

Changes in DNA Methylation in Neoplasia: Pathophysiology and Therapeutic Implications

Valeria Santini, MD; Hagop M. Kantarjian, MD; and Jean-Pierre Issa, MD

Methylation of DNA is a biochemical modification that can influence gene expression and is involved in inactivating one of the two X chromosomes in women. Evidence that has accumulated in the past 10 years suggests that cancer cells usurp this physiologic mechanism and use it to their benefit by inactivating tumor suppressor genes and related proteins. However, the primary structure of the affected proteins remains intact; reversal of abnormalities in DNA methylation may therefore restore the tumor-suppressive function of these genes and provide a novel approach to cancer therapy. Two demethylating drugs, 5-azacytidine and 5-aza-

deoxycytidine, are currently being tested in clinical trials, and several others are in preclinical development. In this article, the biological rationale for targeting aberrant methylation in cancer therapy is reviewed and completed phase I and II trials of this approach, some of which show promise for treatment of hematologic malignancies, are summarized.

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For author affiliations and current addresses, see end of text.

For a glossary of terms, see end of text.

Our increasing knowledge of the molecular pathophysiology of cancer is beginning to find applications in the diagnosis and treatment of various neoplastic diseases. In particular, new therapeutic approaches such as targeted agents, differentiation therapy, and immunotherapy promise to yield substantial clinical benefits with relatively few side effects. Recently, aberrant methylation of the cytosine base within the regulatory area of selected genes was shown to be a very common event in neoplasia; it is thought to contribute to the molecular pathogenesis of the disease through inactivation of tumor suppressor genes (1, 2). This finding has increased interest in use of drugs that can inhibit the process of DNA methylation and restore tumor suppressor gene function as a potential strategy to treat various malignant diseases. Hematopoietic neoplasms in particular have a high degree of aberrant methylation (3), and clinical trials have demonstrated significant activity for hypomethylating drugs in this setting.

We discuss the importance and prevalence of DNA hypermethylation in cancer and review the potential value of hypomethylating agents in the treatment of human neoplasms.

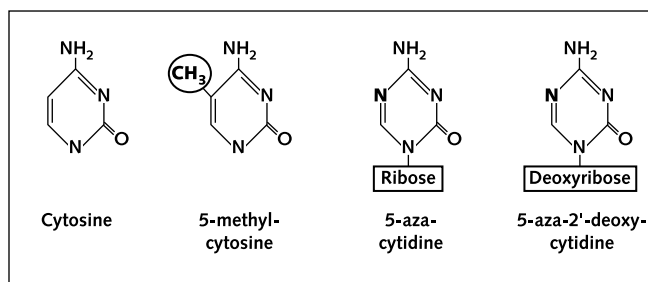
DNA METHYLATION

The presence of 5-methylcytosine in human DNA (4) has genetic and epigenetic effects on cellular development, differentiation, and neoplastic transformation. 5-Methylcytosine differs from cytosine by the presence

of a methyl group at the 5 position of the pyrimidine ring (Figure 1). Methylcytosine is formed after replication by addition of a methyl group to a cytosine already present in the DNA strand. Dramatic changes in overall methylation of DNA occur at different periods of embryogenesis, development, and differentiation to adult cells (5). A wave of demethylation initially erases preset methylation patterns in the first days of embryogenesis. This is followed by several waves of de novo methylation that eventually establish adult patterns of gene methylation. In differentiated cells, methylation patterns change relatively little and are perpetuated after DNA replication through the high affinity of DNA methyltransferase for hemimethylated DNA (6) (Figure 2). Unlike cytosine, 5-methylcytosine is a relatively unstable base because its spontaneous deamination leads to uracil. Through evolution, such mutations have resulted in a relative depletion of 5-methylcytosine in human DNA, and they are a major cause of germ-line mutations in inherited disease and of somatic mutations in neoplasia (7).

The functions of DNA methylation in mammalian cells remain poorly defined. Early speculation that attributed a global transcriptional regulation role to cytosine methylation (8) has not yet been confirmed experimentally. In bacteria, methylation plays a role in defense against genomic invasion by foreign DNA sequences (9). In mammalian cells, most normal methylation takes place within highly repeated transposable elements, and it has been proposed that such methylation

Figure 1. Structure of cytosine, 5-methylcytosine, and hypomethylating 5-methylcytidine analogues.



also plays a role in genome defense by suppressing the potentially harmful effects of expression at these sites (10). This hypothesis was questioned recently (11). Regardless of its global functions, one unequivocal role for DNA methylation is in irreversible gene inactivation in selected cases, such as imprinted genes (12) and genes on the inactivated X chromosome (13).

CpG ISLAND METHYLATION AND GENE SILENCING

In mammalian DNA, normal methylation is restricted to cytosine followed by guanosine (the CpG dinucleotide). These CpG sites are rarer in the human genome than their predicted frequency, presumably because they are eliminated during evolution through C to T mutations of methylcytosine (14). The human genome, however, also contains small regions of DNA called “CpG islands,” in which the frequency of CpG is normal or higher than expected (14). About half of all human genes (including most housekeeping genes) have CpG islands in their 5'-promoter regions. Of note, the promoter regions containing CpG islands are in fact usually unmethylated in normal tissues, regardless of the transcriptional status of the gene.

CpG island methylation is associated with changes in chromatin organization and consequent repression of gene transcription (1). In normal tissues, CpG island methylation is limited to exceptional situations, such as imprinted alleles (12) and genes on the inactive X chromosome (13). These well-studied exceptions to the rule of absent methylation at CpG islands suggest that, once established, gene silencing by CpG island methylation is physiologically irreversible during the lifetime of affected cells. A direct correlation between CpG island methylation and inhibited gene transcription is sup-

ported by the facts that 1) cells in which silencing occurs are usually transcriptionally competent for the affected genes (as demonstrated by normal expression of the unmethylated alleles and exogenously inserted unmethylated promoters), 2) demethylation by pharmacologic (15) or genetic (16) manipulation results in reactivation of gene expression, and 3) in vitro methylation substantially reduces gene expression in reporter experiments (1). The mechanism of CpG island-associated gene silencing appears to involve binding of specific methylated DNA binding proteins, followed by recruitment of a silencing complex that includes histone deacetylases (Figure 3) (17, 18).

ABERRANT CpG ISLAND METHYLATION IN CANCER

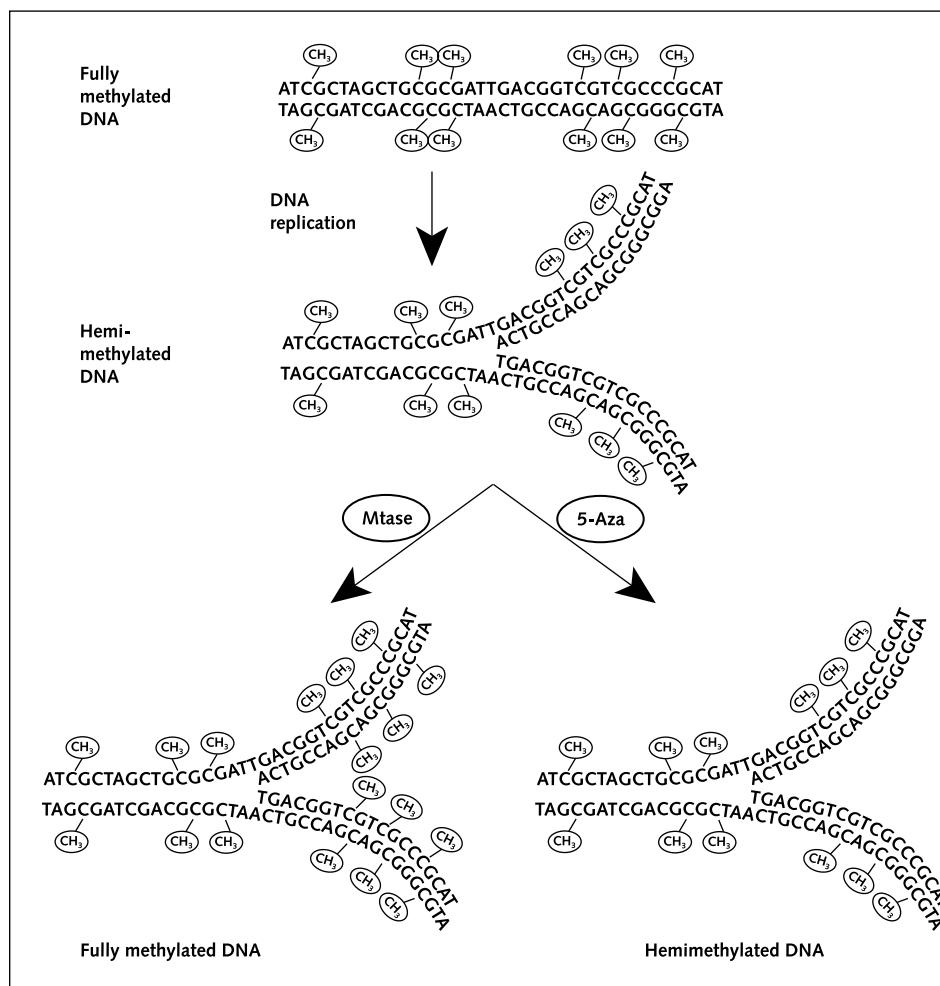
Neoplastic cells often have simultaneous global DNA hypomethylation, localized hypermethylation that involves CpG islands, and increased levels of DNA methyltransferase activity (1). Hypomethylation was initially postulated to play a role in carcinogenesis through activation of oncogenes (19), but this hypothesis has not been experimentally confirmed. Hypomethylation has been linked to chromosomal instability in vitro (20), and it may play such a role in neoplasia. Aberrant CpG island hypermethylation in cancer, in contrast, is clearly associated with transcriptional silencing of gene expression, and increasing experimental data suggest that it plays an important role as an alternate mechanism by which tumor suppressor genes are inactivated in cancer (1, 2).

