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Valproic Acid at Therapeutic Plasma Levels May Increase 5-Azacytidine Efficacy in Higher Risk Myelodysplastic Syndromes

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Abstract

Purpose: Epigenetic changes play a role and cooperate with genetic alterations in the pathogenesis of myelodysplastic syndromes (MDS). We conducted a phase II multicenter study on the combination of the DNA-methyltransferase inhibitor 5-azacytidine (5-AZA) and the histone deacetylase inhibitor valproic acid (VPA) in patients with higher risk MDS.

Experimental Design: We enrolled 62 patients with MDS (refractory anemia with excess blasts, 39 patients; refractory anemia with excess blasts in transformation, 19 patients; and chronic myelomanocytic leukemia (CMML), 4 patients) and an International Prognostic Scoring System (IPSS) rating of Intermediate-2 (42 patients) or high (20 patients). VPA was given to reach a plasma concentration of >50 μg/mL, then 5-AZA was added s.c. at 75 mg/m^2 for 7 days in eight monthly cycles.

Results: The median overall survival was 14.4 months. At a median follow-up of 12 months (range, 0.7-21.0), the disease progressed in 20 patients, with 21% cumulative incidence of progression. Of 26 patients who completed eight cycles, 30.7% obtained complete or partial remission, 15.4% had a major hematologic improvement, whereas 38.5% showed stable disease. Drug-related toxicity was mild. Favorable prognostic factors for survival were IPSS Intermediate-2 and plasma VPA of ≥50 μg/mL (log rank = 0.013 and 0.007, respectively). Analysis of polymorphisms important for the metabolism of the drugs used in the trial showed that carriers of the CYP2C19*2 variant of cytochrome P450 required higher VPA doses to achieve the target VPA plasma concentration of 50 μg/mL on day 1 of 5-AZA treatment ($P = 0.0021$).

Conclusion: Our data show that the 5-AZA/VPA combination is active and safe in patients with MDS with a poor prognosis. Achievement of VPA therapeutic levels may indeed increase 5-AZA efficacy.

Epigenetic transcriptional silencing of genes important for carcinogenesis, including cell cycle, apoptosis, DNA repair, and detoxification are likely to contribute to the leukemogenic event underlying myelodysplastic syndromes (MDS) and acute myeloid leukemias (AML).

The transcriptional silencing of known or candidate tumor suppressor genes depends not only on DNA methylation, but also on additional epigenetic events such as bimethylation and trimethylation of histone lysine residues and recruitment of methylated DNA-binding proteins (1). Methyl CpG-binding

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Translational Relevance

Epigenetic treatment has recently gained great interest as a new and effective treatment option for patients with myelodysplastic syndrome. The role of combination therapies remains to be defined. In this article, we report the results of a phase II multicenter study from the Italian Cooperative Group GIMEMA on the combination of the DNA-methyltransferase inhibitor 5-azacytidine (5-AZA) and the histone deacetylase inhibitor valproic acid (VPA) in patients with higher risk myelodysplastic syndrome. We show that patients achieving VPA concentrations of ≥50 μg/mL have a favorable outcome, indicating that the association of VPA may indeed improve the response to azacytidine. We also did pharmacogenetic analyses demonstrating that carriers of the CYP2C19*2 allele, an enzyme important for the metabolism of VPA, required higher VPA doses to achieve a VPA concentration of >50 μg/mL. This underlines the necessity to include pharmacogenetic analyses into future trials on epigenetic treatment to define patient-tailored approaches.

proteins are also often part of large corepressor complexes which recruit histone deacetylases (HDAC) and histone-methyl transferase on target promoter sequences (2, 3).

Several tumor suppressor genes have been shown to be hypermethylated in MDS and the frequency of methylation has been associated with unfavorable prognosis (4, 5). The potential reversibility of DNA and chromatin modifications favored the development of two classes of epigenetic drugs which inhibit enzymatic activities involved in epigenetic silencing, DNA methyltransferase (DNMT) and HDAC, respectively. Among DNMT inhibitors (DNMTi), azacytidine (5-azacytidine; Vidaza, Celgene, Corp.), and decitabine (5-aza-2′-deoxycytidine; Dacogen, MGI Pharma, Inc.) are available in the clinical setting and have been approved for the treatment of MDS. Both agents are incorporated after cellular uptake into newly synthesized RNA and DNA, respectively. Then, the 5-azacytosine rings covalently bind DNMT1 and the resulting adducts are excised from the DNA, ubiquitinated and targeted to the proteosome for degradation (6). This induces loss of methylation in one of the DNA daughter molecules, blocks DNA synthesis, and elicits the re-expression of silenced genes. Azacytidine also induces RNA degradation and inhibition of protein synthesis. The multiple effects on DNA, RNA, and proteins, like the mere formation of DNA/DNMTi adducts, also account for the cytotoxicity observed during treatment (7, 8).

