Profile of azacitidine

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Abnormal DNA methylation patterns have been described in different tumor types, including myelodysplastic syndromes and acute myeloid leukemia. DNA hypermethylation in CpG-rich promoters leads to transcriptional repression of several genes, contributing to cancer development. Demethylating agents are able to revert epigenetic silencing induced by hypermethylation showing anticancer affects both *in vitro* and *in vivo*. Azacitidine, a DNA-methyltransferase inhibitor approved by the US Food and Drug Administration for the treatment of myelodysplastic syndrome, resulted in reduced transfusion dependence and risk of leukemic transformation and improved quality of life and survival when compared with best supportive care in patients with myelodysplastic syndromes. Future development will include combination therapy with histone deacetylase inhibitors, such as valproic acid, and the development of new oral demethylating agents.

Recent studies have shown that cancer is a genetic and epigenetic disease. During tumorigenesis, gene silencing by epigenetic changes has the same importance and is strictly linked to genetic lesions. DNA methylation and histone modifications regulate chromatin packaging and determine both genomic stability and transcriptional repression of several genes, including those involved in cell-cycle control, apoptosis, DNA repair and differentiation. Aberrant use of these mechanisms may offer a survival advantage for cancer cells [1–3].

DNA methylation is the addition of a methyl group to a cytidine nucleotide in the context of a CpG dinucleotide, where cytidine is followed by guanidine. The CpG dinucleotides are strongly under represented in the mammalian genome because of the spontaneous deamination of the relatively unstable base 5-methylcytosine, but their density is particularly high in specific DNA regions, called CpG islands that include promoter and 5′-coding regions of several genes and repetitive DNA regions, such as *Alu* repeats and endoparasitic sequences. CpG methylation of gene promoters results in transcriptional repression and definitive gene silencing. This drastic and mitotically stable mechanism of gene regulation plays a physiologic role during embryogenesis and differentiation and, in particular, conditions such as X-chromosome inactivation in women and genome imprinting. On the other hand, CpG methylation in the context of repetitive DNA regions is thought to be essential to suppress the harmful effects of the myriad of retrotransposons ('jumping genes') that litter the human genome, contributing to genome stability [4].

Upon CpG methylation by one out of three major DNA-methyltransferases (DNMTs), such as DNMT1, -3a and -3b, the regional chromatin structure changes. Methylated CpGs bind to methylated DNA-binding proteins (MBPs), such as MeCP2, which contains a methyl CpG-binding domain and transcriptional repression domains. MBPs then recruit corepressors, including histone deacetylase (HDAC) that remove acetyl groups from H3 and H4 histones and renders the chromatin compact, the heterochromatin, inaccessible to transcription factors [5].

Even though DNA methylation plays a pivotal role in preventing transcription of noncoding and dangerous DNA regions, what addresses DNMTs to the CpG islands within the promoter of a specific gene has not yet been elucidated. Two mechanisms were suggested to account for different methylation patters: an exclusion of access to methylation sites by proteins bound to specific DNA regions, such as transcription factors; or a methylation targeting mechanism steered by a sequence-specific binding protein [4]. Recently, the role of double stranded (ds)RNAs was suggested: synthetic short interfering (si)RNAs (21–25-nucleotide RNA molecules) targeted to CpG islands of E-cadherin promoter were shown to repress expression of the *E-cadherin* gene at the transcriptional level by means of DNMT-dependent methylation of DNA [6].

Keywords: azacitidine, demethylation agents DNA methylation, myelodysplastic syndrome

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Two distinct alterations of normal DNA methylation patterns occur in cancer: global hypomethylation and gene-specific (often even cancer-specific) promoter hypermethylation. Hypomethylation may result in potentially harmful expression of inserted viral DNA and repeated elements, weak transcriptional repression of normally silent regions of the genome, chromosomal instability and loss of genetic integrity [7–9]. Conversley, DNA hypermethylation leads to silencing of tumor-suppressor genes, including cell-cycle inhibitors, inducers of apoptosis, DNA-repair genes, transcription factors, cell-adhesion mediators, hormonal receptors and detoxifiers. Promoter hypermethylation could affect both alleles of the gene or may represent a second hit that fully inactivates genes with one mutated allele (loss of heterozygosity, [LOH]) [1,2].

Overall, methylation status in the human genome has been studied using two-dimensional gel electrophoresis restriction landmark genomic scanning (RLGS), which showed that aberrant CpG-island methylation in cancer has a non-random, but tumor-specific pattern, and identified specific methylator phenotypes [10].

Similar to other neoplasms, the DNA methylation profile of hematologic malignancies is frequently characterized by global hypomethylation and simultaneous hypermethylation of selected CpG island gene promoters. CpG-island hypermethylation has been shown to induce epigenetic silencing of some tumor-suppressor genes, favoring tumorigenesis. Hypermethylation can be reversed by demethylating agents. Aberrant DNA hypermethylation is thought to be relevant for leukemogenesis [11,12].

Genes commonly inactivated in myeloid malignancies and the myelodysplastic syndrome (MDS) include $p15^{INK4B}$, death-associated protein kinase (*DAP-kinase*), *SOCS-1*, *E-Cadherin*, *ER* and *RAR-β* [13].

The $p15^{INK4B}$ gene encodes a protein that negatively regulates the cell cycle by inhibiting cyclin-dependent kinases 4 and 6, and controls the progression from G1 to S phase. $p15^{ink4B}$ expression is upregulated during *in vitro* granulocytic and megakaryocytic differentiation of normal $CD34^+$ hematopoietic progenitors [14,15]. Inactivation of the $p15^{INK4B}$ genes by homozygous deletion has been detected in many cancers, but rarely occurs in myeloid malignancies. Promoter hypermethylation was shown to be an alternative pathway of *p15* inactivation. Aberrant methylation of CpG islands in the $p15^{INK4B}$-promoter region commonly occurs in MDS, such as refractory anemia with excess blasts (RAEB) and is associated with loss of $p15^{INK4B}$ expression [14–17]. Patients with $p15^{IKN4B}$ methylation at diagnosis had a significantly shorter survival than those with a normal methylation pattern. Using a methylation-specific polymerase chain reaction (MS-PCR), $p15^{INK4B}$ methylation was not detectable throughout the course of 'low-risk' MDS, while 'high-risk' MDS ranged from 23% at diagnosis to 30% at advanced stages. Acquisition of $p15^{INK4B}$ methylation during follow-up mirrored disease progression. $p15^{INK4B}$ methylation ranged from 60 to 75% in acute myeloid leukemia (AML), evolving from previous MDS. Patients with $p15^{INK4B}$ gene methylation at diagnosis, or follow-up, had a significantly higher chance of disease progression to AML than those without. This suggests that $p15^{INK4B}$ gene methylation might play an important role, not only in disease progression, but also in the early development of some high-risk MDS, also indicating *p15* methylation as a marker of leukemic transformation in MDS [16,17].

