DNA methylation and cancer therapy: new developments and expectations

Manel Esteller

Purpose of review
In addition to having genetic causes, cancer can also be considered an epigenetic disease. The main epigenetic modification is DNA methylation, and patterns of aberrant DNA methylation are now recognized to be a common hallmark of human tumors. One of the most characteristic features is the inactivation of tumor-suppressor genes by CpG-island hypermethylation of the CpG islands located in their promoter regions. These sites, among others, are the targets of DNA-demethylating agents, the promising chemotherapeutic drugs that are the focus of this article.
Recent findings
Four exciting aspects have recently arisen at the forefront of the advancements in this field: first, the development of new compounds with DNA-demethylating capacity that are less toxic (for example, procaine) and may be administered orally (for example, zebularine); second, a better knowledge of the molecular mechanisms underlying the action of these drugs for particular genes and throughout the genome; third, the establishment of more reliable techniques to measure the effects of these drugs in clinical samples, such as high-performance capillary electrophoresis; and fourth, a decisive effort in the clinical trials that has merited the approval of 5-azacytidine by the U.S. Food and Drug Administration for the treatment of myelodysplastic syndrome.
Summary
We are at the dawn of an era when epigenetic drugs will be an important weapon in our arsenal in the war against cancer. Hematological malignancies have provided a promising starting point, but studies will surely extend to all solid tumors. However, we need to continue our research to develop more specific DNA-demethylating agents, to understand their biologic effects, and to determine whether they may be successfully combined with other epigenetic drugs, such as the inhibitors of histone deacetylases, and classic chemotherapy compounds.

Keywords
DNA methylation, DNA-demethylating drugs, tumor-suppressor genes, 5-azacytidine, 5-aza-2′-deoxycytidine, zebularine, procaine, myelodysplastic syndrome

Curr Opin Oncol 17:55–60. © 2004 Lippincott Williams & Wilkins.

Cancer Epigenetics Laboratory, Spanish National Cancer Centre (CNIO) Madrid, Spain

Correspondence to Manel Esteller, MD, PhD, Cancer Epigenetics Laboratory, Molecular Pathology Program, Spanish National Cancer Centre (CNIO), Melchor Fernandez Almagro 3, Madrid 28029, Spain
Tel: 34 91 2246940; fax: 34 91 2246923; e-mail: mesteller@cnio.es

Current Opinion in Oncology 2005, 17:55–60

1040-8746

Introduction

Cancer is not only a polygenetic disease, but also a polyepigenetic disease. The inheritance of information based on gene-expression levels independent of the underlying DNA nucleotide sequence is known as epigenetics, as opposed to genetics, which is concerned with information transmitted on the basis of the gene sequence. The main epigenetic modification in humans is methylation of the nucleotide cytosine (C) when it precedes a guanine (G), forming the dinucleotide CpG. It is thought that about 6 to 8% of all cytosines are methylated in normal human DNA. Great effort has been devoted in recent years to understanding the establishment and relevance of aberrant DNA methylation patterns in human tumors. It is known that two apparently opposite phenomena coexist in the cancer cell: a profound loss of global 5-methylcytosine genomic content against a background of discrete areas of dense hypermethylation [1–5]. Overall hypomethylation takes place predominantly in repetitive DNA and endoparasitic sequences [6] and has been linked to the generation of chromosomal instability [7,8•] and nuclear disorganization [9•]. On the other hand, hypermethylation occurs in the CpG islands located in the promoters of certain tumor-suppressor genes such as $p16^{INK4a}$, BRCA1, or hMLH1 [1–5]. The methylation-associated silencing of these genes is a specific cancer marker, affects genes in all cellular pathways, and has a tumor-type-specific profile [2–4]. These hypermethylated sites, among others, are targeted by DNA-hypomethylating agents, thereby opening up new avenues for cancer treatment [10,11]. In fact, 5-azacytidine (Vidaza, Pharmion Corporation, CO, USA) has been recently approved by the U.S. Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome (http://www.fda.gov/bbs/topics/news/2004/NEW01069.html). This article

will describe the origins of this field and the directions in which it may head in the coming years.