HDAC inhibitors (HDACi) on the other hand, induce hyperacetylation of lysine residues in the histone tails, which contributes to shifting the chromatin structure to a transcriptionally active state (8). Additionally, a growing number of non-histone proteins, like heat shock proteins, shuttle proteins, and transcription factors, are acetylated by HDAC, inducing growth arrest, differentiation, or apoptosis *in vitro* and *in vivo* (8). HDACi have shown activity in MDS and AML *in vivo* and among them, vorinostat (Zolinza; Merck) has been approved for recurrent cutaneous T-cell lymphoma (9).

DNMTi and HDACi have been shown to synergize *in vitro* to induce the expression of oncosuppressor genes and apoptosis (10). Several phase I studies have investigated the activity of this combination *in vivo* (11–13).

We conducted a multicenter phase II study on the combination of the DNMTi 5-azacytidine (5-AZA), the HDACi valproic acid (VPA), and all-trans retinoic acid (ATRA) in patients with intermediate-2/high-risk MDS, according to the International Prognostic Scoring System (IPSS). We were interested in the clinical activity of the DNMTi/HDACi combination and in the pharmacogenetic profile associated with treatment response. Similar to previous reports, the study also included patients with refractory anemia with excess blasts (RAEB) in transformation (RAEBT) and CMML, in which azacytidine had been proven effective (11–14). Because demethylating agents have been shown to restore sensitivity to differentiation-inducing agents (15), ATRA was added to unresponsive patients after four 5-AZA/VPA cycles.

Patients and Methods

Eligibility and treatment. The multicenter study GIMEMA MDS0205 (EudraCT no. 2005-004811-31) included 62 patients from 17 Italian Hematology Centers. Patients' ages were 18 years or older, with a diagnosis of RAEB or RAEBT according to the French-American-British classification criteria, and an IPSS score of Intermediate-2 or High (16). The protocol also included patients with a diagnosis of chronic myelomonocytic leukemia according to the modified French-American-British criteria, and a WBC count of ≤13 × 10^9/L. Bone marrow morphology was centrally reviewed before enrollment.

Table 1. Patient characteristics

	n (%)
Sex	
Male	43 (69.4)
Female	19 (30.6)
Age	
Median (range)	69.6 (52.9-83.2)
Diagnosis	
RAEB	39 (62.90)
RAEBT	19 (30.65)
CMML	4 (6.45)
MDS history	
De novo	60 (97)
Therapy-related	2 (3)
IPSS score	
Intermediate-2	42 (67.74)
High	20 (32.26)
Karyotype	
Chromosome 7	8
Chromosome 5	3
Complex	11
Normal	15
Other	25
Bone marrow blasts (%)	
Median (range)	16 (6.0-32.5)
Hemoglobin (g/dL)	
Median (range)	9.0 (5.9-14.5)
Platelets (10^9/L)	
Median (range)	54.0 (4.0-653.0)
WBC (10^9/L)	
Median (range)	2.7 (0.7-34.0)

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Table 2. Treatment response

	After four cycles (n = 41)	After eight cycles (n = 26)
Hematologic improvement	12 (29.3%)	4 (15.4%)
Stable disease	20 (48.9%)	10 (38.5%)
Failure	4 (9.7%)	4 (15.4%)
CR	1 (2.4%)	3 (11.5%)
PR	4 (9.7%)	5 (19.2%)

Other inclusion criteria were an Eastern Cooperative Oncology Group performance status score of 0 to 2, and adequate hepatic and renal functions. The primary objective of the study was to assess efficacy as complete remission (CR) or partial remission (PR) rates for the combination of VPA and 5-AZA (Vidaza, Celgene) ± ATRA in the treatment of MDS.

VPA was given orally on day 0 at 600 to 1,500 mg daily to reach a final plasma concentration of >50 μg/mL, then 5-AZA was added s.c. at a standard dose of 75 mg/m^2 daily, 7 days for eight cycles, every 4 weeks. In case of minor response, stable disease or failure after four cycles, ATRA was added at 30 mg/m^2 orally daily, on days 8 to 27 for four cycles. Treatment was continued in responding patients until response persisted.

