

EPIGENETIC AND HEMATOLOGICAL MALIGNANCIES

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RESUMEN •

En el 2003 las neoplasias hematológicas condicionaron 106,200 casos nuevos en los Estados Unidos. En México, constituyeron aproximadamente el 5.6% de las 56,213 muertes relacionadas a cáncer en el 2001. Aún cuando han surgido nuevos fármacos en las últimas décadas y se han diseñado nuevos esquemas para el tratamiento de estas neoplasias, ajustados de acuerdo con factores pronósticos, los resultados obtenidos hasta ahora distan de ser los óptimos. Por lo anterior, era indispensable redirigir la investigación de esta área. En la actualidad se conoce que los cambios epigenéticos, principalmente la metilación y desacetilación de histonas, son pasos definitivos involucrados en la carcinogénesis. Esto se demuestra por el desequilibrio presente en la metilación de citosinas en diferentes tumores humanos; se sabe que hasta el 90% de las neoplasias hematológicas tienen por lo menos un gen metilado. Se ha descrito un patrón de metilación similar en leucemias/linfomas diferente del señalado para el mieloma múltiple. En esta revisión analizamos los cambios epigenéticos conocidos durante la génesis y transformación de padecimientos hematológicos, así como también el tratamiento epigenético estándar actual, particularmente en síndromes mielodisplásicos.

Palabras clave: Neoplasias Hematológicas, Epigenética, Metilación.



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ABSTRACT •

HEMATOLOGICAL MALIGNANCIES conditioned nearly 106,200 new cases in the United States of America in 2003. In Mexico, they constituted approximately 5.6 % out of 56,213 cancer related deaths in 2001. Although new drugs have emerged in the last decades, and combined schedules were developed in an effort to treat such malignancies, even after being adjusted by prognostic factors, the results of the chemotherapy regimens are not optimal. Therefore, a different research approach was mandatory. Up to date, it is known that epigenetic changes, mainly DNA methylation and histone deacetylation, are definitive steps in carcinogenesis. This is demonstrated by the finding of an imbalance on cytosine methylation in human neoplasms, in fact 90% of hematologic neoplasms show at least one gene methylated. A similar DNA methylation pattern has been described in lymphomas/leukemias, but it is different in multiple myeloma. In this review we analyzed the epigenetic changes known to occur during the genesis and transformation of hematological diseases, as well as, the current standard of care with epigenetic therapy, particularly in myelodysplastic syndromes.

Keywords: Hematological Neoplasms, Epigenetic, Methylation.

HEMATOLOGICAL MALIGNANCIES •

Hematological malignancies conditioned 106,200 new cases in the United States of America, and were diagnosed as lymphoma (61,000), leukemia (30,600) or multiple myeloma (14,600) in 2003. These neoplasms were in such country the second most common cause of cancer deaths, affecting approximately 57,000 people in the same year (1). In Mexico, hematological neoplasms constituted approximately 5.6% from 56,213 cancer related deaths, and the acute lymphoblastic leukemia was the most frequent neoplasm in pre-scholar and < 14 years patients (2).

Lymphoid/ hematopoietic disorders are divided primarily into lymphomas, leukemias and plasma cell dyscrasias. Lymphomas are further classified into two major subtypes, Hodgkin's lymphoma and non-Hodgkin lymphoma, whereas plasma cell dyscrasias are classified into two major subtypes: a) multiple myeloma and related malignancies, and b) monoclonal gammopathy of undetermined significance (MGUS); finally, leukemias are also divided into two major subtypes, acute and chronic leukemia, and the first group is subclassified as primary or secondary/treatment-related, when it is developed either after alkylating treatment or it is the transforming result from a myelodysplastic syndrome (3)

EPIGENETICS •

Epigenetic changes have been a focus for research in all medical areas. They are defined as alterations taking place during development and cell proliferation, without any change in gene sequence, but do modify gene expression. They play a major role in a diversity of biological processes such as embryonic development, cancer biology, and immune system response, among many others. The two most widely studied epigenetic changes are DNA methylation and histone acetylation. (4,5).

DNA METHYLATION •

Genomic methylation is essential for healthy cells and organs. If methylation patterns are not prop-

erly established or maintained, disorders as diverse as mental retardation, immune deficiency, and sporadic or inherited cancers may follow. (6). Therefore, DNA methylation constitutes one of the most commonly occurring epigenetic events taking place in mammalian genome. This change, although heritable is reversible (5).

DNA methylation is a covalent chemical modification that occurs at the cytosine ring, resulting in the addition of a methyl (CH₃) group at the carbon 5 position. The human genome is not methylated uniformly and contains regions of unmethylated segments interspersed with the methylated region.

In contrast to the remainder of the genome, smaller regions of DNA, called CpG islands have distinctive properties. These regions are unmethylated, and GC-rich. Approximately one half of all genes in humans have CpG islands, and these are present in both housekeeping genes and genes with tissue-specific patterns of expression (4).

DNA methylation is brought by a group of enzymes known as DNA methyltransferases (DNMT). The DNMTs known to date are DNMT1, DNMT1b, DNMT1o, DNMT1p, DNMT2, DNMT3A, DNMT3b. Additionally, the other machinery of methylation includes demethylases, methylation centers, triggering DNA methylation, and methylation protection centers (6,7).

Gene silencing by DNA methylation has been considered to be permanent in non-embryonic cells, only reversible pharmacologically during cell division. Interestingly, new findings in lymphocytes may challenge this paradigm of irreversibility (8).

ACETYLATION •

Chromatin is made up of nucleosomes, which are particles consisting of DNA associated with an octamer of two molecules each of the core histone proteins (H2A, H2B, H4 and H4), around

which 146 base pairs of DNA are wound. Chromatin structure is plastic, and chromatin remodeling can lead to activation or repression of transcription. In vivo, histone acetylation depends on the balance between the enzymes with histone acetylase activity and enzymes that deacetylate histones, histone deacetylase (HDAC). Acetylated histones associate preferentially with transcriptionally activated chromatin; such histone acetylation may decrease the affinity of histone binding to DNA through partial neutralization. Histone acetylation may also facilitate binding of transcription factors to the promoters and disrupt higher order chromosome structure, promoting transcription. Agents that inhibit HDACs lead to maintenance of histones in the hyperacetylated state and promote transcription of a variety of genes. In the resting cell, DNA is wound tightly around these basic core histones, excluding the binding of the enzyme RNA polymerase II, which activates the formation of messenger RNA. This conformation of the chromatin structure is described as closed, and is associated with the suppression of gene expression (9). Gene transcription only occurs when the chromatin structure is opened up, with unwinding of DNA so that RNA polymerase II and basal transcription complexes can now bind to the naked DNA to initiate transcription (10).