DNA-demethylating agents: old, new, and recycled

Most current DNA-demethylating agents block the action of DNA methyltransferases (DNMTs), whose expression levels are usually moderately elevated in human tumors. The genetic inactivation of two DNMTs, DNMT1 and DNMT3b, induces demethylation of all known hypermethylated tumor-suppressor genes [12,13•] and remarkably slow growth [12]. DNMTs have two binding sites: one for the cytosine residue and another for *S*-adenosyl-methionine. It is expected that chemicals that tightly bind any of these pockets will reduce the methylation rate because of competitive inhibition.

The cytidine and 2′-deoxycytidine analogs are the most extensively studied members of this class. All of these compounds only inhibit DNMTs when incorporated into double-strand DNA [11], but the mechanism is slightly different, depending on the presence of a hydrogen atom bound at the 5 position of the pyrimidine ring. In analogs that lack this atom, the intermediate of the methylation reaction cannot undergo elimination to release the enzyme, and so the latter becomes covalently trapped. In other cases, for instance 5,6-dihydro-5-aza-cytidine, the enzyme forms a very stable complex with the analog, reducing the turnover. In all cases, nucleoside or deoxynucleoside analogs are transformed into the corresponding deoxynucleotide triphosphate inside the cell. Nucleosides are first converted into diphosphate nucleotides, which are then reduced and, after phosphorylation, are finally incorporated into DNA during the S phase. Cells lacking any of the enzymes involved in the synthesis of triphosphate deoxynucleotide derivatives are resistant to these compounds, and analogs that do not bind any of these enzymes cannot be incorporated into DNA, because they are inactive.

The first analog tested to determine whether it was an inhibitor of DNA methylation was 5-azacytidine [14]. This substance causes covalent arrest of DNMTs, resulting in cytotoxicity, and tumors with increased levels of these enzymes are expected to be more sensitive to the drug [14]. 5-Azacytidine was tested for its usefulness as an antileukemic drug before its demethylating activity was known [15]. Even these first studies reported its interference at very low concentrations (less than 0.1 μM) with RNA processing, tRNA methylation, and protein synthesis, because of its preferential incorporation into RNA *in vivo* and in cultured cells. Treatment with equimolar amounts of both cytidine and 5-azacytidine inhibits the incorporation of the latter into nucleic acids, resulting in no alteration of the cell cycle either *in vivo* or *ex vivo*. 5-Azacytidine is degraded by a nucleoside deaminase, so cells that express this enzyme strongly are less sensitive to this compound [15]. As a consequence, 5-azacytidine is much less frequently used in methylation studies.

The analog 5-aza-2′-deoxycytidine (Decitabine, SuperGen Inc., CA, USA) is one of the most commonly used demethylating drugs in assays with cultured cells. It overcomes the major incorporation of 5-azacytidine into RNA and reduces its side effects. Indeed, 5-aza-2′-deoxycytidine is only incorporated into DNA. However, it has been shown that cytidine deaminase can degrade 5-aza-2′-deoxycytidine to 5-aza-2′-deoxyuridine, resulting in the complete loss of DNMT inhibition. The high level of cytidine deaminase in liver and spleen may reduce the half-life of this compound to 15 to 20 minutes when measured *in vivo* [16]. A phase I clinical trial has suggested that deamination is the major pathway involving this compound [17].

Zebularine [1-(beta-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one] is another cytidine analog that has recently been developed. Similarly to 5-azacytidine, it was first tested as an anticancer agent rather than as a DNA demethylating agent [18]. Initially designed as a cytidine deaminase inhibitor, it has been shown to form a covalent complex with DNA methyltransferases (such as Hha I) [19], to deplete human DNMTs [20•], and, most importantly, to act as a real DNA demethylating agent inducing reactivation of hypermethylated genes in yeast models and of $p16^{INK4a}$ in bladder cancer cells [21••]. Furthermore, zebularine has also shown promising antitumoral effects in mouse xenograft [21••]. Perhaps the most interesting feature of this DNA demethylating agent, compared with 5-azacytidine and 5-aza-2′-deoxycytidine, is that it is chemically stable and of low toxicity, being the first drug in its class that can be given orally. As a drawback, it requires high doses, up to 1 g kg^{-1} body weight in the mouse model, making its use in human clinical trials rather difficult. However, an opportunity has arisen for its use in combination with other DNA-demethylating drugs, because it has been demonstrated that sequential treatment with 5-aza-2′-deoxycytidine followed by zebularine hindered the remethylation of tumor-suppressor genes [20•].

