Treatment of poor-risk myelodysplastic syndromes and acute myeloid leukemia with a combination of 5-azacytidine and valproic acid

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Abstract 5-azacytidine (AZA) has become standard treatment for patients with higher-risk myelodysplastic syndrome (MDS). Response rate is about 50% and response duration is limited. Histone deacetylase (HDAC) inhibitors are attractive partners for epigenetic combination therapy. We treated 24 patients with AZA (100 mg/m², 5 days) plus valproate (VPA; continuous dosing, trough serum level 80–110 μg/ml). According to WHO classification, 5 patients had MDS, 2 had MDS/MPD, and 17 had acute myeloid leukemia (AML). Seven patients (29%) had previously received intensive chemotherapy, and five had previous HDAC inhibitor treatment. The overall response rate was 37% in the entire cohort but significantly higher (57%) in previously untreated patients, especially those with MDS (64%). Seven (29%) patients achieved CR (29%) and two PR (8%), respectively. Hematological CR was accompanied by complete cytogenetic remission according to conventional cytogenetics in all evaluable cases. Some patients also showed complete remission according to FISH on bone marrow mononuclear cells and CD34+ peripheral blood cells, as well as by follow-up of somatic mitochondrial DNA mutations. Four additional patients achieved at least marrow remissions. Factors influencing response were AML (vs. MDS), marrow blast count, pretreatment, transfusion dependency, concomitant medication with hydroxyurea, and valproic acid (VPA) serum level. This trial is the first to assess the combination of AZA plus VPA without additional ATRA. A comparatively good CR rate, relatively short time to response, and the influence of VPA serum levels on response suggest that VPA provided substantial additional benefit. However, the importance of HDAC inhibitors in epigenetic combination therapy can only be proven by randomized trials.

Introduction

In recent years, epigenetic therapy has become a treatment option for patients with higher-risk myelodysplastic syndrome (MDS) who are not considered candidates for intensive induction chemotherapy or allogeneic stem cell transplantation (SCT). The demethylating agent 5-azacytidine (AZA) can achieve substantial survival benefit for patients with higher-risk MDS and patients with acute myeloid leukemia (AML) who have a bone marrow blast count of 20–30% (RAEB-T according to the FAB classification) (Fenaux et al. 2009). Although complete response (CR) rates are not higher than 10–20% (Fenaux et al. 2009; Silverman et al. 1994; Silverman et al. 2002, and Silverman et al. 2006), almost half of the patients with intermediate-II or high-risk disease according to IPSS (Greenberg et al. 1997) show hematological improvement. Responses are usually seen...
only after several treatment cycles. Lengthy time to response is problematic for patients with an aggressive course of disease, particularly patients with AML. Results from phase II trials with azacytidine or decitabine suggest that only about one third of such patients respond (Lubbert et al. 2008; Maurillo et al. 2008). To further improve remission rates, time to response and response duration, combinations of AZA with other agents are being evaluated.

Since epigenetic treatment aims at reversing pathological gene silencing, and DNA methylation cooperates with histone modification to control gene expression, it appears logical to combine AZA with inhibitors of histone deacetylases. Preclinical studies suggest that pharmacologic targeting of both, DNA methyltransferases (DNMT) and histone deacetylases (HDAC), may result in synergistic anticancer activity (Bhalla 2005; Yang et al. 2005).

In 2001, two independent groups showed that the antiepileptic drug valproic acid (VPA) also has HDAC inhibitory activity and induces differentiation of malignant myeloid cells, an ability that is enhanced by all-trans retinoic acid (ATRA) (Göttlicher et al. 2001; Phiel et al. 2001). Stimulated by these findings, we studied the clinical effect of VPA at serum concentrations of 50–100 μg/ml in 23 patients with AML or MDS as monotherapy or in combination with (ATRA) (Kuendgen et al. 2004). The pilot study yielded an overall response rate of 35%. Interestingly, response rate was 44% for patients receiving VPA monotherapy, while none of five patients receiving VPA+ATRA from the start responded. Responses were more frequent in lower-risk MDS, but some patients with higher-risk MDS showed a decrease of their elevated blast count. Follow-up of 122 patients confirmed the higher response rates achieved in low-risk MDS. Only few patients with high-risk MDS benefited from VPA monotherapy or VPA+ATRA. Based on our experience with VPA (Kuendgen et al. 2004; Kuendgen et al. 2006, and Kuendgen and Gattermann 2007) and AZA (Fenaux et al. 2009), we embarked on evaluating the combination of the two drugs in patients with MDS and AML.

