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Review

Reactivation of Epigenetically Silenced Genes by DNA Methyltransferase Inhibitors

Basic Concepts and Clinical Applications

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ABSTRACT

Hypermethylation of tumor suppressor genes is one of the most consistent hallmarks of human cancers. This epigenetic alteration has been associated with gene silencing and thus represents an important pathway for generating loss-of-function mutations. In this review, we survey the available literature on systematic, genome-wide approaches aimed at the identification of epigenetically silenced loci. These studies uncovered a variety of diverse genes, but a common signature for epigenetic reactivation has not been identified. Nevertheless, DNA methyltransferase inhibitors have shown significant clinical benefits, mostly in the therapy of leukemias. Recent analyses revealed substantial drug-induced methylation changes that can now be used as endpoints for the further refinement of clinical treatment schedules. Further optimization of epigenetic cancer therapies should be feasible through the use of novel DNA methyltransferase inhibitors with improved specificity. Rational design of epigenetic inhibitors might provide the foundation for a broader use of these drugs in the treatment of cancer.

Epigenetic mechanisms play a fundamental role in the interpretation of genetic information.[1] Depending on its particular epigenetic modification pattern, a gene can be expressed or silenced. Epigenetic modifications thus represent an integral mechanism for the control of complex gene expression patterns. One prominent example for an epigenetic control mechanism is the covalent modification of histones.[2] Histones can be modified at various amino acid residues by acetylation, phosphorylation, methylation and ubiquitination[3] and it has been suggested that particular combinations of these modifications constitute an "epigenetic code" for the regulation of gene expression.[2] Another prominent epigenetic modification is the methylation of cytosine residues in genomic DNA.[4] About 4% of the cytosines are usually methylated in mammalian genomic DNA[5] and it has been shown that this methylation is essential for mouse development.[6] In addition, it has also been shown that DNA methylation plays an essential role in several epigenetic phenomena, including genomic imprinting,[7] X-chromosome inactivation,[8] and retroelement silencing.[9]

CpG dinucleotides represent the consensus target sequences of DNA methylation in differentiated mammalian cells, but only a relatively small fraction of these sequences becomes methylated.[10] CpG dinucleotides can be clustered in CpG islands[11,12] which are often associated with promoter regions and remain unmethylated for most genes. The signals that determine whether a particular CpG-containing sequence becomes methylated have not been determined yet, but it has been suggested that protein-protein interactions between DNA methyltransferases and chromatin-associated proteins might play an important role.[13] As a consequence, DNA methylation is not evenly distributed over the genome. While the majority of repetitive elements are usually heavily methylated, gene-specific methylation appears to be rather restricted.

Genomic DNA methylation patterns can be drastically altered in human tumors.[14] Due to the selective forces driving carcinogenesis, tumor cells are characterized by specific epigenetic changes that promote uncontrolled cellular proliferation.[15] This explains the remarkable consistency of gene hypermethylation across all forms of human cancers.[16] When hypermethylation affects the CpG islands of genes it can trigger their stable repression and thus has consequences that are functionally equivalent to genetic mutations.[17] The acquisition of such an "epimutation" can therefore make an important contribution to cellular transformation. Over the past few years, the number of known epimutations has vastly increased. Prominent examples for genes "hit" by hypermethylation-induced silencing include the cell-cycle regulators *p15* and *p16*, the mismatch repair gene *MLH1*, as well as the apoptosis effector gene *Apaf-1*.[18-20] These epimutations constitute a tumor-specific epigenetic program that is reflected by the typical characteristics of human cancer

DNA methylation is catalyzed by DNA methyltransferases, a family of enzymes that comprises DNMT1, DNMT2, DNMT3A and DNMT3B in human cells.[21] To analyze the functional role of individual DNA methyltransferases in hypermethylation-induced gene silencing, homologous recombination has been used to disrupt DNMT1 and DNMT3B in the colorectal cancer cell line HCT116.[22,23] Lack of DNMT1 or DNMT3B had little effect on the DNA methylation pattern. However, a double knockout cell line lacking both enzyme activities showed a very strong reduction in 5-methylcytosine content.[23] The effects of DNA methyltransferase knockouts on genomic DNA methylation patterns have been analyzed by two approaches:[24] Differential methylation hybridization, which detects methylated regions in the genome through a CpG island microarray, and amplification of inter-methylated sites, which amplifies anonymous DNA sequences with a differential methylation pattern. It was demonstrated that cells lacking both DNMT1 and DNMT3B undergo a substantial loss of DNA methylation in the promoter region of tumor suppressor genes.[24] This effect could not be seen in single DNMT knockout cell lines, and it was therefore concluded that different DNA methyltransferases cooperate in the epigenetic regulation of gene silencing.