The use of the nucleoside analogs in clinical trials has been limited by their side effects, such as thrombocytopenia and neutropenia, which are probably caused by cytotoxic effects associated with the incorporation of the drugs into DNA independently of their DNA-hypomethylation value. This has encouraged the search for inhibitors of DNA methylation that are not incorporated into DNA. The drug procainamide, approved by the FDA for the treatment of cardiac arrhythmias, has been proposed as a nonnucleoside inhibitor of DNA methylation [22]. Procainamide causes global DNA hypomethylation [22] and restores expression of the detoxifier

gene GSTP1 in prostate cancer cells in which it has been silenced by hypermethylation [23]. This action is thought to be mediated by binding of procainamide to GC-rich DNA sequences [24]. Recently, procaine, a drug approved by the FDA for use as a local anesthetic, has also been found to have DNA hypomethylation and growth-inhibitory activity [25••]. Both procaine and procainamide are derivatives of 4-aminobenzoic acid, but the former is the ester with 2-(diethylamino)ethanol and the latter is the amide with 2-(diethylamino)ethylamine. Procaine acts as an inhibitor of DNA methylation in breast cancer cells, causing global genomic DNA hypomethylation and demethylation and reactivation of tumor-suppressor genes with hypermethylated CpG islands [25••]. This effect is associated with, and possibly mediated by, strong procaine binding to CpG-rich DNA [25••]. Procaine also simultaneously suppresses growth in these breast cancer cells with the occurrence of demethylating events [25••].

Finally, it is worth mentioning that tests have just begun of other agents with potential as DNA demethylating compounds and antitumoral drugs, including several other cytidine and 2-deoxycytidine analogs, such as 5-fluoro-2′-deoxycytidine, fazarabine (Ara-C), pseudoisocytidine, 5,6-dihydro-5-azacytosine, 6-azacyditine, and cytarabine [11]; the antihypertensive drug hydralazine [22,26•]; epigallocathechin-3-gallate, the main polyphenol compound in green tea [27]; the psammaplins, found in marines sponges [28•]; and *S*-adenosylmethionine analogs and competitive inhibitors, such as 5′-amino-5′-deoxyadenosine, sinefungin, L-ethionine, and dihydroxypropyladenine [11].

Mechanisms of action of DNA-demethylating agents and considerations

It is currently thought that the main mechanism of action of DNA-demethylating agents as anticancer drugs is the reactivation of dormant tumor-suppressor genes by the demethylation of their aberrantly hypermethylated promoter-CpG islands. All human primary tumors feature methylation-associated silencing of tumor-suppressor genes (and tumor-suppressor-like genes) that affect all cellular pathways: from cell-cycle inhibitors (for example, $p16^{INK4a}$, $p15^{INK4b}$, and so on) to inducers of apoptosis (for example, DAPK, TMS1, and so on), from DNA repair genes (for example, hMLH1, BRCA1, MGMT, and so on) to cell adhesion (for example, E-cadherin, TIMP2, and so on), and from hormonal receptors (for example, estrogen, progesterone, prolactin, retinoids, and so on) to detoxifiers (for example, GSTP1, PRDX1, and so on) [2,3]. CpG-island hypermethylation plays a role even in familial tumor cases and could be the acquired second-hit necessary to fully inactivate that particular tumor-suppressor gene with a germline mutation in one allele [29]. In some cases, it is tempting to speculate that DNA-demethylating agents can protect against the development of some of these familial tumors by preventing the methylation of the second wild-type allele. This is especially interesting in the context of inherited gastric tumors related to germline mutations in E-cadherin, where the second retained allele is most often shut down by hypermethylation [30].

