

## DNA Methylation and Histone Modifications in Patients With Cancer

*Potential Prognostic and Therapeutic Targets*

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### Summary

Epigenetics, a combination of DNA modifications, chromatin organization, and variations in its associated proteins, configure a new entity that regulates gene expression throughout methylation, acetylation, and chromatin remodeling. In addition to silencing as a result of mutations, loss of heterozygosity, or *classical* genetic events epigenetic modification symbolizes essential early events during carcinogenesis and tumor development. The reversion of these epigenetic processes restoring normal expression of tumor-suppressor genes has consequently become a new therapeutic target in cancer treatment. Aberrant patterns of epigenetic modifications will be, in a near future, crucial parameters in cancer diagnosis and prognosis.

**Key Words:** Cancer epigenetics; histone modifications; DNA methylation; prognosis; therapy.

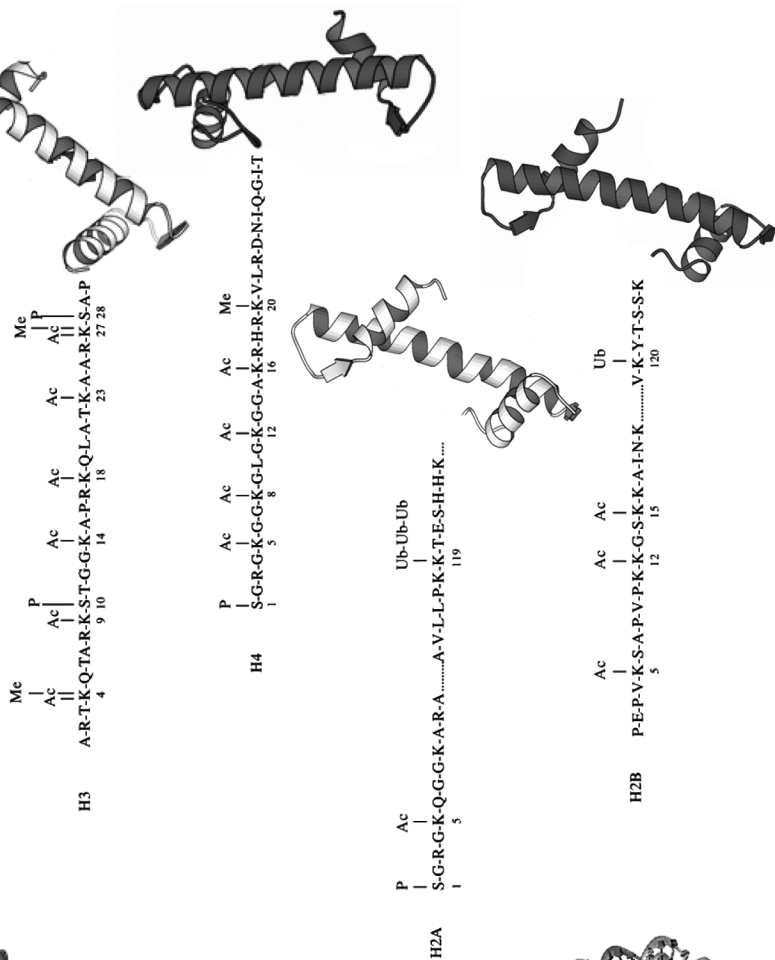
### 1. Introduction

Epigenetics is concerned with the inheritance of information on the basis of differential gene expression, a process separate from genetic inheritance through gene sequence. Epigenetic modifications do not transform the DNA sequence; however, they are heritable and important in gene expression. Different components comprise the safety of the epigenome. Chromatin organization plays an important role in gene-expression regulation by modifying the tertiary structure to an open or accessible (euchromatin) status or to closed and inaccessible configuration (heterochromatin). Nuclear DNA is packaged into nucleosomes, a protein complex around which the DNA helix is wrapped. A nucleosome is a

**A**



**B**



histone octamer core ( $[\text{H3-H4}]_2\text{-}2[\text{H2A-H2B}]$ ). Histone fold domain, present and described in these proteins, is completed by a basic N-terminal and a C-terminal tail (**I**) (**Fig. 1A**).

Remodeling chromatin can be completed in several and unified ways: (1) covalent modification of histones, (2) intrinsic DNA modification, (3) exchange core histones with mutant or modified histone variants, and (4) disrupting DNA–nucleosome contacts. Histone modifications in the basic N-terminal tails, including acetylation, methylation, ubiquitination, phosphorylation, biotinylation, and sumoylation (**Fig. 1B**) (**2–6**), result in changes in the cell transcriptional state. The best studied of these modifications is histone acetylation; this dynamic posttranslational modification is required for active chromatin configuration. Histone acetylation is a chemical equilibrium state. Acetylation is catalyzed by histone acetyltransferases (HAT), and deacetylation is catalyzed by histone deacetylases (HDAC) using acetyl-CoA as universal acetyl-donor (**Fig. 2**).

In general, increased levels of histone acetylation (hyperacetylation) are found in euchromatin, a more open conformation of the nuclear chromatin, in which transcription is held in an active state; however, decreased levels of acetylation (hypoacetylation) are found in the tightly compacted chromatin (heterochromatin) associated with transcriptionally silent genomic regions. Another important histone modification in gene expression is histone methylation. Histone methyltransferases direct site-specific methylation of amino acid residues such as lysine (Lys4 and Lys9) and arginine residues. Methylation of Lys4 is important in maintenance of euchromatin structures, where genes are freely accessible and usually active; in contrast, methylation of H3Lys9 is associated with heterochromatin domains, strap, and inactive (**7**). Lysines can be

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Fig. 1. (*Opposite page*) The histone octamer assembly in the nucleosome and modifications in histone tails. (**A**) Two molecules of each of the four core histone proteins form the histone octamer via formation of one tetramer of H3 and H4 and two dimers of H2A and H2B. Note that the nucleosome containing the two turns of DNA has the N-terminal tails of the eight-histone protein sticking out from the nucleosome like the legs of a spider. The structure of the portion of these N-terminal tails outside of the DNA is not known, and, more importantly, nor is the 30-nm chromatin fiber. Tetramerization occurs via interactions between the C-terminal halves of two histone molecules and results in a twofold axis of symmetry for the tetramer as shown. (**B**) Histone N-terminal tails are exposed from the nucleosomal interior into the aqueous surroundings. Moreover, these core histone tails have been found to be covalently modified; i.e., they can be methylated, acetylated, phosphorylated, and so on, on a single or on sets of N-terminally located serine and lysine residues. Me, methylated; Ac, acetylated; P, phosphorylated; Ub, ubiquitinated.

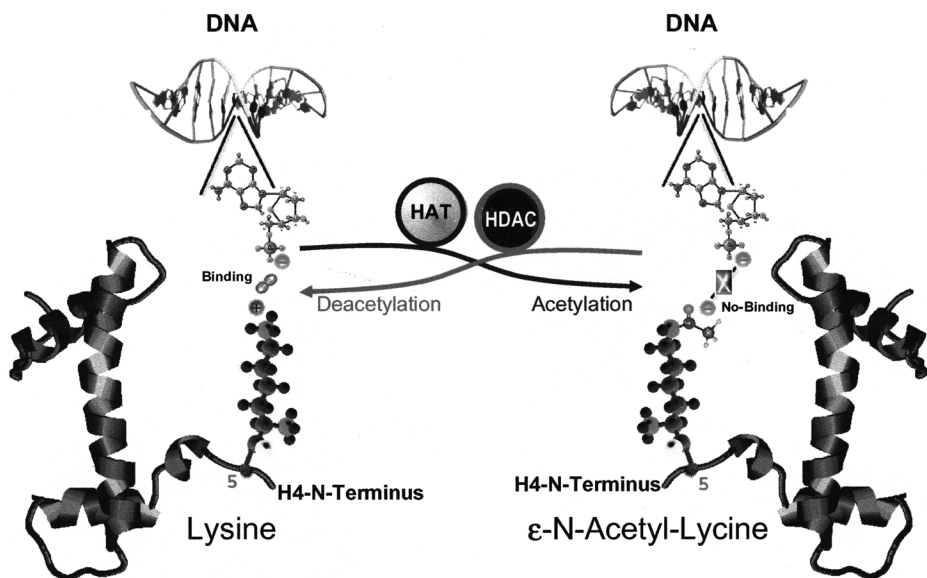


Fig. 2. Equilibrium of steady-state histone acetylation is maintained by opposing activities of histone acetyltransferases and deacetylases. Acetylation is a reversible process, in which histone acetyltransferases (HATs) transfer the acetyl moiety from acetyl coenzyme A to the  $\epsilon$ -amino groups of internal, highly conserved lysine residues. This modification neutralizes the positively charged lysine residues of the histone N termini causing a reduction in the affinity of histone–DNA interactions, leading to increased access of transcription factors to the repressed chromatin template. Therefore, in most cases, histone acetylation enhances transcription while histone deacetylation represses transcription. Histone acetylation is catalyzed by HATs and histone deacetylation is catalyzed by histone deacetylases (denoted by HDACs).

mono-, di- or trimethylated, whereas arginines can be mono- or dimethylated, increasing the complexity of histone modifications.

