Modifying the Epigenome as a Therapeutic Strategy in Myelodysplasia

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The term epigenetics refers to a number of biochemical modifications of chromatin that, without altering the primary sequence of DNA, have a role in genomic regulation and in particular gene expression control. These modifications can occur at the DNA level (i.e., DNA methylation), and affect the chromatin protein scaffold (i.e., histone code modifications), among several others. The study of these modifications is a very active area of research both at the basic and clinical levels. Clinical interest in these epigenetic alterations stems mainly from two observations. First, detection of specific epigenetic alterations could be used to develop cancer biomarkers (e.g., for the early detection or prognostication of cancer). Second, most epigenetic alterations are reversible both in vitro and in vivo, leading the way to the development of new anticancer therapies. This review focuses on the current clinical information regarding different forms of epigenetic therapy in patients with myelodysplastic syndromes (MDS). Basic aspects of DNA methylation or histone code alterations are not covered in detail in this review.

Introduction
The term epigenetics refers to a number of biochemical modifications of chromatin that, without altering the primary sequence of DNA, have a role in genomic regulation and in particular gene expression control. These modifications can occur at the DNA level (i.e., DNA methylation\(^1\)), and/or affect the chromatin protein scaffold (i.e., histone code modifications\(^2\)), among several others (Figure 1). The study of these modifications is a very active area of research both at the basic and clinical levels. Clinical interest in these epigenetic alterations stems mainly from two observations. First, detection of specific epigenetic alterations could be used to develop cancer biomarkers, such as for the early detection or prognostication of cancer. Second, most epigenetic alterations are reversible both in vitro and in vivo, leading the way to the development of new anticancer therapies. This review focuses on the current clinical information regarding different forms of epigenetic therapy in patients with myelodysplastic syndromes (MDS). Due to space limitations, basic aspects of DNA methylation or histone code alterations will not be covered, but will be referred to in a number of recent reviews in both fields for in-depth reading sources to complement the data presented here.

What Is the Epigenome?
The term “epigenome” can be conceptualized in two different ways: (1) a large-scale genome-wide constellation of epigenetic alterations; or (2) the understanding that the different known epigenetic alterations do not function in isolation but cooperate at the molecular level to control the different processes in which they are involved. Both concepts are fundamental when trying to integrate this information. First, it is now well documented that epigenetic alterations, for instance, in the case of DNA methylation, are not isolated but affect simultaneously hundreds of promoter regions.\(^3\) This concept, which for DNA methylation is known as the hypermethylator phenotype,\(^4\) presumes the existence of a basic underlying process that induces the aberrant methylation of multiple promoter regions. On the other hand, histone code alterations, in particular histone acetylation/deacetylation, are significantly more dynamic than DNA methylation.\(^5\) which is considered an irreversible event unless the cell is treated with inhibitors of the DNA methyltransferase (DNMT). Until recently, there was no evidence of a cancer histone code. Recent data suggest the possible existence of specific histone marks associated with a malignant phenotype.\(^6\) Second, it is now clear that all epigenetic events cooperate.\(^7\) It is accepted that there is a close interplay between DNA methylation and specific histone code alterations that render a gene silent. This has significant implications, as it will be predicted that therapeutic interventions targeting both events, for instance, combination epigenetic therapies with a hypomethylating agent and a histone deacetylase (HDAC) inhibitor, may be, in principle, more effective than the single agents (Figure 1).