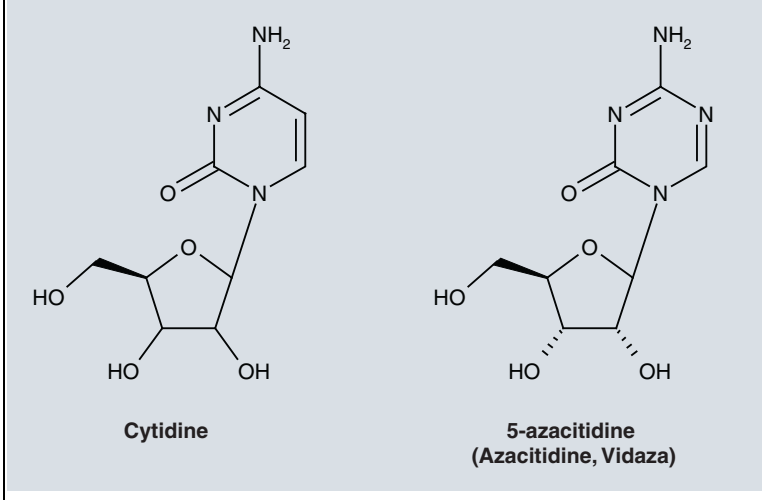
DAP-kinase is a proapoptotic microfilament-bound Ca^{2+}/calmodulin-regulated serine/threonine kinase that participates in a wide array of apoptotic systems initiated by interferon (IFN)-γ, tumor necrosis factor (TNF)-α, activated Fas and detachment from extracellular matrix. After activation by Ca^{2+}/calmodulin, *DAP-kinase* undergoes autophosphorylation and phosphorylates substrates, such as the myosin light chain, cooperating with the cellular apoptotic changes [18]. Loss of *DAP-kinase* expression due to promoter hypermethylation is a common finding in several types of cancer. DAP-kinase promoter was found to be hypermethylated in 47% of MDS and in 27.5% of AML, and was more frequently hypermethylated in AML secondary to therapy for other malignancies (48.3%), than *de novo* AML (22.9%; $p = 0.01$). DAP-kinase promoter methylation correlated to loss of its expression and function, leading to alteration in the apoptotic response, an early event in the transformation pathway to secondary leukemia via myelodysplasia [19].

Several studies showed a mechanistic link between genetic and epigenetic changes in cancer. The PML–RAR-α fusion protein in acute promyelocytic leukemia (APL) induces gene silencing by recruiting both HDACs and DNMT3A to the promoter of RAR-α target gene, *RAR-β2* [20,21]. Consistent with this finding, PML-RAR-α-induced repression of *RAR-β2*

was only partially relieved by treating APL cells with either decitabine, a demethylating agent, or trichostatin A (TSA), a HDAC inhibitor, but simultaneous treatment with decitabine and TSA completely restored *RAR-β2* gene expression [21]. In the same vein, the RUNX1/MTG8 fusion protein, due to the t(8;21)(q22;q22) translocation, was shown to recruit both HDACs and DNMT1 to silence the RUNX1 target gene interleukin (IL)-3, whose expression was synergically restored by the combination of decitabine and depsipeptide, another HDAC inhibitor [22]. Conversely, overexpression of DNMT1 was found in v-src-transfected 3Y1 rat fibroblasts, when compared with fibroblasts transfected with the mock construct, resulting in downregulation of several tumor suppressor genes, that were re-expressed following treatment with decitabine. This experimental model offers a cooperative relationship between oncogenes and tumor-suppressor genes, mediated through promoter hypermethylation [23].

The better understanding of oncogenesis led to the development of drugs against the mechanisms from which cancer arises. As a result, drugs inhibiting the product of genetic lesions, such as inhibitors of constitutionally active mutated tyrosin kinases, have come into clinical practise for different tumor types, such as imatinib for chronic myeloid leukemia (CML). In the same way, in recent years clinical research has focused on drugs affecting chromatin structure, such as demethylating agents and histone deacetylase (HDAC) inhibitors. Progress in this field was rewarded on May 2004 when approval of azacitidine (5-azacitidine, Vidaza™; Pharmion Corporation, CA, USA) was granted by the US Food and Drug Administration (FDA) as the first drug for the treatment of MDSs [24,25].

Figure 1. Azacitidine (5-azacitidine) differs from cytidine with the presence of a nitrogen atom in place of a carbon at position 5 of the pyrimidine ring.