DNA METHYLTRANSFERASE INHIBITORS AS DEMETHYLATING AGENTS

Based on the rationale that hypermethylation-induced gene silencing could be uncovered by gene demethylation and reactivation (Fig. 1), many laboratories have analyzed gene expression patterns in human cancer cells with experimentally reduced DNA methylation levels. To this end, DNA methyltransferase inhibitors,[25] like 5-azacytidine, 5-aza-2'-deoxycytidine and, to a lesser extent, zebularine, have found widespread use. All three compounds need to be metabolized and phosphorylated to deoxynucleotide triphosphates in order to become incorporated into DNA. DNA methyltransferases recognize the modified bases as natural substrate, but fail to resolve a covalent reaction intermediate,[26] which results in the degradation of the covalently trapped enzymes. DNA demethylation is a direct consequence of enzyme trapping because of ongoing DNA replication in the presence of diminished DNA methyltransferase levels. After several rounds of DNA replication, this becomes detectable as a substantial decrease in the genomic methylation level.

5-azacytidine is one of the best-known DNA methyltransferase inhibitors and has been used both in the laboratory and in the clinical practice for more than 20 years. The compound also represents the first established epigenetic drug, since it gained FDA approval for the treatment of myelodysplastic syndrome in May 2004. It was originally developed as a cytotoxic agent[27] and its demethylating activity was only discovered through its ability to influence cellular differentiation.[28] 5-azacytidine is a ribose nucleoside and thus needs to be metabolized into a deoxyribonucleoside-triphosphate before it can be incorporated into DNA. Indeed, a major fraction of the drug becomes incorporated into RNA and thereby interferes with protein biosynthesis.[29] This effect most likely plays a major role in the cytotoxicity of 5-azacytidine.

5-aza-2'-deoxycytidine is the deoxyribose analogue of 5-azacytidine and a promising new drug for the treatment of myelodysplastic syndrome and other leukemias. This compound is a more potent hypomethylating agent, but it does not become incorporated into RNA. However, high doses of 5-aza-2'-deoxycytidine still induce

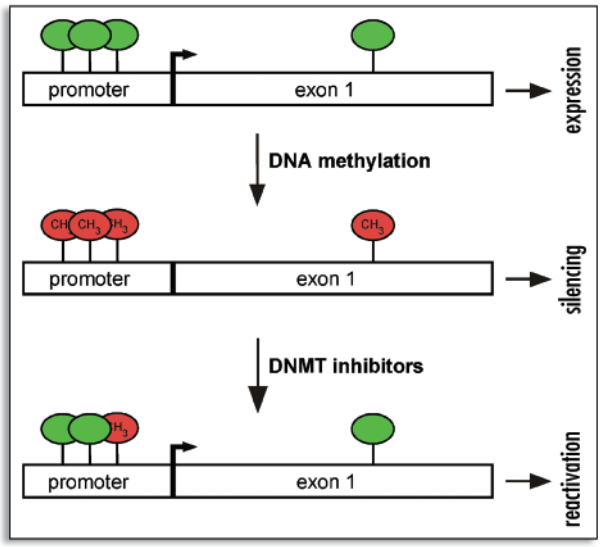

Figure 1. Establishment and reversion of epigenetic mutations. In healthy cells, tumor suppressor genes are unmethylated and expressed at normal levels. In cancer cells, hypermethylation of the promoter (and/or exon 1) region leads to gene silencing. Treatment with DNA methyltransferase inhibitors (DNMT inhibitors) removes the majority of 5-methylcytosine (CH_3), and can reactivate gene expression.

effects.[30] It was also shown that the differentiation inducing ability of decitabine in cultured fibroblasts had a narrow dose window, which provided a first indication that lower doses might induce demethylation with lower levels of overall cytotoxicity.[28] Both 5-azacytidine and 5-aza-2'-deoxycytidine are unstable in aqueous solutions,[31,32] which has limited their clinical application.

The more recently discovered nucleoside inhibitor zebularine[33] is a stable cytidine analog that has shown promising characteristics in several in vitro assays as well as in mouse models.[34-36] Cancer cell lines that responded to zebularine by DNA demethylation showed a complete depletion of the DNMT1 methyltransferase and a partial depletion of DNMT3A and DNMT3B.[36] Gene expression profiling revealed that zebularine specifically affected the gene expression patterns of human cancer cells, even if the number of genes upregulated by drug treatment was rather small (Table 1). Interestingly, zebularine was also shown to be active in mice when administered orally,[34] which suggested novel treatment modalities for epigenetic therapies. However, a recent study has demonstrated very low oral bioavailability in monkeys[37] and the clinical value of zebularine still remains to be determined.