Aberrant CpG island methylation in cancer was initially described for the calcitonin (21) and *MyoD* (22) genes. These two genes are not thought to play a tumor suppressive role in cancer, but these findings prompted additional investigations into the process. The first tumor suppressor gene shown to be inactivated by hypermethylation was the *RBI* gene, in which methylation appeared to be a clear alternate to mutations and deletions for eliminating expression of functional protein (23). Several additional tumor suppressor genes have since been shown to be similarly inactivated in some cancers, including *VHL* (24), *P16* (25), E-cadherin (26), and *hMLH1* (27). For most of these genes, hypermethylation appears to provide a similar selective advantage as genetic inactivation and is usually associated with absence of coding region or promoter mutations of involved alleles.

The list of genes that display hypermethylation-associated inactivation in some sporadic cancers has grown long (Table 1). Multiple cellular systems can be affected by this process, including cell growth and differentiation, cell cycle control, and DNA repair, as well as angiogenesis and invasion. However, hypermethylation in cancer is not invariably associated with repressed transcription. In some cases, the involved CpG island is not in the promoter of the genes (28). In other cases, methylation involves genes that are not normally

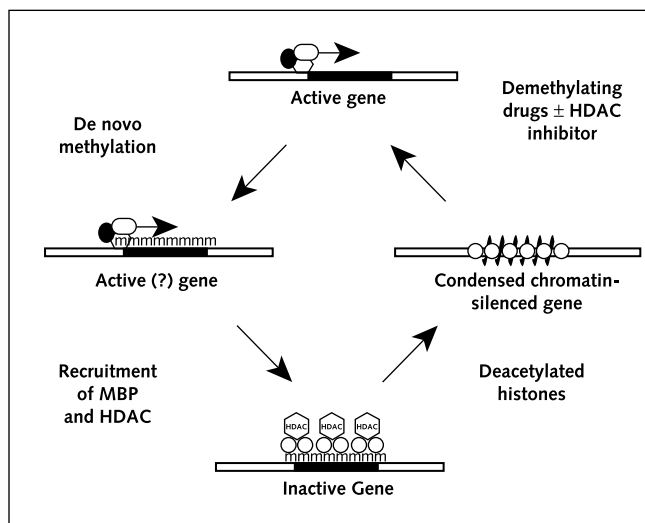
expressed in the diseased tissues (29). In still others, methylation is relatively sparse, and although it can easily be detected experimentally, it does not lead to substantial decreases in gene expression. Aberrant methylation in cancer therefore functions as a mechanism of generating molecular diversity in neoplasia. In a manner analogous to mismatch repair defects in cancer, methylation defects affect many different loci, only some of which are pathophysiologically relevant to the neoplastic process (30).

Figure 2. The maintenance methylation process.



Top. Some (but not all) cytosine-followed-by-guanosine (CpG) sites carry a methyl (CH₃) group at the 5' position of cytosine. Both strands of DNA are methylated ("fully methylated"). **Middle.** Immediately after DNA replication, the newly formed strand is unmethylated (no CH₃ groups), resulting in hemimethylated double-stranded DNA, which rapidly attracts one of several methyltransferase (*Mtase*) enzymes. **Bottom.** Through the action of methyltransferase enzymes, methyl groups are added symmetrically to hemimethylated CpG sites (*left*), resulting in fully methylated DNA. In the presence of cytidine analogues (*5-Aza*), methyltransferases are depleted, and the process of remethylation after replication is inhibited, leading to DNA that remains hemimethylated (*right*). With subsequent rounds of replication in the absence of methyltransferases, DNA becomes progressively more demethylated, until most DNA molecules are completely unmethylated.

Figure 3. Effects of methylation and histone deacetylation on gene expression and silencing.



Initially (top), genes (black boxes) are unmethylated, and the promoter region is occupied by transcription factors (ovals) that direct production of messenger RNA (arrows). De novo methylation, by itself, has a minimal effect on gene expression (left). However, methylated DNA (m) attracts methyl-binding proteins (MBP), such as MeCp2 (bottom). These methyl-binding proteins in turn attract a protein complex that contains histone deacetylases (HDAC). At this point, synthesis of messenger RNA is inhibited and no functional protein can be made from the gene. Through the action of methyl-binding proteins and histone deacetylases, the DNA structure changes to a compact, “condensed chromatin” configuration (right), which results in permanent inhibition of messenger RNA and protein production (silencing). Hypomethylating agents can reverse this silenced state and restore messenger RNA and protein expression (top). Histone deacetylase inhibitors act synergistically with hypomethylating agents to restore functional gene expression.

The causes of aberrant methylation in cancer remain poorly defined. Both hypomethylation and methyltransferase activation can occur in cells that are induced to proliferate (31), and it is not clear whether the observed changes in malignant cells simply reflect cell cycle deregulation. De novo CpG island methylation, however, is not a feature of proliferating cells, and it appears to represent a true pathologic event in neoplasia. For many genes, hypermethylation begins in normal tissues during the process of aging (32), which may partially explain the dramatic increase in cancer incidence associated with aging. Other genes are methylated exclusively in malignant cells and are presumed to arise from rare chance events that lead to gene inactivation and a selective advantage for affected cells (1, 2). Recent data in multiple neoplasms suggest that some cancers have a high degree of de novo methylation compared

with others (33), and specific genetic defects or exposure events may explain these differences. In particular, acute and chronic leukemias have a high degree of aberrant CpG island methylation; genes involved include the cell-cycle regulator *p15* (34), the *p53* homologue *p73* (35), the drug-resistance gene *MDR1* (36), *ER* (37), and *HIC1* (38). Therefore, hematologic malignant conditions present unique opportunities for studying the clinical implications of aberrant methylation.

RATIONALE FOR USE OF METHYLATION INHIBITORS IN NEOPLASIA

Cytosine methylation is a biochemical modification of DNA that does not result in a nucleotide (or genetic) change. Methylation silencing of gene expression is therefore an epigenetic process that may be modulated by biochemical or biological manipulation. Indeed, pharmacologic inhibition of methyltransferase in vitro has been shown to result in reactivation of gene expression for genes silenced either physiologically or pathologically (39). The concentrations of hypomethylating agents required for this effect can readily be achieved in vivo (40). Because CpG island methylation is not a common finding in normal cells, cytosine methylation provides a highly specific target for pharmacologic therapy. Little evidence indicates that demethylation causes substantial changes in gene expression in normal cells, where the major targets would be imprinted genes and X-chromosome genes (in women). In theory, demethylation of imprinted genes would not be a major issue in most adult cells because imprinted genes tend to be expressed primarily during embryogenesis and include both growth promoters and growth suppressors (12). Reactivation of silenced X-chromosome genes could be a drawback for this approach in women; however, X-chromosome inactivation may be most important during embryogenesis. In fact, treatment with the methylation inhibitor 5-deoxyazacytidine has been shown to significantly prolong the doubling time of neoplastic cell lines but not normal fibroblast cell lines (41). Inhibition of cytosine methylation by pharmacologic or biological strategies has been shown to prevent or reverse the onset of neoplasia in various animal models (42, 43); these studies prompted renewed interest in the clinical uses of hypomethylating agents in humans.

Table 1. Genes That Are Hypermethylated in Sporadic Cancers*

Gene	Function	Type of Tumor
Familial cancer		
<i>APC</i>	Signal transduction	Colon cancer
<i>BRCA1</i>	DNA repair	Breast cancer
E-cadherin	Adhesion and metastasis	Common cancers
<i>hMLH1</i>	DNA mismatch repair	Colon, gastric, and endometrial cancer
<i>P16/CDKN2A</i>	Cell cycle regulation	Common cancers
<i>RB1</i>	Cell cycle regulation	Retinoblastoma
<i>VHL</i>	Unknown	Renal-cell cancer
Other		
Androgen receptor	Growth and differentiation	Prostate cancer
c-ABL	Tyrosine kinase	Chronic myelogenous leukemia
Endothelin receptor B	Growth and differentiation	Prostate cancer
Estrogen receptor α	Growth and differentiation	Common cancers
<i>FHIT</i>	Unknown	Esophageal cancer
GST-pi	Detoxification	Prostate cancer
<i>MDR1</i>	Drug transport	Acute leukemias
<i>O6-MGMT</i>	DNA repair	Common cancers
<i>P14/ARF</i>	Cell cycle regulation	Colon cancer
<i>P15/CDKN2B</i>	Cell cycle regulation	Malignant hematologic disease
Progesterone receptor	Growth and differentiation	Breast cancer
Retinoic acid receptor β	Growth and differentiation	Colon and breast cancer
<i>THBS1</i>	Angiogenesis inhibition	Colon cancer, glioblastoma multiforme
<i>TIMP3</i>	Metastasis	Common cancers

* Based on data from references 1–3 and 21–30.