DNA methylation remains the best-studied epigenetic mechanism. Methylation is needed for the normal development of cells because it facilitates static long-term gene silencing and confers genomic stability (8). Abnormal methylation, which confers growth advantages, is tightly connected to cancer development (9). Methylation of cytosines within the CpG dinucleotide (60% of human genes contain a CpG island [10]) by transfer of a methyl group from the methyl donor *S*-adenosylmethionine to the carbon 5 position of cytosines (Fig. 3) is catalyzed by DNA methyltransferases: DNA methyltransferases 1 (DNMT1; responsible for DNA methylation maintenance during cell division, development, and cancer), DNMT3a, and DNMT3b (responsible for *de novo* methylation during early development) (11,12).

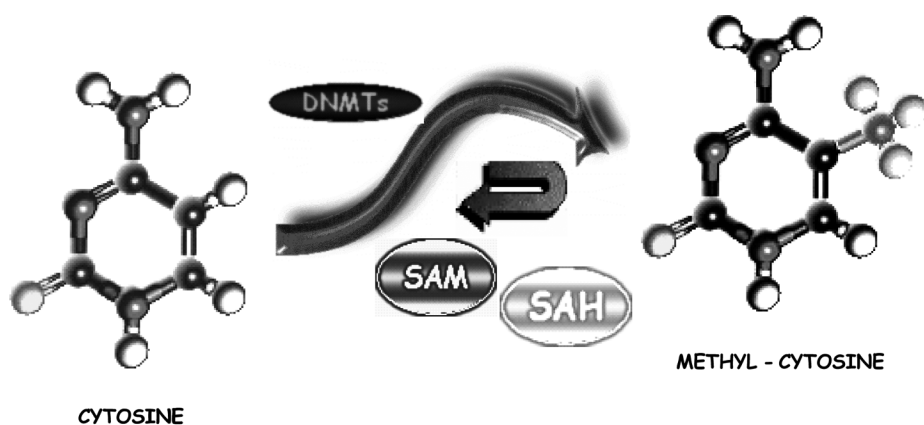


Fig. 3. Epigenetics refers to alterations in gene expression that occur without a change in DNA sequence. The chemistry behind one of these events is simple. It involves the covalent addition of a methyl group to cytosine. Of the four bases that make up DNA—adenine, thymine, cytosine, and guanine—only cytosine has the potential to be methylated in humans and most mammals. The methylation reaction involves flipping the target cytosine out of the intact double helix, so that the transfer of the methyl group from the methyl donor (*S*-adenosylmethionine) can take place. This reaction is catalyzed by the enzyme DNA methyltransferase (DNMTs). Methylation only occurs in cytosines whose 3'-carbon atom is linked by a phosphodiester bond to the 5'-carbon atom of a guanine (CpG dinucleotide). Most CpG dinucleotides are clustered in small stretches of DNA known as CpG islands.

Aberrant methylation patterns associated with cancer appear to be tumor-type specific (13,14).

It is well established that there is a good correlation between methylation state and histone modification. Genes that are methylated are usually related to deacetylated and inactive chromatin, whereas unmethylated promoters and active genes are associated with an open hypoacetylated euchromatin (15). This relationship was, at the beginning, unidirectional; DNA methylation determines histone acetylation status. The molecular event that validates this theory was the MeCP2 discovery. MeCP2 is a methylated DNA-binding protein (MBD) that recruits histone methyltransferase and histone deacetylase activity to the promoter regions of methylation-regulated genes (Jones 1998). However, now it seems to be a bidirectional control, chromatin inactivation recruits DNA methyltransferases to regulatory regions of genes (16,17).

On the basis of our current knowledge, the role of epigenetic events in cancer development, prognosis, and diagnosis is considered to be minor compared with those genetic events. However, nowadays new approaches to cancer therapy

**Table 1**  
**Epigenetic Diseases: Symptoms and Etiology<sup>a</sup>**

Disease	Symptom	Aetiology
ATR-X syndrome	Intellectual disabilities, $\alpha$ -thalassaemia	Mutations in <i>ATR</i> gene, hypomethylation of certain repeat and satellite sequences
Fragile X syndrome	Chromosome instability, intellectual disabilities	Expansion and methylation of CGG repeat in <i>FMR1</i> 5' UTR; promoter methylation
ICF syndrome	Chromosome instability, immunodeficiency	<i>DNMT3b</i> mutations, DNA hypomethylation
Angelman's syndrome	Intellectual disabilities	Deregulation of one or more imprinted genes at 15q11–13 (maternal)
Prader-Willi syndrome	Obesity, intellectual disabilities	Deregulation of one or more imprinted genes at 15q11–13 (paternal)
BWS	Organ overgrowth	Deregulation of one or more imprinted genes at 11p15.5 (e.g. <i>GF2</i> )
Rett syndrome	Intellectual disabilities	<i>MeCP2</i> mutations
$\alpha$ -Thalassaemia (one case)	Anaemia	Methylation of $\alpha$ 2-globin CpG island, deletion of <i>HBA1</i> and <i>HBQ1</i>
Rubinstein-Taybi syndrome	Intellectual disabilities	Mutation in CREB-binding protein (histone acetylation)
Coffin-Lowry syndrome	Intellectual disabilities	Mutation in <i>Rsk-2</i> (histone phosphorylation)

<sup>a</sup>Neurological diseases related with deregulation of imprinted genes, mutations in MBDs (methylation-binding domain proteins), HATs (histone acetyltransferases), or DNMTs (DNA methyl-transferases).

ATR-X syndrome.  $\alpha$ -thalassaemia, mental retardation syndrome; BWS, Beckwith-Wiedemann syndrome; CREB, cAMP-response-element-binding-protein; ICF, immunodeficiency, centromeric region instability, and facial anomalies syndrome; UTR, untranslated region; DNMT, DNA methyl transferase; FMRI, Fragile X mental retardation 1; HBA1, hemoglobin alpha 1; HBQ1, hemoglobin theta 1.

based on epigenetic therapies are emerging, demethylating agents and histone deacetylases inhibitors predominantly.

## 2. Epigenetic Diseases: The Neurological Achilles' Heel

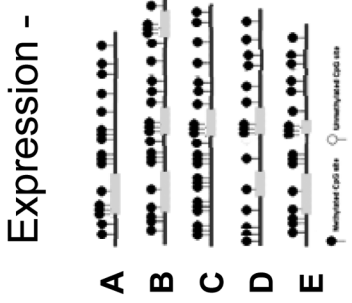
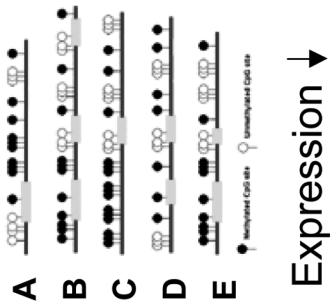
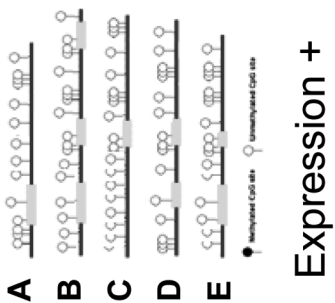
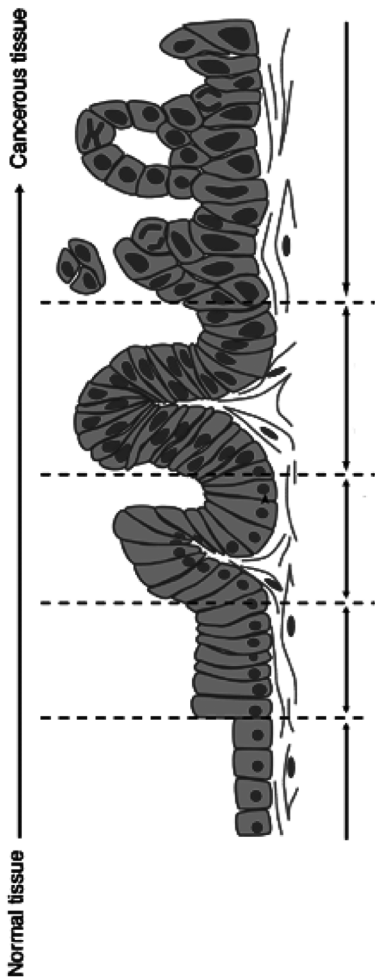
Mutations in genes that affect epigenetic profiles are inheritable or somatic acquired. Hereditary mutations in methyltransferases (DNMTs) or MBDs genes, are the phenomenon behind some human syndrome as ICF syndrome (DNMT3b mutation) or Rett syndrome (MeCP2 mutation). Curiously many of this disease results in mental retardation, chromosomal instability, and learning disabilities (**Table 1**). These new platforms of human disease could be considered as epigenetic diseases.