**Patients and methods**

**Study design**

Primary endpoint of the study was the feasibility and safety of a combination treatment with AZA plus VPA. Secondary endpoints were overall and progression-free survival, as well as hematological response rate according to revised International Working Group (IWG) criteria (Cheson et al. 2006). Study treatment was initiated with AZA 100 mg/m²/day for 5 days every 28 days administered subcutaneously. We chose to investigate a 5-day schedule which is easier to apply than the approved 7-day schedule (75 mg/m²/day for days 1–7) while providing almost the same cumulative dose per cycle. Treatment with VPA was started on day 4. The dosage of daily oral VPA was adjusted to achieve trough serum concentrations between 80 and 110 μg/ml, i.e., in the upper therapeutic range for antiepileptic treatment. Serum VPA levels were measured with a commercially available fluorescence polarization immunoassay (Abbott, Wiesbaden, Germany). Response evaluation was performed by bone marrow cytology, conventional cytogenetics, FISH on bone marrow mononuclear cells (BM-FISH) and CD34⁺ cells in the peripheral blood (PB-CD34-FISH) (Braulke et al. 2010), as well as analysis of somatic mitochondrial DNA mutations (Wulfert et al. 2008). Treatment was continued as long as neither significant side effects nor disease progression occurred. Evaluation of treatment response included all patients who had received at least one complete cycle of study medication and was scheduled at least 1 year after inclusion of the last patient. Two centers participated in the trial, namely the University Hospital of Heinrich-Heine-University, Düsseldorf and the University Hospital of Johann Wolfgang Goethe-University, Frankfurt/Main.

**Inclusion criteria**

Patients included in the study had either

- primary MDS with poor-risk features: bone marrow blast count ≥10% and/or poor-risk karyotype according to IPSS (Greenberg et al. 1997) or
- therapy-related, secondary MDS or AML, or
- AML according to WHO criteria (Harris et al. 1999) or
- chronic myelomonocytic leukemia (CMML-II).

Inclusion criterion for all patients was relapse after, ineligibility for, or refusal of intensive chemotherapy or allogeneic SCT. Ineligibility for intensive treatment was determined by the treating physician after thorough discussion with the patient. Further inclusion criteria included age ≥18 years, Karnofsky index ≥50%, negative pregnancy test, and written informed consent according to the Declaration of Helsinki. Patients were excluded if they were eligible for intensive chemotherapy or allogeneic SCT, had impaired liver function (bilirubin, ALT, and/or AST >2× upper limit of normal) and/or reduced renal function (creatinine ≥2.5× upper limit of normal), previous liver failure, liver or pancreatic disease, or a family history of liver failure, especially after treatment with VPA, porphyria, or any kind of coagulopathy.

**Patient characteristics**

Between March and July 2007, 25 patients were enrolled, of whom 24 received at least one complete cycle of therapy.
and where thus evaluable for response. One patient could not receive a full cycle of vidaza and did not receive VPA due to very rapid disease progression. Median age was 73 years (59–87 years). According to FAB classification (Bennett et al. 1982), 13 patients had MDS and 11 patients suffered from AML. According to WHO (Harris et al. 1999), only 4 patients had MDS, 2 MDS/MPD, and 18 AML (6 de novo, 7 sAML/MDS, 5 AML/MDS therapy related). Only nine patients (37%) had low-risk cytogenetics according to IPSS (Greenberg et al. 1997), all of whom showed a normal karyotype. Four patients (17%) had intermediate-risk and 11 (46%) had high-risk cytogenetics, including seven patients with aberrations of chromosome 7 (2 as single aberration, 2 with one additional abnormality, and 3 as part of a complex karyotype). According to the Grimwade definition for AML (Grimwade et al. 1998), none of our patients had favorable cytogenetics, 12 (50%) had intermediate-risk karyotypes and 10 patients (50%) had adverse cytogenetics. Seventeen patients (71%) were considered ineligible for intensive chemotherapy, because of old age (>75 years) (n=5), concomitant disease (n=6), or adverse cytogenetics in patients >60 years of age (n=8). Seven patients (29%) had previously received one (n=3) or more (n=4) cycles of chemotherapy and had relapsed (n=5) or were refractory (n=2). Five patients had previously received treatment with an HDAC inhibitor (LBH589, n=3; VPA, n=2). Patient characteristics are summarized in Table 1.

Statistical analysis

The product-limit method was used to estimate the probability of survival. Survival was measured from start of treatment to time of death. Survival curves were compared using the log-rank test. Clinical and hematological parameters of patients at the time of treatment were compared using the chi-square test with Yates correction and the Wilcoxon rank-sum test. Parameters that were correlated with response were analyzed using logistic regression and stepwise multivariate regression method of Cox.

