Safety and clinical activity of the combination of 5-azacytidine, valproic acid and all-trans retinoic acid in acute myeloid leukemia and myelodysplastic syndrome


Department of Leukemia, University of Texas MD Anderson Cancer Center, Houston, TX.

Short title: 5-azacytidine, valproic acid and ATRA in leukemia

Key words: 5-azacytidine, valproic acid, ATRA, leukemia, DNA methylation, histone.

Word count: 4708

Abstract 199

Corresponding author:
Guillermo Garcia-Manero MD
Department of Leukemia
Box 428
University of Texas MD Anderson Cancer Center
1515 Holcombe Blvd
Houston, TX 77030
Phone: 713 745 3428
Fax: 713 794 4297
E-mail: ggarciam@mdanderson.org
ABSTRACT

The combination of a DNA hypomethylating agent with a histone deacetylase inhibitor has synergistic antileukemia activity and may restore sensitivity to all-trans retinoic acid (ATRA). We conducted a phase I/II study of the combination of 5-azacitidine (5-AZA), valproic acid (VPA) and ATRA in patients with acute myeloid leukemia or high-risk myelodysplastic syndrome. 5-AZA was administered subcutaneously at a fixed dose of 75 mg/m² daily x 7. VPA was dose-escalated and given orally daily x 7 concomitantly with 5-AZA. ATRA was given at 45 mg/m² orally daily x 5, starting on day 3. Fifty three patients were treated. Their median age was 69 years (range 5-84). The maximum tolerated dose of VPA in this combination was 50 mg/kg daily x 7. Dose limiting toxicity was reversible neurotoxicity. The overall response rate was 42%. In previously untreated older patients the response rate was 52%. Median number of courses to response was 1 (range 1-3). Median remission duration was 26 weeks and median survival has not been reached. A significant decrease in global DNA methylation and induction of histone acetylation were achieved. VPA blood levels were higher in responders (p<0.005). In conclusion the combination studied is safe and has significant clinical activity. This clinical trial was registered at www.clinicaltrials.gov as #NCT00326170.
INTRODUCTION

5-azacytidine (5-AZA) is a cytidine analogue with clinical activity in myelodysplastic syndromes (MDS)\(^1\) and in acute myeloid leukemia (AML)\(^2,3\). 5-AZA is a DNA methyltransferase inhibitor that induces DNA hypomethylation\(^4\) both in vitro\(^6\) and in vivo. DNA methylation is a mechanism of epigenetic regulation of gene transcription\(^6\). It involves the addition of a methyl group to a cytosine when is followed by guanine in the so called cytosine-guanine pairs (CpG). CpGs are underrepresented in the human genome, except in areas known as CpG islands that are located near gene promoters and repetitive elements such as long interspersed nucleotide elements (LINE)\(^7\). DNA methylation is a physiologic process involved in imprinting and other mechanisms of gene dosage control\(^6\). Aberrant DNA methylation of promoter associated CpG islands is frequently detected in cancer\(^7\). Because methylation of promoter associated CpG islands is frequently associated with gene silencing\(^6\), it is considered a functional inactivating mechanism of tumor suppressor genes\(^7\). In contrast to deletions or mutations of tumor suppressor genes, aberrant DNA methylation is reversible both in vitro and in vivo with the use of hypomethylating agents such as 5-AZA or 5-aza-2'deoxycytidine (decitabine)\(^4\). The understanding of the role of 5-AZA as a modulator of DNA methylation has led to rational combination therapies exploiting mechanisms of epigenetic regulation of gene expression. One such mechanism is the biochemical modification of chromatin associated histone proteins, such as histone H3 or H4 acetylation\(^4\). This process is controlled by histone acetyl transferases and deacetylases (HDAC)\(^4,8\). Inhibition of HDAC activity results in histone acetylation that is associated with a permissive gene expression state\(^8\). A number of HDAC inhibitors have shown
activity in leukemia and lymphoma\textsuperscript{8}. The combination of a hypomethylating agent with a HDAC inhibitor has synergistic effects on gene reactivation and leukemia cell kill\textsuperscript{9,10}. VPA, a short chain fatty acid used clinically for the treatment of epilepsy and mood disorders, is a HDAC inhibitor\textsuperscript{11-13}. As a single agent, VPA has modest clinical activity in MDS\textsuperscript{14} and AML\textsuperscript{15}. In combination with decitabine, VPA has synergistic antileukemic activity in vitro\textsuperscript{9} and in a recent clinical study, it was shown to be active in patients with poor risk leukemia\textsuperscript{16}.