Histone acetylation regulates many chromosome functions, such as gene expression and chromosome segregation. Addition of charge-neutralizing acetyl groups to lysine residues on histones disrupts interactions with DNA, resulting in chromatin decompaction, greater access of DNA to transcription factors, and the presence of a transcriptionally active genomic locus. In general, increased levels of histone acetylation (hyperacetylation) are found in more decondensed euchromatin, whereas decreased levels of acetylation (hypoacetylation) are characteristic of more condensed heterochromatin (4).

HDACs play a critical role in the suppression of gene expression by reversing the hyperacetylation

of core histones. Eleven HDACs that deacetylate histones are now recognized in mammalian cells and are classified into two major classes. Class I includes HDAC1, 2, 3, 8 and 11, which bear significant homology to the yeast protein RPD3 and are mainly localized to the nucleus. Class II includes HDAC4, 5, 6, 7, 9 and 10, which are homologous to yeast HAD-1-like enzymes and shuttle between nucleus and cytoplasm. Class I HDACs are widely expressed and are found in most cell types, whereas class II HDACs appear to have a more restricted distribution and may be involved in cellular differentiation. Some HDACs also deacetylate non-histone proteins such as α -tubulin, p53, p65 and MyoD. There is evidence that these different HDACs target different patterns of acetylation and regulate different genes. The different HDACs are also likely to be regulated differently. HDACs interact with corepressor molecules, such as nuclear receptor corepressor, ligand-dependent corepressor, NuRD and mSin3, all of which aid HDACs in gene repression and may provide specificity by selecting which genes are switched off by HDAC (10).

As well as histones, other transcription factors, such as GATA3 and the p65 component of NF- κ B, are targets for acetylation and deacetylation, which thereby modulate their transcriptional activity. Thus, HDACs are also associated with inactive p65 and play a role in the regulation of NF- κ B-mediated gene transcription without altering DNA binding. CBP acetylates specific lysine residues on p65, increasing its binding to DNA and causing transcriptional activation. HDACs reverse this process; HDAC1 and HDAC2 are able to deacetylate acetylated NF- κ B and promote its association with the inhibitor I κ B within the nucleus, in order to promote export into the cytoplasm and, thus, terminate the activity of NF- κ B.

A third class of deacetylases are the atypical nicotinamide adenosine dinucleotide-dependent sirtuins. These proteins deacetylate nonhistone proteins and are thought to play a role in programmed cell death in mononuclear cells (10).

EPIGENETICS AND MALIGNANCIES •

The underlying basis of cancer is a cumulative series of genetic and epigenetic alterations leading to deregulated cell growth. Particular alterations may provide a selective growth advantage to the tumor cell, whether by conferring resistance to therapies, increasing positive growth signals through the activation of oncogenes, or eliminating growth limiting signals through the inactivation of tumor suppressor genes (5). “Mutations” outside the nucleotide sequence occur frequently in human cancer and may contribute to the initiation and malignant progression of tumors. Although genetic mutations involving cytosine methylation were first observed in primary cancers nearly two decades ago, like most controversial ideas in science, it has taken a while to catch on. (6). The fact that changes in the epigenome are potentially reversible makes them important targets for therapeutic intervention, and an exciting goal will be to identify those key steps at which it is possible to reprogram a cancer cell to terminally differentiate or apoptosis rather than proliferate.(11).

DNA METHYLATION AND CANCER •

Abnormalities in DNA methylation have long been associated with cancer. Both hypo- and hypermethylation play a prominent role in carcinogenesis, and their contribution shows scarcely defined boundaries. In cancer cells, both alterations coexist: malignant tumors show global hypomethylation and regional hypermethylation. Whether one must precede the other or whether both should start at the same time remains to be elucidated (4).

Methylation pattern defects include genome wide hypomethylation and localized aberrant hypermethylation of CpG islands. These imbalances can be present together in a single tumor, though the net effect is usually a decrease in total methylation levels. Whether genome hypomethylation and CpG island hypermethylation are linked by a common underlying mechanism or result from distinct abnormalities in the cancer cell is currently unknown.

However, we do know that hypomethylation and hypermethylation occur at specific but distinct sites within the cancer cell genome, suggesting different etiologies. Both defects can precede malignancy, indicating that they are not simply a consequence of the malignant state (6).

Tumor suppressor genes have been described to acquire loss of function mutations or deletions leading to their inability to impede malignant transformation. Alternatively, epigenetic events, such as methylation, represent a distinct mechanism of tumor suppressor gene inactivation. Aberrant gene promoter methylation is associated with gene silencing and is functionally equivalent to a deleted gene.

Through inappropriate silencing of growth regulating genes and simultaneous instability of whole chromosomes, methylation defects help create a chaotic state from which cancer cells evolve. Methylation defects are present in cells before the onset of obvious malignancy and therefore cannot be explained simply as a consequence of a deregulated cancer cell. Researchers are now able to detect with exquisite sensitivity the cells harboring methylation defects, sometimes months or years before the time when cancer is clinically detectable (12).

Furthermore, aberrant methylation of specific genes has been directly linked with the tumor response to chemotherapy and patient survival. Advances in our ability to observe the methylation status of the entire cancer cell genome have let us to the unmistakable conclusion that methylation abnormalities are far more prevalent than expected. This methylomics approach permits the integration of an ever growing repertoire of methylation defects with the genetic alterations catalogued from tumors over the past two decades. (6).