CHEMICAL STRUCTURE AND MECHANISMS OF ACTION OF HYPOMETHYLATING AGENTS

To overcome cytosine arabinoside (ara-C) resistance, a series of cytidine analogues resistant to deamination were synthesized that did not require activation by deoxycytidine kinase (44). Cytidine analogues modified in position 5 of the pyrimidine ring (**Figure 1**)—5-azacytidine, 5-aza-2'-deoxycytidine (decitabine), pseudoisocytidine, and 5'-fluoro-2'-deoxycytidine—are potent inhibitors of DNA methylation. Of these substances, decitabine has the most potent hypomethylating effect (45); it can demethylate 55% of DNA at concentrations 10-fold lower than those of 5-azacytidine. Dihydro-5-azacytidine (46) has been introduced into clinical trials. Arabinofuranosyl-5-azacytosine (fazarabine) showed potent antitumor activity in preclinical models but not in clinical trials (47). The hypomethylating effect of cytidine analogues appears to depend primarily on the presence of an altered C5 position; other cytidine analogues, such as ara-C, 6-azacytidine, and gemcitabine, do not possess this property.

The efficacy of 5-azacytidine and decitabine as antineoplastic agents has been attributed to two distinct mechanisms: cytotoxicity and induction of hypomethylation. Both compounds are activated to a triphosphate

and are degraded by cytidine deaminase. 5-Azacytidine incorporates into RNA and, to a lesser extent, DNA. Decitabine incorporates primarily into DNA. Incorporation into RNA produces disassembly of polyribosomes, defective methylation and acceptor function of transfer RNA, and marked inhibition of protein production. Insertion into DNA results in covalent linking with methyltransferase and blocking of DNA synthesis (48) that ultimately results in direct cytotoxicity (49). 5-Azacytidine is highly cytotoxic to cells in S phase and exerts its action mainly on rapidly dividing cells. Unlike ara-C and 5-azacytidine, decitabine does not block cell progression into S phase, suggesting that its cytotoxic activity may not be self-limiting. This indicates that decitabine would have more powerful cytotoxic in vivo efficacy than ara-C or 5-azacytidine (48, 50).

Both 5-azacytidine and decitabine may also exert an antitumor effect through induction of DNA hypomethylation. By covalently trapping methyltransferase, both agents effectively deplete the cells of functional DNA methylating activity, which results in profound hypomethylation after several rounds of DNA replication (**Figure 2**). Since DNA methylation is associated with suppression of gene expression, 5-azacytidine and decitabine may exert their effects through re-expression of

Table 2. Hypermethylated CpG Islands in Malignant Hematopoietic Disease*

Gene	Methylation Frequency, %
Acute myelogenous leukemia	
<i>P15/CDKN2B</i>	30–70
Calcitonin	>70
<i>MYOD1</i>	50–>80
<i>ER</i>	60
<i>WIT1</i>	50
<i>THBS1</i>	20
<i>CACNA1G</i>	10–20
<i>MDR1</i>	10
<i>P16/CDKN2A</i>	<10
<i>O6-MGMT</i>	<10
<i>LRP6</i>	<10
<i>HIC1</i>	<10
Myelodysplastic syndromes†	
<i>P15/CDKN2B</i>	10–80
Calcitonin	40–80
Chronic myelogenous leukemia†	
<i>c-ABL</i>	25–>90
Calcitonin	20–80
<i>ER</i>	20–50
<i>HIC1</i>	20–50
Acute lymphocytic leukemia	
<i>P15/CDKN2B</i>	40–60
Calcitonin	>70
<i>ER</i>	60–70
<i>MYOD1</i>	50–>80
<i>HIC1</i>	50
<i>P73</i>	20–30
<i>P16/CDKN2A</i>	<10
<i>LRP6</i>	<10
<i>O6-MGMT</i>	<10
Chronic lymphocytic leukemia	
<i>P15/CDKN2B</i>	<10
<i>P16/CDKN2A</i>	<10
Multiple myeloma	
<i>P15/CDKN2B</i>	60
<i>P16/CDKN2A</i>	10–70
Lymphomat	
Calcitonin	50–80
<i>O6-MGMT</i>	25
<i>P16/CDKN2A</i>	10–30
<i>ER</i>	25
<i>P15/CDKN2B</i>	10

* Based on data from references 1–3 and 21–30. CpG = cytosine-followed-by-guanosine site.

† Frequency increases with disease progression.

silenced genes (39). Both decitabine and 5-azacytidine can induce cellular differentiation, expression of several proteins (globins and hepatic enzymes), re-expression of Dr antigens, and erythroid and granulocytic differentiation (51–58). Exposure of murine 10T1/2 embryonic fibroblasts to decitabine induced formation of mature muscle cells or adipocytes (51). Cellular differentiation was observed at concentrations of decitabine that did not inhibit synthesis of protein, RNA, or DNA. Decitabine was 30-fold more potent than 5-azacytidine as an

inducer of differentiation, which occurred at concentrations that inhibited DNA methylation (55). This finding suggests the importance of a modification in the 5 position of the pyrimidine ring for the induction of cellular differentiation.

In vitro, inhibition of DNA methylation by decitabine suppressed growth of human tumor cell lines but not of normal fibroblasts (41). The effect of decitabine on proliferation of L1210 leukemia cells was related to dose and duration of exposure (50). Decitabine showed in vivo antitumor activity against murine leukemias; survival time was up to four times that seen in controls (59). Brown Norway rats carrying acute myelogenous leukemia showed increased survival after treatment with three doses of decitabine (each 5 mg/kg of body weight) compared with animals that received high-dose ara-C. However, rats carrying leukemia cells resistant to ara-C did not have longer survival after decitabine treatment (60). Treatment of rats with tumors resulted in in vivo demethylation and reactivation of tumor suppressor gene expression (41). Finally, decitabine reduced formation of lung tumors induced by carcinogen treatment (42) and intestinal adenomas in a mouse model of familial adenomatous polyposis (43).

RESULTS OF THERAPY WITH HYPOMETHYLATING AGENTS

Induction of Fetal Hemoglobin

Hypomethylating agents have been investigated as a means to reactivate the methylated and silenced fetal hemoglobin gene in patients with thalassemia and sickle-cell anemia. Given as a continuous infusion of 2 mg/kg daily for 7 days every month, 5-azacytidine induced reticulocytosis with fetal hemoglobin-containing cells and little marrow suppression (61). Koshy and colleagues (62) treated eight patients who had sickle-cell anemia with decitabine in a dose-escalation trial, in which therapy was started at 0.15 mg/kg per day, 5 days per week for 2 weeks. All patients responded, and the mean fetal hemoglobin level increased from 3.55% to 13.45%. Maximal fetal hemoglobin levels were attained within 4 weeks from the start of treatment compared with 3 to 12 months in patients receiving hydroxyurea. Although the efficacy of both 5-azacytidine and decitabine in increasing production of fetal hemoglobin is well documented, the potential carcinogenesis and toxicity associ-

Table 3. Treatment of Solid Tumors with 5-Azacytidine and Decitabine*

Location or Type of Tumor	Agent	Patients	Total Dose	Duration and Route of Administration	Patients with Response	Reference
		<i>n</i>		<i>d</i>	%	
Breast	5-Azacytidine	11	300–700 mg/m ²	8 IV	63	65
	5-Azacytidine	31	600 mg/m ²	10 IV	6.4	65
	5-Azacytidine	4	275–850 mg/m ²	10 SC	25	66
	5-Azacytidine	NA	1.6 mg/kg	10 IV	17	67
Ovary	5-Azacytidine	4	275–850 mg/m ²	10 SC	25	66
	Decitabine	24	225 mg/m ²	10 IV	8	69
Colon	5-Azacytidine	27	NA		4	63
	5-Azacytidine	6	300–700 mg/m ²	10 IV	33	64
	5-Azacytidine	4	275–850 mg/m ²	10 SC	0	66
	Decitabine	42	225 mg/m ²	1 IV	0	68
Lung	Decitabine	15	200–600 mg/m ²	1 IV	20	70
Prostate	Decitabine	14	225 mg/m ²	1 IV	16	71
Melanoma	5-Azacytidine	5	300–700 mg/m ²	8 IV	40	64
	Decitabine	18	225 mg/m ²	1 IV	0	68
Mesothelioma	DHAC	40	5 g/m ² per 24 h	1 CI	20	75
	DHAC	41	1500 mg/m ² per d	5 CI	17	76
	DHAC	29	1500 mg/m ² per d	5 CI	17	73

* CI = continuous infusion; DHAC = dihydroazacytidine; IV = intravenously; NA = not available; SC = subcutaneously.

ated with long-term administration has delayed broader applications.