There are syndromes that result in deregulation of imprinted in cluster of same chromosomal location-genes as Angelman's syndrome (AS), a disorder that can be difficult to diagnose, particularly in the first few years of life. Approximately 70% of cases of AS have a deletion of 15q11-q13 in the maternally contributed chromosome. Main characteristics are developmental delay, functionally severe speech impairment, none or minimal use of words, receptive and nonverbal communication skills higher than verbal skills, movement or balance disorder (usually ataxia of gait and/or tremulous movement of limbs), behavioral uniqueness (any combination of frequent laughter/smiling), apparent happy demeanor, easily excitable personality (often with hand flapping movements), hypermotoric behavior, and short attention span.

With the same epigenetic root, but a clinically distinct disorder, is the Prader-Willi syndrome (PWS), a complex disorder, which diagnosis may be difficult to establish on clinical grounds and whose genetic basis is heterogeneous. Approximately 28% of cases of PWS are a result of maternal uniparental disomy. A disorder of chromosome 15 with a prevalence of 1:12,000–15,000 (both sexes, all races). The major characteristics of PWS are hypotonia, hypogonadism, hyperphagia, cognitive impairment, and difficult behaviors.

Finally in these examples, Beckwith-Wiedemann syndrome, and an overgrowth disorder. Wiedemann first recognized it in 1963, and in 1964 by Bruce Beckwith, a pediatric pathologist. Both doctors noted similar characteristics in their patients that were not traceable to other disorders, thereby identifying a new syndrome. The syndrome is usually sporadic, but may be inherited. These children are at risk for developing hypoglycemia and various types of tumors. The clinical picture of this syndrome can vary from mildly to greatly affected. The incidence of BWS has been reported as approx 1:15,000 births. However, exact figures of these kinds of syndromes are impossible to estimate, because so many mildly affected cases are not diagnosed.

Different collections of diseases are related to DNA methyltransferases mutations as ICF syndrome (immunodeficiency-centromeric instability-facial





anomalies) is transmitted as an autosomal recessive trait. It is characterized by immune deficiency in association with unstable paracentromeric heterochromatin instability (extensively related with hypomethylated genomic regions) and facial dysmorphism. Patients are affected by recurrent respiratory infections beginning in childhood. The syndrome directly results from mutations in the gene encoding for DNA-methyltransferase 3B. This may explain the hypomethylation in the pericentromeric repeats observed in the chromosomes of patients.

### 3. Cancer as Epigenetic Disease

A set of human cancers are developed by *de novo* methylations in genes, mainly tumor-suppressor genes, where promoter methylation diminishes or inhibits normal cell expression and thus confers a growth advantage to the tumor cell (**Fig. 4**). Huge expectations have been raised by the large amount of genetic information relating to cancer biology that has been assembled in the past two decades. CpG island hypermethylation of tumor-suppressor genes may be a valuable tool in the essential transfer of research from the “bench” to the “bedside.” The detection of hypermethylation is a “positive” signal that can be accomplished in the context of normal cells, whereas certain genetic changes such as LOH or homozygous deletions are not going to be detected in a background of normal DNA.

In recent years, several groups have extensively mapped from most classes of human neoplasia an increasing number of gene CpG islands aberrantly hypermethylated in cancer (**Table 2; [9]**).

Epigenetics can offer two components to the treatment of cancer: prognostic and predictive factors. Prognostic factors will give us information about the virulence of the tumors. For example, p16INK4a hypermethylation has been linked to tumor virulence in lung and colorectal cancer patients (**18**). The second component is the group of factors that predict response to therapy. For example, the response to cisplatin and derivatives may be a direct function of the methylation state of the CpG island of hMLH1 (**19**). Nevertheless, the most compelling evidence is provided by the methylation-associated silencing of the DNA repair methyltransferase (MGMT) in gliomas and lymphomas, which indicates patients who will be sensitive to chemotherapy with carmustine (BCNU)

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Fig. 4. (*Opposite page*) Epigenetic events in tumor progression, from normal epithelium (normal tissue) to dysplasia and carcinoma (cancerous tissue). Five methylation-controlled-genes (A–E) represented in their promoter regions: ●, methylated CpG; ○, unmethylated CpG. Normal tissue is represented by normal gene expression and an unmethylated state in tumor-suppressor gene promoters; during progression, expression decreases and hypermethylation in promoters increases. Finally, in a cancerous tissue, promoters are extensively methylated and expression is completely inhibited.

**Table 2**  
**Examples of Genes Exhibiting Aberrant Methylation in Cancer**

Evasion of apoptosis	APAF-1
	DAPK
	DLC-1
	p14ARF
	p53
	p73
	SHP1
	TMS1
	TRAIL-R1
	XAF1
Insensitivity to anti-growth signals	CyclinD2
	ER $\alpha$
	LOT1
	p15INK4b
	p16INK4a
	p27KIP1
	p57KIP2
	Pax5
	PTEN
	RAR $\alpha$
Limitless replicative potential	RASSF1A
	pRb
	CDX1
	GATA-4 and -5
	Myf-3
	SOCS-3
Angiogenesis	THBS1
	THBS2
	VHL
Intercellular adhesion and tissue invasion	ADAM23
	E-Cadherin
	H-Cadherin
	CLCA2
	CLDN-7
	laminin-5
	Maspin
	OPCML
	TIMP3
	SLIT2
DNA repair	MLH1
	MGMT
	BRCA1

(Continued)

(20) or cyclofosfamide (21). Three major clinical areas can benefit from hypermethylation-based markers: detection, tumor behavior, and treatment.

### 3.1. DNA Methylation

Epigenetic modifications of DNA do not alter the sequence but are hereditary and involved in gene regulation and transcription. DNA methylation is a very dynamic process but the regulation behind this mechanism is very tight. Aberrant methylation in the CpG island-containing-promoters of genes is usually correlated with gene silencing, however in some cases abnormal methylation patterns could be related to gene activation (22). Global DNA hypomethylation has been reported in several human diseases (13,20). Such global hypomethylations occur mainly in repetitive elements around pericentromeric areas. In cancer, global genome hypomethylation is a common finding but, generally, is associated with specific promoter hypermethylation (23,24). In normal mammalian cells, CpG islands in the regulatory regions of certain genes are not methylated, whereas CpG in the remaining genes are methylated by DNMT1. In cancer cells, global DNA hypomethylation and specific promoter hypermethylation occurs (Fig. 4) (25).

Aberrant patterns of DNA methylation appear to be affected in several pathways: *p53* is the most frequently mutated gene in human cancers, however, *p53* can also become inactivated through methylation-mediated silencing of the tumor-suppressor gene *p14ARF* (26–28), which normally inhibits MDM2, an oncogenic protein that induces *p53* degradation. Moreover, *p73*, a *p53* homolog, has been shown to be hypermethylated in leukemia (29). Hypermethylation of the cell-cycle inhibitor *p16INK4a*, a feature common to many tumors, enables cancer cells to escape senescence and begin to proliferate (30–32). The retinoblastoma gene (*RB*) and the cell-cycle inhibitor *p15INK4b* can also occasionally undergo aberrant methylation (33,34). DNA methylation has a major role in many repair pathways. The consequences of aberrant methylation of repair

**Table 2** (Opposite page)

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BRCA1, breast cancer 1; DAPK, death-associated protein kinase; E-cadherin, epithelial cadherin; GSTP1, glutathione S-transferase p1; MLH1, mutL homolog 1; MGMT, O(6)-methylguanine-DNA methyltransferase; pRb, retinoblastoma; RASSF1a, ras-association domain family 1A; VHL, von Hippel-Lindau; APAF-1, apoptotic protease activating factor 1; DLC1, phosphodynein on microtubules; SHP1, SH2 domain-containing tyrosine phosphatase; TMS1, target of methylation-induced silencing; TRAIL, type II integral membrane protein; XAF1, XIAP-associated factor 1 (XIAP, the X-linked inhibitor of apoptosis); ER, estrogen receptor; PLAGL1/LOT1/ZAC, pleomorphic adenoma of the salivary gland gene like 1; CDX1, homeobox protein CDX-1 (caudal-type-homeobox protein 1); SOCS3, suppressor of cytokine signaling 3; THBS1, thrombospondin 1 precursor; THBS2, thrombospondin 2 precursor; ADAM23, disintegrin and metalloproteinase domain 23; CAV1, caveolin 1; CLCA2, chloride channel, calcium activated, family member 2; CLDN7, claudin 7; OPCML, opioid-binding cell adhesion molecule; TIMP3, tissue inhibitor of metalloproteinase 3; RFC, reduced folate carrier.