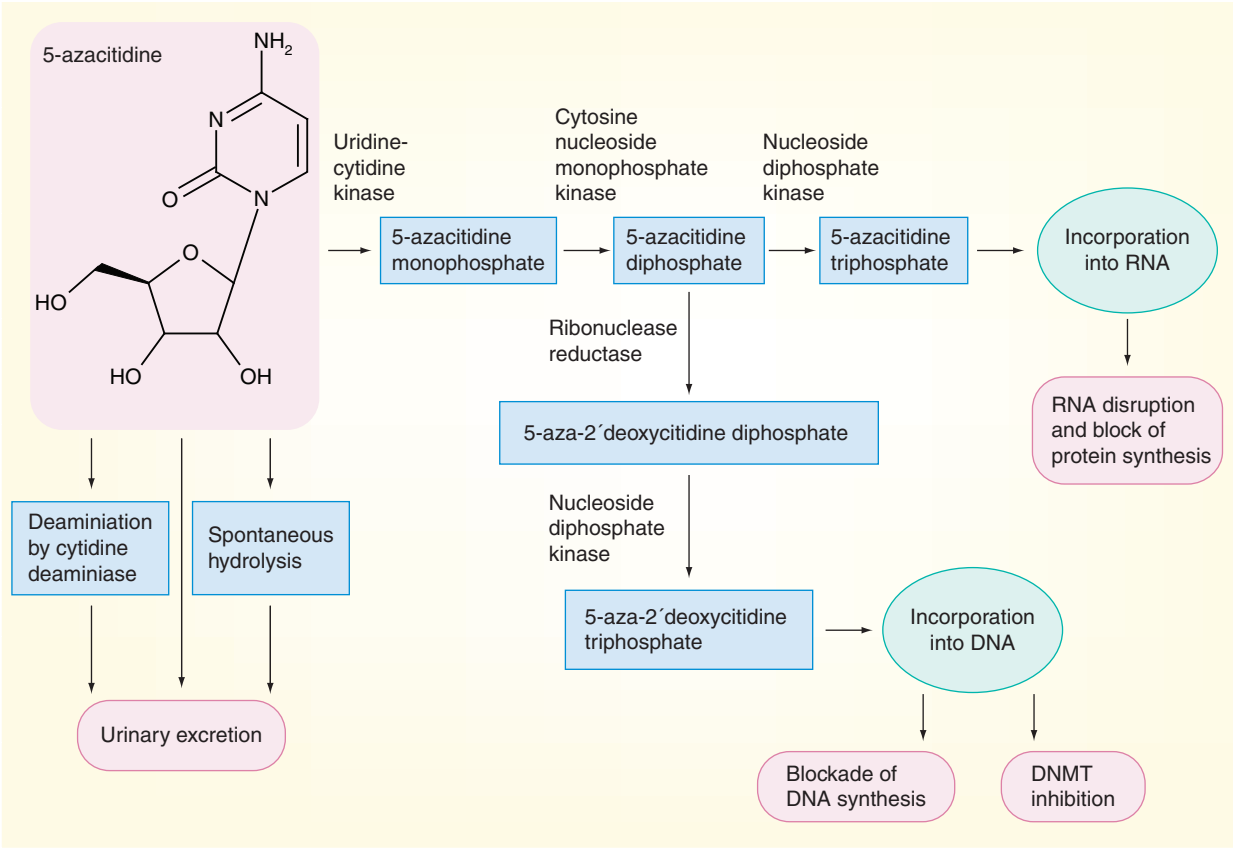


Demethylating agents: mechanism of action & pharmacokinetics

Azacitidine (5-azacitidine), as its homologous decitabine (5-aza-2′-deoxycytidine), is a cytidine analog (azanucleotide) modified in position 5 of the pyrimidine ring with the presence of a nitrogen atom substituting a carbon (**Figure 1**). Azanucleotides are thought to have two distinct mechanisms of action: cytotoxicity at higher doses, resulting from incorporation into RNA and DNA, and DNA demethylation at lower doses, due to DNMT inhibition. Concentration of azacitidine required for maximum inhibition of DNA methylation *in vitro* does not suppress DNA synthesis. Following uptake, azacitidine and decitabine are phosphorylated to a monophosphate derivative by uridine–cytidine kinase and then to diphosphate and triphosphate by pyrimidine monophosphate and diphosphate kinases, respectively. 5-azacitidine triphosphate is incorporated into RNA, disrupts nuclear and cytoplasmatic RNA and inhibits protein synthesis, while 5-aza-2′-deoxycytidine triphosphate is incorporated into replicating DNA, and inhibits DNA synthesis and methylation. Moreover, 5-azacitidine diphosphate is reduced by ribonucleotide reductase to 5-aza-2′-deoxycytidine diphosphate, which is then phosphorylated to 5-aza-2′-deoxycytidine triphosphate and incorporated into DNA (**Figure 2**). As a result, while decitabine-derived triphosphate azanucleotides are incorporated into DNA only, azacitidine-derived triphosphate azanucleotides are incorporated both into DNA and mostly, up to 90%, into RNA [3,11,12,24,25]. This represents the main difference between azacitidine and decitabine. Moreover, while the demethylating effect depends on incorporation of azacitidine-derived deoxyazanucleotides into DNA, dated reports showed that cytotoxicity is mainly due to incorporation into RNA in the G1 phase at low drug concentrations and to incorporation into both RNA and DNA in the G1 and S phases at higher concentrations of azacitidine [26].

The methylation pattern is usually transmitted from a cell to its progeny by the action of DNMTs [4]. After integration into the DNA of

Figure 2. Metabolic pathways of azacitidine after subcoutaneous and intravenous administration [24].



replicating cells, incorporated azanucleotides covalently bind DNMTs that are then targeted for degradation in the proteosome. Absence of DNMTs prevents *de novo* gene methylation during cell division, inducing re-expression of gene silenced by hypermethylation. Since DNA methylation in cancer usually affects tumor-suppressor genes which are genetically intact, their reactivation after demethylation completely restores their normal functions [11,12]. Conversely, demethylating agents were shown to induce in tumor cells, cancer/testis and viral antigens, that may represent a target for humoral and CD8+ T-immune response against tumor, providing opportunities for immunotherapeutic targeting [27,28].

Azacitidine can be administered intravenously or subcutaneously. Maximum plasma concentrations occur 30 min after subcutaneous administration and 11 min after a 10 min intravenous infusion. The mean plasma concentration following intravenous infusion is approximately fourfold higher than that following subcutaneous administration. The bioavailability after subcutaneous administration is 89% of that after intravenous infusion. The plasma half-life is approximately 22 min after intravenous infusion and 41 min after subcutaneous administration. The drug is widely distributed in tissues with a mean distribution volume of 76 l.

Azacitidine is very unstable in aqueous solutions, and after administration it undergoes rapid deamination by cytidine deaminase with subsequent degradation. Azacitidine and its metabolites are mainly excreted by the urinary tract with a mean elimination half-life of about 4 h. There are no data regarding the interaction of azacitidine with other drugs [24,25].

Side effects & toxicity

The main target organs of toxicity after treatment with azacitidine are the bone marrow and gastrointestinal tract. Common side effects reported by patients include nausea, vomiting, diarrhea, constipation, anorexia, injection-site events, arthralgia, cough, dyspnea, headache, weakness, dizziness and insomnia [24,25,29].

Hematologic toxicity includes thrombocytopenia and neutropenia, leading to bleeding and to an increased infection risk. In the Cancer And

Leukemia Group B (CALGB) 9221 trial, standard criteria to evaluate hematologic toxicity used for normal bone marrow were not adaptable due to the patients pretreatment severe cytopenias, related to MDS. Using relative changes in peripheral blood counts compared with those at study entry, grade 3 and 4 leukopenia occurred in 43%, granulocytopenia in 58% and thrombocytopenia in 52% of patients receiving azacitidine. Toxicity was transient and patients usually recovered in time for the next treatment cycle. The highest proportion of myelosuppression occurred during the first two cycles of therapy and decreased in subsequent cycles. In the azacitidine arm, the mean number of red blood cell (RBC) transfusions increased in the first month of treatment but declined thereafter; whereas, the mean number of RBC transfusions remained stable or increased for patients on best supportive care [29].