An imbalance in cytosine methylation is prevalent in human sporadic cancers. Methylation may inactivate one or both alleles of the proven tumor suppressor genes in sporadic cancers and can potentially act as a second hit during the development of hereditary cancer. If methylation imbal-

ances contribute directly to tumor initiation, the alterations should occur in early stages of cancer or in premalignant cells. If the imbalance contributes directly to tumor progression, methylation defects should increase in frequency and/or severity coordinately with increasing malignancy grades. One might also expect that cells harboring functionally important methylation abnormalities could be selected in a manner consistent with the clonal evolution of cancer cells. Finally, there should be a mechanistic explanation linking the methylation change to malignant behavior. Available evidence from premalignant tissues, primary human tumors, and in vitro and in vivo models of cancer support these suppositions (6)

ACETYLATION AND CANCER •

Histone deacetylase inhibitors (HDACIs) are a new class of promising anti-cancer agents which inhibit tumor growth both in vitro and in vivo with very low toxicity toward normal cells. Recently, several HDAC inhibitors have entered Phase I and Phase II clinical trials and demonstrate encouraging anti-tumor activity in a variety of cancer types. The anti-tumor effect of HDACIs was proposed to result from accumulation of acetylated histones leading to activation of genes involved in inhibition of tumor cell growth. Altered activities of histone deacetylases or histone acetyl transferases are indeed involved in different human cancer. HDACIs mechanism of action appears to involve cell cycle arrest, induction of apoptosis and differentiation both in vitro and in vivo. The mechanisms of induction of apoptosis by HDACIs are cell type specific and involve the activation of the intrinsic apoptotic pathways (13).

EPIGENETICS AND HEMATOLOGICAL NEOPLASMS •

Histone acetylation status plays a key role in the regulation of gene transcription and is closely linked to DNA methylation. Decreased histone acetylation, i.e., by increased histone deacetylase activity (HDACs), may also lead to epigenetic silencing of tumor suppressor genes. Inhibition of histone

deacetylation constitutes a new interesting concept in the treatment of hematological malignancies.

It has been demonstrated that hematopoietic malignancies show a methylation profile, where 90 % of malignancies had at least one gene methylated, if compared with non malignant tissues samples. In particular five genes (CDH1, CDH13, CAPK, CRB1 and RARB) are frequently methylated in all tumor types. In general, the methylation patterns of lymphomas and leukemias are similar and have in > 20 % of cases the following methylated genes: CDH1, CDH13, DAPK, CRB1, p15, DcR1, RARB and TIMP3; whereas in multiple myeloma (MM), DcR1 and p16 are methylated at greater frequency, but TIMP3 is infrequently methylated (14).

Aberrant DNA hypermethylation is relevant for leukemogenesis and multiple genes have been evaluated in malignant hematological neoplasms (see Table 1). For instance, during the progression of chronic myelogenous leukemia (CML), the ABL1 promoter of the BCR-ABL fusion gene becomes significantly hypermethylated. An association be-

TABLE 1 •
Genes Hypermethylated in Acute Leukemia

Gene	Chromosome	Function	AML	ALL
ER- α	6q25	Estrogen receptor	70-90	90
E-Cadherin	16q22	Ca dependent intracellular adhesion	32-78	53
CALC 1	11p15	Ca bone resorbtion	50-90	45-90
HIC 1	17p13	Putative tumor suppressor gene	10	50-100
GPR 37	7q31	G-protein-coupled receptor	47	
MDR	7q21	Drug efflux	31	
MINT 1	5q13	CpG island hypermethylated in cancer	16	
MINT 2	2p22	CpG island hypermethylated in cancer	8	
MyoD	11p15	Muscle specific transcription factor	61	
p15	9p21	Cyclin dependent kinase inhibitor	30-90	
p16	9p21	Cyclin dependent kinase inhibitor	0	
PITX2	4q25	Homeotic gene	64	
PTC-A	9q22	WNT signaling	17	
PTC-B	9q22	WNT signaling	11	
SDC4	20q12	Surface heparin sulfate proteoglycan	56	
THBS1	15q15	Angiogenesis inhibitor	25	

AML= Acute Myeloid Leukemia; **ALL=** Acute Lymphoblastic Leukemia

tween methylation of the p15ink4b gene promoter and risk for AML transformation has been documented in MDS; hence, DNA hypermethylation has been suggested as one of the more important therapeutic targets in this disorder (15).

Hypermethylation of the calcitonin gene has been found in 65% of myelodysplastic syndromes (MDS), 43 in 95% of acute leukemias, and is associated with an unfavorable clinical outcome in acute lymphoblastic leukemia (ALL) (16).

Estrogen receptor methylation (ERM) is frequent in adult AML: In a series, (17) 61% of 261 patients had ERM values over 15% and were considered ERM+. ERM decreased with increasing age ($p=0.0001$) and its levels were significantly lower in patients with French-American-British subtypes M4 or M5 AML ($p=0.0019$). Whereas ERM was not associated with a reduced complete remission rate after induction therapy, ERM+ patients had significantly better overall and relapse-free survival.

When the methylation pattern of the CpG islands of the calcitonin, estrogen receptor, E-cadherin, p15, p16, Rb, GST-Pi, and HIC1 genes was investigated in the bone marrow from 9 controls and 20 AML patients, all the control samples were essentially unmethylated for all the eight tumor-related genes studied. In contrast, 19 of 20 (95%) AML samples had an abnormal methylation pattern in at least one gene, and 15 of 20 (75%) had abnormal methylation patterns in two or more of the target genes (15,18). In the same direction, mutations in PASG, a member of the DDM1 subfamily that facilitates DNA methylation, have been identified in 40- 60 % cases of acute myelogenous leukemia and acute lymphoblastic leukemia (19).

Thus, in a subset of AML cases, a methylator phenotype could be hypothesized. Methylation depends on several functional DNA methyltransferases, including DNMT1, DNMT3A, and DNMT3B (20). Mizuno described a 5.3-, 4.4, and 11.7-fold increase in DNMT1, 3A, and 3B, respectively, in AML when compared with normal

bone marrow (Mizuno, 2001). Although CML cells in chronic phase did not show significant changes, cells in blastic transformation showed 3.2-, 4.5-, and 3.4- fold increases in the levels of DNMT1, 3A and 3B, respectively. Using methylation-specific PCR, it was observed that the p15INK4B gene was methylated in 24 of 33 (72%) AML cases. Furthermore, AML cells with methylated p15INK4B tended to express higher levels of DNMT1 and 3B. Thus, DNMTs were substantially overexpressed in leukemia cells in a leukemia type- and stage-specific manner and may contribute to the pathogenesis of leukemia by inducing aberrant regional hypermethylation.

