (69). Interestingly, ectopic expression of MBD2 in liver cells induced the expression of type II *HEXOKINASE*, a gene suppressed by methylation in normal liver cells and induced by demethylation in liver cancer cells (145).

Knockdown of MBD2 blocked tumor growth in vitro and in vivo (146, 147). Blocking MBD2 in breast and prostate cancer cell lines inhibits tumor growth, invasiveness, and metastasis in vivo (131, 142). Antisense oligonucleotides, siRNA inhibitors, and MBD2 antagonists are therefore potential promising antimetastatic candidates.

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DNA Methylation and Demethylation Inhibitors in Other Diseases

The brain is now a fertile ground for DNA methylation research. Emerging data suggest that both early and adult environments affect DNA methylation in the brain, and that DNA methylation is changing in a physiological timescale during memory acquisition, thereby illustrating its dynamic nature (148–150). There are data to suggest that increased DNMT1 expression and hypermethylation of *REELIN* in the cortex might be involved in schizophrenia (151–155). Animal models have provided evidence that hypermethylation of *reelin* could be reversed by pharmacological treatment with HDACi (16, 84). DNMT inhibitors might be of therapeutic utility in schizophrenia. However, the currently approved DNA methylation inhibitor 5-azaC requires DNA synthesis for its action. 5-azaC is a prodrug that has to be phosphorylated to the tri-nucleotide form and incorporated into DNA to trap the DNMT during progression of the DNA replication fork (114, 156) (**Figure 3**). Thus, 5-azaC seems to be of essentially no utility in the brain, where a vast majority of neurons are postmitotic and do not incorporate DNA. Surprisingly, several recent studies attempted to inhibit DNA methylation in the brain using 5-azaC (157), but if this was successful it must have been accomplished through a different, yet unknown mechanism. A possible strategy to achieve demethylation is using HDACis. Indeed, TSA (158), valproate (40), and a benzamide HDACi, MS-275 (16), induced demethylation in the brain (**Figure 2**). Nevertheless, it might be valuable to develop small-molecule DNMT antagonists, which do not require incorporation into DNA and could thus serve as DNA methylation inhibitors even in postmitotic tissues.

Autoimmune diseases are an example of a health state with documented involvement of hypomethylation. Hypomethylation of the DNA in T cells is believed to drive expression of antigens and other genes that stimulate the autoimmune response in lupus (159–162). It was recently shown that DNA in T cells from lupus patients were hypomethylated and, interestingly, the level of hypomethylation correlated with the levels of expression of MBD2 (163). MBD2 inhibitors might be of interest in the treatment of lupus and perhaps other autoimmune diseases. In addition, it might be worthwhile to test whether SAM or MTA would be effective. A similar approach might be of value in other autoimmune and hyperinflammatory diseases.

SUMMARY AND PERSPECTIVES

In summary, chromatin modification and DNA methylation and demethylation machineries are attractive therapeutic targets in cancer and other diseases, however, certain cardinal issues need to be addressed before the full potential in therapy is realized. First, the epigenetic machinery is complex. It is therefore important to understand the differential role of specific isotypes of all the participants (HDACs, HMTases, DNMTs, and demethylases) in the specific disease in question.

Second, disease-specific changes in DNA methylation or histone modification require targeting of histone and DNA modification enzymes to specific genes by specific factors. Targeting these factors is an interesting possibility for drug development.

Third, it is important to understand the exact mechanism through which certain DNA and histone modifying enzymes promote disease. Some of the epigenetic proteins such as DNMT1 are multifunctional proteins. The bona fide enzymatic function might not be exclusively involved in transformation. It is clear, for example, that DNMT1 is involved in cancer through DNA methylation-independent and -dependent mechanisms (95, 112).

Fourth, in addition to DNMTs, the DNA demethylation machinery is emerging as a new target for inhibition of metastasis, one of the most intractable facets of cancer, and for other diseases such as autoimmune disease. The fifth issue relates to the interrelationship between the DNA methylation and chromatin modification machineries. This has several implications. First,

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adverse and long-term effects through DNA methylation changes need to be considered. Second, HDACis could be used as a strategy to block DNA methylation in postmitotic tissues. Third, different combinations of histone and DNA modification inhibitors have synergistic effects, thus providing a promising approach in therapy (164).

Fourth, the emerging importance of DNA methylation in the brain and in mental health calls for the development of DNMT inhibitors that do not require DNA replication for their mode of action. Understanding how some epigenetic agents might act as psychiatric drugs is one of the most exciting new directions in epigenetics.

Although many questions remain open, the DNA methylation and chromatin modification machineries appear to be extremely important targets for novel therapeutics that are bound to have an impact on human disease. These classes of drugs will open new chapters in pharmacology and in our therapeutic arsenal.

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