Solid Tumors

Despite ample literature on the *in vitro* anti-tumor activity of 5-azacytidine and decitabine in epithelial tumors, few clinical trials have been performed in solid tumors. In general, response rates have been low (63–76) (Table 3). Common side effects were nausea, vomiting, diarrhea, and myelosuppression. In a trial involving 30 patients with solid tumors, 5-azacytidine (1 to 24 mg/kg given as an intravenous bolus daily for at least 8 days) induced remission in 7 of 11 patients with breast cancer, 2 of 5 patients with melanoma, and 2 of 6 patients with colon cancer (64). In another trial, of 58 patients with metastatic breast cancer, 2 of 27 (7%) patients receiving 5-azacytidine and 3 of 27 patients (11%) receiving lomustine responded to treatment (65). Among 170 patients treated with 5-azacytidine, 1.6 mg/kg per day for 10 days, antitumor activity was reported in 17% of patients with breast carcinoma, and 21% of patients with lymphoma had “clinical improvement” (67).

Momparler and colleagues (70) treated 15 patients with stage IV non-small-cell lung carcinoma with decitabine, 200 to 600 mg/m² given over 8 hours. Nine of

these patients were evaluable; median survival was 6.7 months, and 3 patients were still alive at 15 months. Plasma concentrations of the drug were in the range needed to induce *in vitro* tumor suppression and gene expression in lung cell lines (70).

Dihydro-5-azacytidine (DHAC) has shown activity in mesothelioma. Samuels and associates (74) treated 36 patients with untreated mesothelioma with DHAC, 1500 mg/m² per day for 5 days by continuous infusion, and cisplatin, 15 mg/m² per day for 5 days. The rate of response to DHAC was 17% (5 of 29 evaluable patients), including 1 patient who experienced complete remission. Addition of cisplatin to the DHAC regimen did not increase the response rate. In another study (75), 40 patients with disseminated malignant melanoma received DHAC, 5 mg/m² as a continuous infusion over 24 hours. Three of 26 “good-risk” patients and 0 of 14 “poor-risk” patients showed a response. The absence of myelosuppression has raised possibilities for DHAC combination regimens in malignant melanoma and mesothelioma (76).

Malignant Hematologic Disease

Acute Leukemia

Salvage Therapy: Studies of 5-azacytidine in salvage therapy for acute myelogenous leukemia are summarized

Table 4. Salvage Therapy for Acute Myelogenous Leukemia with 5-Azacytidine and Decitabine*

Study (Reference)	Patients, <i>n</i>	Total Dose	Duration and Route of Administration	Patients with Response, %
5-Azacytidine				
McCredie et al. (77)	28	400 mg/m ²	5 d IV	28
Karon et al. (78)	37	150–200 mg/m ²	5 d IV	36
Von Hoff et al. (79)	200	150–400 mg/m ²	5 d IV	36
Vogler et al. (80)	45	100–300 mg/m ²	5 d IV	33
Saiki et al. (81)	120	300–750 mg/m ²	5 d IV	9
Winton et al. (82)	128	112–200 mg/m ² (+ amsacrine)	2 d CI	16
Goldberg et al. (83)	53	200 mg/m ² (+ mitoxantrone)	3 d CI	15
Steuber et al. (84)	81	250 mg/m ² (+ amsacrine and etoposide)	2 d CI	0 (35)
Decitabine				
Salvage therapy				
Rivard et al. (85)	9	36–80 mg/kg	36–44 h CI	33
Momparler et al. (88)	6	45–100 mg/kg	40–90 h CI	89
Richel et al. (89)	16	250–1000 mg/m ² (± amsacrine)	6 d/6 h IV	62
Willemze et al. (86)	22	250 mg/m ² (+ amsacrine or idarubicin)	6 d/6 h IV	68
Willemze et al. (87)	63	250 mg/m ² (+ amsacrine or idarubicin)	6 d/6 h IV	39
First-line therapy				
Petti et al. (90)	12	90–120 mg/m ²	3 d/4 h IV	33
Schwartzmann et al. (91)	8	90 mg/m ² (+ daunorubicin)	5 d/4 h IV	75

* CI = continuous infusion; IV = intravenously; SC = subcutaneously.

in Table 4 (77–89). In a review of 200 patients with acute myelogenous leukemia treated with 5-azacytidine in different studies, the cumulative rates of complete and partial remission were 20% and 16%, respectively (79). Of note, the total dose of 5-azacytidine administered was not as important as the infusion schedule: Longer or continuous infusions were more effective than shorter ones (Table 4).

Studies of decitabine as a single agent in refractory or relapsed acute leukemia showed response rates of 33% to 89% (Table 4). Combination of decitabine with conventional therapy yielded higher response rates. Willemze and colleagues (87) conducted a randomized phase II study of decitabine plus either amsacrine (30 patients) or idarubicin (33 patients) in patients with relapse of acute myelogenous leukemia or acute lymphocytic leukemia (87). Twenty-three of 63 patients (36%) achieved complete remission, including 18 of 49 (37%) patients with a first relapse of acute myelogenous leukemia, 2 of 6 (33%) patients with a second relapse of acute myelogenous leukemia, and 1 of 3 (33%) patients with a first relapse of acute lymphocytic leukemia. Eleven patients died during induction of therapy. The most frequent side effects of decitabine were myelosuppression, nausea, vomiting, and diarrhea. When the dose of decitabine was 1250 mg/m² or more per course, neurotoxicities occurred. Rates of complete remission were

27% with decitabine plus amsacrine and 45% with decitabine plus idarubicin. The rate of complete remission was 61% among patients with acute myelogenous leukemia and normal karyotypes. Overall, these data suggest encouraging activity of decitabine in leukemia.

First-Line Therapy: 5-Azacytidine has been studied in combination with amsacrine and etoposide as consolidation therapy for acute myelogenous leukemia after remission (92, 93) or as maintenance therapy (94). Its use did not significantly influence clinical outcome. A small number of patients with acute leukemias were treated with decitabine as part of their first-line therapy. In one study, 12 patients with newly diagnosed acute myelogenous leukemia who were not eligible for intensive chemotherapy (because of old age or myocardial diseases) received decitabine, 90 to 120 mg/m² as 4-hour intravenous infusions daily for 3 consecutive days every 4 to 6 weeks (90) (Table 4). Seven of the patients presented with acute myelogenous leukemia after progression of myelodysplastic syndromes. Of 10 evaluable patients, 3 had complete remission and 1 had partial remission. The kinetics of response and the immunophenotypic patterns suggested differentiation as the basis for clinical response; progressive acquisition of mature myelomonocytic markers before complete clearance of bone marrow blasts was observed (90). Schwartzmann and colleagues (91) treated 8 patients

with newly diagnosed acute myelogenous leukemia with decitabine, 90 mg/m² intravenously over 4 hours daily for 5 days, and daunorubicin, 50 mg/m² for 3 days. Six patients had complete remission (75%) after one or two courses of therapy.

Myelodysplastic Syndromes

5-Azacytidine and decitabine have substantial activity in the treatment of myelodysplastic syndromes (Table 5). Silverman and associates (95) treated 43 patients with myelodysplastic syndromes with 5-azacytidine, 75 mg/m² given by continuous infusion daily for 7 days. Responses were observed in 21 of the 43 patients (49%); 5 (12%) had complete remission, 11 (25%) had partial remission, and 5 (12%) had hematologic improvement (95). Thirty-seven percent of patients had trilineage response, and 33% experienced myelosuppression. The same investigators later used subcutaneous 5-azacytidine, 75 mg/m² daily for 7 days, in 36 patients with myelodysplastic syndromes (96). No bone marrow hypoplasia was induced by this regimen, and the trilineage response rate was 40% (12 of 36 patients, of whom 4 had complete remission and 8 had partial remission); 6 had hematologic improvement. These findings led to a large randomized trial of subcutaneous 5-azacytidine, 75 mg/m² seven times daily every 4 weeks (99 patients), versus observation (92 patients) in 191 patients with high-risk myelodysplastic syndromes. Responses occurred in 63% of patients who received 5-azacytidine (6% had complete remission, 10% had

partial remission, and 47% had hematologic improvement). These results differed significantly from those in the observation group, in which 7% of patients experienced improvement. Median time to leukemia transformation (22 vs. 12 months; *P* < 0.01) and median survival (18 vs. 14 months) were longer in patients who received 5-azacytidine. The absence of significant differences in survival may have been due to the crossover design, which allowed patients whose disease progressed while receiving supportive care to receive 5-azacytidine. The persistent hematologic improvement in a high percentage of patients was accompanied by improved quality of life (97, 98). This study showed that treatment with 5-azacytidine effectively modified the natural history of myelodysplastic syndromes.