Major concerns include the risk of developing secondary malignancies after azacitidine treatment due to global hypomethylation. Secondary tumorigenesis may be caused by the loss of genome integrity and thus to chromosomal instability, or to the re-expression of potentially harmful viral DNA and repeated elements [7–9]. Contradictory data exist on this issue. Studies *in vitro* and in rodents have shown that azacitidine is mutagenic and carcinogenic, as other pyrimidine analogs. Azacitidine caused mutagenic response in bacterial systems [30] and in a tumorigenicity study where 31 out of 70 Fisher rats treated with azacitidine developed a variety of tumor types, including acute leukaemia, malignant reticuloendotheliosis, tumors of testis, skin, bronchus and often multiple tumors [31]. Moreover, azacitidine is embryotoxic and teratogenic in rats at doses of 3 to 12 mg/m^2, inducing embryo loss in female rats and reproductive organ toxicity in males [32]. The immunodeficiency, centromere instability and facial anomalies syndrome (ICF), a recessive autosomal disorder due to mutations in both alleles of the gene encoding DNMT3B, is characterized by the almost complete demethylation of classical satellite pericentromeric DNA, which is normally heavily methylated. In mitogen-stimulated lymphocytes, this causes DNA rearrangements targeted to the centromere-adjacent heterochromatic region, mainly affecting chromosomes 1, 16 and sometimes 9 [8]. Nonetheless, there are no reports in the current literature showing an increased cancer risk for ICF patients, such that in humans hypomethylation alone is not likely to be associated with a strongly increased risk of developing malignancies.

Conversely, in genetically engineered mouse models, mice carrying a hypomorphic DNMT1 allele, with reduced DNMT1 expression to 10% of wild-type levels in all tissue, had substantial genome-wide hypomethylation in all tissues, increased genomic instability and, finally, developed aggressive T-cell lymphoma, suggesting that hypomethylation may induce malignancies by promoting chromosomal instability [9]. However it was shown that pharmacologically induced demethylation is only transient and methylation levels return to normal 2 weeks after cessation of therapy [33].

In patients, there are no reports of secondary malignancies developing following treatment with azacitidine or decitabine.

Myelodysplastic syndromes: orphan diseases looking for a drug

MDS represent a heterogeneous group of clonal diseases of hematopoietic stem cells and are characterized by ineffective hematopoiesis, morphologic aspects of dysplasia, peripheral blood cytopenias, requiring transfusion of blood components and increased risk of developing AML. Approximately 87,000 people worldwide are diagnosed with MDS each year, and since the median age of onset is over 60 years, the overall incidence is increasing, especially with the aging of the population [34]. The French American Britain (FAB) (1982) and the World Health Organization (WHO) (2001) classifications of myeloid neoplasms describe distinct nosographic categories according to blast percentage, lineage involvement, presence of ring sideroblasts or monocytosis (**Table 1**), with different clinical behaviour and risk of leukemic transformation [35]. In 1997, an international workshop introduced an International Prognostic Scoring System (IPSS) for MDS, which identified blast percentage, number and degree of cytopenias and karyotype as the most powerful prognostic factors in MDS (**Table 2**) [36]. Four major prognostic risk groups were identified: low, intemediate (INT)-1, INT-2 and high, with a median overall survival of 5.7, 3.5, 1.2 and 0.4 years respectively. Moreover, 25% of MDS patients develop AML with a median time of 9.4 years for low risk group, 3.3 years for INT-1, 1.1 years for INT-2 and 0.2 years for the high-risk group [36]. Most patients with high-risk MDS usually die within 1 year of the diagnosis for progressive marrow failure, hemorrhage and infection, while low risk patients have a significantly better survival. Since most

Table 1. Myelodysplastic syndromes.

FAB classification (1982)
Refractory anemia
Refractory anemia with ringed sideroblasts
Refractory anemia with excess blasts
Refractory anemia with excess blasts in transformation
Chronic myelomonocytic leukemia
WHO classification (2001)
Refractory anemia
Refractory anemia with ringed sideroblasts
Refractory cytopenia with multilineage dysplasia
Refractory cytopenia with multilineage dysplasia and ringed sideroblasts
Refractory anemia with excess blasts-1
Refractory anemia with excess blasts-2
Myelodysplastic syndrome, unclassified
MDS associated with isolated del(5q)

FAB: French American Britain; WHO: World Health Organisation; MDS: myelodysplastic syndrome

patients are elderly, treatment options are mainly limited to supportive care, such as transfusion of blood component and antibiotics. Hemopoietic growth factors, immmunosuppressive drugs and chemotherapy have not changed the natural history of the disease, and have given no survival benefits. Particularly, high- and low-intensity chemotherapeutic regimens are able to induce complete responses in approximately half of patients with high risk MDS, but remissions are short and long-term disease-free survival remains poor [37]. The only curative therapy demonstrated to date for MDS is high-dose chemotherapy followed by allogenic stem cell transplantation, however, only young patients with an HLA-identical related or HLA-matched unrelated donors are eligible for this option, with high morbility and mortality [38]. Moreover, IPSS directly impacts survival after allogenic stem cell transplantation as well, with a 5-year disease-free survival of 60% for low and INT-1 risk patients, 36% for INT-2 and only 28% for high-risk patients, where the major cause of failure is disease recurrence [39].

These data show that present MDS treatments are not curative for most patients and new therapeutic developments are required. Different studies have shown that MDS and AML are characterized by hypermethylation and silencing of multiple genes, making demethylating drugs a promising therapeutic option for these diseases.

Clinical studies with azacitidine in MDS

Azanucleotides were first used in clinical trials, before the discovery of their demethylating activity and of the importance of epigenetic abnormalities in the pathogenesis of myeloid malignancies. They were considered cytotoxic drugs, and their use at intermediate and high-doses in combination treatment for leukemia had been limited by prohibitive side effects, such as mucositis and CNS toxicity. In 1979, Jones and Taylor first reported the capability of azacitidine to demethylate newly synthesized DNA and to trigger differentiation of cultured mouse embryonic cells. In the late 1980s, clinical studies using low-dose azanucleotides regimens were started for elderly MDS and leukemia patients, not eligible for standard chemotherapy. Preliminary studies showed promising results in high-risk MDS and resistant or relapsed leukemias [11,12]. The Cancer and Leukemia Group B (CALGB) conducted three different trials, which showed 50% efficacy of low-dose azacitidine in MDS patients (**Table 3**) [11,12,24,25]. In the single-arm Phase II trial CALGB 8421, azacitidine was administered as a

Table 2. International prognostic score system for MDS [36].