All-trans retinoic acid (ATRA) is a cell differentiation agent with clinical activity in acute promyelocytic leukemia (APL)\textsuperscript{17} but not in other types of leukemias\textsuperscript{18}. In APL, ATRA releases co-repressors and histone deacetylases and induces the expression of target genes\textsuperscript{19}. In ATRA-resistant APL variants, the use of a HDAC inhibitor can restore sensitivity to ATRA\textsuperscript{19}. In APL, DNA methyltransferases are also recruited to target gene promoters\textsuperscript{20}. ATRA can induce hypomethylation and re-expression of the RAR-\textgreek{beta}\textsubscript{2} gene\textsuperscript{20}. This activity is enhanced by 5-AZA\textsuperscript{20}. Therefore, it is possible that the combination of a hypomethylating agent with a HDAC inhibitor may sensitize ATRA resistant AML to the effects of ATRA.

Based on this information, we designed a study to test whether the combination of 5-AZA, VPA and ATRA is safe and active in patients with high risk MDS and AML.

**METHODS**

**Study group eligibility**

Patients with refractory or relapsed AML or high risk MDS, defined as having 10% or more bone marrow blasts, were included. Untreated patients over 60 years with AML or high risk MDS, who refused or were not candidates for frontline chemotherapy, were
also eligible. Whether a patient was a candidate for intensive chemotherapy was determined by the treating physician after discussion with the patient. Other inclusion criteria included performance status ECOG 2 or less, age 2 years or older, and adequate hepatic and renal functions. Patients must have been off chemotherapy for at least 2 weeks prior to entering the study. Patients with previously untreated core binding factor leukemias, nursing or pregnant females or with active or uncontrolled illnesses, were excluded. The protocol was approved by the MD Anderson Cancer Center (MDACC) ethics committee. All patients signed informed consent following institutional guidelines.

**Treatment**

Treatment consisted of 5-AZA, VPA and ATRA. 5-AZA was given at a fixed dose of 75 mg/m² subcutaneous daily for 7 days (days 1-7). VPA was administered orally daily for 7 days (days 1-7) concomitantly with 5-AZA administration. ATRA was administered at a dose of 45 mg/m² orally daily x 5 days, starting on day 3 of 5-AZA and VPA (days 3-7). Courses of therapy were repeated not earlier than every 3 weeks. Patients were allowed to receive supportive care measures including antibiotics, antiemetics and growth factors as clinically indicated and according to institutional guidelines. In the phase I portion, three different dose levels of VPA were studied: 50, 62.5 and 75 mg/kg orally daily for 7 days. In the phase II, VPA was administered at its maximum tolerated dose (MTD) in combination with 5-AZA and ATRA.

**Response criteria and statistical methods**

A complete response (CR) required the disappearance of all signs and symptoms related to the disease, a bone marrow with ≤ 5% blasts and peripheral blood count with
an absolute neutrophil count $\geq 10^9$/L and platelet count $\geq 100 \times 10^9$/L. A CRp was defined as a CR except for a platelet count increase by 50% to above $30 \times 10^9$/L but less than $100 \times 10^9$/L. A bone marrow (BM) response was defined as bone marrow blast $\leq 5\%$ but without meeting the peripheral blood count criteria for CR or CRp. For a response to be considered, it had to last for at least 4 weeks. Remission duration was calculated from the date of first response until relapse, and survival from start of therapy until death from any cause. Bone marrow aspiration procedures were performed uniformly during course #1 of therapy pretreatment and on days 21 and 28 (+/-3 days). On subsequent courses, they were performed on day 28 of each cycle until documentation of response and thereafter as clinically indicated.