This review focuses on DNA methylation and HDAC inhibition. It is now becoming evident that histone acetylation/deacetylation is just one of multiple biochemical modifications of histone proteins, and perhaps not even the more significant ones.\(^8\) However, because the available forms of epigenetic therapy target only these alterations, they will be the focus of this review.\(^9\)

DNA methylation
DNA methylation is the addition of a methyl group to a cytosine (C).\(^1\) This occurs only when the C is followed by a guanine (G) in the so called CpG pairs (Figure 1). CpG pairs are underrepresented in the human genome but cluster together in the so-called CpG islands. CpG islands can...
be located in the proximity of gene promoter regions (promoter-associated CpG islands) or in other intergenic regions. In a normal cell, most of DNA intergenic repetitive elements are methylated, whereas most promoter-associated regions, except for rare unimprinted genes or genes localized in the X chromosome in females, are not. Physiologic methylation of the intergenic regions is thought to be a process important for genomic stability. Methylation of promoter-associated CpG islands is associated with gene silencing. This again can be physiologic, as in the case of imprinted genes or X-linked genes in females, or may result in aberrant gene silencing. The current paradigm is that aberrant promoter DNA methylation is a functional equivalent to gene silencing by deletion or inactivating mutations and, therefore, serves as an additional inactivating mechanism of tumor suppressor genes. Hereinafter, we will discuss why these molecules are targeted.  

**Histone code**

Histone code refers to the potential combination of multiple biochemical alterations that can be imposed on histone tails. There is a large and growing number of these modifications. These can have either repressive or permissive effects on gene transcription. In particular, histone acetylation can affect both histone H3 and H4 in specific lysine residues. Histone acetylation is controlled by two different sets of enzymatic activities: histone acetyltransferases and HDACs (Figure 1). HDACs are compromised by a large number of proteins grouped in three different classes (class I to III). Current HDAC inhibitors inhibit mainly enzymes from class I and II. Other potential future targets include histone methylation and phosphorylation. Indeed, drugs with aurora kinase inhibitory activity target, in part, this last modification that is fundamental for mitosis control.

**Why Target MDS?**

The question is whether MDS is a disease more amenable to epigenetic therapy than other leukemias/lymphomas or even solid tumors. The answer is empiric in that most of the early success with drugs that eventually have been recognized as hypomethylating agents was first observed in patients with MDS and acute myeloid leukemia (AML). Now, it is accepted that aberrant DNA methylation is as common in solid tumors as it is in hematologic malignancies, and that different subtypes of disease, such as cutaneous lymphomas, are sensitive to the use of HDAC inhibitors, an approach that has been less effective, as single agents, in leukemia. In contrast, leukemias and MDS are sensitive to single-agent hypomethylating agents. Why are there differences in chemosensitivity between solid tumors, so far quite refractory to the use of epigenetic therapy, and leukemias, and why are leukemias more sensitive to hypomethylating agents, and lymphomas to HDAC inhibitors? The answer is unknown at the present time and may not depend on intrinsic molecular epigenetic alterations but more with pharmacologic issues that require dose/schedule optimization. It is also important to realize that, at the present time, most studies have failed to show a relationship between induction of DNA hypomethylation or histone acetylation and clinical response. This suggests that it is possible that neither of these two epigenetic marks are involved in the clinical mechanism of action of these drugs, or that other processes downstream of either DNA hypomethylation or induction of histone acetylation are key.

That said, the current working hypothesis is that reactivation of key genes or network of genes mediates clinical responses. It is thought that the lack of a clear association between induction of hypomethylation and clinical response is related to the fact that most studies so far have been limited to single gene or small gene set analysis. In
part this is due to the lack of MDS cell lines and of a specific immunophenotype of the MDS cell and MDS animal models that could allow easy in vivo or ex vivo analysis. Genes frequently known to be hypermethylated in MDS include RIL,15 calcitonin,16 and p15,17 among several others.18 Experience in AML19 predicts that MDS should be characterized by the methylation of multiple CpG islands. An effort needs to be made to map this DNA methylation target and match this with other genetic alterations in MDS. This work is currently ongoing. Finally, there is no evidence of an altered histone code in MDS. Recent results have failed to identify a HDAC class I or II expression specific profile in human leukemia (H. Yang, manuscript in preparation).