Results

Hematological response

Of the 24 patients evaluable for response, 7 achieved cytological CR (29%) and two had PR (8%), giving an overall response rate of 37%. All these patients showed hematologic improvement. One CR was a CRi (with incomplete recovery of platelets to less than 100,000/μl), and in five cases dysplastic features persisted (CRdys). In

| Characteristic | No. |
|---------------|-----|
| Median age/years (range) | 73 (59–87) |
| Gender (n=) | 19/5 |
| Male/female | |
| Diagnosis (FAB) (n=) | |
| RAEB | 8 |
| RAEB-T | 3 |
| CMML | 2 |
| AML | 11 |
| Diagnosis (WHO) (n=) | |
| RAEB II | 4 |
| CMML II | 2 |
| AML, de novo | 6 |
| sAML/MDS | 7 |
| AML/MDS, therapy-related | 5 |
| IPSS-risk group (n=) | |
| Intermediate II | 5 |
| High | 9 |
| Karyotype-risk (IPSS) (n=) | |
| Good | 9 |
| Intermediate | 4 |
| Poor | 11 |
| Karyotype type risk (AML1) (n=) | |
| Good | 0 |
| Intermediate | 12 |
| Poor | 12 |
| Karyotype (n=) | |
| Normal | 7 |
| Abnormal | 17 |
| Median marrow blast count (%) | 18 (1–80) |
| Pre-treatment (n=) | |
| Intensive chemotherapy | 1 |
| Hydroxyurea | 3 |
| LBH-589 | 3 |
| VPA (+/− ATRA) | 2 |
| Growth factors | 2 |
| None | 2 |
| Transfusion dependency Yes/no | 19/5 |
| Median WBC/μl | 2,245 (400-32,000) |
| WBC>10,000 (n=) | 5 |
| WBC<1,500 | 20 |
| WBC<500 | 7 |
| Median platelet count/μl | 57,000 (5,000–252,000) |
| Platelets<100,000 (n=) | 17 |
| Platelets<30,000 | 9 |

FAB French–American–British classification, WHO World Health organization classification, IPSS International Prognostic Scoring System, AML acute myeloid leukemia, VPA valproic acid, ATRA all-trans retinoic acid, WBC white blood count. 1 Grimwade 1998
addition to these nine patients with CR/PR, four patients showed decreasing marrow blasts (three complete marrow responses and one reduction from 34 to 8%), though without hematologic improvement. These cases were not considered as responders.

Interestingly, patient 14 with sAML/MDS, whose marrow blasts decreased from 22 to 2% and who developed Coombs-negative hemolysis, responded well to erythropoietin which was started after the official end of the study (cycle 24). Despite a relatively high endogenous Epo level of 584 mU/l, the patient became transfusion independent (maximum hemoglobin 13.6 g/dl). After cycle 24, the blast count increased to 22% and then 50%. Surprisingly, peripheral blood counts remained stable and the patient was transfusion-independent for another 8 months despite full-blown relapse.

Cytogenetic response

In the six evaluable patients, hematological CR or PR was always accompanied by complete remission as assessed by conventional cytogenetics (CgCR). The cytogenetic diagnosis of the responders (+21; complex including monosomy 7; +8; del(7q); −7 plus dup(1q); del(20q)) included three poor-risk karyotypes.

Cytogenetic responses were also analyzed by FISH on bone marrow mononuclear cells (BM-FISH) and CD34+ cells in the peripheral blood (PB-CD34-FISH). Only the patient with CRi did not have BM-FISH. In the other five, complete remission was confirmed in four, while in one patient, 6% cells with monosomy 7 persisted among BM-MNC. However, in this patient, no aberrant cells were detectable by PB-CD34-FISH. Among the six patients with CgCR by conventional cytogenetics, four also showed CgCR on analysis of PB-CD34-FISH, while two achieved only CgPR (91 → 5 → 29%, and 79 → 1%).

Patient no. 6 showed an interesting development. Within 2 cycles, he achieved complete cytomorphologic remission. After 5 cycles, he also showed CgCR on conventional cytogenetics and BM-FISH. At that time, 32% of his PB-CD34+ cells still showed del(7q) (from an initial 91%). After 9 cycles, the proportion had fallen to 5%. After the first 2 cycles of treatment, an additional clone characterized by loss of the Y chromosome was detected in the marrow, which had not been described prior to study treatment and increased to 53% of metaphases after 5 cycles. Thereafter, that clone decreased slowly to 0%, in parallel to an increase of del(7q) cells. A third clone was observed after 13 cycles exhibiting complete monosomy 7. Cytologically, the patient showed only a borderline blast count of 5% after 17 cycles. The astonishing observation in this case is that peripheral blood counts remained completely stable, and that the patient had never required transfusions for now 43 treatment cycles despite recurrence of del(7q) and karyotype evolution with development of a second clone with monosomy 7.