IDENTIFICATION OF NOVEL TUMOR SUPPRESSOR GENES BY EXPERIMENTAL DEMETHYLATION

Because of the strong correlation between promoter hypermethylation and gene silencing, demethylating drugs have been repeatedly used for the identification of epigenetically silenced cancer genes. Transcriptional profiling in a variety of inhibitor-treated cancer cell lines revealed a relatively small number of genes that were significantly upor downregulated by demethylating drugs (Table. 1). Two studies suggested that genes in the interferon pathway might be induced by 5-aza-2'-deoxcycytidine treatment,[38,39] but, otherwise, no defined gene expression signatures have been identified. Based on the rationale

Table 1 **Overview of gene expression changes induced by DNMT inhibitors**

Cell Line	Drug	No. of Genes Analyzed	Genes Upreg. (%)	Genes Downreg. (%)	Reference
HT29	5-aza-CdR	4608	0.4	n.d.	38
T24	5-aza-CdR	6600	0.9	0.03	39
LD419	5-aza-CdR	6600	0.5	0.2	39
RKO	5-aza-CdR	10814	0.5	n.d.	41
KYSE30, KYSE410, KYSE520	5-aza-CdR	12599	1.0	n.d.	40
LNCaP	5-aza-CdR	1176	2.0	2.0	77
DU145	5-aza-CdR	1176	1.6	1.8	77
HCT116	5-aza-CdR	8000	0.8	0.6	43
HCT116 (1KO)	5-aza-CdR	8000	0.5	0.3	43
T24, HCT15, CFAPC-1	zebularine	13300	0.1	0.05	36
LD98, LD419, T-1, CCD-1070K	zebularine	13300	0.1	0.05	36
OCI-AML2	5-aza-CdR	22000	0.4	0.4	44

5-aza-CdR: 5-aza-2′-deoxycytidine; n.d., not determined

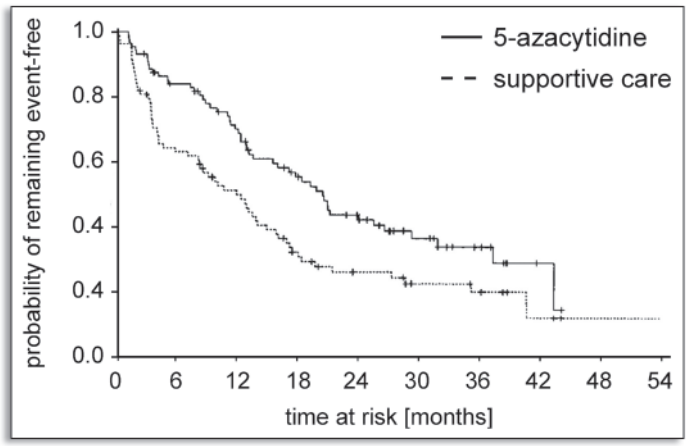

Figure 2. Clinical efficacy of DNA methyltransferase inhibitors in cancer patients. Survival of myelodysplastic syndrome patients treated with 5-azacytidine or supportive care, as determined by Kaplan-Meier analysis. Adapted from reference 48 with permission from the American Society of Clinical Oncology.

in cancer cells, pharmacological inhibition of DNA methyltransferases was also used to uncover novel cancer-related genes. Transcriptional profiling of esophageal squamous cell carcinoma (ESCC) cell lines treated with 5-aza-2'-deoxycytidine identified ten putative tumor suppressor genes that were found to be methylated in primary tumor tissues.[40] Three of these genes, *CRIP-1*, *Apo D* and *NU* were overexpressed in ESCC cells and found to strongly inhibit their ability to grow colonies.[40] This provided an indication that all three genes might function as suppressors of tumor growth. In a similar study, the genes encoding *secreted frizzled-related proteins* (*SFRPs*) were

demethylation and were subsequently found to be hypermethylated in colorectal cancers.[41] More recently, it has been shown that epigenetic silencing of *SFRP* genes plays a functional role in the constitutive activation of the WNT signalling pathway, which further underscored the significance of these events for colorectal carcinogenesis.[42]