Patients were allowed to receive supportive care, including blood transfusions, antibiotics, and antiemetics, as clinically indicated, but not growth factors, except for granulocyte colony-stimulating factor in case of life-threatening infections. The protocol had been approved by the local ethical committees of the participating centers. All patients signed informed consent in accordance with the Declaration of Helsinki, following institutional guidelines.

Response criteria. Hematologic response was defined according to the International Working Group Criteria for MDS (17). A CR required the disappearance of all signs and symptoms related to the disease, bone marrow blasts <5%, and peripheral blood absolute neutrophil count of ≥1^9/L, platelet count of ≥100 10^9/L or more, hemoglobin ≥110 g/L, untransfused. PR included all CR criteria, except for blast counts, which had to decrease to ≥50% of pretreatment values. VPA levels were measured on day 0 of each course of therapy and dosage was adapted accordingly to achieve the target concentration of 50 μg/mL.

Isolation of mononuclear cells and genotyping for enzymatic polymorphisms. Bone marrow mononuclear cells were separated using Ficoll-Paque PLUS gradient centrifugation (Amersham Biosciences AB). Genotyping of GSTP1-Ile105Val, CYP2C19*2, and CYP2C19*3 was done by PCR-RFLP, as previously described (18). We designed a PCR-RFLP technique to study polymorphisms of the cytidine deaminase promoter region (CDA-897C>A and CDA-92A>G). The CDA-451C>T variant and haplotypes for CDA-897, CDA-451, and CDA-92 were defined according to Fitzgerald et al. (19). The CYP3A4-A290G polymorphic variant was studied by a mismatch PCR-RFLP technique, as previously described (20). Oligonucleotide sequences and PCR-RFLP conditions are detailed in Supplementary Table S1.

Statistical analysis. Differences in the distributions of prognostic factors in subgroups were analyzed by χ^2 test and the Wilcoxon or Kruskal-Wallis test for categorical and continuous covariates, respectively. A nonparametric test (Friedman test) was used to compare observations repeated on the same subject. Median follow-up time was estimated by reversing the codes for the censoring indicator in a Kaplan-Meier analysis. Survival was defined as the time from registration to death or date of the last follow-up. Differences in survival were calculated by the log rank test in univariate analysis and by the Cox regression model in multivariable analysis. The probability of cumulative incidence of disease progression or transformation to AML was estimated using the appropriate nonparametric method, considering death as a competing risk and comparing groups by the Gray test. Confidence intervals were estimated (95% CI) using the Simon and Lee method. Logistic regression and Cox proportional hazard regression models were done to examine and check for treatment results and the risk factors affecting CR rate and time to event. All analyses were carried out in SAS 8.02, methods for competing risks were applied using the macro CIN created by the Department of Biostatistics of the St. Jude Children Research Hospital, Memphis, TN.

Results

Study group and treatment response. The study included 62 patients: 43 males and 19 females with a median age of 70 years (range, 53-83 years). According to the French-American-British classification, diagnosis was RAEB for 39 patients (62.90%), RAEBT for 19 patients (30.65%), and CMML for 4 patients (6.45%). Patient characteristics are shown in Table 1. The IPSS was Intermediate-2 for 42 patients and High for 20 patients. A VPA concentration between 45 and 55 μg/mL was reached in a median of 7 days (range, 2-28 days), whereas the median VPA concentration during treatment was 56.6 μg/mL (range, 16.0-285 μg/mL).

A median of six 5-AZA cycles (range, 0-8) were administered. Three patients died before treatment started, 41 patients completed four cycles, whereas 26 patients completed eight cycles and were evaluable for treatment response (Table 2). The median duration for each of the first four cycles was 39 days (range, 31-103 days), whereas the last four cycles were administered every 37.5 days (median range, 30-82 days).

The CR/PR rate in patients which completed eight cycles was 30.7%, and the disease was stable in 38.5% of patients (Table 2). There were four patients who achieved HI. The median time to achieve CR was 5.4 months (range, 2.0-9.8 months). ATRA was

Table 3. Organ toxicity (National Cancer Institute-Common Toxicity Criteria scale)

	Grade 1-2 (events)	Grade 3-4 (events)
Allergy	56	1
Cardiovascular	57	1
Coagulation	19	0
Cutaneous rash	48	0
Gastrointestinal (colitis)	25	2
Gastrointestinal (diarrhea)	22	0
Gastrointestinal (stomatitis)	20	0
Bleeding	13	3
Liver bilirubin	24	0
Liver sGPT	19	0
Neurology (pain)	18	0
Neurology (sensory)	19	1
Neurology (motor)	19	0
Ocular (conjunctivitis)	19	1
Lung (dyspnea)	20	5
Kidney (creatinine)	19	0
Hematology (anemia)	39	14
Hematology (thrombocytopenia)	28	35
Hematology (neutropenia)	20	48

NOTE: Three hundred and thirty-one cycles of therapy.