Of the patients who achieved hematologic remission, only three had somatic mitochondrial DNA mutations suitable for follow-up. Two showed a CR (70 → 0 → 50%, and 67 → 0%) and one had a PR (50 → 5%). Characteristics of responders and patients with marrow remissions are shown in Table 2. Tables 3 and 4 present an overview of treatment details and responses.

Median number of cycles to response was 2 (range 1–9, mean 4) but three patients needed 8 or 9 cycles. Median response duration was 19 months (4–42). Median survival was 9 months, 23 months for responders, 9 months for marrow responders, and 7 months for non-responders (see Fig. 1a).

Potential factors influencing treatment response

Four patients received hydroxyurea (HU) for more than 1 cycle during their study treatment, due to leukocytosis from start of study treatment. One of them had stable disease, two were classified as having progressive disease, and one patient remained stable until HU was discontinued. The latter patient (no. 5) with CMMML-II illustrates that HU may influence the response to epigenetic treatment. He started with a marrow blast count of 15% in March 2007, which was virtually unchanged (18%) prior to cycle 5. Regarding cytogenetics, 100% of mitoses (n=22) showed trisomy 8. After 8 cycles, 1 cycle after HU had been stopped, marrow blasts had decreased to 2%. Cells still harbored trisomy 8 [18/18]. At the same time, platelet transfusion dependency had subsided. After 12 cycles, the patient achieved CgCR according to conventional cytogenetics and BM-FISH (46, XY [24/24]) and PR by PB-CD34-FISH and mitochondrial DNA monitoring. Apparently, study treatment only became successful after HU had been discontinued (see Discussion).

We analyzed other factors possibly related to response, as shown in Table 5. There was no significant influence of age, gender, IPSS, primary vs. secondary MDS, WBC, platelets, and karyotype, although four out of seven patients (57%) with chromosome 7 abnormalities responded, including two with a complex karyotype. One further patient with monosomy 7 and complex karyotype achieved a marrow response. Factors negatively influencing response were AML (vs. MDS), marrow blast count, pretreatment, and transfusion dependency. Of the ten patients who had received previous treatment, only one responded, while 8 out of 14 untreated patients (57%) in the entire group and 7 out of 11 (64%) untreated MDS patients responded. Variables influencing survival were AML (vs. MDS) and transfusion dependency.

The influence of VPA serum levels was also evaluated. Median VPA dosage was 25 mg/kg (14–41). The highest
| No. | Age | Diagnosis (WHO, FAB) | IPSS | Karyotype | Pretreatment | Total no. cycles | Response | Cytogenetic response | Cycles to response | Response duration/months | Dose reductions | Status | Survival |
|-----|-----|----------------------|------|-----------|--------------|-----------------|----------|-------------------|-----------------|----------------------|-----------------|--------|----------|
| D05<sup>a</sup> | 69 | CMML II, CMML RAEB II, RAEB | Int-II | +8 | Hydroxyurea | 23 | CR | CR | 9 | 20 | Aza 75% | Dead | 26 |
| D03<sup>a</sup> | 73 | RAEB II, RAEB | Int-II | Normal | None | 18 | CRdys | na | 2 | 20 | Aza 50% | Dead | 23 |
| D04<sup>a</sup> | 72 | sAML/MDS RAEB | IntII | Complex (incl. −7) del(7q) | None | 6 | CRdys | PR | 1 | 9 | none | Dead | 11 |
| D06<sup>a</sup> | 64 | CMML II, CMML | High | −7, dup 1 (q) del(7q) | None | 43 | CRdys | CR | 2 | 44 | Aza 50% | Alive, ongoing | 44 |
| D17<sup>a</sup> | 67 | sAML/MDS, RAEB-T | High | None | 19 | CRdys | CR | 9 | 16 | Aza 50%, VPA reduction | Dead | 30 |
| F03<sup>a</sup> | 77 | AML de-novo | X | +21 | Intensive chemotherapy interruption | None | Dead | 13 | CRdys | na | 5 | VPA |
| D14<sup>a</sup> | 77 | sAML/MDS RAEB-T | High | Normal | None | 24 | CRdys | na | 5 | 29 | VPA |
| D01<sup>a</sup> | 69 | sAML/MDS RAEB | High | Complex (incl. −7) | None | 4 | PR | Discontinuation | nd | 1 | 19 | VPA |
| D15<sup>a</sup> | 74 | RAEB I, RAEB | Int-II | −7, dup 1 (q) | None | 5 | PR | Discontinuation | Lost to follow up | 19 |
| F02<sup>b</sup> | 87 | AML de-novo | X | Normal | LBH589 | 3 | SD, marrow CR | Discontinuation | na | 2 | VPA interruption | dead | 2 |
| F05<sup>b</sup> | 59 | sAML/MDS RAEB-T | High | Complex (incl. −7) | Intensive | 3 | SD, marrow CR | none | na | 3 | na |
| D11<sup>b</sup> | 74 | t-AML | X | +6,+8 | Epo | 5 | PBSCT -> CR, dead | 13 | none | na | Aza 75% | dead | 9 |
| D13<sup>b</sup> | 72 | sAML/MDS | X | Normal | None | 6 | SD, marrow PR (blast count: 34→8%) | na | na | na | none | dead | 9 |