pathways include microsatellite instability in sporadic colorectal (35,36), endometrial (37,38), and gastric (39) tumors, owing to silencing of the DNA mismatch repair gene *hMLH1*; mutations in *K-RAS* and *p53* caused by hypermethylation of the O6-methylguanine-DNA methyltransferase promoter (40); the prevention of the removal of methyl groups at the O6 position of guanine (41,42); hypermethylation of the mitotic checkpoint gene *CHFR* (43); and inactivation of *BRCA1* in breast and ovarian tumors (44), which prevents the repair of DNA double-strand breaks and causing global gene-expression changes similar to those present in carriers of *BRCA1* germline mutations (45). Other targets of aberrant methylation, the aberrant methylation of androgen receptors occurs in breast and uterus tumors, may render cancer cells unresponsive to treatment with steroid hormones. Some of the other genes that are affected by DNA methylation are the proapoptotic death-associated protein kinase (DAPK), a target of methylation-induced silencing gene; the von Hippel-Lindau gene in kidney tumors and hemangioblastomas; *LKB1/STK11* (a serine-threonine kinase) in hamartomatous neoplasms, the RAS-related gene, *RASSF1*; thrombospondin 1 (an antiangiogenic factor); cyclo-oxygenase 2; *TPEF*, which comprises epidermal growth-factor domains; and glutathione-S-transferase P1 (an electrophilic detoxifier) in tumors of the prostate, breast, and kidney (Table 2).

### 3.2. Histone Acetylation

Chromatin remodeling also plays an important role in the regulation of expression of certain genes. The basic unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped around a histone octamer. Modification of the N-terminal group of lysine in histones by acetylation or deacetylation changes the configuration of nucleosomes. The positive charge on unacetylated lysines in the histones is attracted to the negatively charged DNA producing a compact chromatin state that is repressive for transcription. On the other hand, acetylation of the lysines by histone acetylase removes their positive charge and results in an open chromatin structure, which facilitates gene transcription. HDAC removes the acetyl groups from lysine, which reverses this process and silences gene expression (Fig. 2). Aberrant deacetylation of histones in nucleosomes is probably a result of a dysregulation of the specificity of HDAC and may be associated with neoplastic transformation. For example, gene translocations in some types of leukemia can generate fusion proteins that recruit HDAC and bind to promoters to silence genes involved in differentiation (46).

For many years, epigenetic research focused on DNA methylation; now, a critical role in epigenetic gene control is assigned to histone modifications. Histone tails are targets for covalent posttranslational modifications, such as acetylation, methylation, and phosphorylation (47). Hypoacetylation of histone-3 and -4 are usually associated with transcriptionally inactive genome regions

inside a global structure called heterochromatin. Acetylation levels and acetylation states are regulated by equilibrium of HAT and HDAC (48,49).

### 3.3. Histone Methylation

In addition to acetylation of H3 and H4 tails, methylation in lysine residues (Lys4 and Lys9) of histone 3 has been described (Fig. 1B). The methylation of lysine in histones by specific histone methylases is also implicated in changes in chromatin structure and gene regulation. The methylation of lysine-4 in histone-3 is associated with an open chromatin configuration and gene expression. On the other hand, the methylation of lysine-9 in histone-3 is associated with condensed and repressive chromatin. This histone modification and the acetylation/deacetylation of histones to influence gene expression are called the histone code (47). A hypermethylated promoter is surrounded by methylated lysine-9 in histone-3, whereas an unmethylated promoter is surrounded by methylated lysine-4 in histone-3 (50). Treatment of tumor cells with 5-AZA reduces the level of methylated lysine-9 in histone-3 and increases the level of methylated lysine-4 in histone-3 in the promoter region of genes silenced by aberrant DNA methylation (51).

As an example of cooperativity between chromatin modifications, it is interesting to note that Lys9 in histone-3 is acetylated in euchromatin (active state for gene transcription) but appears to be methylated in regions of gene-expression silencing (52). Methylated K4 and K79 and acetylated K9 and K14 of H3 are associated with transcriptionally active regions. H4 methylated at K20 is present in heterochromatin regions (53). Histone methylation is catalyzed by histone methyltransferases, a family of proteins with affinity for lysines and arginines. Recent studies demonstrate that peptidyl arginine deiminase 4 (PADI4) specifically deiminates arginine residues R2, R8, R17, and R26 in the H3 tail. This deimination by PADI4 prevents arginine methylation by CARM1. These results define deimination as a novel mechanism for antagonizing the transcriptional induction mediated by arginine methylation. (54). Histone methylation could be involved in the replacement of histones during transcription core dislodging (55). Some histone methyltransferases important in cancer are EZH2 (H3K27 histone methyltransferase), overexpressed in prostate and breast cancer (56), and SMYD3 (H3K4 histone methyltransferase) overexpressed in colorectal and hepatocellular carcinomas (57). Notably, there is a dynamic relationship between DNA methylation and histone modifications. Low levels of histone acetylation and H3K9 methylation recruit DNMT1 and DNA methylation to regulatory regions.

## 4. Epigenetic Therapy of Cancer

Tumorigenesis is known to be a multistep process in which defects in various cancer genes accumulate (58,59). It is now clear that genetic alterations in human cancers will not provide a complete answer of genomic alterations

behind tumor development, progression, or metastasis. Epigenetic factors cause changes in mechanisms contributing to the malignant phenotype (**Fig. 4**). Epigenetic causes of human diseases, especially of cancer, has given impetus to the development of new therapies for reversing the processes involved. Inhibitors of DNA methylation were the first molecules to appear on the market. To date, several compounds that inhibit DNA methylation are being used in both in vitro and in vivo studies. Clinical trials have shown an incredible decrease in global methylation and specifically demethylation of tumor-suppressor-promoter-CpG-island cancer cells, recovery of the normal expression levels of these genes, and restoration of the normal phenotype. In this field, compounds such as 5-azacytidine, 5-aza-2'-deoxycytidine, zebularine, procainamide, and so on are emerging as powerful and nontoxic tools for cancer therapy.

Knowledge of CpG-island hypermethylation of tumor-suppressor genes may be an important tool in the essential transfer of research from laboratory to clinical practice. In contrast to genetic markers, in which mutations occur in various sites and can be of very different types, promoter hypermethylation occurs only within CpG islands. Furthermore, hypermethylation is a positive signal that can be observed at background levels in normal cells, whereas particular genetic changes, such as loss of heterozygosity and homozygous deletions, cannot be detected so easily. The impetus for DNA methylation studies in cancer has come from two sources. The first is the identification of well-recognized tumor-suppressor genes that undergo methylation-mediated silencing in human cancer, e.g., *BRCA1*, *hMLH1*, *p16INK4a*, *VHL* (**Table 3**) (**60,61**). The second is the emergence of a new technology to study DNA methylation, one based on bisulphite modification coupled with PCR techniques (**62**).

The study of pigenetic silencing in the last years has been on histone modifications, acetylation, methylation, and phosphorylation of histone tails, but it has already begun a shift to new transcription regulation mechanisms, those catalyzed by two groups of proteins HATs and HDACs. As a result, inhibitors of HDACs are growing as a promising therapeutic compound; inhibition of deacetylation as “word play” increases acetylation levels and maintains or remodels the chromatin to an open or gene-activation state. Some of these newly activated-genes are tumor-suppressor genes and cancer-negative-selected genes. HDAC inhibitors reduce cell growth and induce differentiation and apoptosis. Some of the classic and commonly used compounds in this field are butyric acid, valproic acid, suberoylanilide hydroxamic acid (SAHA), depsipeptide, and so on.