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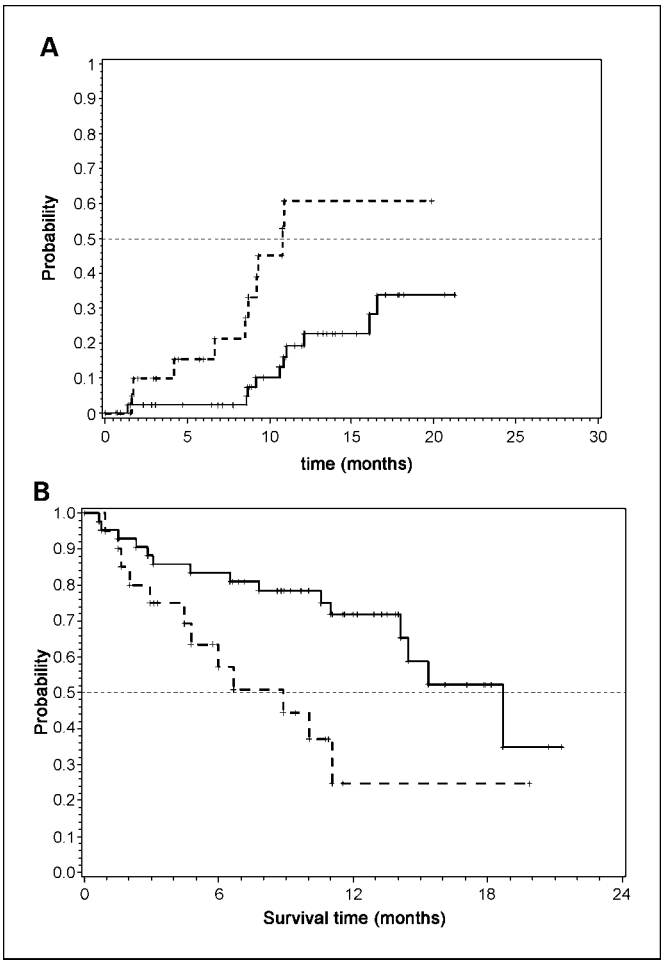

Fig. 1. Survival by the IPSS score. MDS patients with an Intermediate-2 IPSS score (*continuous line*) had a significantly better progression-free (*A*) and overall (*B*) survival (log rank = 0.006 and 0.013, respectively), than patients with a high IPSS (*broken line*).

added in 11 patients on cycle 5, without significant clinical benefit (data not shown). Of 36 patients who did not complete eight cycles, 3 died before treatment started, 2 refused treatment, 12 discontinued treatment for toxicity, 13 for disease progression, and 6 for medical decision.

Transfusion needs decreased from a median of 3 RBC units/mo (range, 0-8) to 1.5/mo (range, 0-10) after four cycles, and 0/mo after treatment completion (range, 0-7; $P = 0.165$). The disease progressed in 20 patients, with 21% cumulative incidence of progression at 10 months (95% CI, 20.4-21.6 months). The median follow-up was 12 months (range, 0.7-21.0). On an intent to treat basis, 32 patients (58.7%; 95% CI, 51.3-67.1) were alive at 12 months and 3 patients died before treatment started, whereas 27 died due to disease progression (9 patients, 33.4%), infections (11 patients, 40.7%), hemorrhage (2 patients, 7.4%), cardiovascular complications (3 patients, 11%), and other causes (2 patients, 7.4%).

Toxicity. Patients were evaluable for toxicity for a total of 331 treatment cycles. The number of grade 3 to 4 toxicities during therapy is shown in Table 3 ($n = 30$ patients). The most frequent toxicity was hematological with 97 events (anemia, 14; thrombocytopenia, 35; and neutropenia, 48), resulting in four grade 1 to 2 and four grade 3 to 4 fever of unknown origin (FUO).