Wijermans and colleagues treated elderly patients with high-risk myelodysplastic syndromes with decitabine, 40 to 50 mg/m² given over 24 hours for three days every 6 weeks and later with 15 mg/m² given over 4 hours every 8 hours for 3 days every 6 weeks (in both cases, the total dose was 120 to 150 mg/m² per course) (99, 100). An update on the first 125 patients in three studies was reported recently (101). The median patient age was 70 years; risk according to the International Prognostic Scoring System was intermediate-1 in 35 patients, intermediate-2 in 38 patients, and high in 52 patients. Of 121 evaluable patients, 59 (49%) responded to therapy: 24 (20%) had complete remission, 12 (10%) had partial remission, and 23 (19%) had hematologic improvement. Response rates were 58% in the high-risk

Table 5. Treatment of Myelodysplastic Syndrome and Chronic Myelogenous Leukemia in Blastic Phase with 5-Azacytidine and Decitabine*

Disease	Study (Reference)	Patients	Total Dose	Duration and Route of Administration	Patients with Response	
		<i>n</i>	mg/m ²		%	
Myelodysplastic syndrome	5-Azacytidine	Silverman et al. (95)	43	75–150	7 d CI	49
		Silverman et al. (96)	36	75	7 d SC	40
		Silverman et al. (97)	99	75	7 d SC	63
	Decitabine	Wijermans et al. (99)	29	40–55	3 d CI	54
		Wijermans et al. (100)	66	45	4 h IV daily × 3	48
Chronic myelogenous leukemia in blastic phase	5-Azacytidine	Schiffer et al. (103)	27	150 (+ etoposide)	5 IV	58
		Dutcher et al. (104)	40	150 (+ mitoxantrone)	5 IV	23
	Decitabine	Kantarjian et al. (105)	47	100–200	5 IV	Blastic phase: 25 Accelerated phase: 53
		Sacchi et al. (106)	31	100–200	5 IV	26

* CI = continuous infusion; IV = intravenously; SC = subcutaneously.

group, 39% in the intermediate-1 risk group, and 45% in the intermediate-2 risk group. Ten patients (8%) died during therapy. Cytogenetic abnormalities disappeared in 15 patients with chromosomal abnormalities who had complete remission. Median duration of response was 9 months; median survival was 15 months (19 months in the intermediate-1 risk group, 13 months in the intermediate-2 risk group, and 14 months in the high-risk group).

The methylation status of *p15* before and after decitabine therapy was recently analyzed in a subset of these patients. Hypermethylation at three cytosine residues in the 5' region of the *p15* gene was detected in 13 patients (59%), and efficient reduction of methylation accompanied or preceded suppression of bone marrow blasts and improvement of cytopenias. However, responses to decitabine also occurred in the absence of *p15* hypermethylation. This suggests that *p15* is one but not the only molecular target of pharmacologic demethylation in myelodysplastic syndromes or that decitabine induces antimyelodysplastic activity through other mechanisms in addition to hypomethylation induction (102).

Chronic Myelogenous Leukemia

Twenty-seven patients with chronic myelogenous leukemia in the myeloid blastic phase were treated with 5-azacytidine (150 mg/m² per day intravenously in three divided doses for 5 days) and etoposide (75 mg/m² per day for 5 days). One patient had a complete response and 15 had a partial response (58% overall response rate) (Table 5). Median survival was 231 days among responders and 73 days among nonresponders (103). Dutcher and associates (104) administered mitoxantrone, 12 mg/m² per day for 3 days, and 5-azacytidine, 150 mg/m² per day for 5 days, to 40 patients with chronic myelogenous leukemia in accelerated or blastic phase. The overall response rate was 23% (5 patients had complete response, 2 had partial response, and 2 had hematologic improvement).

In a phase II trial of decitabine, 500 to 1000 mg/m² per course, for treatment of chronic myelogenous leukemia in accelerated and blastic phase, 2 of 20 (10%) patients with blastic phase disease achieved complete hematologic response (1 of whom had disappearance of Ph-positive cells) and 3 (15%) had hematologic improvement (overall response rate, 25%). Of 17 patients with accelerated-phase disease, 6 reverted to second

chronic phase (of whom 2 had suppression of Ph-positive cells); the overall objective hematologic response was 53% (105). In 162 adults with nonlymphoid chronic myelogenous leukemia in the blastic phase who received first salvage therapy, the response rate was similar among decitabine-treated patients (26%) and those treated with conventional high-dose chemotherapy (28%) (106), which consisted of high-dose cytarabine with or without anthracyclines. Median survival with decitabine therapy was 29 weeks compared with 21 weeks with intensive chemotherapy and 22 weeks with other single agents. Among patients 50 years of age or older, survival was significantly longer with decitabine therapy compared with intensive chemotherapy ($P < 0.01$). The positive effect of decitabine therapy was confirmed as an independent treatment variable by multivariate analysis (106).

CONCLUSIONS AND FUTURE PERSPECTIVES

The widespread DNA hypermethylation observed in neoplasia has prompted renewed interest in hypomethylating agents in the treatment of cancer, particularly as a novel method of inducing differentiation and stopping growth. Of the hypomethylating agents currently being tested in clinical trials, decitabine appears promising, not as "another ara-C," but as an effective drug (perhaps complementary to ara-C) with a unique mechanism of action and an acceptable toxicity profile. Reports of the efficacy of decitabine, both as salvage and first-line therapy for acute myelogenous leukemia, myelodysplastic syndromes, and chronic myelogenous leukemia in the blastic phase, are encouraging and are the beginning steps to a broad area of research.

An important issue that must be carefully evaluated is the *in vivo* mechanism of action and ideal dose of decitabine. The demonstrated efficacy of the drug in chronic myelogenous leukemia and myelodysplastic syndromes could be related to cytotoxicity or induction of hypomethylation (or both); studies are needed to clarify this issue. This knowledge is especially important because *in vitro*, decitabine exerts maximal demethylation at low doses but causes cytotoxicity at higher doses. Thus, low-dose regimens may take advantage of the hypomethylating effects of the drug, allowing more frequent or long-term administration while avoiding cytotoxic side effects.

Other promising areas of research include 1) combination of hypomethylating agents with other drugs that could potentiate the differentiation response, such as histone deacetylase inhibitors (107) and retinoic acid analogues (108); 2) regimens combining decitabine with disease-specific active agents, such as topotecan in myelodysplastic syndromes (109) and interferon- α in chronic myelogenous leukemia (110); and 3) development of alternate strategies for inhibition of DNA methyltransferase, such as use of antisense compounds (111). In addition, the crystalline structure of several methyltransferases has been reported (112), and computer modeling may help identify novel potent inhibitors of this enzyme. Finally, recent data from methylation profiling of human neoplasms has identified patients whose tumors have particularly high levels of CpG island methylation (30, 33). Patients whose neoplasms evolve along “hypermethylator” phenotypes may respond more favorably to hypomethylating agents, leading to use of such agents to treat only neoplasms in which hypermethylation has silenced tumor suppressor genes. Ultimately, development of strategies for achieving gene-specific demethylation may result in tailored therapy based on the unique methylation fingerprint of an individual patient’s neoplasms.

GLOSSARY

Acetylation: Addition of an acetyl coenzyme A group to a protein.

Chromatin: DNA packaged with associated proteins (histones). The degree of packaging affects gene activity.

Epigenetic: Changes in gene expression that are clonal but not associated with changes in the nucleotide sequence of involved genes.

Histones: Proteins that participate in the packaging of DNA into chromatin fibers.

Imprinting: A process in which only one of the two normal copies of a gene is expressed (functional). Usually, the expressed copy is determined on the basis of which parent it was derived from.

Methylation: The addition of a methyl (CH₃) group to a molecule (DNA, RNA, or protein).

Methyltransferase: An enzyme that can catalyze the methylation reaction.

Promoter: A region of DNA that is responsible for regulation of gene expression. A given promoter is typically located at the beginning of the gene it regulates.

Silencing: Irreversible inactivation of a gene by suppression of gene transcription.

Transposable elements: DNA sequences that are capable of moving from one site to another (so-called “jumping genes”) and that are present in numerous copies throughout the human genome. These elements resemble viral DNA and are commonly referred to as “parasitic DNA.”

From the University of Texas M.D. Anderson Cancer Center, Houston, Texas; and University of Florence, Florence, Italy.

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Requests for Single Reprints: Hagop M. Kantarjian, MD, M.D. Anderson Cancer Center, Leukemia Department, Box 61, 1515 Holcombe Boulevard, Houston, TX 77030.

Current Author Addresses: Dr. Santini: Department of Hematology, University of Florence, Viale Morgagni 85, Florence, Italy.

Drs. Kantarjian and Issa: M.D. Anderson Cancer Center, Leukemia Department, Box 61, 1515 Holcombe Boulevard, Houston, TX 77030.