These genes are genetically intact and so reactivation completely restores their normal function, as has been demonstrated for the mismatch repair gene hMLH1 and the MDM2-inhibitor $p14^{ARF}$ [31,32]. This release of gene silencing by the demethylating agent is not definitive, and, given sufficient time, that particular tumor-suppressor gene will be completely shut down again by methylation. However, the temporal window for overexpression of multiple tumor-suppressor genes could have been enough to induce cell death or to have enhanced the action of another chemotherapy drug. In the context of the latter issue, promising experiments have been undertaken in which the use of demethylating agents is combined with more classic antineoplastic drugs, such as cisplatin. It is also a matter of dose: higher doses of these DNA-demethylating drugs act as cytotoxics, independently of their hypomethylating capacity, whereas lower doses act as cytostatics, which are more dependent on their capacity to restore the expression of hypermethylated tumor-suppressor genes. Furthermore, the demethylating effect of 5-aza-2-deoxycytidine seems to be universal, affecting all human cancer cell lines [33••], although this may not be the case for its cytotoxic capacity [34]. This is the conclusion that may be drawn from cancer-cell-line and mouse-tumor models, although we really do not know the molecular and cellular responses of patients with cancer in their entirety.

One obstacle of all the current DNA-demethylating agents has been the lack of specificity of the drugs used [11]. Demethylating agents cause global hypomethylation, and we cannot reactivate solely the particular gene we would wish to [11]. If we assume that only tumor-suppressor genes were hypermethylated, this would not be a great problem. However, we do not know if we have disrupted essential methylation at certain sites that could cause further problems for the patients. Mouse-model studies are currently inconclusive: the first studies that crossed a deficient DNA methylation mouse with a strain prone to colon cancer revealed an adenoma-protective effect [35], whereas most recent reports suggest that mice with hypomorphic alleles of DNMTs are more susceptible to lymphomagenesis and chromosomal instability [8•]. In any case, these latter findings have not been observed in humans [36,37], in whom a completely different pattern is noted. By this we do not mean constantly deficient DNA methylation, as occurs in the mouse model, but that we are giving a short-duration treatment to a patient with cancer who would have a bad outcome if left untreated. Furthermore, we should re-

member that the tumors of these patients have already lost 40 to 50% of their total 5-methylcytosine content [29,38•], and so they have probably attained the lowest levels of 5-methylcytosine compatible with the life of the cancer cell, and so the treatment with the DNA-demethylating agent could be the ideal last straw that causes these cells to die.

Thus, at lower doses, which avoid excessive toxicity to the patients, DNA-demethylating drugs restore the expression of hypermethylated tumor-suppressor genes. This feature can be enhanced if we remember that DNA methylation is just one of several levels involved in epigenetic silencing. Histone modification is also important, specifically, in this case, histone deacetylation that associates with gene silencing. Histone-deacetylase inhibitors are also widely tested for their potential as anticancer drugs [39–41]. However, what makes the case more interesting is that the use of both types of drug, DNA-demethylation agents and histone-deacetylase inhibitors, causes a synergism in the reactivation of hypermethylated tumor-suppressor genes [42]. These *in vitro* observations have also prompted the development of several clinical trials to test their power in patients.

Finally, it is worth considering that the anticancer features of DNA-demethylating drugs can also be due to other mechanisms than the restoration of the expression of classic tumor-suppressor genes. For example, 5-aza-2′-deoxycytidine alone can induce, by mechanisms that are not fully understood, the re-expression of certain silenced tumor-suppressor genes that do not have apparent CpG island hypermethylation, such as the pro-apoptotic gene APAF-1 [43] and the cell-cycle inhibitor $p19^{INK4d}$ [44]. Indeed, there is a category of genes, which include the MAGE, GAGE, and LAGE families, whose CpG islands are normally methylated (unlike what would be predicted by the classic dogma): some of these genes become demethylated in cancer cells, but others remain heavily hypermethylated [45]. In this scenario, treatment with a DNA-demethylating agent will induce the expression of all these MAGE, GAGE, and LAGE genes, which are well-known antigen inducers of the immune response, and the patient will perhaps have been provided with another line of defense, thanks to the immune system [45]. In this regard, it is known that, under normal circumstances, DNA-demethylating agents can induce autoimmune diseases by the induction of antigens that are usually repressed by DNA methylation [46].