*na not applicable, nd not done

<sup>a</sup> Responders
<sup>b</sup> Marrow remissions only
trough level was 86 μg/ml (58–122). Median VPA trough level over the whole treatment period was 68 μg/ml (44–97), due to necessary dose reductions. A serum level >70 μg/ml was achieved after a median of 4 weeks. The likelihood of response was influenced by median ($p=0.004$) and maximum VPA levels ($p=0.007$). The time to reach a VPA level above 70 μg/ml also provided prognostic information ($p=0.02$). The strongest predictive factor ($p=0.00005$) was the VPA level achieved within the first 2–3 weeks of treatment. The influence of median VPA serum levels on survival is illustrated by Fig. 1c and Table 5. On the other hand, VPA dosage (absolute amount or dose/kg) was not related to response or survival. On multivariate analysis, median VPA level was the only independent prognostic factor for response ($p=0.015$), while MDS vs. AML ($p=0.031$) and transfusion dependency ($p=0.032$) were the only independent parameters influencing survival.

**Side effects**

Adverse events are summarized in Table 6. Most patients had transient central nervous system (CNS) side effects, necessitating dose reductions or temporary treatment interruption in eight and cessation of therapy in two patients. In one patient, Coombs-negative hemolysis occurred. It remained unclear whether this was related to one of the study drugs. Discontinuation of VPA was without effect, and hemolysis did not respond to steroids. Two patients developed a rash after AZA but were able to continue treatment with concomitant steroids. Myelosuppression occurred in all patients, especially during the initial

| Table 3 Characteristics of response and treatment |
|-----------------------------------------------|
| Characteristic                          | No. (range) |
| Cytologic CR/PR                        | 7/2         |
| Marrow response CMR/PRMR               | 3/1         |
| Hematologic improvement                |             |
| HI-N                                    | 6           |
| HI-P                                    | 9           |
| HI-E                                    | 5           |
| Cytogenetic response (conventional) CR/PR | 6/0         |
| FISH response (BM) CR/PR/not done      | 4/1/1       |
| FISH response (CD34, pB) CR/PR         | 4/2         |
| Mitochondrial mutations CR/PR          | 2/1         |
| Median survival                        |             |
| All patients                           | 9.5 months  |
| Responders vs. marrow response vs. nonresponders | 23 vs. 9 vs. 7 months |
| Median number of cycles                | 5(2–43)     |
| Median number of cycles to response    | 2(1–9)      |
| Dose reduction vidaza                  | 9           |
| 75%                                     | 5           |
| 50%                                     | 4           |
| Median dosage VPA mg/kg                | 25 (14–41)  |
| Maximum VPA level (median)/μg/ml       | 86 (58–122)$^a$ |
| Median VPA level (median)/μg/ml        | 68 (44–97)$^a$ |
| Median time to VPA level >70 μg/ml     | 4 weeks$^b$ |
| Patients with significant VPA side effects leading to: | 13 |
| Dose reduction/Treatment break/cessation VPA | 7/5/3 |

$^a$ not done, $n=1$

$^b$ never acheived, $n=1$

| Table 4 Response details |
|--------------------------|
| Patient | Cytologic response (blast count) (%) | Hematological improvement | Cytogenetic response | Response FISH-BM (%) | Response FISH-CD34 (%) | Mito response (%) |
|---------|--------------------------------------|---------------------------|----------------------|----------------------|-----------------------|------------------|
| F03     | CRi                                  | HI-P                      | CR                   | nd                   | CR                    | na               |
| D01     | PR (5)                               | HI-N, HI-P, HI-E          | nd                   | nd                   | nd                    | nd               |
| D03     | CRdys                                | HI-N, HI-P, HI-E          | na                   | na                   | na                    | na               |
| D04     | CRdys                                | HI-N, HI-P, HI-E          | CR                   | CR                   | na                    | na               |
| D05     | CR                                   | HI-N, HI-P                | CR                   | CR                   | PR (1)                | PR (5)           |
| D06     | CRdys                                | HI-P                      | CR                   | CR                   | PR (5)                | na               |
| D14     | CRdys                                | None                      | na                   | na                   | na                    | na               |
| D15     | PR (5)                               | HI-P, HI-E                | CR                   | PR (6)               | CR                    | CR               |
| D17     | CRdys                                | HI-N, HI-P, HI-E          | CR                   | CR                   | CR                    | CR               |
| F02     | SD (CMR)                             | None                      | na                   | na                   | na                    | na               |
| F05     | SD (CMR)                             | None                      | None                 | nd                   | None                  | None             |
| D11     | SD (CMR)                             | None                      | None                 | None                 | None                  | None             |
| D13     | SD (PMR)                             | None                      | na                   | na                   | na                    | None             |