An elegant study by Di Croce established a link between genetic and epigenetic changes in leukemogenesis (21). The author demonstrated that in acute promyelocytic leukemia (APL) the leukemia-promoting PML-RARA fusion protein induces gene hypermethylation and silencing by recruiting DNMT1 and DNMT3A to the promoter of its target gene RAR β 2. A previous study showed that an additional oncogenic transcription factor induces aberrant hypermethylation of target gene promoters.

The mechanism of transcription repression exerted by PML-RARA was the recruitment of HDAC complex (22). Consistent with this finding, Di Croce showed that PML-RARA-induced repression of RAR β 2 was only partially relieved by either 5-aza-2'-deoxycytidine or TSA treatment of APL cells (21). Notably, only simultaneous treatment with 5-aza-2'-deoxycytidine and TSA completely restored RAR β 2 gene expression. Thus, all these observations, taken together, suggest the intriguing scenario in which the newly methylated CpGs become docking sites for methyl-binding proteins, which in turn interact with both HDAC complexes and DNMTs, finally leading to the spreading of hypermethylation to the neighboring DNA regions (23).

Myelodysplastic syndromes include a heterogeneous group of clonal myeloid stem cell disor-

ders characterized by peripheral cytopenias and dysplasia of bone marrow progenitor cells. This genetic disorder is characterized by cytogenetic abnormalities in approximately 50- 60 % of patients. A clonal evolution associated with progressive bone marrow failure and transformation to AML occurs in 10- 60 % of cases, depending of the MDS subtype and cytogenetic pattern, evolution that may be conditioned by p15 gene methylation (24). Recently, somatic loss of function-mutations in ATRX, and RAD54-like protein, have been identified in a rare form of myelodysplasia, which is a preleukemic blood disorder. Alpha thalassaemia myelodysplastic syndrome (ATMDS) is a clonal myeloid disorder associated with dramatic down regulation of alpha globin gene expression (25).

CLL is characterized by numerous genetic alterations including frequent deletions of chromosomal segments 13q14, 11q22-q23, 6q21, 17p13, and trisomy of chromosome 12. It is intriguing to speculate that hypermethylation and chromosomal instability are somehow correlated; however evidence from the literature linking these two events is rare. More convincing at this time is the finding of hypomethylation of centromeric and satellite repeat sequences, a phenomenon leading to chromatin decondensation and chromosomal fragility (6, 26). The methylation events that we have identified occur throughout the genome without preferential clustering and in line with a previous report on brain tumors demonstrating that aberrant methylation and genetic alterations do not coincide (27).

THERAPEUTICS •

Since, the reversible nature of the epigenetic aberrations constitutes a very attractive therapeutic target and as such, a number of inhibitors of DNA methylation and histone deacetylase are currently being evaluated in cancer therapy, either alone or in combination, as it is clear that these drugs have a synergistic effect upon gene expression and tumor growth (Table 2).

EFFECTS OF HDAC INHIBITORS ON LEUKEMIA CELLS •

HDACs are seen as a potential target for cancer treatment. The anticancer mechanism of HDAC inhibitor is dependent on the regulation of gene expression, large experiments in vivo and in vitro have confirmed that there different kinds of anticancer mechanisms: 1) block cell cycle and promote cell differentiation, 2) induce cell apoptosis, 3) inhibit angiogenesis (28) and to sensitize cells to chemotherapy or radiation therapy (4). However, the HDAC-dependent mechanisms accounting for the observed and rather selective modulation of gene.

Extensive research of the effect in vitro of many HDAC inhibitor for different tumor cell lines showed that HDAC inhibitor can lead many leukemia cell lines to different grade of differentiation, apoptosis and block cell circle at G0~G1 period or G2~M period. The effect depends on the type of cell lines, different drugs and action time, which maintain local HDAC in a variety of hematologic lineage-specific gene promoters. This HDAC dependent transcriptional repression appears as a common pathway in the develop-

TABLE 2 •
Genes Hypermethylated in Acute Leukemia

DNA Hypomethylation Agents	
1. Deoxycytidine analogs:	• 5-azacytidine, 5-aza-2-deoxycytidine, 1-B-D-arabinosil 5-azacytosine, dihydro-5-azacytidine.
2. Nucleic acid-based:	• MG98 antisense oligonucleotide
3. Cytidine deaminase analogs:	• zebularine
4. Non-nucleoside analogs:	• (-) epigallocatechin-3gallate, procaine, procainamide, hydralazine.
Histone Deacetylase Inhibitors	
1. Small molecular weight carboxylates:	• sodium butyrate, valproic acid, sodium phenylbutyrate and pivaloyloxymethyl butyrate.
2. Hydroxamic acids:	• SAHA, trichostatin A,
3. benzamides:	• CI-994, MS-275
4. Epoxyketones:	• Trapoxin B, 2-amino-8-oxo-9,10 epoxydecanoid acid.
5. Cyclic peptides:	• Apicidin, depsipeptide.
6. Hybrid molecules:	• CHAP 31, CHAP 50

ment of leukemia and could constitute an important target for new therapeutic agents.

HDAC inhibitors enhance the apoptosis-inducing potential of TRAIL in leukemia cells (HL60, Jurkat, K562, and U937) through multiple mechanisms, which can up-regulate DR4, DR5, Bak, Bax, Bim, Noxa and PUMA, down-regulate IAPs, Mcl-1, Bcl-2, Bcl-XL and cFLIP, release mitochondrial proteins (cytochrome c, Smac/DIABLO and Omi/Htr2) to the cytosol, induce p21WAF1/CIP1 and p27KIP1, activate caspase-3 and cleave poly (ADP-ribose) polymerase (PARP).