Links between DNA methylation and histone acetylation necessarily favor dual therapies, combining DNA methylation inhibitors with HDAC inhibitors. This synergy was profoundly studied in combinations of 5-AZA-CdR and trichostatin A (TSA) (**63–65**). Four major clinical areas can potentially benefit from hypermethylation-based markers: neoplasm detection, studies of tumor

**Table 3**  
**Hypermethylated Genes in Cancer, Role in Tumor Development and Tumor Type**

SITE	GENE: role in tumor development
Brain	<i>MGMT</i> : DNA repair and drug resistance
Breast	<i>APC</i> : cytoskeletal reorganization <i>BRCA1</i> : DNA repair <i>E-Cadherin</i> : proliferation, invasion and metastasis <i>ER</i> : hormone response <i>GSTP1</i> : detoxification <i>RASSF1A</i> : control of cell proliferation
Colon	<i>hMLH1</i> : DNA mismatch repair
Endometrium	<i>hMLH1</i> : DNA mismatch repair
Esophageal	<i>APC</i> : cytoskeletal reorganization
Gastric	<i>E-Cadherin</i> : proliferation, invasion and metastasis <i>hMLH1</i> : DNA mismatch repair
Head and Neck	<i>p16</i> : cyclin dependent kinase inhibitor
Kidney	<i>RASSF1A</i> : control of cell proliferation
Leukemia	<i>p15</i> : activation of cell proliferation
Lymphoma	<i>p15</i> : activation of cell proliferation
Lung	<i>APC</i> : cytoskeletal reorganization <i>p16</i> : cyclin dependent kinase inhibitor <i>DAPK1</i> : Suppression of apoptosis. <i>MGMT</i> : DNA repair and drug resistance <i>RASSF1A</i> : control of cell proliferation
Nasopharyngeal	<i>RASSF1A</i> : control of cell proliferation
NHL	<i>p16</i> : cyclin dependent kinase inhibitor
Oligodendroglioma	<i>Rb</i> : DNA replication and cell division
Ovarian	<i>BRCA1</i> : DNA repair <i>hMLH1</i> : DNA mismatch repair <i>RASSF1A</i> : control of cell proliferation
Prostate	<i>ER</i> : hormone response <i>GSTP1</i> : detoxification
Retinoblastoma	<i>Rb</i> : DNA replication and cell division
Renal	<i>GSTP1</i> : detoxification <i>VHL</i> : RNA stability
Squamous cell carcinoma	<i>p15</i> : activation of cell proliferation
Thyroid	<i>E-Cadherin</i> : proliferation, invasion and metastasis

APC, adenomatous polyposis coli; BRCA1, breast cancer 1; CDKN2A/p16, cyclin-dependent kinase 2A; DAPK1, death-associated protein kinase 1; ER, estrogen receptor; GSTP1, glutathione S-transferase Pi 1; hMLH1, mut L homolog 1; MGMT, 0-6 methylguanine-DNA methyltransferase; RASSF1, ras-association domain family member; Rb, retinoblastoma; VHL, von Hippel-Lindau; GIT, gastrointestinal tract; NHL, non-Hodgkin's lymphoma.

**Substances that reduce DNMTs expression :** antisense DNMTs cDNA oligonucleotides and constructs

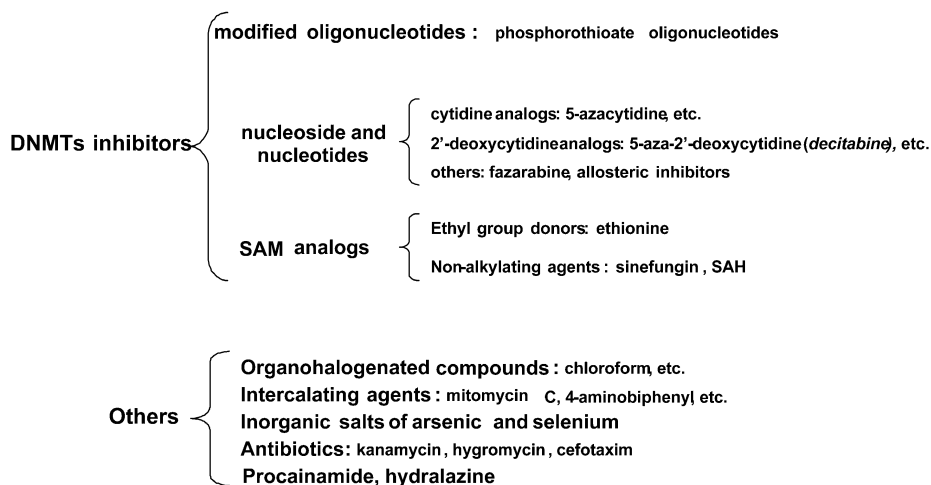


Fig. 5. DNA methylation inhibitors may be used in cancer therapy to modulate hypermethylation of genes and to reactivate antiproliferative, apoptotic, and differentiation-inducing genes in cancer cells. Although some compounds have been proposed for use as DNA methylation inhibitors, these compounds are chemically instable, have weak potency, and can generate toxic metabolites, thus preventing their use as therapeutic agents. Compounds are divided in three categories; substances that directly reduce DNMTs (DNA methyl-transferases), expression, and inhibitors of DNMT activity and others.

behavior, prediction of treatment response, and the development of therapies that target methylated tumor-suppressor genes.

#### 4.1. DNA Methylation in Cancer Therapy

Epigenetic modifications are reversible, whereas genetic modifications are not. This feature makes epigenetic modifications a target for new human therapies. Demethylating agents are “on the crest of the wave” in pharmaceutical development of portfolio molecules (Fig. 5).

For several years we have been able to reactivate hypermethylated genes in vitro. One obstacle to the transfer of this technique to human primary cancers is the lack of specificity of the drugs used. Since demethylating agents such as 5-azacytidine or 5-aza-2-deoxycytidine (decitabine) (66) inhibit DNMTs and cause global hypomethylation, we cannot reactivate only the particular gene we are targeting. New chemical inhibitors of DNA methylation are being introduced, such as zebularine, and provide us with more hope, but the nonspecificity problem persists. If we consider that only tumor-suppressor genes are



hypermethylated, this would not be a great problem. However, we do not know if we have disrupted some essential methylation at certain sites, and global hypomethylation may be associated with even greater chromosomal instability (67). Another drawback is the toxicity to normal cells, a phenomenon that was in fact observed with the initial higher doses. However, these compounds and their derivatives have been used in the clinic with some therapeutic benefit, especially in hematopoietic malignancies (68,69).

Methylation-associated silencing affects many genes in all existing cellular pathways (13,61). As examples of DNA methylation markers of poor prognosis, we can mention that the death-associated protein kinase, p16INK4a hypermethylation, has been linked to tumor virulence in lung and colorectal cancer patients (61). Not all hypermethylation events are bad: in neuroblastoma, the CpG island hypermethylation of *HOXA9* is associated with poor survival, but the hypermethylation of *RARB2* is an excellent marker of good outcome (70).

However, one of the most attractive possibilities is the establishment of clusters of CpG island hypermethylation in human tumors with prognostic value (70). Studying more than 150 neuroblastomas and using an unsupervised hierarchical cluster analysis of all tumors based on methylation of 10 genes, we separated the three clinically relevant groups of tumors (70). CpG island hypermethylation has been used as a tool to detect cancer cells in broncoalveolar lavage (71), lymph nodes (72), sputum (73), urine (74), semen (75), ductal lavage (76), and saliva (77). Thus, we have shown its versatility across multiple tumor types and environments (Table 4).

It was possible to screen for hypermethylated promoter loci in serum DNA from lung cancer patients (78), as well as from a broad spectrum of tumor types (79,80), some screening even using semiquantitative and automated methodologies. The detection of DNA hypermethylation in serum or biological fluids of cancer patients (and even patients at risk of cancer) should encourage academic, governmental, and private agencies to create consortiums of different institutions (and even countries) to develop comprehensive studies to validate the use of these markers in the clinical environment. CpG island hypermethylation could be used as a predictor of response to treatment. The methylation-associated silencing of the DNA repair MGMT in human cancer provides the most compelling evidence. The MGMT protein (O6-methylguanine DNA methyltransferase) is directly responsible for repairing the addition of alkyl groups to the guanine base of the DNA (81). MGMT-promoter hypermethylation predicts a good response to chemotherapy, greater overall survival, and longer time to progression in glioma patients treated with BCNU (20). The potential of MGMT methylation to predict the chemoresponse of human tumors to alkylating agents is not limited to BCNU-like alkylating agents; it also extends to other drugs such as cyclophosphamide (21). This has been demonstrated in diffuse large

**Table 4**  
**Detection of Cancer in Body Fluids Using DNA Methylation as Marker<sup>a</sup>**

SPECIMEN	TUMOR TYPE	GENE	METH. (%)
Serum/plasma	Prostate	GSTP1	72
		APC	47
	NSCL	p16	73
		DAPK	73
		MGMT	73
		GSTP1	73
		p16	23
	Colorectal	p16	38
	Esophageal	APC	27
	Liver	p15	81
	Head and neck	p16	42
		DAPK	42
		MGMT	42
		GSTP1	42
Urine	Prostate	GSTP1	36
Ejaculates	Prostate	GSTP1	50
Sputum	NSCL	CDKN2A	50
Ductal lavage	Breast	Cyclin D2	85
		RAR-β	85

<sup>a</sup>Tumor type, gene involved, specimen of fluid, and percentage of methylation.

Meth., methylation; GSTP1, glutathione *S*-transferase Pi 1; APC, adenomatous polyposis coli; CDKN2A/p16, cyclin-dependent kinase 2A; DAPK1, death-associated protein kinase 1; MGMT, O-6 methylguanine-DNA methyltransferase.

cell lymphomas treated with cyclophosphamide, where MGMT hypermethylation was the strongest predictor of overall survival and time to progression, and was far superior to classic clinical factors such as the international prognostic index (21). Finally, gene inactivation by promoter hypermethylation may be the key to understanding the loss of hormone response of many tumors. The inefficacy of the antisteroids estrogen–progesterone–androgen-related compounds such as tamoxifen, raloxifene, or flutemide, in certain breast, endometrial, and prostate cancer cases may be a direct consequence of the methylation-mediated silencing of their respective cellular receptors (*ER*, *PR*, and *AR* genes) (61,82).