References

1. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res.* 1998;72:141-96. [PMID: 0009338076]
2. Jones PA, Laird PW. Cancer epigenetics comes of age. *Nat Genet.* 1999;21:163-7. [PMID: 0009988266]
3. Issa JP, Baylin SB, Herman JG. DNA methylation changes in hematologic malignancies: biologic and clinical implications. *Leukemia.* 1997;11(Suppl 1):S7-11. [PMID: 0009130685]
4. Weissbach A. A chronicle of DNA methylation (1948-1975). *EXS.* 1993;64:1-10. [PMID: 0008418945]
5. Razin A, Shemer R. DNA methylation in early development. *Hum Mol Genet.* 1995;4 Spec No:1751-5. [PMID: 0008541875]
6. Bestor TH, Verdine GL. DNA methyltransferases. *Curr Opin Cell Biol.* 1994;6:380-9. [PMID: 0007917329]
7. Zingg JM, Jones PA. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. *Carcinogenesis.* 1997;18:869-82. [PMID: 0009163670]
8. Razin A, Riggs AD. DNA methylation and gene function. *Science.* 1980;210:604-10. [PMID: 0006254144]
9. Noyer-Weidner M, Trautner TA. Methylation of DNA in prokaryotes. *EXS.* 1993;64:39-108. [PMID: 0008380352]
10. Simmen MW, Leitgeb S, Charlton J, Jones SJ, Harris BR, Clark VH, et al. Nonmethylated transposable elements and methylated genes in a chordate genome. *Science.* 1999;283:1164-7. [PMID: 0010024242]
11. Bestor TH, Tycko B. Creation of genomic methylation patterns. *Nat Genet.* 1996;12:363-7. [PMID: 0008630488]

12. Barlow DP. Gametic imprinting in mammals. *Science*. 1995;270:1610-3. [PMID: 0007502071]
13. Goto T, Monk M. Regulation of X-chromosome inactivation in development in mice and humans. *Microbiol Mol Biol Rev*. 1998;62:362-78. [PMID: 0009618446]
14. Bird AP. CpG-rich islands and the function of DNA methylation. *Nature*. 1986;321:209-13. [PMID: 0002423876]
15. Jones PA. Altering gene expression with 5-azacytidine. *Cell*. 1985;40:485-6. [PMID: 0002578884]
16. Li E, Beard C, Jaenisch R. Role for DNA methylation in genomic imprinting. *Nature*. 1993;366:362-5. [PMID: 0008247133]
17. Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, et al. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet*. 1998;19:187-91. [PMID: 0009620779]
18. Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, et al. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature*. 1998;393:386-9. [PMID: 0009620804]
19. Feinberg AP, Vogelstein B. Hypomethylation of ras oncogenes in primary human cancers. *Biochem Biophys Res Commun*. 1983;111:47-54. [PMID: 0006187346]
20. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. *Nature*. 1998;395:89-93. [PMID: 0009738504]
21. Baylin SB, Hoppener JW, de Bustros A, Steenbergh PH, Lips CJ, Nelkin BD. DNA methylation patterns of the calcitonin gene in human lung cancers and lymphomas. *Cancer Res*. 1986;46:2917-22. [PMID: 0003009002]
22. Jones PA, Wolkowicz MJ, Rideout WM 3rd, Gonzales FA, Marziasz CM, Coetzee GA, et al. De novo methylation of the MyoD1 CpG island during the establishment of immortal cell lines. *Proc Natl Acad Sci U S A*. 1990;87:6117-21. [PMID: 0002385586]
23. Sakai T, Toguchida J, Ohtani N, Yandell DW, Rapaport JM, Dryja TP. Allele-specific hypermethylation of the retinoblastoma tumor-suppressor gene. *Am J Hum Genet*. 1991;48:880-8. [PMID: 0001673287]
24. Herman JG, Latif F, Weng Y, Lerman MI, Zbar B, Liu S, et al. Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci U S A*. 1994;91:9700-4. [PMID: 0007937876]
25. Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, et al. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat Med*. 1995;1:686-92. [PMID: 0007585152]
26. Kanai Y, Ushijima S, Hui AM, Ochiai A, Tsuda H, Sakamoto M, et al. The E-cadherin gene is silenced by CpG methylation in human hepatocellular carcinomas. *Int J Cancer*. 1997;71:355-9. [PMID: 0009139867]
27. Kane MF, Loda M, Gaida GM, Lipman J, Mishra R, Goldman H, et al. Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res*. 1997;57:808-11. [PMID: 0009041175]
28. Bender CM, Gonzalgo ML, Gonzales FA, Nguyen CT, Robertson KD, Jones PA. Roles of cell division and gene transcription in the methylation of CpG islands. *Mol Cell Biol*. 1999;19:6690-8. [PMID: 0010490608]
29. Ahuja N, Li Q, Mohan AL, Baylin SB, Issa JP. Aging and DNA methylation in colorectal mucosa and cancer. *Cancer Res*. 1998;58:5489-94. [PMID: 0009850084]
30. Toyota M, Issa JP. CpG island methylator phenotypes in aging and cancer. *Semin Cancer Biol*. 1999;9:349-57. [PMID: 0010547343]
31. Szyf M, Kaplan F, Mann V, Giloh H, Kedar E, Razin A. Cell cycle-dependent regulation of eukaryotic DNA methylase level. *J Biol Chem*. 1985;260:8653-6. [PMID: 0004019444]
32. Issa JP. Aging, DNA methylation and cancer. *Crit Rev Oncol Hematol*. 1999;32:31-43. [PMID: 0010586353]
33. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A*. 1999;96:8681-6. [PMID: 0010411935]
34. Herman JG, Civin CI, Issa JP, Collector MI, Sharkis SJ, Baylin SB. Distinct patterns of inactivation of p15INK4B and p16INK4A characterize the major types of hematological malignancies. *Cancer Res*. 1997;57:837-41. [PMID: 0009041182]
35. Kawano S, Miller CW, Gombart AF, Bartram CR, Matsuo Y, Asou H, et al. Loss of p73 gene expression in leukemias/lymphomas due to hypermethylation. *Blood*. 1999;94:1113-20. [PMID: 0010419905]
36. Kantharidis P, El-Osta A, deSilva M, Wall DM, Hu XF, Slater A, et al. Altered methylation of the human MDR1 promoter is associated with acquired multidrug resistance. *Clin Cancer Res*. 1997;3:2025-32. [PMID: 0009815593]
37. Issa JP, Zehnbaauer BA, Civin CI, Collector MI, Sharkis SJ, Davidson NE, et al. The estrogen receptor CpG island is methylated in most hematopoietic neoplasms. *Cancer Res*. 1996;56:973-7. [PMID: 0008640788]
38. Issa JP, Zehnbaauer BA, Kaufmann SH, Biel MA, Baylin SB. HIC1 hypermethylation is a late event in hematopoietic neoplasms. *Cancer Res*. 1997;57:1678-81. [PMID: 0009135007]
39. Bender CM, Zingg JM, Jones PA. DNA methylation as a target for drug design. *Pharm Res*. 1998;15:175-87. [PMID: 0009523301]
40. Chabot GG, Momparler RL. Pharmacokinetics of 5-aza-2'-deoxycytidine in animals and man: relevance to clinical trials. In: Momparler PL, de Vos D, eds. *5-Aza-2'-Deoxycytidine: Preclinical and Clinical Studies*. Haarlem, the Netherlands: PCH Publications; 1990:105-55.
41. Bender CM, Pao MM, Jones PA. Inhibition of DNA methylation by 5-aza-2'-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer Res*. 1998;58:95-101. [PMID: 0009426064]
42. Lantry LE, Zhang Z, Crist KA, Wang Y, Kelloff GJ, Lubet RA, et al. 5-Aza-2'-deoxycytidine is chemopreventive in a 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone-induced primary mouse lung tumor model. *Carcinogenesis*. 1999;20:343-6. [PMID: 0010069475]
43. Laird PW, Jackson-Grusby L, Fazeli A, Dickinson SL, Jung WE, Li E, et al. Suppression of intestinal neoplasia by DNA hypomethylation. *Cell*. 1995;81:197-205. [PMID: 0007537636]
44. Momparler RL. 5-Aza-2'-deoxycytidine: an overview. In: Momparler PL, de Vos D, eds. *5-Aza-2'-Deoxycytidine: Preclinical and Clinical Studies*. Haarlem, the Netherlands: PCH Publications; 1990:9-15.
45. Creusot F, Acs G, Christman JK. Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2'-deoxycytidine. *J Biol Chem*. 1982;257:2041-8. [PMID: 0006173384]
46. Powell WC, Avramis VI. Biochemical pharmacology of 5,6-dihydro-5-azacytidine (DHAC) and DNA hypomethylation in tumor (L1210)-bearing mice. *Cancer Chemother Pharmacol*. 1988;21:117-21. [PMID: 0002450689]
47. Goldberg RM, Reid JM, Ames MM, Sloan JA, Rubin J, Erlichman C, et al. Phase I and pharmacological trial of flazarabine (Ara-AC) with granulocyte colony-stimulating factor. *Clin Cancer Res*. 1997;3:2363-70. [PMID: 0009815635]
48. Jones PA, Taylor SM. Cellular differentiation, cytidine analogs and DNA methylation. *Cell*. 1980;20:85-93. [PMID: 0006156004]
49. Juttermann R, Li E, Jaenisch R. Toxicity of 5-aza-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc Natl Acad Sci U S A*. 1994;91:11797-801. [PMID: 0007527544]
50. Covey JM, Zaharko DS. Effects of dose and duration of exposure on 5-aza-