In chronic myelocytic leukemia (CML) the activity of the Bcr-Abl tyrosine kinase is known to activate a number of molecular mechanisms, which inhibit apoptosis. SAHA induced apoptosis in BV-173 cells, which involves decreased protein expression levels of Bcr-Abl, c-Myc and HDAC3. Depsipeptide can up-regulate IL-3 gene expression of AML1/ETO positive leukemia cell, and IL-3 is essential signal transduction regulating gene for normal haematopoiesis. Apicidin might induce apoptosis of HL-60 cell through selective induction of Fas/Fas ligand, resulting in the release of cytochrome c from the mitochondria to the cytosol and subsequent activation of caspase-9 and caspase-3(29).

Low dose of sodium butyrate and Trichostatin can induce K562 cell line differentiation. They can block K562 cell cycle in different stage, but the differentiation both through inducing P21 and cyclin D3 expression. The up regulation of death receptors and inhibition of cFLIP by HDAC inhibitors will increase the ability of TRAIL to induce apoptosis, due to enhance activation of caspase-8, cleavage of Bid, release of mitochondrial proteins to the cytosol, and subsequent activation of caspase-9 and caspase-3. The link between altered HDAC activity and tumorigenesis is probably best demonstrated in acute promyelocytic leukemia (APL). HDAC inhibitor can induce many lymphocytic leukemia differentiation and apoptosis (30,31).

Chronic lymphocytic leukemia cell and myeloma cell are sensitive to HDAC inhibitors, either SAHA can induce diffuse large cell lymphoma and Hodgkin disease cell lines apoptosis.

In general, HDAC inhibitors are at most at the early stages of clinical development. Among them, SAHA has shown in a recently reported phase I study of 73 patients with advanced cancer a complete response in a patient with transformed diffuse large B-cell lymphoma for 17 months, three partial responses in B-cell lymphoma, laryngeal cancer, and papillary thyroid cancer, and prolonged stabilization in patients with renal carcinoma.

The strong interplay between DNA hypermethylation and histone deacetylation for silencing and modulating the expression of a number of cancer-related genes predicts not only a synergy in gene expression at global and individual gene levels, but also an antitumoral activity. For instance, combinations of decitabine with trichostatin A or depsipeptide synergistically reactivate silenced tumor suppressor genes including MLH1, TIMP3, CDKN2B, CDKN2A, ARHI, gelsolin, and maspin and increased the level of tumor cell apoptosis. Thus, a logical step forward is to combine a demethylating with a histone deacetylase inhibitor for cancer treatment.

HYPOMETHYLATING AGENTS •

Inhibitors of DNA methylation have demonstrated the ability to inhibit hypermethylation, restore suppressor gene expression, and exert antitumoral effects in *in vitro* and *in vivo* laboratory models. Several demethylating agents are being evaluated in preclinical and clinical studies (table 2). The classical demethylating agents comprise the analogs of deoxycytidine: 5-azacytidine, 5-aza-2-deoxycytidine, 1- β -D-arabinofuranosil- 5-azacytosine, and dihydro-5-azacytidine. 5-azacytidine and its analog are the most studied and were developed over 30 years ago as classical cytotoxic agents, but were subsequently discovered to be effective DNA methylation inhibitors; these were

tested as such in several phase II studies against solid tumors demonstrating very modest activity. To the contrary, their antileukemic activity was very promising and both are being revived as a consequence of their demonstrated inhibitory activity upon DNA methylation and gene-reactivating function. Currently, 5-azacytidine is Federal Drug Administration (FDA)-approved to be used against myelodysplastic syndrome, and the hydrosoluble analog 5-aza-2-deoxycytidine is being tested in a variety of solid tumors, as DNA demethylating agent. As a second category of demethylating agents, we note the antisense oligonucleotide MG98 against the 3' untranslated region of DNMT1 mRNA, which codes for the enzyme DNA methyltransferase 1 that is responsible for maintenance of DNA methylation (32).

The fact that deoxycytidine analogs such as current cytotoxic agents are not only carcinogenic but also exhibit neutropenia as their dose-limiting toxicity even when used at doses required for demethylation has renewed interest in finding effective and less toxic demethylating agents. Zebularine is a new oral cytidine analog originally synthesized as a cytidine deaminase inhibitor that has been shown to cause demethylation and reactivation of a silenced and hypermethylated p16 gene in human bladder tumor cells grown in nude mice.

There is another class of so-called "old drugs" whose demethylating activity upon gene promoters of tumor suppressor genes was recently highlighted. Procainamide, a non-nucleoside inhibitor of DNA methyltransferases approved for treatment of cardiac arrhythmias, can demethylate the GSTP1 promoter, a common somatic genome change in human prostate cancer and reactivates in vitro and in nude mice the expression of the gene.

A related drug, procaine, has also the ability of demethylating and reactivating tumor suppressor gene expression, such as the RAR β 2 gene in a breast cancer cell line effect that is accompanied by growth-inhibitory actions.

Our group has shown in vitro and in vivo promoter demethylation and tumor suppressor gene transcriptional reactivation mediated by the antihypertensive compound hydralazine, a well-tolerated drug devoid of the common side effects of cytotoxic chemotherapy agents (33).

Decitabine has also employed in combination with other anticancer agents, such as anthracyclines or, more recently, histone deacetylation inhibitors, e.g. phenylbutyrate. The initial phase II studies were performed in patients with relapsed and resistant leukemia, in combination with either amsacrine (120 mg/twice daily for 3-6 days) or idarubicin (12 mg/m² for 3 days).

The most important study was conducted by the European Organization for Research and Treatment of Cancer (EORTC). 5-aza-2'-deoxycytidine, combined with either amsacrine or idarubicin (34) was administered to patients with acute myeloid or lymphocytic leukemia in relapse. Sixty-three patients received 5-aza-2'-deoxycytidine 125 mg/m² (decitabine) as a 6 h infusion every 12 h for 6 days (total dose 1500 mg/m²), in combination with either amsacrine 120 mg/m² as a 1-h infusion on days 6 and 7 (n=30) or idarubicin 12 mg/m² as a 15-min infusion on days 5, 6 and 7 (n=33). Twenty-three patients (36.5%) achieved complete remission (8 of 30 patients treated with amsacrine and 15 of 33 treated with idarubicin). Complete remission was achieved by 51% of patients with more than a 1-year interval between initial diagnosis and start of therapy and in only 15.4% of patients with an interval shorter than 1 year.