The most frequent events related to VPA was grade 1 to 2 neurotoxicity ($n = 6$ patients), mainly somnolence and confusion, followed by fatigue, which were transient and reversible. Severe toxicity leading to treatment discontinuation was observed in 12 patients (7 due to infectious, 3 to cardiovascular complications, and 2 for neurologic toxicity).

Prognostic factors for outcome. We were interested in the prognostic factors playing a significant role for overall survival. The IPSS score (High versus Intermediate-2) maintained its prognostic relevance. Transformation into AML or progression occurred in 20 patients and were significantly more frequent in patients with a high IPSS when compared to those with Intermediate-2 IPSS scores (at 10 months, 45.0% versus 10.2%; $P = 0.006$). A high IPSS score was also a significant negative prognostic factor for survival (median survival, 8.9 versus 18.7 months; HR, 2.69; 95% CI, 1.23-5.87; $P = 0.013$; Fig. 1). At univariate analysis, further factors associated with survival were VPA serum concentration (≥50 μg/mL versus <50 μg/mL; HR, 0.35; 95% CI, 0.16-0.77), median survival 18.7 versus 10 months ($P = 0.009$; Fig. 2), hemoglobin (as a continuous variable; HR, 0.67; 95% CI, 0.50-0.89; $P = 0.006$) and platelet counts (as a continuous variable; HR, 0.99; 95% CI, 0.981-0.999; $P = 0.024$). In this line, VPA serum concentration also predicted the achievement of CR or PR (as a continuous variable; median, 66 versus 53 μg/mL; $P = 0.04$). Older age as a continuous variable had a trend towards worse survival (HR, 1.046; 95% CI, 0.994-1.101; $P = 0.08$), whereas karyotype and addition of ATRA (11 patients) did not influence response or survival.

The multivariate analysis confirmed the prognostic role of IPSS score (High versus Intermediate-2; HR, 2.778; 95% CI, 1.054-7.316; $P = 0.039$), of hemoglobin concentration (HR, 0.678; 95% CI, 0.46.2-0.996; $P = 0.047$), and VPA serum concentration (HR, 1.001; 95% CI, 1.000-1.002; $P = 0.007$) considered as continuous variables.

Genomic polymorphisms. Analysis of the polymorphisms important for metabolism of the drugs used in the trial showed that a variant of cytochrome P450 (CYP450), CYP2C19*2, influenced the VPA dose necessary to achieve the target VPA concentration of 50 μg/mL on day 1 of AZA treatment. Carriers of this single nucleotide polymorphism required higher VPA doses compared with wild-type subjects (median, 609 versus 600 mg; range, 600-1,400 versus 600-1,000; $P = 0.0021$). Other enzymatic polymorphisms, including CYP3A4-A290G, GSTP1-Ile105Val, and cytidine deaminase (CDA-92A>G, CDA-451C>T, and -897C>A) did not play a role as predictors of toxicity or response.

Discussion

In this multicenter study, we show that epigenetic therapy, combining the DNMTi5-ZA with the HDACi VPA, is active and associated with a high response rate in patients with MDS and unfavorable prognosis.

Azacytidine, as a single agent, was shown to be effective in patients with MDS (21, 22). These data have been confirmed in a phase III, multicenter, randomized, prospective trial which included 358 patients with MDS, demonstrating the superiority of azacytidine over three conventional care regimens (median

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overall survival, 24.4 versus 15 months; ref. 23). The synergistic activity of azacytidine and the HDACi VPA, in an attempt to restore sensitivity to the differentiating effect of ATRA, was tested in 49 refractory/relapsed or *de novo* AML and 4 MDS patients (24). A 42% overall response rate was observed, with most responses at the VPA dose of 50 mg/kg, after a median of one course. Blum et al. obtained a 44% response with decitabine alone or combined with VPA in 25 patients with AML (12). VPA at 15 to 25 mg/kg did not improve the response to decitabine, but induced dose-limiting encephalopathy at plasma levels within or just above 120 μg/mL, the upper limit of the therapeutic range (12). The German MDS study group treated 25 poor-risk MDS and AML patients with 5-AZA at an increased dose of 100 mg/m^2/d for 5 days combined to VPA, to achieve serum levels of 80 to 110 μg/mL. The response rate was 33% and the median survival was 8 months (13). Myelosuppression seemed to be more severe than with 5-AZA alone and most patients had transient central nervous system side effects leading to a dose reduction or transient discontinuation. Similar remission rates were reported in AML patients treated using the 5-AZA/VPA combination (25, 26).