Reactivating genes with DNA demethylating agents is an encouraging discovery with respect to avoiding toxic effects. However, it is important to note that the hypermethylation of CpG islands occurs in conjunction with the action of methyl-binding proteins, histone hypoacetylation, and histone methylation, which all contribute to formation of a closed chromatin state and transcriptional silencing (83). Several clinical trials to study these and other mechanisms in patients with cancer are underway in United States and Europe. In such studies, it is essential that the clinical and molecular parameters of response are well defined. Quantitative measurement of 5-methylcytosine DNA after treatment, by use of high-performance capillary electrophoresis (84,85), is an excellent surrogate marker to validate efficacy, as well as demethylation of CpG islands in tumor-suppressor genes, such as p15INK4b (86).

#### 4.2. DNA Methylation Inhibitors as Therapy

Demethylating agents such as 5-aza-cytidine or 5-aza-2-deoxycytidine inhibit DNA methyltransferases and cause global hypomethylation (87). Furthermore, the demethylating effect of 5-aza-2-deoxycytidine seems to be universal, affecting all human cancer cell lines (68). New inhibitors of DNA methylation are being introduced, e.g., procainamide, but the issue of nonspecificity still persists (Fig. 5). Another problem is that at high doses, these agents seem to have toxic effects on normal cells. But despite their drawbacks, these compounds and their derivatives have achieved some therapeutic success in the clinic, especially in hemopoietic disorders such as myelodysplastic syndrome and acute myeloid leukemia (68,69).

One of the most promising clinical scenarios for the use of demethylating drugs is acute promyelocytic leukemia (APL), which is largely caused by transcriptional disruption induced by the PML-RAR $\alpha$  translocation. Combined treatment with inhibitors of histone deacetylases, inhibitors of DNA methylation, and differentiating factors (arsenic trioxide may have all three functions) has achieved moderate success in several patients with APL (88). 5-Aza-2'-deoxycytidine alone can also induce the reexpression of silenced, but not hypermethylated, tumor-suppressor genes, such as the proapoptotic gene *APAF1* (89). Although the mechanisms underlying this effect are not fully understood, this drug is known to have additional cytotoxic effects, other than those resulting from demethylation, which potentiate the killing capabilities of demethylating compounds, and thus increase their effectiveness in cancer treatment.

The discovery that lower doses of 5-azacytidine associated with inhibitors of HDACs may also reactivate tumor-suppressor genes was encouraging (63). Several phase I trials to test this strategy in human cancer patients are underway. 5-Aza-2'-deoxycytidine alone can even induce reexpression of certain silenced tumor-suppressor genes that do not have an apparent CpG island

hypermethylation, such as *APAF-1* (89). These new findings have proved very attractive to several pharmacological and biotech companies, and they are now studying how to accomplish demethylation of cancer cells using novel approaches such as antisense constructs or ribozymes against the DNMTs.

### **4.3. Histone Acetylation Platform for Cancer Therapy**

The core histones are N-terminal tails covalently modified by acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and biotinylation (3,90,91). Modifications of specific histone residues (Lys, Arg, Ser) are essential for specific proteins interactions important in gene regulation, chromatin condensation, and remodeling and structure (92). Chromatin-modifying enzymes are necessary to generate an open chromatin conformation that permits transcription factors and cofactors positive accessibility to target sequences. Modification of the highly charged lysine or arginine residues in the N-terminal histone tails is one mechanism of remodeling control (Figs. 1 and 2). These residues are susceptible to modifications by acetylation and/or methylation (47). This phenomenon appears to be controlled by a set of new emerging enzyme termed as HDACs, together with the ongoing family of HATs.

#### *4.3.1. HDACs as Therapeutic Targets*

HDACs modify chromatin by removing acetyl groups from N-terminal tail of histones and from other proteins such as p53 or tubulin (93,94). The HDAC family is divided in Zn-dependent (class I and II) and Zn-independent (class III) enzymes. Class I and II are the most extensively studied inside the HDAC family proteins. Expression profile of each member is tissue dependent and disease-dependent, critical aim in the development of new therapeutic strategies. Abnormal expression of HDACs is frequent in hematological malignancies. RAR-PML, for instance, could recruit HDACs and cause transcriptional repression and no differentiation (95).

#### *4.3.2. HDACs as Therapeutic Molecules*

The importance of histone modifications in cancer is illustrated by the marked antitumor activity of different inhibitors of HDAC, both in animal models and in preliminary clinical trials (46,96). The molecular mechanism of action of HDAC inhibitors is related to their activation of a subset of genes that can produce cell cycle arrest and induce differentiation or apoptosis in tumor cells (46,96).

Inhibition of HDAC includes natural and synthetic molecules. The naturally occurring antifungal antibiotic TSA was one of the first HDAC inhibitor compounds identified as having antiproliferative activity. Agents identified as HDAC inhibitors can be divided into different structural categories: hydroxamates (such as SAHA and TSA), short-chain fatty acids (such as valproic acid),

cyclic peptides (such as depsipeptide), and benzamides (MS-275). HDAC inhibitors cause differentiation, cell-cycle arrest in G1 and/or G2 and apoptosis in cultured transformed cells and tumors in animals that arise from both hematological and solid tumors. The mechanisms of genetic silencing by HDACs are associated with activation of selected genes (97). Activation of these silenced genes by inhibition of HDACs contribute to repression of tumor cell growth. In practice, the results of treatment with HDAC inhibitors differ by cell type in having both activate and repressive effects (98,99). However new hypotheses about the mechanism of HDAC inhibitors involvement in cancer are emerging; for example, in HepG2 cells, HDACs increased p21WAF1/CIP1 expression not through changes in chromatin structure or by enhancing promoter activity, but by mRNA stabilization (100). All these findings together indicate that HDAC inhibitor treatment results in changes in chromatin structure and an increase of susceptibility to transcription factors, RNA polymerase, or topoisomerases and mRNA stabilization.

Naturally occurring and synthetic HDAC inhibitors are now of interest to pharmaceutical companies because of their great potential use against cancer and other human pathologies. These compounds can be classified according to their chemical nature and mechanism of inhibition as follows (Fig. 6):

#### 4.3.2.1. HYDROXAMIC ACIDS

This is probably the broadest set of HDAC inhibitors. Most of the chemicals in this group are very potent but reversible inhibitors of class I/II HDACs. Among these compounds we find TSA, which was one of the first HDAC inhibitors to be described (101) and is widely used as a reference in research in this field. However, its toxicity to patients and lack of specificity for certain HDACs has motivated the search for other substances. The design of many synthetic drugs has been inspired by TSA structure: from the simplicity of SAHA to the latest drugs including NVP-LAQ-824 (102,103) and PXD-101 (104).

#### 4.3.2.2. CARBOXYLIC ACIDS

There are few drugs in this group: butanoic (105), valproic (106,107), and 4-phenylbutanoic (108). Despite being much less potent than the hydroxamic acids and their pleiotropic effects, these are currently among the best studied HDAC inhibitors: valproic acid and phenylbutyrate have already been approved for use in treating epilepsy and some cancers, respectively, whereas butanoic acid is undergoing clinical trials (109,110).

#### 4.3.2.3. BENZAMIDES

MS-275 and some of its derivatives inhibit HDACs in vitro at micromolar concentrations, but the mechanism is not clearly understood. MS-275 and *N*-acetyldinaline are undergoing clinical trials (111–113).