Prognostic variables	Low	INT-1	INT-1	INT-2	INT-2
Score	0	0.5	1	1.5	2
Bone marrow blasts (%)	<5	5–10		11–20	21–30
Cytopenias	0/1	2/3			
Karyotype*	Good	Intermediate	Poor		

** Good: normal, -Y, del(5q), del(20q); Poor: complex (>3 abnormalities) or chromosome 7 anomalies; Intermediate: other abnormalities.*
INT: Intermediate; MDS: Myelodysplastic syndrome.

Table 3. Response rates in azacitidine-treated patients in all three CALGB trials*.

	CALGB 8421	CALGB 8921	CALGB 9221	
			Azacitidine arm	*Azacitidine after crossover*
Type of study	Phase II	Phase II	Phase III	Phase III
Mode of administration‡	i.v.	s.c.	s.c.	s.c.
Evaluable patients	43	68	99	49
Complete response	5 (12%)	8 (12%)	7 (7%)	5 (10%)
Partial response	11 (25%)	10 (15%)	16 (16%)	2 (4%)
Improvement	5 (12%)	18 (27%)	37 (37%)	16 (33%)
Overall response	21 (49%)	36 (53%)	60 (60%)	3 (47%)

** Data reported in this table were published in Silverman's original paper and in previous reviews [12,29]. Recently published data, submitted to the US FDA for marketing apporval of azacitidine, show lower response rates [24,25].*

‡Azacitidine was administered in all three trials at the starting dose of 75 mg/m^2/day for 7 days every 4 weeks [24,25,29].

CALGB: Cancer And Leukemia Group B trial; i.v.: Intravenous; s.c.: Subcutaneous.

continuous intravenous infusion, at 75 mg/m^2/day for 7 days every 28 days (total dose 525 mg/m^2 per course), to 43 patients with RAEB and RAEB-T. Responses (complete, partial or improved) occurred in 49% of patients, with 37% having trilineage responses, either complete or partial [12]. Complete remission was achieved by 5 of 43 patients and partial remission by 11 patients. Patients who did not respond after 4 months were discontinued from the study. Median survival for all patients was 13.3 months, while median remission duration was 14.7 months. Interestingly, the best response was observed after a mean of 3.8 treatment courses (range 2–11 courses), indicating that azacitidine may need repeated applications to achieve maximum efficacy. A subsequent Phase II trial (CALGB 8921), using subcutaneous daily bolus injection of azacitidine at the identical dose and schedule on ambulatory basis also produced a response in about 50% of patients, with 27% having trilineage responses. A mild activity, without significant myelosuppression, was demonstrated in MDS using lower azacitidine doses (from 10 mg/m^2/day to 35 mg/m^2/day, by continuous intravenous infusion for 14 days) [12].

The crucial study that demonstrated the efficacy of azacitidine for the treatment of MDS was the Phase III, randomized, controlled trial, CALGB 9221 [25]. A total of 191 patients with MDS were randomized in two arms, one receiving azacitidine 75 mg/m^2/day given subcutaneously for 7 days every 28 days (n = 99), and the other receiving best supportive care only (n = 92). Patients in the supportive arm were allowed to cross over to azacitidine treatment after 4 months, in case of disease progression (n = 49). MDS was defined by the FAB criteria. Patients with RAEB (n = 66), RAEB-t (n = 45) and chronic myelomonocytic leukemia (CMMoL) (n = 14) were enroled. To enter the study, patients with refractory anemia (RA) (n = 37) and RA with ringed syderoblasts (RARS) (n = 8) had to meet at least one of these additional criteria: symptomatic anemia requiring RBC transfusions for at least 3 months before study entry, or platelet counts of 50 ´ 10^9/l or less or a significant clinical hemorrhage requiring platelet transfusions, or neutropenia with an absolute neutrophil count less than < 1 ´ 10^9/l and an infection requiring intravenous antibiotics. The study also included 19 patients with AML, one with unclassifiable leukemia, and one with undefined MDS. Patients with therapy-related MDS were eligible if they were cancer-free for at least 3 years and had not received radiation or chemotherapy for 6 months. Both arms received transfusions and antibiotics as required, but no hemopoietic growth factors were allowed. If no beneficial effect was demonstrated after two cycles and no significant toxicity had occurred, the dose of azacitidine was increased by 33%. Patients were assessed after the fourth cycle. Those who achieved complete response (CR) continued azacitidine for three more cycles, while those with partial response (PR) or hematologic improvement continued on azacitidine until CR or relapse. Rigorous response criteria were applied (**Table 4**). Responses occurred in 60% of patients on azacitidine (7% CR, 16% PR, 37%

Table 4. Response criteria for azacitidine-treated MDS patients [29]

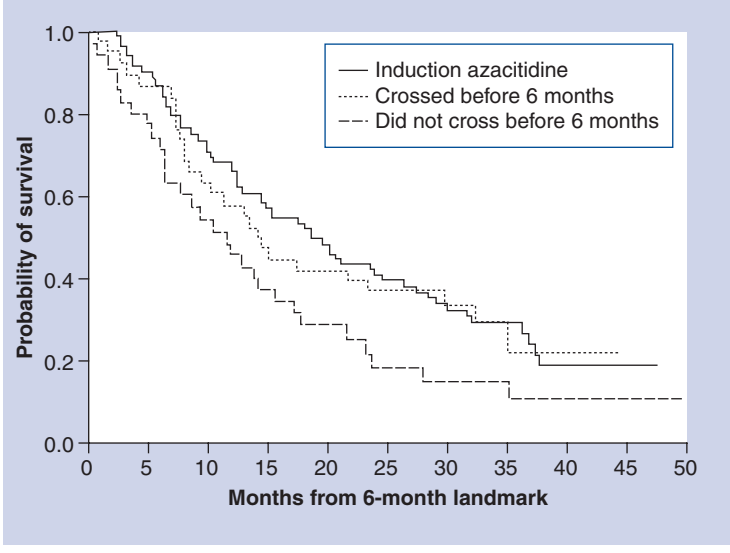
Target area	Complete response	Partial response	Improvement
Bone marrow	Normal, or <5% blasts and some dyshematopoietic features.	≤50% of initial bone marrow blasts.	
Peripheral blood	Complete normalization of the peripheral blood count.	Trilineage response*.	Monolineage/bilineage response‡.