In our prognostically unfavorable, elderly, higher risk MDS patients (also including three patients who died before treatment started), the median overall survival on an intent to treat basis was 14.4 months. Toxicity leading to treatment discontinuation was observed in 12 patients. The survival rate was inferior to that reported by the AZA-001 international trial (23), and is more similar to data reported by the Cancer and Leukemia Group B on high-risk MDS/WHO-AML (21, 22).

A significantly superior survival (18.7 months) was observed for patients reaching a plasma VPA concentration of >50 μg/mL. This indicates that the addition of the HDACi VPA to azacytidine improves the response. Similarly, in the M.D. Anderson series (24), significantly higher response rates were observed in patients reaching higher VPA concentration, but there was no association between the dose administered and response. This points out that specific targeting of VPA plasma levels should be used instead of doses based on weight, and that factors influencing plasma VPA concentration may play a significant role.

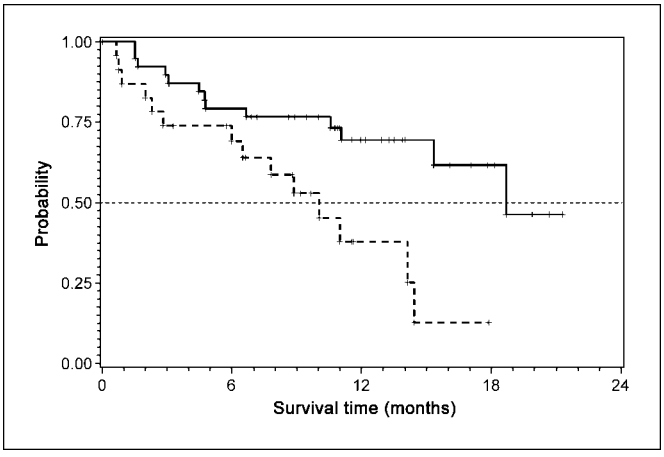

Fig. 2. The plasma VPA concentration significantly affects overall survival. The achievement of a VPA concentration of >50 μg/mL (*continuous line*) on the first day of 5-AZA treatment (median, on day 12; range, 3-69) was associated with superior overall survival when compared with lower VPA concentrations (*broken line*, log rank = 0.007).

At present, we do not have any valuable parameter, either disease-associated or patient-associated, predictive of response to DNMTi or to the combination of DNMTi and HDACi. None of the methylation studies done thus far during hypomethylating treatment have found reliable predictors of response (12, 24). Soriano et al. found a significant decrease in global methylation by day 7 of treatment, but the degree of hypomethylation did not differ between responders and nonresponders (24). This may be due to the presence of genome-wide hypomethylation concomitant with specific tumor suppressor gene hypermethylation in MDS and AML. Prospective biological and clinical studies are warranted to define a patient-tailored approach. We studied the pharmacogenomic profile of patients, and in particular, the genomic polymorphisms of GSTP1, of the CYP450 enzyme, which has been shown to be important for the hepatic metabolism of psychotropic drugs, and of cytidine deaminase, which is important for the inactivation of azacytidine. The polymorphisms CYP3A4-A290G and GSTP1-Ile105Val did not play a role as predictors of toxicity or response. Similarly, no effects were shown for the cytidine deaminase promoter region polymorphisms CDA-92A>G, CDA-451C>T, and -897C>A, which have been shown to significantly decrease CDA activity, especially when combined with haplotype 3 (19).

On the other hand, carriers of the CYP2C19*2 variants of CYP450 (slow metabolizers; ref. 27) required higher VPA doses to achieve the target VPA concentration of 50 μg/mL on day 1 of AZA treatment, which had a significant prognostic role for survival. The definition of this enzymatic polymorphism may help to identify patients with a favorable profile and define a patient-tailored approach.

The addition of the differentiating agent ATRA to azacytidine and VPA in 11 patients did not improve the response rate, indicating that demethylation may not be able to restore impaired differentiation in MDS. Larger patient numbers may be required to answer this issue.

In conclusion, our study shows good efficacy and tolerability of the combination therapy in higher risk MDS, but many issues are still open. In fact, there is no consensus on which schedule and dose of both drugs should be used, nor do we possess biomarkers to predict prognosis. It is equally possible that aside from epigenetic effects, DNMTi and HDACi exert their activity through epigenetically independent induction of DNA damage, requiring better determination of the drug targets in patients with MDS.

Disclosure of Potential Conflicts of Interest

Some of the authors have received honoraria from the speakers' bureau of Celgene.

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