In the phase I of the study, cohorts of 3 patients were entered at each dose level. If no Grade 3 or higher non hematological toxicity was observed, VPA was escalated to the next dose level. If one patient developed such toxicity, three more patients were accrued at that dose level. If two or more patients developed Grade 3 or higher non-hematological toxicity, that dose of VPA was considered too toxic. The MTD was defined as the dose below the one producing dose limiting toxicity (DLT) in 2 or more patients. Ten patients were to be treated at the MTD to confirm the rate of toxicity.

Toxicity was graded using the National Cancer Institute Common Toxicity Criteria (NCI CTC version 3).

The phase II was designed to evaluate the activity of the combination. The study would stop early if the probability was less than 10 % that the response rate (CR + CRp) was at least 30%. The probability of a patient response was assessed in cohorts of 10
patients. The study was to stop if there were 0 responses in the first 10 patients, 3 or fewer responses of 20 or 5 or less in 30 patients. Otherwise, the maximum number of patients that could be treated during the phase II was 40. Patients treated during the phase I portion of the study at the MTD of VPA could be included in the phase II analysis.

Groups were compared using the two sample t-test. Survival and relapse-free survival were reported using Kaplan Meier curves. All p-values were two-sided; statistical significance was declared if p was below 0.05. Statistica 6.1 software was used to perform these calculations.

**Cytogenetic analysis**

Cytogenetic analysis was performed in the clinical cytogenetics laboratory at MDACC. Baseline cytogenetic analysis was performed prior to therapy, and subsequently with each course of therapy when possible in patients with abnormal cytogenetics as previously described\(^{16}\).

**Detection of VPA levels**

Free and bound VPA levels were measured using commercially available assays on days 0, 5 and 7 of each course of therapy as previously described\(^{16}\).

**Isolation of human mononuclear cells**

Peripheral blood human mononuclear cells were used to perform DNA methylation and mRNA studies. Peripheral blood specimens from patients were drawn into heparin (30 U/ml) containing tubes. Mononuclear cells were separated using Ficoll-Paque™ PLUS gradient centrifugation (Amersham Biosciences AB, Uppsala, Sweden). The monocyte-enriched fraction was collected and washed twice with calcium and magnesium free
phosphate-buffered saline (PBS). After centrifugation, PBS was removed and the samples were immediately frozen at minus 80 ºC.

**Analysis of DNA Methylation**

To study the dynamics of DNA hypomethylation during treatment, we sequentially studied LINE methylation, a marker of global DNA methylation\(^\text{16}\). To do so we used a bisulfite pyrosequencing assay\(^\text{16}\). The details of this technique have been described elsewhere\(^\text{16}\).

**Analysis of Histone acetylation**

Histone H3 and H4 acetylation was analyzed by Western blot using antibodies directed against acetylated histone H3 and H4 as previously described\(^\text{16}\). In brief, proteins were isolated from peripheral blood mononuclear cells by sonication, using a lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 1 mM PMSF), and posteriorly quantitated. Thirty µg of protein from each cell lysate were separated in 12% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to Immobilon-P Transfer Membranes (Millipore, Billerica, MA). Membranes were blocked in 3% nonfat milk and probed overnight at 4ºC with 1:2000 dilution of acetylated histone H3 antibody (Upstate Biotechnology, Waltham, MA). Membranes were washed three times with PBS containing 0.1% Tween 20 (PBS-T) and incubated with an anti-rabbit peroxidase-conjugated secondary antibody (1:2000, Amersham Biosciences, England, UK) for 1 hour at room temperature. Membranes were washed three times in PBS-T and the film was developed by using enhanced chemoluminescence ECL (Amersham Pharmacia Biotech, Piscataway, NJ). β-actin (1:5000, Sigma, St Louis, MO) was used as internal control. HL-60 cells treated with VPA were used as positive controls.
Real-time PCR analysis of p21CIP1 and p15 mRNA gene expression