The clinical use of DNA-demethylating agents

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

Several phase-I/II trials have been developed for solid tumors. Some of them involve a single agent, such as 5-aza-2′-deoxycytidine, in the treatment of non–small-cell lung carcinoma (achieving survival of more than 5 years in 1 of 15 cases) [47], or the combination with classic chemotherapeutic drugs such as cisplatinum in advanced cervical cancer (yielding 40% partial responses) [48]. However, it is in the field of hematological malignancies where DNA-demethylating agents have had their greatest success so far.

After the report of an initial 50% overall response rate with 40% complete remission in patients with high-risk myelodysplastic syndrome using 5-aza-2-deoxycytidine in a 3-day schedule [49], subsequent studies also found overall response rates of 49 to 54% with 23 to 29% complete responses [50,51]. A different scheduling model involving intravenous administration of 5-aza-2′-deoxycytidine for 1 hour over 10 to 20 days has also been successfully used [52••]. This treatment was well tolerated overall with few nonhematopoietic side effects. Elevation in liver function tests were observed in 6 of 50 cases (12%) and febrile episodes were noted in 26 of 50 patients (52%) [52••]. Interestingly, the response to this DNA demethylating drug seems to be better in those patients displaying high-risk cytogenetic abnormalities [37]. Furthermore, 5-aza-2′-deoxycytidine has a clinically significant and often long-lasting effect on the platelet count in a substantial number of high-risk patients with myelodysplastic syndrome [53•]. For 5-azacytidine, initial studies demonstrated a significant number of complete and partial remissions in patients with myelodysplastic syndrome [54]. These encouraging results prompted the development of a phase III comparative trial comparing 5-azacytidine with supportive care [55]. Most importantly, significant differences in favor of the DNA-demethylating-drug group were observed in relation to the rate of remission and quality of life of these patients [55]. The most common toxicity was transient myelosuppression, and patients usually recovered in time for the next treatment cycle [55]. Infection was thought to have been related to treatment in 20% of patients. The definitive support for an epigenetic treatment of hematological malignancies was provided in

2004 with the approval by the FDA of the use of 5-azacytidine for the treatment of all myelodysplastic syndrome subtypes (http://www.fda.gov/bbs/topics/news/2004/NEW01069.html), including refractory anemia, refractory anemia with ringed sideroblasts if accompanied by neutropenia or thrombocytopenia transfusions, refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, and chronic myelomonocytic leukemia.

Finally, I would like to mention two other issues. First, another line of investigation worth exploring is the combination of DNA-demethylating agents with histone-deacetylase inhibitors: the epigenetics double-bullet. The overall low profile of toxicity of histone-deacetylase inhibitors, such as sodium phenyl butyrate, valproic acid, LAQ814, SAHA, and MS275 [39–41], has also encouraged the pursuit of these studies. For example, the sequential administration of 5-azacytidine and phenylbutyrate in patients with myelodysplastic syndrome and acute myelogenous leukemia was well tolerated, and 2 of 11 patients had significant hematopoietic improvement [56]. Second, it is extremely important to define the parameters of response, clinically and molecularly. In the latter case, the demethylation of the CpG islands of tumor-suppressor genes, such as has recently been demonstrated for $p15^{INK4b}$ [57] and the measurement of the 5-methylcytosine DNA levels after treatment using high-performance capillary electrophoresis [58,59], which can be used for blood samples, could be excellent surrogate markers for testing the molecular efficacy of every newly developed DNA-demethylating drug or proposed drug administration schedule.