Experience with DNA Hypomethylating Agents

At the present time, two DNA hypomethylating agents have been approved for patients with MDS in the US: 5-azacitidine13 and 5-aza-2′-deoxycytidine.20 5-azacitidine is approved for all French-American-British (FAB) MDS subtypes and 5-aza-2′-deoxycytidine is approved for patients with intermediate-1 or higher risk by the International Prognostic Score (IPSS).21 5-azacitidine was initially developed by the Cancer and Leukemia Group B (CALGB) group in a number of sequential studies that led to the development of the CALGB 9221 study.13 This was a randomized study of 5-azacitidine versus supportive care with a crossover design and a parallel quality of life questionnaire.22 Results are summarized in Table 1. 5-azacitidine was associated with an increased response rate and time to AML transformation and a trend towards improved overall survival compared with supportive care. Quality of life analysis indicated that this therapy was associated with an improvement in the quality of life of patients treated with 5-azacitidine instead of supportive care. The results of this study have been reanalyzed recently using modern response criteria23 (Table 1). At the American Society of Hematology 2005 meeting in Atlanta, Silverman et al presented very significant results of an analysis of several CALGB trials of 5-azacitidine.24 These data suggested that, despite the relatively low CR rate with 5-azacitidine, this intervention was associated with a significant improvement in overall survival and disease-free survival, in particular in patients with an estimated pretreatment survival of less than 1.2 years.24 Also, important data were presented regarding the dynamics of response from the CALGB studies that indicated that 75% to 90% of responses are observed between courses 4 and 6 of therapy, respectively, with a plateau on the response rate after that.24 The implications of this information are of importance, as they suggest that we should continue this type of therapy for at least 4 to 6 courses to achieve maximal effect before discontinuing therapy. Also, data were presented at the American Society of Hematology 2005 meeting indicating that 5-azacitidine therapy was associated with improvement in bleeding and rates of infectious complications.25

There are several limitations to the CALGB studies. First, because the CALGB 9221 study was conducted in the early 1990s, routine cytogenetic information was only obtained in a subset of patients; therefore, it is not possible to analyze responses by IPSS21 criteria. Second, at that time there was limited knowledge regarding the relationship between DNA hypomethylation and use of 5-azacitidine; therefore, there is limited information regarding the molecular effects of this therapy in that trial. Also, supportive care measures have improved since that study was conducted. Recent studies have suggested that response rates with current modern supportive care techniques, such as the use of growth factors, significantly improve response rates with 5-azacitidine and that shorter 5-day schedules may have the same activity and perhaps less myelosuppression than more intense (7- or 10-day) schedules.26,27 Recently, an intravenous formulation of 5-azacitidine has been approved. This may allow continuing therapy in patients who developed skin complications with chronic subcutaneous 5-azacitidine use. A large multicenter study of 5-azacitidine evaluating the effects of this agent on survival has been completed, and results are expected soon.

Table 1. Clinical experience with 5-azacitidine in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) in both untreated and relapsed/refractory patients.

<table>
<thead>
<tr>
<th>CALGB 922113</th>
<th>Updated CALGB 922123</th>
<th>5-azacitidine and phenylbutyrate</th>
<th>5-azacitidine and valproic acid</th>
<th>5-azacitidine and valproic acid in untreated patients</th>
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<tbody>
<tr>
<td>Phase</td>
<td>3</td>
<td>3</td>
<td>1/2</td>
<td>1/2</td>
</tr>
<tr>
<td>N</td>
<td>99</td>
<td>99</td>
<td>29</td>
<td>53</td>
</tr>
<tr>
<td>CR, no. (%)</td>
<td>7 (7)</td>
<td>10 (10)</td>
<td>4 (14)</td>
<td>12 (22)</td>
</tr>
<tr>
<td>PR, no. (%)</td>
<td>16 (16)</td>
<td>1 (1)</td>
<td>1 (3)</td>
<td>3 (6)</td>
</tr>
<tr>
<td>HI, no. (%)</td>
<td>37 (37)</td>
<td>36 (36)</td>
<td>6 (21)</td>
<td>7 (13)</td>
</tr>
<tr>
<td>OR, no. (%)</td>
<td>60 (60)</td>
<td>47 (47)</td>
<td>11 (38)</td>
<td>22 (41)</td>
</tr>
</tbody>
</table>

* Includes patients with AML and relapsed and refractory disease.