P21CIP1 and p15 mRNA expression was conducted as previously described16. In summary, total cellular RNA was extracted using Trizol (Invitrogen, Carlsbad, CA). Two to three µg of total RNA were used for reverse transcription (RT) reactions using the Moloney murine leukemia virus RT enzyme (Invitrogen, Carlsbad, CA). Levels of p21CIP1, and p15 mRNA expression were analyzed using real-time PCR. Primers and probes were purchased from Applied Biosystems and analyzed using an Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR reactions were performed using 20X Assays-On-Demand Gene Expression Assay Mix (contained unlabeled PCR primers and TaqMan probes) and TaqMan Universal PCR Master mix (Applied Biosystems, Foster City, CA). PCR conditions were 95ºC for 10 min and then 95ºC for 15 sec and 60ºC for 1 min repeated for 40 cycles. Experiments were performed in triplicate. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. Quantitative values were obtained from the cycle number (C_T Value) at which the increase in fluorescent signal associated with an exponential accumulation of PCR products was detected. The amount of target gene was normalized to the endogenous reference GAPDH to obtain the relative threshold cycle (ΔC_T) and then related to the C_T of the control to obtain the relative expression level (2^-ΔΔC_T) of target gene.

RESULTS

Study group

Fifty-three patients were treated. Patient characteristics are shown in Table 1. Nineteen patients were treated during the phase I portion of the study, and 34 during the phase II,
which included 10 patients that were an expansion cohort treated at the MTD. Thirty-three patients (62\%) were older than 60 years and had previously untreated disease. These patients refused or were not considered candidates for frontline intensive chemotherapy. Thirty-four patients (64\%) had poor-risk cytogenetics.

**Valproic acid dose escalation and toxicities**

Three dose levels of VPA (50, 62.5 and 75 mg/kg orally daily for 7 days) were explored in combination with 5-AZA and ATRA. No grade 3-4 non-hematologic toxicity attributable to 5-AZA or ATRA was observed at any dose level. The number of patients who experienced Grade 3-4 toxicities related to therapy during the first course of therapy is shown in Table 2. We assessed toxicities in cohorts of three patients. The starting dose of VPA was 50 mg/kg. One of the first 3 patients experienced a DLT (Grade 3 neurotoxicity), and 3 more patients were enrolled in that cohort. Subsequently, the dose was escalated to 62.5 mg/kg orally daily for 7 days. The first 3 patients enrolled in this cohort did not experienced DLTs and the dose of VPA was further increased to 75 mg/kg orally daily for 7 days. One of the first 3 patients at this dose level experienced a DLT. Three more patients were enrolled at this dose level with one additional patient developing toxicity. Subsequently, VPA was decreased to 62.5 mg/kg orally daily for 7 days. DLTs were observed in 2 additional patients at this dose level, and VPA was decreased again to 50 mg/kg which was declared as the MTD after treating 10 patients at this dose level. Subsequently, a total of 34 patients were treated in the phase II at the MTD, including 10 patients in the expanded phase I cohort at the MTD. Table 3 summarizes the most frequent toxicities during course 1 and thereafter. Most frequent events included neurotoxicities, mainly somnolence and confusion,
followed by fatigue. Toxicities were transient and reversible. Only 3 out of 53 patients (5%) died during induction, none of them was previously untreated. Overall, there were no differences in terms of toxicities between the previously untreated group and those patients that had received prior therapy, nor between responding patients and those that did not achieve a response.

Because the entry criteria for the study allowed patients to be off prior chemotherapy for just 2 weeks, we analyzed the impact of prior therapy on this group of patients. Of the 20 previously treated patients, only 3 were treated less than 1 month after their prior 3rd, 4th and 5th salvage attempts. All were taken off because of no response. No toxicity was observed. The median time after last therapy was 2 months (range 0.5 to 72). Therefore, prior therapy did not seem to have an impact on response or toxicity.

**Clinical activity**

Responses are summarized in Table 4. An overall response rate of 42% was observed. This included 12 (22%) CRs, 3 (5%) CRps and 7 (13%) BM responses. In the subgroup of 33 previously untreated patients over 60 years, 11 (33%) CRs, 3 (9%) CRps and 3 (9%) BM responses were demonstrated for an overall response rate of 52%. In this subgroup, nine CRs occurred at the VPA dose of 50 mg/kg, one at 62.5mg/kg and 1 at 75mg/kg. The 3 BM responses in this subgroup were observed at 50 mg/kg. In the subgroup of previously treated patients, one CR was seen at the 50 mg/kg dose. Three BM responses were observed at the VPA dose level of 50 mg/kg and 1 at 75 mg/kg. With a median follow up of 21 weeks, the median survival in patients achieving CR or CRp has not been reached (Figure 1). Median remission duration was 26 weeks (Figure 2).
Overall, the median number of courses of therapy administered was 2 (1-10+). In the untreated patients over 60 years it was 3 (1-10+). The median number of courses to first response was 1 (range 1-3). Among 4 patients with high risk MDS, 2 achieved CR and 2 a bone marrow response.