** Trilineage response: > 50% restitution of the initial deficit from normal in all three peripheral-blood counts and elimination of all blood transfusion requirements.*

‡ Monolineage or bilineage response: ≥ 50% restitution of the initial deficit from normal in one or two peripheral blood counts.

improved) and in 5% (improved) of those receiving supportive care ($p < 0.0001$). Most patients responded by the third or fourth month. Median time to leukemic transformation or death was 21 months for azacitidine versus 12 for supportive care ($p = 0.007$). Transformation to AML occurred as the first event in 15% of patients on azacitidine and 38% of patients receiving supportive care ($p = 0.001$), suggesting that azacitidine may prevent leukemic transformation. This is consistent with a low-dose cytotoxic effect and with the biologic response-modifying activity of azacitidine. Eliminating the confounding effect of early cross-over to azacitidine, a landmark analysis after 6 months showed median survival of an additional 18 months for azacitidine and 11 months for supportive care ($p = 0.03$) (Figure 3). The quality-of-life analysis, assessed by the European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionaire C30 and by the Mental Health Inventary (MHI), showed that patients initially treated with azacitidine had a significant improvement over time in fatigue, dyspnea, physical functioning, and physiologic distress, compared with those receiving supportive care only [40]. In conclusion, azacitidine treatment, compared with supportive care, results in a significantly higher response rate, improved quality of life, reduced risk of leukemic transformation and improved survival. This study demonstrates for the first time that the natural history of MDS may be changed by a non-intensive drug treatment and establishes azacitidine as an important therapeutic option for MDS.

Figure 3. Survival curves of the CALGB 9221 trial, comparing azacitidine treatment, cross-over with azacitidine before 6 months, and supportive care [29].



Data submitted to the FDA for marketing approval of azacitidine were recently revised and reviewed [24,25]. Global response rate (CR + PR) among all 270 patients treated with azacitidine in the three completed CALGB trials was 15.2%, while the response rate among 238 patients with MDS was 15.1%. Response rates were similar in subjects with all MDS subtypes and with AML. Median duration of the responses was at least 9 months. In addition to patients with CR or PR, approximately 19% of azacitidine-treated patients had an hematologic improvement and two thirds of transfusion-dependent patients became transfusion independent with improvement response. Transformation to AML occurred in 44% of patients in the observation arm, in 14% of patients randomized to azacitidine and 12% of patients who crossed over azacitidine after being randomized to the observation arm. So far azacitidine treatment could not be shown to result in a significant survival benefit because of the cross-over of some patients from the obervation arm to the azacitidine arm.

A multicenter, randomized, open-label Phase III trial, AZA PH GL2003 CL001, sponsored by Pharmion Corporation (CA, USA), is ongoing and aims to compare subcutaneous azacitidine (75 mg/m^2/day given subcutaneously for 7 days every 28 days) plus best supportive care, versus conventional regimens plus best supportive care, in patients with MDS. Eligible patients are randomized in a 1:1 ratio to either azacitidine or a conventional care regimen. Conventional regimens include one of the following options: best supportive care only, or low dose Cytarabine (20 mg/m^2/day subcutaneously 14 days every 28 to 42 days) plus best supportive care, or standard chemotherapy plus best supportive care. This study includes patients with a diagnosis of RAEB or RAEB-T according to the FAB classification, with an IPSS score of INT-2 or high.

An additional trial conducted by Pharmion is examining alternate dosing regimens of azacitidine that may make drug administration more convenient for patients. The regimens under investigation are: 75 mg/kg/day for 5 days, no treatment for 2 days, and then 75 mg/kg/day for 2 days; 50 mg/kg/day for 5 days, no treatment for 2 days, and then 50 mg/kg/day for 5 days; and 75 mg/kg/day for 5 days [101].

Studies combining demethylating agents with other drugs

Since DNMTs and HDACs were shown to cooperate in gene silencing, clinical studies on the combination of HDAC inhibitors and demethylating agents in hematological diseases have been encouraged. Two clinical National Cancer Institute-sponsored studies have tested the combination of azacitidine and sodium phenylbutyrate (PB) [41,42]. In the study of the Memorial Sloan Kettering Cancer Center, the treatment schedule entailed subcutaneous injections of azacitidine for 7 consecutive days (75 mg/m^2/day), similar to the Cancer and Leukemia Group B (CALGB) schedule, followed by 5 days of intravenous PB (200 mg/kg/day), repeated on a 21–28 day schedule, depending on tolerability and response. A total of six patients with MDS/secondary AML received at least one cycle of therapy (range: 1–3); a reduction of bone marrow blast counts as well as increased myeloid maturation was observed in four patients. One patient with leukemia who relapsed following bone marrow transplantation (BMT), had a complete elimination of bone marrow blasts after one cycle of therapy, and, subsequently underwent a second allogeneic BMT. Peripheral blood and bone marrow samples were collected before azacitidine, on day 8 (at completion of azacitidine, and before beginning PB), and at the completion of PB. An increase in histone acetylation was consistently detected in peripheral blood and bone marrow samples collected after PB administration. Selected genes commonly silenced (e.g., *p15^{INK4b}* in myelogenous leukemia) were analyzed for methylation and expression. Treatment was relatively well tolerated, with mild adverse reactions including fatigue, nausea, vomiting and local tenderness at injection sites associated with azacitidine, and transient somnolence and drowsiness associated with PB. This ongoing study will evaluate the safety and potential antitumor efficacy of this combination, and its effects on gene methylation and histone deacetylation [41].

A second study combining azacitidine and PB was reported by the Johns Hopkins' Baltimore Group. Sequential administration of azacitidine and PB to re-express transcriptionally silenced genes was initated in patients with MDS and AML. The initial azacitidine dose was also 75 mg/m^2/day subcutaneously, in this study given for 5 days, followed by PB at 375 mg/kg/day, intravenous continuous infusion, days 5 to 12 repeated every 28 days. Dose deescalation to determine the minimal azacitidine dose associated with significant demethylation continues (current dose level 50 mg/m^2/day). A total of 11 patients have been treated in 39 courses. The combination was well tolerated with 1:6 patients at the highest dose level developing dose limiting toxicity (fatigue). No unexpected clinical toxicities have been observed to date. Two patients had significant hematopoietic improvement. The primary laboratory end point is inhibition of methylation. Baseline methylation activity was highly variable. Of two patients with high baseline activity, one had significant inhibition (85% of baseline levels) following treatment with azacitidine (50 mg/m^2/day). Two of five patients with lower baseline levels of activity also showed inhibition (20 and 30%) following treatment with 75 mg/m^2/day. Histone acetylation was increased over baseline in four out of 11 patients investigated. In addition, two patients had significant detectable acetylation which persisted during the PB infusion. Increases in acetylation were detected within 4 h of initiation of PB infusion and persisted throughout the infusion. In nine patients, sequential measurements of

p15^{INK4B} promoter methylation by a newly developed PCR-based assay were performed. All had measurable methylation of the *p15* promoter exceeding 10% of available CpG sites (normal <2%). *p15* methylation density was higher in patients with AML or RAEB-t compared with patients with lower-grade MDS. In three patients during treatment with azacitidine and PB, *p15* methylation levels decreased to 19%, 45% and 56% respectively. *p15* methylation increased in one patient (in association with disease progression) and was stable in five patients. Baseline methylation density did not predict for the extent of demethylation in response to azacitidine and PB [42].