Patients with normal cytogenetics had a higher complete remission rate (61%) than those with abnormal cytogenetics (15.8%). With this high-dose decitabine schedule, digestive tract and hematologic toxicity was prolonged compared to that produced by standard induction schedules. The median disease-free survival was approximately 8 months, with only 20% of patients in remission for longer than 1 year. At this dose decitabine may be considered a good antileukemic agent, similar to cytarabine, but with considerable toxicity.

Preliminary results of a small phase II trial also combining an intensive schedule of decitabine with daunorubicin (35) given as first-line induction therapy to patients with acute myeloid leukemia (except FAB M3), have been reported. Decitabine was given as a 4-h intravenous infusion at the dose of 90 mg/m² daily, days 1-5, while daunorubicin was administered at the dose of 50 mg/m² on days 1-3.

A maximum of two courses were given with an interval of 4-6 weeks. Eight patients were enrolled and six of them were evaluable for toxicity and response. The main toxic effects were bone marrow suppression, mucositis, nausea and vomiting, and alopecia. All six patients achieved complete remission after one (5 cases) or two (1 case) courses.

Decitabine has recently been employed in patients with advanced MDS in combination with phenylbutyrate. Sodium phenylbutyrate (PB) is an aromatic fatty acid with cytostatic and differentiating activity against malignant myeloid cells (ID50, 1-2mM). A number of mechanisms have been proposed for the antitumoral effect of PB, including glutamine depletion and inhibition of cholesterol synthesis. The clinical activity of PB at low concentrations (0.25-0.5 mM) may be explained by its effect on histone acetylation. At these doses, like butyric acid, PB has an inhibitory activity on histone deacetylase, inducing histone H3 and H4 acetylation. Like other histone deacetylase (HDAC) inhibitors, PB synergizes in vitro with retinoids in the induction of differentiation and cell cycle arrest of myeloid leukemia cells. Furthermore PB, again like other HDAC inhibitors, synergizes with demethylating agents (36). Patients with myelodysplasia (n=11) and AML (n=16) were treated with PB as a 7-day continuous infusion, repeated every 28 days, in a phase I dose-escalation study (37). The maximum tolerated dose was 375 mg/kg/day; higher doses led to dose-limiting reversible neurocortical toxicity. At the maximum tolerated dose, PB was extremely well tolerated, with no significant toxicities.

Recent demonstrations of the important role of histone acetylation in the regulation of gene expression and the recruitment of histone deacetylase enzymes by several fusion genes involved in acute leukemias have led to the speculation that agents that inhibit histone deacetylase may be useful in the treatment of neoplasms. Inhibition of histone deacetylase may explain the changes in bone marrow CFU, hemograms, and cells seen in patients treated with PB, despite submillimolar plasma concentrations (38).

Both studies demonstrate that the sequential administration of a first generation demethylating agent and HDAC inhibitors is feasible, and give preliminary evidence of an effect on the methylated targeted gene promoter, as also described with decitabine (23).

The DNA hypomethylating pyrimidine analogues 5-azacytidine and 5-aza-2-deoxycytidine (decitabine) may reduce hypermethylation and induce re-expression of key tumor suppressor genes in MDS. The effect of azacytidine was evaluated in a randomized phase III trial (39). Azacytidine treated patients showed a better overall response compared to those treated with supportive care only (60% vs 5%) and a longer time to progression to AML or death, but none overall survival advantage. These results led to the licensing of azacytidine in the US in 2004, while the decision from the European authorities is still pending. The effect of azacytidine on survival and AML evolution is currently being evaluated in an international randomized phase III trial of patients with Int-2 and high-risk MDS. Patients in the control arm are treated with "doctor's choice"; supportive care, low-dose cytosine arabinoside (ara-C) or induction chemotherapy. The Nordic MDS Group is investigating the effect of azacytidine given as long-term maintenance treatment in CR after induction chemotherapy in patients with high-risk MDS and AML following MDS (40).

One clinically available oral HDAC inhibitor, valproic acid given in combination with all-trans

retinoic acid, was recently reported to have an effect in a limited number of high-risk MDS and leukemia patients. Several HDAC inhibitors, such as MS 275, SAHA, and depsipeptide, are being evaluated in clinical trials for MDS and other hematological malignancies (41).

According to the recent preliminary report of a US trial of 170 patients, AML-free survival, but not overall survival, was longer in the decitabine group, which also showed improved quality of life (42). The median number of courses to response has been noted to 3–4 with both azacytidine and decitabine. Accordingly, comparison of studies involving these drugs may be compromised if patients are taken off study at varying times. The ability to give multiple courses of decitabine may be increased by the development of low-dose schedules and is being investigated.

Recently, 54 patients were treated in a phase I/II study with decitabine (fixed dose of decitabine (15 mg/m²) and escalating orally doses of valproic acid (VPA) for 10 days. Twelve (22%) patients had objective response, including 10 (19%) complete remissions (CRs) and 2 (3%) CRs with incomplete platelet recovery (CRp). Among 10 elderly patients with acute myelogenous leukemia or myelodysplastic syndrome, 5 (50%) had a response (4CRs, 1CRp's). Major cytogenetic response was documented in 6 of 8 responders. Remission duration was 7.2 months (range, 1.3-12.6+ months). Overall survival was 15.3 months (range, 4.6-20.2+ months) in responders. Transient DNA hypomethylation and global histone H3 and H4 acetylation were induced, and were associated with p15 reactivation. These results suggest that this combination of epigenetic therapy in leukemia was safe and active, and was associated with transient reversal of aberrant epigenetic marks (43).