Abbreviations: N, number; CR, complete remission; PR, partial remission; HI, hematologic improvement; OR, overall response. HI may include complete marrow responses.
5-aza-2′-deoxycytidine initially followed a parallel development strategy to that of 5-azacitidine. Initial studies in Europe indicated that this agent was also active in elderly patients with MDS and AML. In these studies, 5-aza-2′-deoxycytidine was administered every 8 hours daily for 3 days. The positive early results led to a randomized phase 3 study of 5-aza-2′-deoxycytidine versus supportive care in the U.S. This study, in contrast to the CALGB 9221 study, did not have a crossover design and used more modern supportive care measures. Results are shown in Table 2. Treatment with this agent was associated with increased response rates, a positive effect on transfusion requirements, and a trend toward a longer median time to AML transformation or death compared with patients on the supportive care alone. That said, no effect was observed in terms of survival on an intent-to-treat analysis. The lower response rate, compared with that of other schedules of 5-aza-2′-deoxycytidine discussed later, and the limited effect on survival observed in this study could be explained by two observations. First, 43 patients treated on that study who were randomized to the 5-aza-2′-deoxycytidine arm received less than 2 cycles of therapy. Of importance, responders in the study required more than 2 cycles, or 3.3 months, of therapy to achieve response. It is therefore possible that if more patients could have been treated beyond 2 cycles of therapy, a greater fraction would have responded, which could have translated into longer survival. The reason not to continue beyond 2 cycles of therapy was not clear in a large fraction of these 43 patients, including 6 patients who never received therapy. Limited data exist in terms of the dynamics of DNA hypomethylation induction with this schedule of 5-aza-2′-deoxycytidine. A large randomized study is currently evaluating the effect of 5-aza-2′-deoxycytidine on survival in patients with MDS. Results are expected next year.

### Table 2. Clinical experience with 5-aza-2′-deoxycytidine in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) in both untreated and relapsed/refractory patients.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Randomized 3-day (%)</th>
<th>5-day schedule (%)</th>
<th>Combination with valproic acid (%)</th>
<th>Combination with valproic acid in untreated patients (%)</th>
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</thead>
<tbody>
<tr>
<td>N</td>
<td>89</td>
<td>95</td>
<td>53</td>
<td>10</td>
</tr>
<tr>
<td>CR, no. (%)</td>
<td>8 (9)</td>
<td>32 (34)</td>
<td>10 (19)</td>
<td>4 (40)</td>
</tr>
<tr>
<td>PR, no. (%)</td>
<td>7 (8)</td>
<td>1 (1)</td>
<td>2 (4)</td>
<td>1 (10)</td>
</tr>
<tr>
<td>HI, no. (%)</td>
<td>12 (13)</td>
<td>13 (13)</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>OR, no. (%)</td>
<td>27 (30)</td>
<td>46 (48)</td>
<td>12 (23)</td>
<td>5 (50)</td>
</tr>
</tbody>
</table>

* Includes patients with AML and relapsed and refractory disease. Abbreviations: N, number; CR, complete remission; PR, partial remission; HI, hematologic improvement; OR, overall response.