$nd$ not done, $na$ not applicable
cycles. Treatment had to be delayed in several patients after the first or second cycle, and dose reductions of AZA were made in seven cases. One patient died of pneumonia after the first cycle.

**Discussion**

Our study of AZA plus VPA confirms that this combination is feasible and efficacious with an ORR of 37% and a CR rate of 29% in patients with higher-risk MDS and AML. However, since our study was not randomized, the additive value of VPA cannot be determined. AZA as monotherapy significantly prolongs survival of patients with higher-risk MDS (Fenaux et al. 2009). Still, about 50% of patients do not respond, and response duration is limited despite continued administration. In order to augment the effect of AZA on epigenetic regulation of gene expression, the most promising approach was to combine the demethylating agent with an HDAC inhibitor (Yang et al. 2005).

Most combination studies to date have used VPA, which requires higher concentrations than newer HDAC inhibitors but is relatively selective for class-I-HDACs and leads to degradation of HDAC 2 (Göttlicher et al. 2001).

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Table 5 Comparison of characteristic responders vs. non-responders and influence of different characteristics on response rate and survival

| Characteristics                  | Responders N=8 | Nonresponders incl. marrow CR N=16 | p response rate | p survival |
|----------------------------------|----------------|------------------------------------|----------------|-----------|
| Median age                       | 72             | 74                                 | n.s.           | n.s.       |
| Sex                              | 78% male       | 80% male                           | n.s.           | n.s.       |
| Karyotype risk (IPSS)            | 44% high       | 53% high                           | n.s.           | n.s.       |
| Normal/abnormal KT               | 22% normal     | 33% normal                         | n.s.           | n.s.       |
| Chromosome 7 abnormality         | 44% Chr 7      | 27% Chr 7                          | n.s.           | n.s.       |
| IPSS score                       | 50% high       | 83% high                           | n.s.           | n.s.       |
| MDS vs. AML                      | 89% MDS        | 33% MDS                            | 0.02           | 0.0052     |
| Prim vs. sek.                    | 13% sek        | 33% sek                            | n.s.           | n.s.       |
| Treatment vs. no pretreatment    | 11% yes        | 60% yes                            | 0.03           | n.s.       |
| Mean marrow blast count          | 15             | 40                                 | 0.009          | n.s.²      |
| WBC/μl                           | 3,000          | 3,900                              | n.s.           | n.s.       |
| Transfusion dependency           | 56%            | 93%                                | 0.047          | 0.014      |
| Platelet count/μl                | 42             | 76                                 | n.s.           | n.s.³      |
| Median VPA level (mean)/μg/ml    | 77             | 62                                 | 0.004          | 0.03³      |
| Maximum VPA level (mean)/μg/ml   | 101            | 80                                 | 0.007          | 0.04⁰f     |
| Time to level >70 μg/ml          | 3              | 7                                  | 0.02           | n.s.⁶      |
| VPA level week 2/3               | 79             | 46                                 | 0.00005        | 0.0012³b   |
| VPA dosage (mg)                  | 1,800          | 2,066                              | n.s.           | n.s.¹      |
| VPA dosage/kg                    | 23             | 27                                 | n.s.           | n.s.¹      |

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cutoff (median):

| a 70 years | b 20% marrow blasts | c 3,500/μl | d 60,000/μl | e 70 μg/ml | f 80 μg/ml | g 5 weeks | h 50 μg/ml | i 2,000 mg | j 25 mg/kg |

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Fig. 1 Survival from start of treatment, a for the entire cohort, b responders vs. nonresponders, landmark analysis at 1 year (at 2 years, 44% of responders are alive compared to 0% of non-responders) c) according to VPA serum level \(\geq 70\) μg/ml