To compare the effects of pharmacological and genetic inactivation of DNA methyltransferases, transcriptional profiles of knockout cells were compared to those obtained from inhibitor-treated cells.[43] Interestingly, the effects of 5-aza-2'-deoxycytidine appeared fully established after 24 hours and more closely resembled those of histone deacetylase inhibitor (TSA) treatment than the effects of DNA methyltransferase knockouts. These results cannot be explained by the covalent trapping of DNA methyltransferases, followed by passive demethylation of DNA. Rather, it seems likely that active demethylation is triggered by the pharmacological inhibitors through an upstream mechanism involved in the regulation of both DNA methylation and histone deacetylation. Another surprising finding was the substantial fraction of genes found to be downregulated after exposure to 5-aza-2'-deoxycytidine. In contrast to the prevailing view, this suggested that hypomethylation may also be associated with gene silencing. Consistently, demethylation of the APM2 promoter region closely coincided with its transcriptional silencing.[43]

The validity of the results obtained with cell lines was confirmed by a study that used primary cells from leukemia patients after treatment with 5-aza-2'-deoxycytidine ex vivo or in vivo.[44] The transcriptional profiles obtained in these experiments were comparable to those obtained in cultured cell lines, which suggested that the molecular responses in cells and in patients might be similar. However, only half of the induced genes contained putative CpG islands in their 5' promoter region and only a fraction of those showed changes in their methylation patterns. This result provides a good starting point for a review of the complex data on drug-induced methylation changes in patient material.

THE USE OF DEMETHYLATING DRUGS IN CLINICAL TRIALS

Both 5-azacytidine and 5-aza-2'-deoxycytidine have been used in dozens of clinical trials for more than 20 years.[45,46] In agreement with standard procedures, most early trials used the drugs at concentrations that were close to the maximum tolerated dose. The clinical results were generally disappointing and included severe toxicities related to prolonged myelosuppression. More recently, treatment schedules have been adjusted to increase drug tolerance,[47] which has allowed the completion of several effective low-dose trials and provided the foundation for an increased clinical interest in DNA methyltransferase inhibitors. A randomized, controlled phase III trial of low-dose 5-azacytidine vs. supportive care in myelodysplastic

drug-treated patients (Fig. 2). Similar results appear to have been obtained in a phase III clinical trial of 5-aza-2'-deoxycytidine.[49] In addition, promising clinical responses have also been reported in phase I trials of low-dose 5-aza-2'-deoxycytidine in other leukemias, including AML and CML.[50,51] These results suggest that the demethylation induced by azanucleoside drugs might be of general benefit for the reversion of epigenetic lesions in cancer patients.

Drug-induced methylation changes have only recently been analyzed in the clinical setting. One of the first such studies focused on the methylation of the p15 tumor suppressor gene in the bone marrow of 5-aza-2'-deoxycytidine-treated patients with myelodysplastic syndrome.[52] The results suggested demethylation in nine out of 12 patients and also provided evidence of p15 reactivation by immunohistochemistry in four patients. It remains a possibility that these changes are at least partially attributable to the expansion of nonclonal cells in responding patients, but gene reactivation was also shown in morphologically dysplastic cells from patients that were not in complete remission. This indicated a significant potential of the drug to activate gene expression in tumors and also suggested a direct involvement of DNA demethylation.[52] However, there appeared to be no correlation between p15 demethylation and clinical response to decitabine in a larger phase I study of decitabine in MDS and AML patients.[50] Overall similar results were also obtained in a phase I study of 5-azacytidine in Epstein-Barr virus-associated tumors.[53] While these tumors showed substantial demethylation in the EBV promoter, reactivation of EBV expression could only be observed in one case and there appeared to be no detectable clinical response.