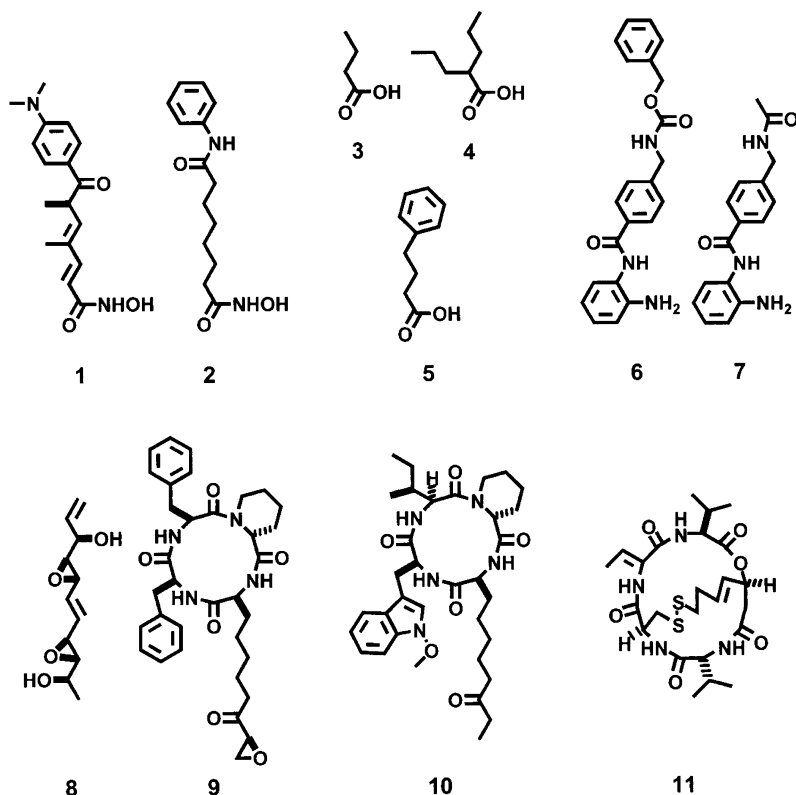


Fig. 6. Examples of chemicals included in the different HDAC inhibitor groups (*see* text). (1) TSA, (2) SAHA, (3) butanoic acid, (4) valproic acid, (5) 4-phenylobutanoic acid, (6) MS-275, (7) N-acetyldinaline, (8) depeudecin, (9) trapoxin A, (10) apicidin, and (11) depsiptide FK228.

#### 4.3.2.4. EPOXIDES

The only HDAC inhibitors in this set of compounds are a number of natural products with significant *in vitro* activity, such as depeudecin, trapoxin A (**114**).

#### 4.3.2.5. OTHERS

Depsiptide FK228 (a fungal metabolite) is also undergoing clinical trials, but the mechanism by which it inhibits classical HDACs *in vitro* remains unknown. Apicidin A is another fungal metabolite that is able to inhibit HDACs in many organisms, from protozoa to humans, at micromolar concentrations. Apicidins B and C (also natural products) have the same structure, differing from apicidin A by a single residue. Trapoxins are also cyclic tetrapeptides that are closely related to apicidins. However, the main difference between the two

**Table 5**  
**Mammalian HATs and HDACs<sup>a</sup>**

HDAC	HAT
<b>Class I</b>	<b>GNAT family</b>
HDAC1	PCAF
HDAC2	GCN5L2
HDAC3	<b>CREBBP family</b>
HDAC8	CREBBP
<b>Class II</b>	EP300
HDAC4	<b>MYST family</b>
HDAC5	HTATIP
HDAC6	ZNF220
HDAC7	HB01
<b>Sirtuins</b>	MORF
SIRT1	MYST1
SIRT2	<b>TAFII 250 family</b>
SIRT3	TAFII 250
SIRT4	<b>SRC family</b>
SIRT5	ACTR
SIRT6	SRC1
SIRT7	SRC3
	NCOA2
	<b>Other HATs</b>
	TCF2
	GTF3C1

<sup>a</sup>HATs are divided into six different families (GNAT, CREBBP, MYST, TAFII, SRC, and others) and HDAC in three classes (class I, class II, and sirtuins).

ACTR, activin receptor; CREBBP, CREB-binding protein; EP300, e1a-binding protein p300; GCN5L2, general control of amino-acid synthesis 5-like 2; GNAT, GCN5-related acetyltransferase; GTF3C1, general transcription factor 3c, polypeptide 1; HAT, histone acetyltransferase; HBO, histone acetyltransferase binding to ORC; HDAC, histone deacetylase; HTATIP, HIV tat interactive protein; HIV tat, human immunodeficiency virus type I transacting transcription factor; MORF, MOZ-related factor; MOZ, monocytic leukemia zinc finger protein; MYST, MOZ, YBF2/SAS3, SAS2, TIP60 protein family; NCOA2, nuclear receptor coactivator 2; ORC, original recognition complex; PCAF, EP300/CREBBP-associated factor; SIRT, sirtuin; SRC, steroid receptor coactivators; TAF, TATA box-associated factors; TCF2, transcription factor 2; ZNF220, zinc finger protein 220.

groups of substances is that the former bears epoxyketone functionality rather than an alkylketone functionality, which makes the compound much less stable under physiological conditions (*115–118*).

#### 4.3.3. HATs as Therapeutic Targets

The addition of acetyl groups from universal donor acetyl-CoA on lysine residues placed on histone tails is catalyzed by HATs (**Table 5**). Thus, histone

tails acetylation is the best-characterized of histone modifications, demonstrating a positive association between acetylation levels and gene expression profiles. In addition, acetylated histones mean actively transcribed regions of chromatin (119). HATs represent an active group of proteins important in replication, apoptosis, repair, and cell cycle. This crucial function in several cellular mechanisms makes HAT damage an important step in human diseases. In cancer, hematological malignancies with chromosomal translocation express chimeric HAT proteins that gain functions (120). In such solid tumors as breast, colon, and gastric cancers, mutation in HAT genes have also been reported (119). Nonfunctional CBP is the main cause of the Rubinstein-Taybi syndrome (121) and is in the clinical etiology of neurological disorders such as Huntington disease, Alzheimer's disease, and muscular atrophy (119,122).

HATs specific inhibitors or activators remain on portfolio molecules although much is known about substrates and mechanisms. To date, few molecules have been identified without clinic applicability (Lysyl.CoA, H3-CoA-20, and anacardic acid [120]).

## 5. Epigenetic Diagnosis

Such mapping of DNA methylation has highlighted the existence of a unique profile of hypermethylated CpG islands that defines each tumor type (Table 2) (60,61). Several groups are currently attempting to define the DNA methylation signature (methylo type) of each type of human cancer. Only methylation markers that are always unmethylated in normal cells can be used for methylation profiling, but by combining three or four methylation markers, we can extract the greatest possible amount of useful information, because hypermethylation events at different loci are unrelated (60).

For epigenetic markers to be clinically useful, ways of detecting hypermethylation in the CpG islands of tumor-suppressor genes that are quick, non-radioactive, and sensitive are required, such as methylation-specific PCR (123). Methylation-specific primers should be developed in stringent conditions with the inclusion of positive and negative controls to avoid false-positive results. CpG island hypermethylation has been used as a tool to detect cancer cells in several types of biological fluids and biopsy samples. It was possible to screen for hypermethylated promoter loci in DNA from the serum of patients with lung cancer (78). Thus, DNA hypermethylation has proved its applicability in the detection of wide range of tumor types.

The promoter hypermethylation of CpG islands in tumor-suppressor genes occurs early in tumorigenesis. But the presence of aberrant CpG island methylation alone does not necessarily indicate an invasive cancer because premalignant or precursor lesions can also carry this epigenetic marker. This finding has implications for early detection of cancer, especially in people with a high inherited



risk, because patterns of CpG island hypermethylation are the same between familial and sporadic cancers (124). Aberrant DNA methylation has been found up to 3 yr before diagnosis of lung cancer in individuals, such as uranium miners and smokers, who have been exposed to large amounts of carcinogens (73).

Standardization and validation of techniques for detecting changes in methylation are vital. Detection of DNA hypermethylation in the biological fluids of patients with cancer and those at risk of cancer should lead to comprehensive studies to justify the use of these markers in the clinic, through the establishment of multidisciplinary consortiums (Table 4).

One of the most important steps for conferring on CpG island hypermethylation a critical role in the origin and progression of a tumor is the demonstration of biological consequences of the inactivation of that particular gene. A good example is provided by the DNA repair genes *hMLH1*, *MGMT*, and *BRCA1*, in which methylation-associated inactivation may change the entire genetic environment of the cell. In the first case, there is a lack of mutations in the mismatch repair genes in sporadic tumors, and the main cause of the presence of microsatellite instability in the sporadic cases of colorectal, endometrial, and gastric cancer is the transcriptional inactivation of *hMLH1* by promoter hypermethylation (125,126). In the second case, the DNA repair gene O6-methylguanine DNA *MGMT* removes the promutagenic O6-methylguanine from the DNA. However, the DNA repair gene *MGMT* can be transcriptionally silenced by promoter hypermethylation in primary human tumors (127). Most importantly, these *MGMT*-methylated tumors accumulate a considerable number of G-to-A transition mutations, some of them affecting key genes such as *K-ras* and *p53* (81). Finally, in the case of *BRCA1*, its hypermethylation-associated inactivation (44) produces the same profound disruption of expression profiles as do the *BRCA1* germline mutations (128).

One of the most critical steps in giving CpG island methylation of a particular gene its true value is the fact that it should occur in the absence of gene mutations. Both events (genetic and epigenetic) abolish normal gene function and their coincidence in the same allele would be redundant. There are multiple examples but three are worth mentioning. First, the cell cycle inhibitor p16INK4a in one allele of a few colon and bladder cancer cell lines has a genetic mutation while the other is wild-type: p16INK4a hypermethylation occurs only on the wild-type allele, whereas the mutated allele is kept unmethylated (129,130). A second example is that of APC, the gatekeeper of colorectal cancer, which is mutated in the vast majority of colon tumors. When APC methylation occurs in that type, it is clustered in the APC wild-type cases (131). Finally, in tumors from families that harbor a germline mutation in tumor-suppressor genes, only those tumors that still retain one wild-type allele undergo CpG island hypermethylation (124).