- 2'-deoxycytidine cytotoxicity for L1210 leukemia in vitro. *Cancer Treat Rep.* 1984;68:1475-81. [PMID: 0006210141]
51. Taylor SM, Jones PA. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell.* 1979;17:771-9. [PMID: 000090553]
 52. Pinto A, Attadia V, Fusco A, Ferrara F, Spada OA, Di Fiore PP. 5-Aza-2'-deoxycytidine induces terminal differentiation of leukemic blasts from patients with acute myeloid leukemias. *Blood.* 1984;64:922-9. [PMID: 0006206904]
 53. Creusot F, Acs G, Christman JK. Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2'-deoxycytidine. *J Biol Chem.* 1982;257:2041-8. [PMID: 0006173384]
 54. Attadia V, Fusco A, Di Fiore PP, Pinto A. Effects of 5-aza-2'-deoxycytidine on erythroid differentiation and globin synthesis of the human leukemic cell line K 562. In: Momparler PL, de Vos D, eds. *5-Aza-2'-Deoxycytidine: Preclinical and Clinical Studies.* Haarlem, the Netherlands: PCH Publications; 1990:89-103.
 55. Attadia V. Effects of 5-aza-2'-deoxycytidine on differentiation and oncogene expression in the human monoblastic leukemia cell line U-937. *Leukemia.* 1993;7(Suppl 1):9-16. [PMID: 0007683359]
 56. Momparler RL, Laliberte J. Induction of cytidine deaminase in HL-60 myeloid leukemic cells by 5-aza-2'-deoxycytidine. *Leuk Res.* 1990;14:751-4. [PMID: 0001700230]
 57. Pinto A, Zagonel V, Attadia V, Bullian PL, Gattei V, Carbone A, et al. 5-Aza-2'-deoxycytidine as a differentiation inducer in acute myeloid leukaemias and myelodysplastic syndromes of the elderly. *Bone Marrow Transplant.* 1989;4 Suppl 3:28-32. [PMID: 0002483349]
 58. Momparler RL, Bouchard J, Samson J. Induction of differentiation and inhibition of DNA methylation in HL-60 myeloid leukemic cells by 5-AZA-2'-deoxycytidine. *Leuk Res.* 1985;9:1361-6. [PMID: 0002417065]
 59. Covey JM, Zaharko DS. Comparison of the in vitro cytotoxicity (L1210) of 5-aza-2'-deoxycytidine with its therapeutic and toxic effects in mice. *Eur J Cancer Clin Oncol.* 1985;21:109-17. [PMID: 0002578963]
 60. Richel DJ, Colly LP, Lurvink E, Willemze R. Comparison of the anti-leukaemic activity of 5-aza-2'-deoxycytidine and arabinofuranosyl-cytosine in rats with myelocytic leukaemia. *Br J Cancer.* 1988;58:730-3. [PMID: 0002465015]
 61. Ley TJ, DeSimone J, Noguchi CT, Turner PH, Schechter AN, Heller P, et al. 5-Azacytidine increases gamma-globin synthesis and reduces the proportion of dense cells in patients with sickle cell anemia. *Blood.* 1983;62:370-80. [PMID: 0006191799]
 62. Koshy M, Dorn L, Bressler L, Molokie R, Lavelle D, Talischy N, et al. 2-deoxy 5-azacytidine and fetal hemoglobin induction in sickle cell anemia. *Blood.* 2000;96:2379-84. [PMID: 11001887]
 63. Moertel CG, Schutt AJ, Reitemeier RJ, Hahn RG. Phase II study of 5-azacytidine (NSC-102816) in the treatment of advanced gastrointestinal cancer. *Cancer Chemother Rep.* 1972;56:649-52. [PMID: 0004119908]
 64. Weiss AJ, SJE, Mastrangelo MJ, Laucius JF, Bellet RE. Phase I study of 5-azacytidine (NSC-102816). *Cancer Chemother Rep.* 1972;56:413-9.
 65. Cunningham TJ, Nemoto T, Rosner D, Knight E, Taylor S, Rosenbaum C, et al. Comparison of 5-azacytidine (NSC-102816) with CCNU (NSC-79037) in the treatment of patients with breast cancer and evaluation of the subsequent use of cyclophosphamide (NSC-26271). *Cancer Chemother Rep.* 1974;58:677-81. [PMID: 0004138966]
 66. Bellet RE, Mastrangelo MJ, Engstrom PF, Strawitz JG, Weiss AJ, Yarbrow JW. Clinical trial with subcutaneously administered 5-azacytidine (NSC-102816). *Cancer Chemother Rep.* 1974;58:217-22. [PMID: 0004133839]
 67. Weiss AJ, Metter GE, Nealon TF, Keanan JP, Ramirez G, Swaiminathan A, et al. Phase II study of 5-azacytidine in solid tumors. *Cancer Treat Rep.* 1977;61:55-8. [PMID: 0000067894]
 68. Abele R, Clavel M, Dodion P, Brunsch U, Gundersen S, Smyth J, et al. The EORTC Early Clinical Trials Cooperative Group experience with 5-aza-2'-deoxycytidine (NSC 127716) in patients with colo-rectal, head and neck, renal carcinomas and malignant melanomas. *Eur J Cancer Clin Oncol.* 1987;23:1921-4. [PMID: 0002449354]
 69. Sessa C, ten Bokkel Huinink W, Stoter G, Renard J, Cavalli F. Phase II study of 5-aza-2'-deoxycytidine in advanced ovarian carcinoma. The EORTC Early Clinical Trials Group. *Eur J Cancer.* 1990;26:137-8. [PMID: 0001691012]
 70. Momparler RL, Bouffard DY, Momparler LF, Dionne J, Belanger K, Ayoub J. Pilot phase I-II study on 5-aza-2'-deoxycytidine (Decitabine) in patients with metastatic lung cancer. *Anticancer Drugs.* 1997;8:358-68. [PMID: 0009180389]
 71. Thibault A, Figg WD, Bergan RC, Lush RM, Myers CE, Tompkins A, et al. A phase II study of 5-aza-2'-deoxycytidine (decitabine) in hormone independent metastatic (D2) prostate cancer. *Tumori.* 1998;84:87-9. [PMID: 0009619724]
 72. Walters RS, Theriault RL, Holmes FA, Hortobagyi GN, Esparza L. Phase II trial of fazarabine (ARA-AC, arabinosyl-5-azacytosine) in metastatic breast cancer. *Invest New Drugs.* 1992;10:43-4. [PMID: 0001376723]
 73. Curt GA, Kelley JA, Fine RL, Huguenin PN, Roth JS, Batist G, et al. A phase I and pharmacokinetic study of dihydro-5-azacytidine (NSC 264880). *Cancer Res.* 1985;45:3359-63. [PMID: 0002408749]
 74. Samuels BL, Herndon JE 2nd, Harmon DC, Carey R, Aisner J, Corson JM, et al. Dihydro-5-azacytidine and cisplatin in the treatment of malignant mesothelioma: a phase II study by the Cancer and Leukemia Group B. *Cancer.* 1998;82:1578-84. [PMID: 0009554537]
 75. Creagan ET, Schaid DJ, Hartmann LC, Loprinzi CL. A phase II study of 5,6-dihydro-5-azacytidine hydrochloride in disseminated malignant melanoma. *Am J Clin Oncol.* 1993;16:243-4. [PMID: 0007687819]
 76. Vogelzang NJ, Herndon JE 2nd, Cirincione C, Harmon DC, Antman KH, Corson JM, et al. Dihydro-5-azacytidine in malignant mesothelioma. A phase II trial demonstrating activity accompanied by cardiac toxicity. *Cancer and Leukemia Group B. Cancer.* 1997;79:2237-42. [PMID: 0009179072]
 77. McCredie KB, Bodey GP, Burgess MA, Guterman JU, Rodriguez V, Sullivan MP, et al. Treatment of acute leukemia with 5-azacytidine (NSC-102816). *Cancer Chemother Rep.* 1973;57:319-23. [PMID: 0004127393]
 78. Karon M, Sieger L, Leimbrock S, Finklestein JZ, Nesbit ME, Swaney JJ. 5-Azacytidine: a new active agent for the treatment of acute leukemia. *Blood.* 1973;42:359-65. [PMID: 0004125239]
 79. Von Hoff DD, Slavik M, Muggia FM. 5-Azacytidine. A new anticancer drug with effectiveness in acute myelogenous leukemia. *Ann Intern Med.* 1976;85:237-45. [PMID: 0000060073]
 80. Vogler WR, Miller DS, Keller JW. 5-Azacytidine (NSC 102816): a new drug for the treatment of myeloblastic leukemia. *Blood.* 1976;48:331-7. [PMID: 0000060156]
 81. Saiki JH, Bodey GP, Hewlett JS, Amare M, Morrison FS, Wilson HE, et al. Effect of schedule on activity and toxicity of 5-azacytidine in acute leukemia: a Southwest Oncology Group Study. *Cancer.* 1981;47:1739-42. [PMID: 0006164472]
 82. Winton EF, Hearn EB, Martelo O, Presant CA, Adler S, Vogler WR, et al. Sequentially administered 5-azacytidine and amnarsine in refractory adult acute leukemia: a phase I-II trial of the Southeastern Cancer Study Group. *Cancer Treat Rep.* 1985;69:807-11. [PMID: 0002410119]
 83. Goldberg J, Gryn J, Raza A, Bennett J, Browman G, Bryant J, et al. Mitoxantrone and 5-azacytidine for refractory/relapsed ANLL or CML in blast crisis: a leukemia intergroup study. *Am J Hematol.* 1993;43:286-90. [PMID: 0007690519]
 84. Steuber CP, Krischer J, Holbrook T, Camitta B, Land V, Sexauer C, et al. Therapy of refractory or recurrent childhood acute myeloid leukemia using