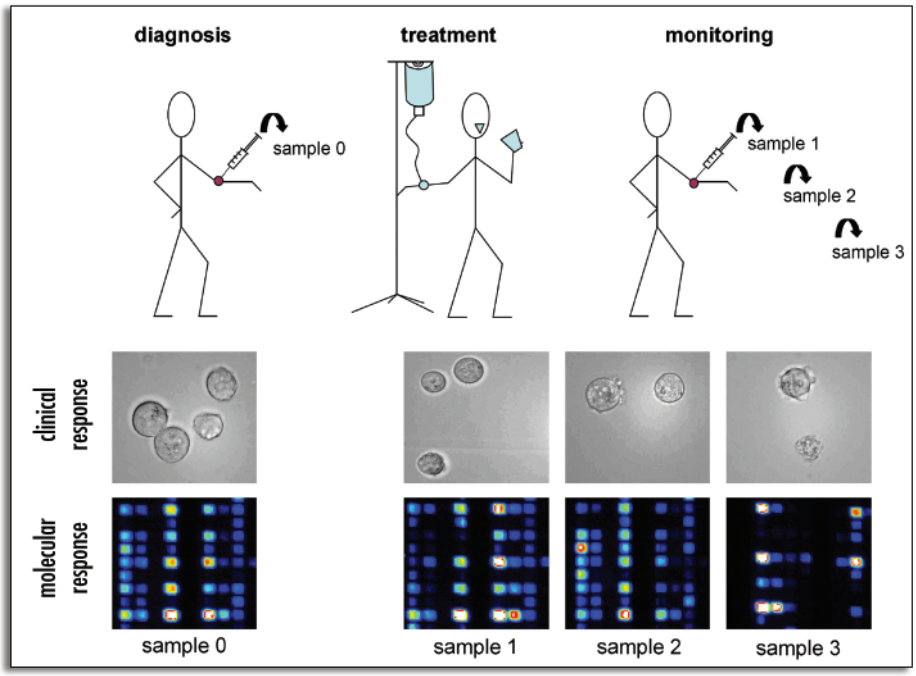

Figure 3. Epigenetic cancer therapy. After diagnosis, cancer patients can be treated with DNA methyltransferase and/or histone deacetylase inhibitors. Treatment response is being monitored at clinical and molecular levels by analysis of tumor and peripheral blood samples. Correlations between clinical and molecular parameters will be important for the optimization of clinical treatment schedules.

Biological responses might be maximized by changes in the drug delivery schedule, and for this reason different schedules are currently being tested. In some of these clinical trials, the clinical responses are being analyzed in parallel with the changes in genomic DNA methylation patterns (Fig. 3). One such example is provided by a recent phase I study of continuous 5-aza-2'-deoxycytidine infusion in a small group of patients with refractory solid tumors.[54] While the patient group was too small to assess the clinical benefit of the treatment schedule, all patients showed indications for demethylation in blood samples. This effect could be observed either by quantitative PCR-based methylation analysis of the MAGE-1 promoter or by HPLC analysis of genomic DNA. Importantly, it could also be shown that DNA methylation reverted to pretreatment levels within four weeks, which indicated that drug-induced demethylation is transient. These findings are in agreement with an analysis of drug-induced demethylation effects observed after 5-aza-2'-deoxycytidine treatment of MDS patients.[55] Here, the analysis of global DNA methylation levels from serial bone marrow samples indicated strong demethylation after the fourth treatment cycle, while karyotype normalization had occurred after the second treatment cycle. This indicated an important role of drug-mediated cytotoxicity, followed by a substantial demethylation of nonclonal cells. Again, DNA methylation returned to pretreatment levels several weeks after the last treatment cycle, which confirmed the transient nature of the demethylating effect.

The dynamics of drug-induced demethylation will be important for the design of combination trials that address the potential synergy between demethylating drugs and other chemotherapeutic agents. Experiments with mouse xenograft tumors have shown a strong chemosensitizing effect for 5-aza-2'-deoxycytidine when the drug was administered a few days before standard cytotoxic drugs.[56] This effect is presumably due to the epigenetic reactivation of apoptosis effector and DNA repair genes. 5-aza-2'-deoxycytidine has also been shown to be effective in a clinical combination study with the tyrosine kinase inhibitor imatinib mesylate (Gleevec).[51] A complete hematological response after decitabine treatment was seen in up to 50% of the Gleevec-resistant patients in the chronic phase of the disease. Furthermore, the demethylating effect was assessed by methylation analysis of LINE-1 elements as well as p15 tumor suppressor gene. Surprisingly, the degree of LINE-1 hypomethylation at the end of therapy was higher in patients who did not subsequently respond to therapy. This has been interpreted to reflect the possibility that hypomethylated cells die more rapidly, while cells resistant to therapy can withstand higher degrees of hypomethylation.[51]

Lastly, it will also be critical to design treatment schedules that maximize the reversion of epigenetic mutations with minimal influences on epigenetic effects required for normal cellular functions.[57] This is an important aspect in light of the finding that strong and continuous demethylation in animal and cellular models have been shown to promote genome instability and thereby induce tumor formation.[58,59] A recent analysis used Apc^{min} mice with three different levels of the DNA maintenance methyltransferase DNMT1 to model the degree of hypomethylation observed in tumors (Yamada intestinal tumorigenesis but promotes microadenoma formation in

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