### Dose/Schedule Optimization of Hypomethylating Agents

Initial data from the Jones group indicated a dose response curve in terms of the effects of this class of drugs and cell differentiation that was eventually correlated with their hypomethylating effects. Following this observation, a number of trials have been developed to study lower-dose schedules of hypomethylating agents in leukemia. A pioneer of this type of study was a phase 1 low-dose biological study of 5-aza-2′-deoxycytidine for patients with advanced leukemia or MDS. In that study, the drug was administered daily over 1 hour via an intravenous infusion. This was repeated daily for 10, 15 or 20 days; the starting dose was 10 mg/m². No significant nonhematologic toxicity was observed up to doses of 15 mg/m² daily for 20 days; paradoxically, the highest number of responses were observed at intermediate dose levels such as 15 mg/m² intravenously for 10 days. Dose/schedules below or above these dose levels were associated with significantly lower response rates. Based on this data, a randomized Bayesian phase 2 study of 5-aza-2′-deoxycytidine was conducted in patients with MDS. Three arms were studied: 10 mg/m² intravenously for 10 days; 20 mg/m² intravenously for 5 days; and 20 mg/m² subcutaneously for 5 days. The total dose was identical in all three arms: 100 mg/m² per course. The study was designed to end early if any arm was likely to be associated with a complete remission rate of less than 20%. Both the 10-day intravenous and 5-day subcutaneous schedules were terminated early, indicating that the 5-day intravenous schedule may be superior to the other two schedules of 5-aza-2′-deoxycytidine administration. Also, the response rates observed with this trial appear to be superior to those observed with the initial randomized 3-day schedule of 5-aza-2′-deoxycytidine (Table 2). Because of the limitations of the initial study mentioned above, this observation needs to be confirmed. A confirmatory multicenter trial studying the 5-day schedule has been completed. 5-aza-2′-deoxycytidine administration was associated with transient global and gene-specific induction of DNA hypomethylation.

There is less experience with single-agent 5-azacitidine dose/schedule optimization based on DNA methylation dynamics. That said, two recent studies have indicated a potential role for dose improvement in MDS. In a study using 5-azacitidine post–allogeneic stem cell transplantation by De Lima et al at M.D. Anderson Cancer Center (MDACC), doses as low as 26 mg/m² of 5-azacitidine were able to induce global hypomethylation. Second, in a recently completed phase 1 study of low-dose 5-azacitidine with valproic acid in solid tumors by Kurzrock et al at MDACC, it was recently demonstrated that doses as low as 20 mg/m² were able to induce global DNA methylation in peripheral blood from these patients.
with data from Gore et al at Johns Hopkins University, these indicate that it could be possible to further optimize the dose/schedule of 5-azacitidine as well.

**Toxicities Associated with Hypomethylating Therapy**

The toxicity profile of 5-azacitidine and 5-aza-2′-deoxycytidine are not identical. Emesis and skin rashes are more frequently associated with 5-azacitidine. Myelosuppression is a characteristic of both agents. At the present time, it is unclear whether this complication is more pronounced with one agent than the other. But it is known that early myelosuppression may be associated with early drug interruption and/or longer intervals between repeated cycles of therapy. Delaying cycles of therapy in patients with residual disease may be associated with lower response rates. Data from our group and others have indicated that administration of both 5-azacitidine and 5-aza-2′-deoxycytidine followed by supportive care with colony-stimulating factors and prophylactic antibiotics improve historic response rates in this patient population. Furthermore, 5-azacitidine has been administered in combination with valproic acid every 3 weeks without an increase in toxicity and a response rate of more than 50% in untreated patients. It also should be noted that the overall induction mortality rate with this type of agent is extremely low (less than 5%) with both agents despite myelosuppression. In a recent report, it was suggested that administration of 5-aza-2′-deoxycytidine treatment was associated with an improvement in survival compared with standard cytarabine-based induction therapy in patients with MDS. This was attributed in part to the lower mortality observed with 5-aza-2′-deoxycytidine that allowed for repeated courses of therapy and therefore response.

Another potential toxicity associated with hypomethylating therapy is the development of secondary malignancies. Animals in which DNMT activity is depleted both by genetic targeting and pharmacologic manipulation have been shown to develop secondary tumors probably related to the development of genomic instability. The experience at MDACC with several hundred patients treated with either 5-azacitidine or 5-aza-2′-deoxycytidine has been reviewed, and no evidence of increased cytogenetic alterations or development of secondary cytogenetic alterations or secondary tumors has been observed. Longer follow-up is needed to fully assess this issue.