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vary considerably. All trials except ours added ATRA to the treatment regimen, although Voso et al. (2009) did so only in non- or suboptimal responders. Craddock et al. (2008) added theophylline as a fourth substance. Various AZA dosages have been used. In our trial, we chose a novel dosing regimen for AZA (100 mg/m²/day for 5 days) which avoids the “weekend problem” while providing an overall dose of AZA (500 mg/m²/cycle) that is very similar to the approved regimen (525 mg/m²/cycle). The higher dose intensity of AZA in our schedule may have contributed to the increased myelosuppression compared to our previous experience with AZA monotherapy. However, the combination with VPA, as well as unfavorable patient characteristics, represent other potentially important factors. Two previous studies (Soriano et al. 2007; Raffoux et al. 2010) used a high-dose intermittent schedule for VPA. We chose to apply VPA on a continuous schedule. The doses administered by us and the serum trough levels achieved were higher than in the Italian study (Voso et al. 2009). Despite major differences in theses three treatment regimens, the significant influence of VPA serum levels on response rate and survival in our study was the same as that observed by Soriano et al. and Voso et al. Also in line with previous observations, we found no influence of VPA dosage, probably due to pharmacogenomic differences between patients, as suggested by Voso et al. (2009). As far as typical side effects of HDAC inhibitors, like CNS or gastrointestinal symptoms are concerned, we observed no increased incidence or severity during combination treatment with AZA.

Combination trials have also been performed using decitabine as a demethylating agent. A randomized trial adding VPA intermittently to decitabine in one of the two arms (Issa et al. 2008) yielded response rates (52% vs. 43%) that failed to show a statistically significant difference, probably due to the limited number of patients included (n=67).

The overall response rate in our trial was 37%, which is lower than expected from previous trials with AZA monotherapy (Fenaux et al. 2009). However, our series included a high proportion of AML patients, particularly patients pretreated with intensive chemotherapy and/or HDAC inhibitors. Although VPA can be beneficial for patients with AML, the majority of patients included in the abovementioned clinical trials had a relatively low marrow blast count (Fenaux et al. 2009; Sudan et al. 2006). In contrast, the French ATU program included 184 patients with relapsed/refractory AML and generated a response rate of only 13% (Itzykson et al. 2009). In our study, the response rate according to IWG criteria was 57% for all untreated patients (MDS and AML) and 64% for untreated patients with MDS. The other combination trials mentioned above reached comparable response rates. Phase II trials are often difficult to compare, due to varying patient and response characteristics, which are given in Table 7. For example, Voso and coworkers decided to analyze responses only in patients who completed 8 cycles. Some studies, in contrast to our trial, classified marrow responses as response rather than stable disease. Furthermore, IWG criteria, which are currently considered the gold standard for response evaluation, are different for MDS and AML.

A relatively large proportion of our patients achieved CR (29% overall and 55% of previously untreated patients with MDS). Hematological CR was accompanied by complete cytogenetic remission in all evaluable cases. All long-time hematologic improvements were associated with marrow remissions, while not all marrow remissions produced hematologic improvement. An improved CR rate with combination therapy has been described in previous trials (Soriano et al. 2007). Whether improved CR rates were due to the addition of VPA, a slightly increased daily dosage of AZA, or other causes is not entirely clear from these phase II studies. The same is true for time to response, which appeared shorter than with AZA monotherapy in the study reported by Soriano et al. That observation is in accordance with the median time to response in our trial (only 2 cycles). However, even though combination therapy may achieve earlier responses in a proportion of patients, it does not seem to obviate the need for prolonged treatment. While five of our patients responded quite early, after only 1 or

| Side effects grades 1/2 | Frequency |
|------------------------|-----------|
| Local skin reaction    | 25        |
| Nausea                 | 24        |
| Diarrhea               | 19        |
| Constipation           | 17        |
| Leukocytosis           | 15        |
| Fatigue                | 14        |
| Hypokalemia            | 13        |
| Somnolence             | 12        |
| Fever                  | 10        |
| Influenza-like symptoms| 10        |

| Side effects grade 3/4 | Frequency |
|------------------------|-----------|
| Neutropenic fever      | 12        |
| Pneumonia              | 10        |
| Febrile neutropenia    | 4         |
| Septicemia             | 4         |
| Dyspnea                | 3         |
| Somnolence             | 3         |
| Catheter-side infection| 3         |
| Neutropenia            | 3         |
| Pancytopenia           | 3         |
| Confusion              | 3         |
2 cycles, three other patients needed 8 or 9 cycles. Like AZA monotherapy, combination therapy with AZA plus VPA requires perseverance, and it is now generally recommended that patients who achieve at least stable disease should be kept on treatment as long as possible.

An interesting finding in our study was that patients can remain transfusion-free with stable peripheral blood counts despite showing a relapse in the bone marrow. We found that increases in medullary blasts or aberrant cells may occur without being followed by clinically relevant progression/relapse. Actually, the two patients with the longest treatment benefit had cytogenetic or even cytomorphological full-blown relapse. These findings illustrate that epigenetic treatment may require changes in our concept of evaluating treatment success. Achieving CR is not paramount, since it is not a prerequisite for obtaining a substantial survival benefit. The AZA-001 trial (Fenaux et al. 2010) made it clear that patients whose best response was hematologic improvement had the same increase in life expectancy as patients who achieved CR or PR.