Conclusion

The approval by the FDA of 5-azacytidine for the treatment of myelodysplastic syndrome is welcome news not only for eager epigenetic researchers, but also, and most importantly, for the oncological patients themselves. It is a great advance, but above all it symbolizes a new wave of future epigenetic drugs and indications. We should understand these compounds in the context of a transcriptional therapy for cancer, for which we already have a successful precedent: the treatment of acute promyelocytic leukemia with all-*trans* retinoic acid. We must now extend the clinical trials with 5-azacytidine and 5-aza-2-deoxycytidine to solid tumors and stimulate the translational use of new drugs, such as zebularine and procaine. Furthermore, we should not stop there but look for more specific DNA-demethylating agents. As always, epigenetic drugs are not magic bullets, but their great antitumoral potential, alone and in combination with other widely used chemotherapy regimens, needs to be tested to assess how their value could be greatly enhanced to the benefit of patients.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • Of special interest
- •• Of outstanding interest

1 Jones PA, Laird PW: Cancer epigenetics comes of age. Nat Genet 1999, 21:163–167.

2 Esteller M, Corn PG, Baylin SB, et al.: A gene hypermethylation profile of human cancer. Cancer Res 2001, 61:3225–3229.

3 Esteller M: CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene 2002, 21:5427–5440.

4 Herman JG, Baylin SB: Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003, 349:2042–2054.

5 Feinberg AP, Tycko B: The history of cancer epigenetics. Nat Rev Cancer 2004, 4:143–153.

6 Ehrlich M: DNA methylation in cancer: too much, but also too little. Oncogene 2002, 21:5400–5413.

7 Chen RZ, Pettersson U, Beard C, et al.: DNA hypomethylation leads to elevated mutation rates. Nature 1998, 395:199889–199893.

8 • Eden A, Gaudet F, Waghmare A, et al.: Chromosomal instability and tumors promoted by DNA hypomethylation. Science 2003, 300:455.
In a mouse model, the presence of hypomorphic alleles of DNMT1 is associated with the development of lymphomas and chromosomal instability.

9 • Espada J, Ballestar E, Fraga MF, et al.: Human DNA methyltransferase 1 is required for maintenance of the histone H3 modification pattern. J Biol Chem 2004, Jun 25.
This article shows that cancer cells deficient in DNMT1 present aberrant nuclear structures.

10 Leone G, Voso MT, Teofili L, et al.: Inhibitors of DNA methylation in the treatment of hematological malignancies and MDS. Clin Immunol 2003, 109:89–102.

11 Villar-Garea A, Esteller M: DNA demethylating agents and chromatin-remodelling drugs: which, how and why? Curr Drug Metab 2003, 4:11–31.

12 Rhee I, Bachman KE, Park BH, et al.: DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 2002, 416:552–556.

13 • Paz MF, Wei S, Cigudosa JC, et al.: Genetic unmasking of epigenetically silenced tumor suppressor genes in colon cancer cells deficient in DNA methyltransferases. Hum Mol Genet 2003, 12:2209–2219.
This article demonstrates that the double inactivation of DNMT1 and DNMT3b induces a massive reactivation of previously dormant methylated tumor-suppressor genes.

14 Jones PA, Taylor SM: Cellular differentiation, cytidine analogs and DNA methylation. Cell 1980, 20:85–93.

15 Li LH, Olin EJ, Buskirk HH, et al.: Cytotoxicity and mode of action of 5-azacytidine on L1210 leukemia. Cancer Res 1970, 30:2760–2769.

16 Ho DH: Distribution of kinase and deaminase of 1-beta-D-arabinofuranosylcytosine in tissues of man and mouse. Cancer Res 1973, 33:2816–2820.

17 Rivard GE, Momparler RL, Demers J, et al.: Phase I study on 5-aza-2′-deoxycytidine in children with acute leukemia. Leuk Res 1981, 5:453–462.

18 Driscoll JS, Marquez VE, Plowman J, et al.: Antitumor properties of 2(1H)-pyrimidinone riboside (zebularine) and its fluorinated analogues. J Med Chem 1991, 34:3280–3284.

19 Zhou L, Cheng X, Connolly BA, et al.: Zebularine: a novel DNA methylation inhibitor that forms a covalent complex with DNA methyltransferases. J Mol Biol 2002, 321:591–599.