A recent report about the sequencial treatment of MDS patients with azacitidine and PB shows that treatment with azacitidine decreased *p15* methylation in 50% of patients and *p15* expression increased in patients in whom methylation was decreased. Interestingly, azacitidine increased global acetylation of histones H3 and/or H4, evaluated by western analysis, and acetylation was further increased following PB administration. However, the mechanism underlying histone acetylation in response to azacitidine remains to be elucidated [43].

These studies demonstrate that the sequential administration of a 'first generation' demethylating agent and HDAC inhibitors is feasible, and give preliminary evidence of an effect on the methylated targeted gene promoter.

Other demethylating drugs

Decitabine (5-aza-2′-deoxycytidine, SuperGen, Inc., CA, USA) is one of the most commonly used demethylating drugs *in vitro* and *in vivo*. Upon its exclusive integration into DNA, decitabine should theoretically be more powerful as demethylating and less cytotoxic than azacitidine which is mostly incorporated into tRNA and mRNA. Moreover decitabine was only weakly mutagenic in 10T1/2 cells and V79 cells, and in animal models of carcinogenesis it has been chemopreventive rather than carcinogenic. Decitabine was shown to induce terminal *ex vivo* differentiation of leukemic blasts and a considerable activity of high-dose decitabine was demonstrated in leukemic patients [3,11,12]. Decitabine at low doses was shown to induce trilineage responses in about half of patients affected by high-risk MDS, including also CR and PR. In a recent update about 162 uniformly decitabine-treated MDS patients with a median age of 70 years, the response rate was 49%. A remarkable effect on platelet counts was observed, with a significant platelet increase in 42% of patients after one cycle of therapy and in 63% of patients receiving at least two cycles. The median response duration was 36 weeks. Major cytogenetic responses were observed in 31% of patients with abnormal cytogenetics after a median of three courses (range: 2–6). Myelosuppression is the most common side effect [44]. An European Organisation for Research and Treatment of Cancer (EORTC)-German MDS Study Group randomized Phase III trial (EORTC protocol 06011) is ongoing and aims to assess a difference in terms of overall survival between low-dose decitabine (15 mg/m^2 intravenously in 4 h infusion three times a day for 3 days every 6 weeks, total dose 135 mg/m^2/course) and supportive care (control arm) in elderly patients with primary MDS with blast percentage higher than 10% or high risk cytogenetics, secondary MDS or CMML, who are not eligible for intensive care. Other clinical trials with decitabine are ongoing for MDS, AML, CML and solid tumors [101].

Zebularine [1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one] is another cytidine analog that acts as both cytidine deaminase and DNMT inhibitor [45]. Continuous application of zebularine to T24 human bladder cancer cells induces and maintains p16 gene expression and sustains demethylation of its 5′ region for over 40 days, preventing remethylation [46]. Zebularine is chemically stable, has minimal cytotoxicity both *in vitro* and *in vivo* and, in contrast to azacitidine and decitabine, it could be given orally. More interestingly, since cytidine deaminase represents the main pathway of azanucleotide degradation, it could be theoretically given together with azacitidine or decitabine to prolong their half-lifes and to increase their activities [47]. Therefore, zebularine is potential candidate for clinical utilization as a chemotherapeutic agent, also in combination use, to reverse abnormal hypermethylation and reactivate regulatory genes in cancer.

Procainamide and procaine, derivatives of 4-aminobenzoic acid approved by the FDA as antiarrhytmic and local anesthetic respectively, were found to induce DNA hypomethyaltion by binding to CpG-rich DNA regions but without incorporation into DNA [48]. Procainamide causes global hypomethylation and

restores expression of the detoxifier GSTP1 in prostate cancer cells in which it has been silenced by hypermethylation [49]. Procaine was shown to inhibit DNA methylation and cell growth in breast cancer cells and to induce reactivation of several tumor suppressor genes [50]. Due to their well known and favorable clinical profile, procaine and procainamide seem to be excellent anticancer candidate to be used in clinical trials.

Finally, several other agents are under investigation such as DNA demethylating agents and antitumor drugs. A brief list of such compunds includes: other cytidine and 2′-deoxycytidine analogs, as 5-fluoro-2′-deoxycyditine, fazarabine and cytarabine; the antihypertensive drug hydralazine; the main polyphenol compound present in green tea, epigallocatheni-3-gallate; the psammaplins, found in marine sponges; and s-adenosyl-methionine analogs and competitive inhibitors, such as 5′amino-5′-deoxyadenosine, sinefungin, L-ethionine and dihydroxypropyladenine [3].

Other clinical applications for demethylating drugs

Azacitidine and decitabine were studied in solid tumor with modest results so far, despite the proven role of DNA methylation abnormalities in almost all kinds of cancers. Wider experience exists in this field for decitabine, alone or in combination with chemotherapy, but only short-lasting, partial responses were observed in different studies [33]. Although these first data are not encouraging, more extensive use of demethylating agents should be evaluated, particularly in combination with HDAC inhibitors.

The demethylating agents, azacitidine and decitabine, were studied as therapeutic agents in hemoglobinopathies. Fetal hemoglobin (HbF) production is downregulated during extra-uterine life by DNA methylation of the γ-globin chain gene promoter. Although recognizing different pathogenesis, the severity of β-thalassemia and sickle cell disease (SCD) were shown to be ameliorated by increasing HbF levels. HbF increase can improve the imbalance between α- and non-α-globin chain, which is the cause of ineffective erythropoiesis and reduced circulating RBC life. Moreover, HbF decreases polymerization of sickle hemoglobin (HbS), increases its solubility and prevents red cell sickling. The first studies conducted in anemic baboons treated with azacitidine at a dose of 2 to 4 mg/kg/day for 5 days per week for 2 weeks, showed an increase in HbF levels up to 70% of total hemoglobin, with mild toxicity. Similar results were observed in two patients with sickle cell disease treated with azacitidine according to the same schedule, without substantial toxicity and with improvement in hematological parameters. Moreover, three patients with end-stage β-thalassemia treated with 1 to 2 mg/kg/day azacitidine, 4 days a week, once a month, showed a increase in total hemoglobin (3–5 g/dl) and two of them, treated for 30 months, remained transfusion-independent for all the duration of treatment. Despite these promising early results, further trials with azacitidine were discouraged due to concerns regarding its potential carcinogenicity. Decitabine has more widely entered in clinical trials for the treatment of SCD and β-thalassemia, obtaining similar results for azacitidine [51,52].