**Use of Histone Deacetylase Inhibitors in MDS**

HDAC inhibitors have been used with modest success in patients with leukemia, mainly AML. The only HDAC inhibitor that has been extensively studied in MDS is valproic acid. Valproic acid is a fatty acid chain derivative with HDAC inhibitory activity, but millimolar concentrations are needed to achieve this effect. In an initial study in 18 patients with MDS, a response rate of 37% (1 partial response and 6 hematologic improvements) was observed. This was not enhanced by the addition of all-trans retinoic acid (ATRA) in patients who had not initially responded to valproic acid. In a follow up report of 43 patients with MDS, 35% achieved a response (1 partial response and 15 hematologic improvements). Although there is no information regarding specific histone code alterations in MDS, HDAC inhibitors, such as vorinostat, LBH589, or MGCD0103, that are significantly more potent histone deacetylase (HDAC) inhibitors in vitro than valproic acid, several studies are now being studied as single-agent HDAC inhibition in patients with lower-risk MDS.

**Combination Strategies**

*In vitro*, the combination of a hypomethylating agent and an HDAC inhibitor have been shown to have a synergistic effect both in terms of leukemia cell killing, and also in terms of gene reactivation. In an initial study by Cameron et al, a sequence in which the DNA hypomethylating agent was used first followed by the HDAC inhibitor was reported to be optimal for gene reactivation. Follow-up studies in leukemia cell lines have demonstrated a synergistic anti-leukemia effect of this type of combination, but in a sequence-independent fashion. Indeed, sequencing is difficult to model in leukemia clinical trials because the need for repeated courses of therapy. Based on this concept, a number of clinical trials have been conducted by several groups using either 5-azacitidine or 5-aza-2′-deoxycytidine in combination with agents such as phenylbutyrate, MS-275, valproic acid, and more recently MGCD0103. Studies with vorinostat and LBH589 are ongoing or planned as well as with other HDAC inhibitors. Overall, the response rate with this type of combination epigenetic therapy appears to be superior to those of each single agent (Tables 1 and 2). Furthermore, it also seems that the rapidity of response may be accelerated with this type of therapy. In both a study with 5-aza-2′-deoxycytidine and valproic acid and the follow-up study with 5-azacitidine and valproic acid and ATRA, the median time to response was 1 course (range, 1 to 3 courses), in contrast with the data discussed above of 4 to 6 courses for single-agent 5-azacitidine. The value of this type of combination needs to be demonstrated in randomized studies. Several of these studies with either MS-275, valproic acid are already ongoing and, with MGCD0103, are planned to start this year. The results of these trials will have a significant effect on our understanding of this type of therapy in human leukemia.

**Future Developments**

The most important needs in the development of epigenetic therapy are: (1) the discovery of predictive biomarkers of response; (2) the development of newer DNA hypomethylating agents; and (3) the development of new alternative forms of epigenetic modifiers.
Biomarkers
At the present time, in most studies conducted so far there has been no relationship between induction of DNA methylation and/or histone acetylation and response. Several groups are trying to identify key genes whose reactivation is associated with response. This discovery will have a profound effect in the future development of this type of therapy.

New hypomethylating agents
Although we are experiencing the development of a large number of new HDAC inhibitors, it seems that there is a relatively small effort in the development of newer hypomethylating agents. One such development is the advent of an oral formulation of 5-azacitidine. This enteric-coated formulation can be absorbed by humans. A phase I study initiated accrual in summer 2007. An oral form of a hypomethylating agent may allow chronic low-dose dosing and may represent a significant improvement for patients with MDS.

Other epigenetic targets
Currently most efforts are focusing on DNA hypomethylation and histone acetylation, but it is clear that other epigenetic alterations, such as histone methylation, may be as dominant as these two others and that inhibition of the catalytic processes that control histone methylation/demethylation may in the future evolve into major forms of epigenetic therapy.

Finally, not every patient with MDS has the same natural history. A risk-oriented approach using either single agents or combinations targeting different patient risk groups needs to be studied prospectively. Approaches could include very low doses of hypomethylating agents, or HDAC inhibitors for lower-risk MDS patients and combination strategies for higher-risk patients.

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