20 • Cheng JC, Weisenberger DJ, Gonzales FA, et al.: Continuous zebularine treatment effectively sustains demethylation in human bladder cancer cells. Mol Cell Biol 2004, 24:1270–1278.
It shows that the combination of 5-aza-2′-deoxycytidine and zebularine is highly effective in maintaining the demethylation status of a tumor-suppressor gene.

21 •• Cheng JC, Matsen CB, Gonzales FA, et al.: Inhibition of DNA methylation and reactivation of silenced genes by zebularine. J Natl Cancer Inst 2003, 95:399–409.
The first study showing the use of an oral DNA demethylating agent that restores the methylation of a tumor-suppressor gene and has antitumoral effects.

22 Cornacchia E, Golbus J, Maybaum J, et al.: Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity. J Immunol 1988, 140:2197–2200.

23 Lin X, Asgari K, Putzi MJ, et al.: Reversal of GSTP1 CpG island hypermethylation and reactivation of pi-class glutathione S-transferase (GSTP1) expression in human prostate cancer cells by treatment with procainamide. Cancer Res 2001, 61:8611–8616.

24 Thomas TJ, Messner RP: Effects of lupus-inducing drugs on the B to Z transition of synthetic DNA. Arthritis Rheum 1986, 29:638–645.

25 •• Villar-Garea A, Fraga MF, Espada J, et al.: Procaine is a DNA-demethylating agent with growth-inhibitory effects in human cancer cells. Cancer Res 2003, 63:4984–4989.
The first demonstration that the local anesthetic drug procaine also has DNA demethylation and antitumoral activities.

26 • Segura-Pacheco B, Trejo-Becerril C, Perez-Cardenas E, et al.: Reactivation of tumor suppressor genes by the cardiovascular drugs hydralazine and procainamide and their potential use in cancer therapy. Clin Cancer Res 2003, 9:1596–1603.
A new study pinpoints the DNA demethylating capacity of the old drug hydralazine.

27 • Fang MZ, Wang Y, Ai N, et al.: Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. Cancer Res 2003, 63:7563–7570.
The first demonstration that these compounds may be able to reactivate hypermethylated tumor-suppressor genes.

28 • Pina IC, Gautschi JT, Wang GY, et al.: Psammaplins from the sponge Pseudoceratina purpurea: inhibition of both histone deacetylase and DNA methyltransferase. J Org Chem 2003, 68:3866–3873.
The first evidence of the DNA demethylation capacity of these compounds.

29 Esteller M, Fraga MF, Guo M, et al.: DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet 2001, 10:3001–3007.

30 Machado JC, Oliveira C, Carvalho R, et al.: E-cadherin gene (CDH1) promoter methylation as the second hit in sporadic diffuse gastric carcinoma. Oncogene 2001, 20:1525–1528.

31 Herman JG, Umar A, Polyak K, et al.: Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA 1998, 95:6870–6875.

32 Esteller M, Cordon-Cardo C, Corn PG, et al.: p14ARF silencing by promoter hypermethylation mediates abnormal intracellular localization of MDM2. Cancer Res 2001, 61:2816–2821.

33 •• Paz MF, Fraga MF, Avila S, et al.: A systematic profile of DNA methylation in human cancer cell lines. Cancer Res 2003, 63:1114–1121.
A study that shows that all cancer cell lines are sensitive to the DNA-demethylating effect of 5-aza-2′-deoxycytidine.

34 Nieto M, Samper E, Fraga MF, et al.: The absence of p53 is critical for the induction of apoptosis by 5-aza-2′-deoxycytidine. Oncogene 2004, 23:735–743.

35 Laird PW, Jackson-Grusby L, Fazeli A, et al.: Suppression of intestinal neoplasia by DNA hypomethylation. Cell 1995, 81:197–205.

36 Yang AS, Estecio MR, Garcia-Manero G, et al.: Comment on "Chromosomal instability and tumors promoted by DNA hypomethylation" and "Induction of tumors in nice by genomic hypomethylation". Science 2003, 302:1153.