Expert commentary

The results of CALGB trial 9221 demonstrated that the natural history of MDS could be changed by a single agent therapy. Azacitidine, given subcutaneously for 7 days every 4 weeks, induced clinical responses in about 60% of patients, including complete and partial responses, improved quality of life and survival, and reduced the risk of leukemic transformation, with an outstanding toxicity profile [29]. This is particularly significant when considering that for a long time supportive care has been the standard treatment for most MDS patients, and that less then 5% are eligible for allogenic bone marrow transplantation, at the price of high transplant-related morbility and mortality. Demethylating agents are one of the most promising agents in the treatment of MDS and open new biologic and therapeutic scenarios.

It remains to identify which patients are likely to respond to azacitidine. For this purpose, DNMT levels have been investigated [53,54]. From a clinical point of view, these authors are of the opinion that the methylation status of single selected genes [10] and genome-wide methylation levels could be useful to predict response to demethylating agents [55]. Some data exist for *p15*, whose methylation frequency increases during the progression from low to high risk MDS and to AML. *p15* was shown to be demethylated and reinduced in bone marrow mononuclear cells

from MDS patients after *in vivo* treatment with demethylating agents, paralleling clinical response [43,56]. A major point in favor of the use of azacitidine is the strong reduction of proportion of patients transformed to acute leukemia in the group treated with azacitidine and this could be related to *p*15 demethylation. Moreover, an extended study on azacitidine treated patients in the context of CALGB 9221 demonstrated that MDS patients with normal cytogenetics or who develop normal karyotype after demethylating treatment, present a significantly longer survival when compared to patients with abnormal karyotype that remained abnormal [57].

Furthermore, demethylating agents are able to restore normal hematopoiesis in MDS patients with both complete and partial trilineage responses reducing the requirement of blood and platelet transfusion. Increase in platelet count is frequently the first sign of a good response to demethylating therapy, as shown for decitabine [58].

Another unanswered question involves the use of demethylating agents as maintenance therapy after achievement of CR or PR. *In vitro* and *in vivo* studies showed that the demethylating effect of azanucleotides on different cancer cell types is transient and that the previous methylation status is slowly restored, probably due to the mechanistic link between genetic and epigenetic lesions and to the action of environmental signals [23,33,59]. Moreover, demethylating agents act only on cycling cells, without affecting resting neoplastic stem cells, so it is our belief that long-term treatment may be necessary.

Outlook

In the next few years we will see an extensive use of demethylating agents in hematological malignancies, due to the high frequency of methylating lesions in these diseases and the already demonstrated efficacy of this treatment. Clinical trials including azacitidine will bloom, encouraged by the recent promising results in MDS, and will involve also other myeloid and lymphoid neoplasias. In particular, the recent evidence of DNMT recruitment by different fusion proteins in AML, opens the way to the use of azacitidine in specific leukemic subsets, in combination with other drugs [21,22]. Moreover, a recent report showed an overall response rate of 53% (including CR and PR) in a group of 15 patients with newly diagnosed AML and treated with out-patient azacitidine 75 mg/m^2/day given subcutaneously for 7 days every 28 days as primary induction therapy with good tolerance [60]. This result encourages a wider use of demethylating agents to treat AML patients, particularly those who cannot undergo more aggressive chemotherapic regimens.

In the last decades, advances in understanding the multistep pathogenesis of MDS led to experimental use of new compounds acting at different levels and provided some responses in MDS patients. These new compounds include anti-angiogenetic factors, receptor tyrosine kinase inhibitors, farnesyl transferase inhibitors and pharmacologic differentiators [34]. Azacitidine should also be compared and combined with these drugs, and interactions between these new drugs should be studied. The most interesting development of epigenetic therapy in myeloid malignancies is the combination of demethylating agents with HDAC inhibitors. As described, epigenetic gene silencing occurs by the cooperation of DNA methylation and histone deacetylation, leading to chromatin packaging and transcriptional repression [61,62]. HDAC inhibitor showed anticancer and differentiating activity *in vitro* and *in vivo* [63,64], and first data concerning the combination of azacitidine and PB were promising [41,42]. Other HDAC inhibitors, with well known pharmacokinetics and toxicity profile, such as valproic acid, or newly synthesized, such as depsispeptide, suberoyl anilide hydroxamic acid and MS-275, will be tested in MDS, in combination to azacitidine, aiming at increasing the proportion of responders and the remission duration. Moreover, pharmacokinetics studies on the combination of azacitidine to other drugs need to be performed, although some data exist with PB [65].

Finally, new oral demethylating agents, such as zebularine, procainamide, and procaine should be tested on MDS patients, for the easier out-patient management, and should be compared to azacitidine, in terms of demethylating activity and clinical responses. Moreover the combination of zebularine with azacitidine needs to be tested, since zebularine was shown to inhibit cytidine-deaminase, the main enzymatic pathway involved in the degradation of azacitidine, and may possibly increase azacitidine anticancer activity [66]. In conclusion, azacitidine, alone or in combination, is a good candidate to become standard therapy for MDS.

Highlights

- Gene silencing by epigenetic changes plays a pivotal role during tumorigenesis and is strictly linked to genetic lesions.
- Demethylating agents, such as azacitidine, are able to revert epigenetic silencing induced by promoter hypermethylation, showing anticancer activity both *in vitro* and *in vivo*.
- Aberrant promoter hypermethylation of several genes is a frequent finding in myelodysplastic syndromes (MDS) and acute myelogenous leukemia.
- Azacitidine, a DNA methyltransferase inhibitor, used as a single agent, was able to reduce transfusion dependence and risk of leukemic transformation and to improve quality of life and survival when compared with supportive care in patients with MDS.
- On May 2004 the US Food and Drug Administration (FDA)-approved azacitidine as the first drug for the treatment of MDS.
- Clinical trials combining azacitidine and histone deacetylase inhibitors, such phenobarbital or valproic acid, represent the future development of epigenetic therapy of myeloid malignancies.

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