- amsacrine and etoposide with or without azacitidine: a Pediatric Oncology Group randomized phase II study. *J Clin Oncol*. 1996;14:1521-5. [PMID: 0008622066]
85. Rivard GE, Momparler RL, Demers J, Benoit P, Raymond R, Lin K, et al. Phase I study on 5-aza-2'-deoxycytidine in children with acute leukemia. *Leuk Res*. 1981;5:453-62. [PMID: 0006173545]
86. Willemze R, Archimbaud E, Muus P. Preliminary results with 5-aza-2'-deoxycytidine (DAC)-containing chemotherapy in patients with relapsed or refractory acute leukemia. The EORTC Leukemia Cooperative Group. *Leukemia*. 1993;7(Suppl 1):49-50. [PMID: 0007683357]
87. Willemze R, Suci S, Archimbaud E, Muus P, Stryckmans P, Louwagie EA, et al. A randomized phase II study on the effects of 5-aza-2'-deoxycytidine 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):S24-7. [PMID: 0009130688]
88. Momparler RL, Rivard GE, Gyger M. Clinical trial on 5-aza-2'-deoxycytidine in patients with acute leukemia. *Pharmacol Ther*. 1985;30:277-86. [PMID: 0002433702]
89. Richel DJ, Colly LP, Kluin-Nelemans JC, Willemze R. The antileukaemic activity of 5-Aza-2 deoxycytidine (Aza-dC) in patients with relapsed and resistant leukaemia. *Br J Cancer*. 1991;64:144-8. [PMID: 0001713050]
90. Petti MC, Mandelli F, Zagonel V, De Gregoris C, Merola MC, Latagliata R, et al. Pilot study of 5-aza-2'-deoxycytidine (Decitabine) in the treatment of poor prognosis acute myelogenous leukemia patients: preliminary results. *Leukemia*. 1993;7 Suppl 1:36-41. [PMID: 0007683355]
91. Schwartzmann G, Fernandes MS, Schaan MD, Moschen M, Gerhardt LM, Di Leone L, et al. Decitabine (5-Aza-2'-deoxycytidine; DAC) plus daunorubicin as a first line treatment in patients with acute myeloid leukemia: preliminary observations. *Leukemia*. 1997;11 Suppl 1:S28-31. [PMID: 0009130689]
92. Volger WR, Weiner RS, Moore JO, Omura GA, Bartolucci AA, Stagg M. Long-term follow-up of a randomized post-induction therapy trial in acute myelogenous leukemia (a Southeastern Cancer Study Group trial). *Leukemia*. 1995;9:1456-60. [PMID: 0007544851]
93. Rees JK, Gray RG, Wheatley K. Dose intensification in acute myeloid leukaemia: greater effectiveness at lower cost. Principal report of the Medical Research Council's AML9 study. MRC Leukaemia in Adults Working Party. *Br J Haematol*. 1996;94:89-98. [PMID: 0008757514]
94. Zittoun R, Jehn U, Fiere D, Haanen C, Lowenberg B, Willemze R, et al. Alternating v repeated postremission treatment in adult acute myelogenous leukemia: a randomized phase III study (AML6) of the EORTC Leukemia Cooperative Group. *Blood*. 1989;73:896-906. [PMID: 0002645950]
95. Silverman LR, Holland JF, Weinberg RS, Alter BP, Davis RB, Ellison RR, et al. Effects of treatment with 5-azacytidine on the in vivo and in vitro hematopoiesis in patients with myelodysplastic syndromes. *Leukemia*. 1993;7 Suppl 1:21-9. [PMID: 0007683352]
96. Silverman LR, Holland JF, Demakos EP, Gattani A, Cuttner J. 5-azacytidine in myelodysplastic syndromes (MDS): the experience at Mount Sinai Hospital, New York. *Leuk Res*. 1994;18:21.
97. Silverman LR, Demakos EP, Peterson B, Odchimar-Reissig R, Nelson D, Kornblith AB, Stone R, et al. A randomized controlled trial of subcutaneous azacytidine (AZA C) in patients with myelodysplastic syndromes (MDS): a study of the cancer and leukemia group B, American Society of Clinical Oncology 34th Meeting, Los Angeles [Abstract]. *Proceedings of American Society of Clinical Oncology*. 1998;17:14a.
98. Kornblith AB, Herndon JE, Silverman LR. The impact of 5-azacytidine on the quality of life of patients with myelodysplastic syndrome (MDS) treated in a randomized phase III trial of the Cancer and Leukemia Group B. *Proceedings of the American Society of Clinical Oncology*. 1998;77:49a.
99. Wijermans PW, Krulder JW, Huijgens PC, Neve P. Continuous infusion of low-dose 5-Aza-2'-deoxycytidine in elderly patients with high-risk myelodysplastic syndrome. *Leukemia*. 1997;11(Suppl 1):S19-23. [PMID: 0009130687]
100. Wijermans P, Lübbert M, Verhoef G, Bosly A, Ravoet C, Andre M, et al. Low-dose 5-aza-2'-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. *J Clin Oncol*. 2000;18:956-62. [PMID: 10694544]
101. Wijermans P, Luebbert M, Verhoef G, et al. DNA demethylating therapy in MDS—the experience with 5-aza-2'-deoxycytidine (decitabine) [Abstract]. *Blood* 1999;94(Suppl 1):306a.
102. Daskalakis M, Nguyen TT, Wijermans P, et al. Reduction of p15 hypermethylation in bone marrow cells from patients with MDS following treatment with a methyltransferase inhibitor, decitabine [Abstract]. *Blood*. 1998;94:306a.
103. Schiffer CA, DeBellis R, Kasdorf H, Wiernik PH. Treatment of the blast crisis of chronic myelogenous leukemia with 5-azacitidine and VP-16-213. *Cancer Treat Rep*. 1982;66:267-71. [PMID: 0006173123]
104. Dutcher JP, Eudey L, Wiernik PH, Paietta E, Bennett JM, Arlin Z, et al. Phase II study of mitoxantrone and 5-azacytidine for accelerated and blast crisis of chronic myelogenous leukemia: a study of the Eastern Cooperative Oncology Group. *Leukemia*. 1992;6:770-5. [PMID: 0001379312]
105. Kantarjian HM, O'Brien SM, Keating M, Beran M, Estey E, Giralt S, et al. Results of decitabine therapy in the accelerated and blastic phases of chronic myelogenous leukemia. *Leukemia*. 1997;11:1617-20. [PMID: 0009324279]
106. Sacchi S, Kantarjian HM, O'Brien S, Cortes J, Rios MB, Giles FJ, et al. Chronic myelogenous leukemia in nonlymphoid blastic phase: analysis of the results of first salvage therapy with three different treatment approaches for 162 patients. *Cancer*. 1999;86:2632-41. [PMID: 0010594858]
107. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet*. 1999;21:103-7. [PMID: 0009916800]
108. Cote S, Momparler RL. Activation of the retinoic acid receptor beta gene by 5-aza-2'-deoxycytidine in human DLD-1 colon carcinoma cells. *Anticancer Drugs*. 1997;8:56-61. [PMID: 0009147612]
109. Lopez-Baena M, Mateos S, Pinero J, Trinidad Ortiz, Cortes F. Enhanced sensitivity to topoisomerase inhibitors in synchronous CHO cells pre-treated with 5-azacytidine. *Mutat Res*. 1998;421:109-16. [PMID: 0009748527]
110. Dore BT, Momparler RL. Effects of 5-aza-2'-deoxycytidine and interferon-alpha on differentiation and oncogene expression in HL-60 myeloid leukemic cells. *Anticancer Drugs*. 1992;3:281-7. [PMID: 0001381972]
111. MacLeod AR, Szyf M. Expression of antisense to DNA methyltransferase mRNA induces DNA demethylation and inhibits tumorigenesis. *J Biol Chem*. 1995;270:8037-43. [PMID: 0007713905]
112. Cheng X, Kumar S, Posfai J, Pflugrath JW, Roberts RJ. Crystal structure of the HhaI DNA methyltransferase complexed with S-adenosyl-L-methionine. *Cell*. 1993;74:299-307. [PMID: 0008343957]