37 Lubbert M, Wijermans P, Kunzmann R, et al.: Cytogenetic responses in high-risk myelodysplastic syndrome following low-dose treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine. Br J Haematol 2001, 114:349–357.

38 •• Fraga MF, Herranz M, Espada J, et al.: A mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors. Cancer Res 2004, 64:5527–5534.
The first demonstration that there is a progressive loss of 5-methylcytosine DNA content in tumorigenesis.

39 Johnstone RW, Licht JD: Histone deacetylase inhibitors in cancer therapy: is transcription the primary target? Cancer Cell 2003, 4:13–18.

40 Richon VM, Zhou X, Secrist JP, et al.: Histone deacetylase inhibitors: assays to assess effectiveness in vitro and in vivo. Methods Enzymol 2004, 376:199–205.

41 Villar-Garea A, Esteller M: Histone deacetylase inhibitors: understanding a new wave of anticancer agents. Int J Cancer, in press.

42 Cameron EE, Bachman KE, Myohanen S, et al.: Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 1999, 21:103–107.

43 Soengas MS, Capodieci P, Polsky D, et al.: Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature 2001, 409:207–211.

44 Zhu WG, Dai Z, Ding H, et al.: Increased expression of unmethylated CDKN2D by 5-aza-2′-deoxycytidine in human lung cancer cells. Oncogene 2001, 20:7787–7796.

45 Xiao J, Chen HS: Biological functions of melanoma-associated antigens. World J Gastroenterol 2004, 10:1849–1853.

46 Sekigawa I, Okada M, Ogasawara H, et al.: DNA methylation in systemic lupus erythematosus. Lupus 2003, 12:79–85.

47 Momparler RL, Bouffard DY, Momparler LF, et al.: Pilot phase I-II study on 5-aza-2′-deoxycytidine (Decitabine) in patients with metastatic lung cancer. Anticancer Drugs 1997, 8:358–368.

48 Pohlmann P, DiLeone LP, Cancella AI, et al.: Phase II trial of cisplatin plus decitabine, a new DNA hypomethylating agent, in patients with advanced squamous cell carcinoma of the cervix. Am J Clin Oncol 2002, 25:496–501.

49 Pinto A, Zagonel V, Attadia V, 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.

50 Wijermans P, Lubbert M, Verhoef G, 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, 18:956–962.

51 Wijermans PW, Luebbert M, Verhoef G: Low dose decitabine for elderly high risk MDS patients: who will respond? Blood 2002, 100:96a.

52 •• Issa JP, Garcia-Manero G, Giles FJ, et al.: Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2′-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 2004, 103:1635–1640.
A study that proves that low doses of 5-aza-2′-deoxycytidine are effective in myeloid malignancies.

53 • van den Bosch J, Lubbert M, Verhoef G, et al.: The effects of 5-aza-2′-deoxycytidine (Decitabine) on the platelet count in patients with intermediate and high-risk myelodysplastic syndromes. Leuk Res 2004, 28:785–790.
5-aza-2′-deoxycytidine affects platelet response and overall survival in myelodysplastic syndrome.

54 Silverman LR, Holland JF, Weinberg RS, 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):21–29.

55 Silverman LR, Demakos EP, Peterson BL, et al.: 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.

56 Camacho LH, Ryan HJ, Chanel P, et al.: Transcription modulation: a pilot study of sodium phenylbutyrate plus 5-azacytidine. Blood 2001, 98(suppl 1):460.

57 Daskalakis M, Nguyen TT, Nguyen C, et al.: Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-Aza-2′-deoxycytidine (decitabine) treatment. Blood 2002, 100:2957–2964.

58 Fraga MF, Uriol E, Borja Diego L, et al.: High-performance capillary electrophoretic method for the quantification of 5-methyl 2′-deoxycytidine in genomic DNA: application to plant, animal and human cancer tissues. Electrophoresis 2002, 23:1677–1681.

59 Paz MF, Avila S, Fraga MF, et al.: Germ-line variants in methyl-group metabolism genes and susceptibility to DNA methylation in normal tissues and human primary tumors. Cancer Res 2002, 62:4519–4524.