Although the epigenetic treatment with hypomethylating agents is the standard of care in myelodysplastic syndromes, response rates remain low. Therefore a new approach was evaluated in a randomized trial, where 95 adults with ad-

vanced MDS or chronic myelomonocytic leukemia were included: 1) decytabine 20 mg/m², IV, for 5 days, 2) 20 mg/m² daily for 5 days, given in 2 subcutaneous doses daily for 5 days; or 3) 10 mg/m², IV, for 10 days. All received 100 mg/m², per course. Fifty three (60%) achieved an improvement, including a 34% complete response rate. Response of thrombocytopenia was particularly relevant. Among 68 patients with pretreatment platelets counts less than 100 × 10⁹/L, 333 (49%) achieved platelets counts of at least 100 × 10⁹/L. The degree of hypomethylation did not correlate with responses, suggesting that downstream effects are also key for decitabine activity. The fact that responses tend to occur slowly suggests that these early changes in DNA methylation and gene expression cannot be explained by clonal changes in cell composition (44).

Whether additional therapy or new schedules will be developed to improve the response rate in myelodysplastic syndromes, as well as, the addition of this kind of treatment to target drugs, either to improve the response rate or to reintroduce a drug after secondary resistance, will be the focus of research in the following years.

REFERENCES •

1. Jemal A, Murray T, Samuels A. Cancer statistics. 2003. *CA-Cancer J Clin* 2003; 53: 5-26 •
2. Registro histopatológico de neoplasias malignas. Secretaria de Salud. México. 2001. <http://www.dgepi.salud.gob.mx/diveent/RHNM.htm> •
3. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J. Lymphoma classification- from controversy to consensus: the REAL and WHO Classification of lymphoid neoplasms. *Ann Oncol*. 2000; 11 Supp 1: 3-10 •
4. Dueñas-González A, Lizano M, Candelaria M, Cetina Lucely, Arce C, Cervera E. Epigenetics of cervical cancer. An overview and therapeutic perspectives. *Molecular Cancer* 2005, 4: 38 •
5. Das P, Singal R. DNA methylation and Cancer. *J Clin Oncol*. 2004; 22: 4632-4642 •
6. Costello J, Plass C. Methylation matters. *J. Med. Genet*. 2001; 38: 285-303 •
7. Szyf M. Targeting DNA methylation in cancer. *Ageing Res Rev*. 2003; 1: 229-328 •
8. Hayslip J, Montero A. Tumor Suppressor Gene Methylation In Follicular Lymphoma: A Comprehensive Review. *Molecular Cancer* 2006, 5: 44 <http://www.Molecular-Cancer.Com/Content/5/1/44> •
9. Gilbert J, Gore SD, Herman JG, Carducci MA. The clinical application of targeting cancer through histone acetylation and hypomethylation. *Clin Cancer Res*. 2004; 10: 4589-4596 •
10. Barnes PJ, Adcock IM, Ito K. Histone acetylation and deacetylation: importance in inflammatory lung diseases. *Eur Respir J*. 2005; 25: 552-563 •