## 6. Epigenetic Prognosis

The most compelling evidence for predicting treatment response is provided by the methylation-associated silencing of O6-methylguanine-DNA methyltransferase. This protein is responsible for the removal of alkyl groups from guanine, which is the preferred point of DNA attack of several alkylating agents used in cancer treatment, such as carmustine, nimustine, procarbazine, streptozotocin, and temozolamide. Thus, tumors that lack function of O6-methylguanine-DNA methyltransferase owing to hypermethylation (**40**) are more sensitive to the action of alkylating agents, because there is no pathway to repair the damage these drugs cause.

In a study of patients with glioma who were treated with carmustine, we found that hypermethylation of the O6-methylguanine-DNA methyltransferase promoter was indicative of a good response to chemotherapy, greater overall survival, and longer time to progression (**20**). The potential of O6-methylguanine-DNA methyltransferase for predicting the response of tumors to chemotherapy is not limited to carmustine-like alkylating agents, but also extends to drugs such as cyclophosphamide (**61**). This capability has been shown for diffuse large-cell lymphomas treated with cyclophosphamide, where hypermethylation of O6-methylguanine-DNA methyltransferase was the strongest predictor of overall survival and time to progression, and was far better than classic clinical factors such as the international prognostic index (**121**). More studies are needed to clarify this issue as the findings may have a direct effect on treatment of cancer.

Gene inactivation by promoter hypermethylation may be a crucial step in the loss of hormone responsiveness of many tumors. The lack of effectiveness of antisteroidal drugs, such as tamoxifen, raloxifene, and flutemide, in some patients with breast, endometrial, and prostate cancer may be a direct consequence of methylation-mediated silencing of their respective cellular receptors. A similar explanation can be applied to the lack of success with preventive retinoid treatment. It may be that premalignant lesions become insensitive to retinoids because of epigenetic silencing of genes that are crucial to the retinoid response, particularly the retinoic acid receptor 2 (RAR 2) (**132,133**), and the cellular retinol-binding protein I (CRBPI) (**134**). We have shown that supplementation of dietary retinoids prevents the aberrant methylation of RAR 2 and CRBPI in colorectal tumorigenesis (**134**); DNA demethylating drugs can be given, if necessary, to improve treatment.

## 7. Clinical Applications

Most current DNA-demethylating agents (**Fig. 5**) block the action of DNMTs (**135**). The cytidine and 2-deoxycytidine analogs of cytosine are the most extensively studied drugs. The first analog tested to determine whether

it was an inhibitor of DNA methylation was 5-azacytidine. The second analog reported was 5-aza-2-deoxycytidine (decitabine), one of the most commonly used demethylating drugs in assays with cultured cells. All of these compounds only inhibit DNMTs when incorporated into double-strand DNA (135). Zebularine (1-[beta-D-ribofuranosyl]-1,2-dihydropyrimidin-2-one) is another cytidine analog that has recently been developed (136,137). Perhaps the most interesting feature of this DNA-demethylating agent, compared with 5-azacytidine and 5-aza-2-deoxycytidine, is that it is chemically stable and of low toxicity, being the first drug in its class that can be given orally (136,137). The use of the nucleoside analogs in clinical trials has been limited by their side effects, such as thrombocytopenia and neutropenia, which are probably a result of cytotoxic effects associated with the drug's incorporation into the DNA independently of their DNA-hypomethylation value. This has encouraged the search for inhibitors of DNA methylation that are not incorporated into DNA. In this category, the drugs procainamide and procaine, approved by the FDA for the treatment of cardiac arrhythmias and as a local anesthetic, respectively, also act as nonnucleoside inhibitors of DNA methylation (138,139). The demethylating effect of 5-aza-2-deoxycytidine seems to be universal, affecting all human cancer cell lines (85). This is the conclusion that may be drawn from cancer-cell-line and mouse-tumor models, although we really do not know the molecular and cellular responses of cancer patients in their entirety.

Two phases in the clinical use of DNA-demethylating agents can be outlined. The first was during the 1970s and 1980s when high, and frequently significantly toxic, doses of 5-azacytidine and 5-aza-2-deoxycytidine were used to treat leukemia. At this time, their hypomethylating properties had not been fully recognized. The second period is marked by the acceptance of the idea that low doses of these drugs will induce cell differentiation and stop the growth of cancer cells by restoring the expression of silent tumor-suppressor genes.

Several phase I/II trials have been developed for solid tumors. However, it is in the field of hematological malignancies where DNA-demethylating agents have had their greatest success so far. Studies have found overall response rates of 40–54%, with 23–29% complete responses using 5-aza-2-deoxycytidine (decitabine) in myelodysplastic syndrome (68,69,140). For 5-azacytidine, a similar scenario can be drawn with a significant number of complete and partial remissions in myelodysplastic syndrome patients (141,142). The definitive support for an epigenetic treatment of hematological malignancies was provided in 2004, with the approval by the FDA of the use of 5-azacytidine (Vidaza) for the treatment of all myelodysplastic syndrome subtypes (<http://www.fda.gov/bbs/topics/news/2004/NEW01069.html>).

**Table 6**  
**Histone Deacetylase Inhibitors in Preclinical and Clinical Development<sup>a</sup>**

Clinical Trials	HDAC Inhibitor	Family
+	Acid bishydroxamic	Hydroxamic acid
+	Acid (CBAH)	Hydroxamic acid
	Oxamflatin	Hydroxamic acid
+	Suberoylamilide	Hydroxamic acid
	SAHA	Hydroxamic acid
+	Trichostatin a (TSA)	Hydroxamic acid
+	Butyrate	Short-Chain-Fatty-Acid
+	Valproic acid	Short-Chain-Fatty-Acid
	Phenylacetate	Short-Chain-Fatty-Acid
	Apicidin	Cyclic Tetrapeptide
	Trapoxin A	Cyclic Tetrapeptide
+	Depsipeptide	Tetrapeptide
	Depudecin	Epoxide
+	MS275	Benzamide
+	LAQ824	

<sup>a</sup>Histone acetylation and deacetylation is modulated by the interplay between histone acetyltransferases (HATs) and histone deacetylases (HDACs), and imbalances of this process cause multisystem diseases and cancers. Biochemical structures are indicated. “+” Denotes a phase I or II clinical trials.

It is clear from in vitro and preclinical studies that HDACs have great potential as anticancer drugs, but their value will be established by the ongoing clinical trials (**Table 6**).

Multiple phase I and phase II clinical trials with many HDACs have now been completed, and others are being initiated. A phase I trial for depsipeptide in patients with postthymic lymphoma unresponsive to chemotherapeutic regimens showed partial and complete clinical responses with minimal side effects (**143**). Another phase I trial of depsipeptide in patients with refractory neoplasms yielded biologically active serum concentrations of the drug and provided a recommended dose for the phase II trials (**144**). Clinical trials of valproic acid (Mount Sinai School of Medicine), MS-275 (National Cancer Institute), and SAHA (Memorial Sloan-Kettering Cancer Center) are currently in their final stages. In the case of SAHA, a phase I trial has shown that is well

tolerated, induces histone acetylation and has antitumor activity in solid and hematological tumors (145).

Clinical trials have also been undertaken to examine the combination of HDAC inhibitors with DNA-demethylating agents, such as decitabine (5-azacytidine). A treatment scheme for acute myelogenous leukemia (AML) patients entailing subcutaneous injections of 5-azacytidine for seven consecutive days followed by 5 d of iv phenylbutyrate was well tolerated, and a reduction in bone marrow blasts and increased myeloid maturation were observed (146). A similar study initiated at the Johns Hopkins University of patients with myelodysplastic syndrome and AML also indicated good tolerance, and significant hematopoietic improvements were observed in several patients.

## 8. Future Directions

The main goal in biomedical cancer research is to find therapies that can reverse silencing in human diseases. Epigenetic therapy places new drug discovery in a critical role. Here, it is important to remember that epigenetic diseases are developed by abnormal hypermethylation of CpG island-containing-promoters that seem to be particularly frequent in cancer cells, so DNA demethylating agents or histone deacetylating inhibitor compounds specifically targets cancer cells and certain kind of cancers. Now is just the beginning of our understanding of epigenetic causes of human diseases but is at the same time the beginning of new families of antitumoral compounds. There is much to learn about enzymes involved in the epigenetic pathways and the tight regulation of their activities, but this question will provide new opportunities for cancer therapy. The list of all possible histone modifications is not yet complete. In the future, the manipulation of the epigenetic landscape may indeed prove to be a key element of cancer therapy.

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