Even though a number of patients in our trial achieved hematological CR, most of them showed persistent dysplastic features, as it is often the case after chemotherapy in MDS as well. This was even true for patient 17, who was in complete remission according to conventional cytogenetics, PB-CD34-FISH, BM-FISH, and mitochondrial mutation analysis. This patient relapsed relatively quickly after 16 cycles. This suggests that in some cases, the available monitoring tools may detect only the most advanced, dominant MDS clone without revealing an underlying preleukemic clone that may be responsible for the dysplastic features.

As in previous studies, favorable responses were observed in patients with chromosome 7 abnormalities (Raj et al. 2007). The response rate may not be better than with a normal karyotype or trisomy 8, but this group of patients is clearly doing worse with conventional therapies. In contrast to Mufti et al. (2009), we even observed responses in two of three patients who had chromosome 7 abnormalities as part of a complex karyotype (survival time 11 and 19 months, respectively).

An interesting aspect of our study relates to the possible interaction of hydroxyurea with study medication. In vitro studies suggest that inhibition of ribonucleotide reductase by HU (Choi et al. 2007) interferes with conversion of 5-azacytidine to 5-aza-2'-deoxycytidine, thereby decreasing incorporation of the false nucleotide into DNA and diminishing its hypomethylating effect. Although it has not been proven that DNA hypomethylation is

Table 7 Overview of different combination studies with VPA and AZA

| Patient | This study | Soriano et al. | Voso et al. | Raffoux et al. | Craddock et al. |
|---------|------------|----------------|-------------|---------------|----------------|
| Drugs   | VPA, 5-Aza | VPA, Aza, ATRA | VPA, Aza, ATRA<sup>a</sup> | VPA, Aza, ATRA | VPA, Aza, ATRA, theophylline |
| Dosage VPA | Median dosage 25 mg/kg continuously | 50–75 mg/kg 7 days | 600–1,500 mg continuously | 35–50 mg/kg 7 days | ng |
| Dosage AZA | 100 mg/m<sup>2</sup> 5 days | 75 mg/m<sup>2</sup> 7 days | 75 mg/m<sup>2</sup> 7 days | 75 mg/m<sup>2</sup> 7 days | 75 mg/m<sup>2</sup> 5 days |
| No. of pts. | n=25, evaluable 24 | n=53 | n=62, 26 evaluable<sup>b</sup> | n=65 | n=45 |
| Median age | 73 years | 69 years | 70 years | 72 years | 66 years |
| No. AML/MDS | 75%/25<sup>c</sup> | 92%/8% | 31%/69<sup>d</sup> | 85%/15% | 80%/20% |
| Pretreatment | n=10 (42%) | n=20 (38%) | ng | n=13 (20%) | n=30 (67%) |
| ORR | 37% | 27<sup>e</sup> | 31% | 26% | 33% |
| CR/PR/Hi/marrow response | 7/2/0/4 | 27/0/0/7 | 3/5/0/7 | 14/3/ng/ng | 7/8/ng/ng |
| Median cycles to response | 2 (1–9) | 2 (1–3) | 5 (2–10) months<sup>g</sup> | ng | 2 (1–6) |
| OS | 9 months (23 in responders) | 6.5 months (14+ in responders) | 14.4 months | 12.4 months (19.5 in responders) | ng |

<sup>a</sup> only in non-responders  
<sup>b</sup> at least 8 cycles to be evaluable  
<sup>c</sup> 46%/54% according to FAB  
<sup>d</sup> 100% according to FAB  
<sup>e</sup> 42% including marrow response  
<sup>f</sup> 12.5% of non-responders had Hi-erythroid  
<sup>g</sup> Time to CR
the only mechanism behind the clinical efficacy of AZA, the clinical course of our patient who achieved remission only after cessation of HU appears to underscore the relevance of HU interference with AZA conversion, thus pointing to the importance of AZA getting incorporated into DNA. It is worth mentioning that three further patients who received HU during study treatment were non-responders. Our trial was the first to investigate the combination of AZA plus VPA without additional ATRA. Although the response rate was not higher than would be expected with AZA monotherapy, the heterogeneity of the patient population, with a significant proportion of pretreated AML patients, should be taken into consideration. The CR rate was favorable compared with previously published trials, and some of the responses appeared to occur faster than with single-agent AZA. These observations as well as the significant influence of VPA serum levels on response suggest that the HDAC inhibitor contributes to treatment success. However, the true value of HDAC inhibitors in this kind of epigenetic combination therapy can only be determined by randomized trials.

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