11. Gibbons Richard J. Histone modifying and chromatin remodeling enzymes in cancer and dysplastic syndromes *Human Molecular Genetics*, 2005; 14: 85-92 •
12. Fahrner JA, Eguchi S, Herman JG, Baylin SB. Dependence of histone modifications and gene expression on DNA hypermethylation in cancer. *Cancer Res*. 2002; 62: 7213-7218 •
13. Muhlethaler Mottet A, Flahaut M, Bourlout KB, Auderset K, Meier R, Joseph JM, Gross N. Histone deacetylase inhibitors strongly sensitise neuroblastoma cells to TRAIL-induced apoptosis by a caspases-dependent increase of the pro- to anti-apoptotic proteins ratio. *BMC Cancer*. 2006; 6: 214 •
14. Takahashi T, Shivapurkar N, Reddy J, Shigematsu H, Miyajima K, Suzuki M, Toyooka S, Zochbauer-Muller S, Drach J, Parikh G, Zheng Y, Feng Z, Kroft SH, Timmons C, McKenna RW, Gazdar AF. DNA methylation. *Clin Cancer Res*. 2004; 10: 2928-2935 •
15. Melki JR, Vincent PC, Clark SJ. Concurrent DNA hypermethylation of multiple genes in acute myeloid leukemia. *Cancer Res*. 1999; 59: 3730-3740 •
16. Roman J, Castillejo JA, Jimenez A, Bornstein R, Gonzalez MG, del Carmen-Rodriguez M. Hypermethylation of the calcitonin gene in acute lymphoblastic leukaemia is associated with unfavourable outcome. *Br J Haematol*. 2001; 113: 329-338 •
17. Li Q, Kopecky KJ, Mohan A, Willman CV, Appelbaum FR, Weick JK. Estrogen receptor methylation is associated with improved survival in adult acute myeloid leukemia. *Clin Cancer Res*. 1999; 5: 1077-1084 •
18. Melki JR, Vincent PC, Brown RD, Lark SJ. Hypermethylation of E-cadherin in leukemia. *Blood*. 2000; 95: 3208-3213 •
19. Lee DW, Zhang K, Ning ZQ, Raabe EH, Tintner S, Wieland R, Wilkins BJ, Kim JM, Blough RI, Acres RJ. Proliferation-associated SNF-2 like gene (PASG): a SNF-2 family member altered in leukemia. *Cancer Res*. 2000; 60: 3612-3622 •
20. Mizuno SI, Chijiwam T, Okamura T, Akashi K, Fukumaki Y, Niho Y. Expression of DNAMethyltransferases DNMT1, 3A, 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. *Blood*. 2001; 97: 1172-1179 •
21. Di Croce L, Raker VA, Corsaro M, Fazi F, Fanelli M, Faretta M. Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science* 2002; 295: 1079-1082 •
22. Grignani F, De Matteis S, Nervi C, Tomassoni L, Gelmetti V, Ciocce M. Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. *Nature*. 1998; 391: 815-818 •
23. Leone G, Teofilii L, Voso MT, Lübbert M. DNA Methylation and Demethylating Drugs in Myelodysplastic Syndromes and Secondary Leukemias. *Haematologica* 2002; 87:1324-1341 •
24. Uchida T, Kinoshita T, Nagai H, Nakahara Y, Saito H, Tota T. Hypermethylation of the p15/INK4B gene in myelodysplastic syndromes. *Blood*. 1997; 4: 1403-1409 •
25. Gibbons RJ, Pellagatti A, Carrick D, Wood WG, Malik N, Ayyub H, Langford C, Boultonwood J, Wainscoat JS, Higgs DR. Identification of acquired somatic mutations in the gene encoding chromatin-remodeling factor ATRX in the alpha-thalassemia myelodysplasia syndrome (ATMDS). *Nat Genet*. 2003; 34: 446-449 •
26. Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science*. 2003; 300: 455 •
27. Rush LJ, Raval A, Funchain P, Johnson AJ, Smith L, Lucas DM, Bembea M, Liu TH, Heerema NA, Rassenti L, Liyanarachchi S, Davuluri R, Byrd JC, Plass C. Epigenetic profiling in chronic lymphocytic leukemia reveals novel methylation targets. *Cancer Res*. 2004; 64: 2424-2433 •
28. Gaofeng Bi1 and Guosheng Jiang .The Molecular Mechanism of HDAC Inhibitors in Anticancer Effects. *Cellular & Molecular Immunology*. 2006; 3: 285-290 •
29. Rossetti S, Hoogeveen AT, Liang P, Stanciu C, van der Spek P, Sacchi N. A distinct epigenetic signature at targets of a leukemia protein. *BMC Genomics* 2007, 8: 38 •
30. Gebhard C, Schwarzfischer L, Pham T, Schilling E, Klug M, Andreesen R, Rehli M. Genome-Wide Profiling of CpG Methylation Identifies Novel Targets of Aberrant Hypermethylation in Myeloid Leukemia. *Cancer Res* 2006; 66: 6118- 6128 •
31. Di Croce L. Chromatin modifying activity of leukaemia associated fusion proteins. *Hum Mol Genet*. 2005 15:14 •
32. Garcia-Manero G, Bueso-Ramos C, Danie J, Williamson J, Kantarjian H, Issa J- DNA Methylation Patterns at Relapse in Adult Acute Lymphocytic Leukemia *Clinical Cancer Research* 2002; 8 1897-1903 •
33. Chavez-Blanco A, Segura-Pacheco B, Perez-Cardenas E, Taja-Chayeb L, Cetina L, Candelaria M, Cantu D, Gonzalez-Fierro A, Garcia-Lopez P, Zambrano P, Perez-Plasencia C, Cabrera G, Trejo-Becerril C, Angeles E, Duenas-Gonzalez A. Histone acetylation and histone deacetylase activity of magnesium valproate in tumor and peripheral blood of patients with cervical cancer. A phase I study. *Mol Cancer*. 2005; 4: 22 •
34. Wilemze R, Suci S, Archimbaud E; Muus, Stryckmans P, Lowagie EA, Berneman Z, Tjean M, Wijermans P, Dohner H, Jehn U, Labar B, Jaskovic B, Dardenne M. A randomized phase II study of the effects of 5-Aza-2'deoxyctidine combined with either amsacrine or idarubicin in patients with relapsed acute leukemia: an EORTC Leukemia Cooperative Group phase II study (06893). *Leukemia*. 1997; 11 Suppl 1: S24-S27 •
35. Fernandes MS, Schaan MD; Moschen M, Gerhardt LM, Di Leone L. Decytidine (5-aza-2'deoxyctidine, DAC) plus daunorubicin as a first line treatment in patients with acute myeloid leukemia, preliminary observations. *Leukemia*. 1997; 11 Suppl 1: 28-31 •
36. Egger G, Aparicio AM, Escobar SG, Jones PA. Inhibition of histone deacetylation does not block resiliencing of p16 after 5-aza-2'-deoxyctidine treatment. *Cancer Res*. 2007; 67: 346-353 •
37. Gore SD; Weng LJ, Zhai S, Figg WD, Donehower RC, Dover GJ. Impact of the putative differentiating agent sodium phenylbutyrate on myelodysplastic syndromes and acute myeloid leukemia. *Clin Cancer Res*. 2001; 7: 2330-2339 •
38. Scott SA, Lakshimikuttyamma A, Sheridan DP, Sanche SE, Geyer CR, DeCoteau JF. Zebularine inhibits human acute myeloid leukemia cell growth in vitro in association with p15INK4B demethylation and reexpression. *Exp Hematol*. 2007; 35: 263-273 •
39. Silverman LR, Demakos EP, Peterson BL. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. *J Clin Oncol*. 2002; 20: 2429-2440 •
40. Koistinen P, Raty R, Tala M, Jantunen E; Koivunen E, Nousiainen T, Peliemi TT, Remes K, Ruutu T, Savolainen ER, Siitonen T, Silvennoinen R, Volin L, Elonen E; for the Finnish Leukaemia Group. Long term outcome of intensive chemotherapy for adults with de novo acute myeloid leukaemia (AML): the nationwide AML-92 study by the Finnish Leukaemia Group. *Eur J Haematol*. 2007 Mar 28 •
41. Hellström-Lindberg E. Update on Supportive Care and New Therapies: Immunomodulatory Drugs, Growth Factors and Epigenetic-Acting Agents *Hematology Am Soc Hematol. Educ Program*. 2000: 110-132 •
42. Saba HI, Rosenfeld CS, Issa J. Clinical benefit and survival endpoints from a phase III trial comparing decitabine (DAC) vs supportive care (SC) in patients with advanced myelodysplastic syndrome. *Proc AM Soc Clin Oncol*. 2005; 23: abstr 6543 •
43. Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, Yang H, Rosner G, Verstovsek S, Rytting M, Wierda WG, Ravandi F, Koller C, Xiao L, Faderl S, Estrov Z, Cortes J, O'Brien S, Estey E, Bueso-Ramos C, Fiorentino J, Jabbour E, Issa JP. Phase 1/2 study of the combination of 5-aza-2'deoxyctidine with valproic acid in patients with leukemia. *Blood*. 2006; 108: 3271-3279 •
44. Kantarjian H, Oki Y, Garcia-Manero G, Huang W, O'Brien S, Cortes J, Faderl S, Bueso-Ramos C, Ravandi F, Estrov Z, Ferrajoli A, Wierda W, Shan J, Davis J, Giles F, Saba H, Issa JP. Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. *Blood*. 2007; 109: 52-57 •