**Cytogenetic responses**

Pretreatment cytogenetic characteristics are shown in Table 5. Five out of 8 patients (62%) with poor risk cytogenetics had a response. Four of 11 responders with informative cytogenetics achieved a cytogenetic response (36%). Two had a complete cytogenetic response, and 2 a partial cytogenetic response.

**Valproic acid levels**

VPA levels were measured sequentially pretreatment on day 0, and prior to drug administration on days 5 and 7. Free (mg/L) and bound (µg/mL) VPA levels during cycle 1 are shown in Table 6. The median free and bound VPA levels on days 5 and 7 were significantly higher in patients achieving a response. Median free VPA level on day 5 in responders was 36.8 mg/L (range 29.4-61.6 mg/L) whereas it was 21.6 (range 4.2-46.4) in non responders (p<0.005). Median bound VPA level on day 5 was 132 mcg/ml (69-202 mcg/ml) in responders and 104 mcg/ml (26-165 mcg/ml) in nonresponders (p<0.005). A similar pattern was observed on day 7 (Table 6). Despite the association between VPA blood levels and response, there was no association between dose level and response.

**Induction of Histone Acetylation**
Histone H3 and H4 acetylation was evaluated using western blots on days 0, 2, 7 and 21 in 48 patients. Histone acetylation was detected in 26 patients (54%). The frequency of histone acetylation per dose level is shown in Figure 3. There was no correlation between VPA dose level and histone acetylation or histone acetylation and response.

**DNA methylation Analysis**

To study the DNA methylation dynamics during treatment, global DNA methylation was analyzed on days 0, 2, 7 and 21. We used the LINE pyrosequencing assay which is a surrogate marker of global methylation\textsuperscript{21}. We found a significant decrease in global methylation by day 7, the last day of treatment, with a return towards baseline by day 0 of the next cycle as shown in figure 4. The degree of global DNA hypomethylation achieved during treatment was not different between responders and non responders.

**Induction of p21 and p15 mRNA expression**

We measured the expression of p21 and p15 mRNA pretreatment and sequentially on days 7 and 21 in 43 patients with available samples. Results are shown on figure 5. Although a modest but significant increment on p15 and p21 mRNA expression was detected, this was not associated with clinical response.

**Subset analysis of pediatric patients**

Three patients ages 5, 15 and 18 were treated in this study. All of them had relapsed or refractory AML. A 15 years old patient achieved a bone marrow response during therapy but died after 9 weeks. No significant toxicities were observed in this age group. Two of the patients had evidence of histone acetylation. No difference in the dynamics of DNA hypomethylation was observed between children and adults (data not shown).
DISCUSSION

In this study, we demonstrated that the combination of 5-AZA, VPA at 50 mg/kg orally daily for 7 days, and ATRA was safe. This combination was also active and associated with induction of global DNA hypomethylation and histone acetylation. We defined the MTD of VPA to be 50 mg/kg orally for 7 days. This is consistent with our prior experience with decitabine and VPA, where a dose of 50 mg/kg orally for 10 days could be tolerated\(^{16}\). VPA administration at these doses was associated with reversible neurotoxicity which resolved shortly after drug interruption. No significant toxicities related to 5-AZA or ATRA administration were observed. In particular, no evidence of acute cell differentiation syndrome (i.e. ATRA syndrome) was observed in any patient. The overall treatment mortality was 5%: 3 patients, all of them with relapsed/refractory disease, died during induction. The combination of 5-AZA, VPA and ATRA, was clinically active: the overall response rate was 42%, including 12 CRs (22%). In the subset of patients over 60 years with previously untreated disease, the response rate was 52% with no induction deaths. Their median survival has not been reached. Therefore, the overall response and toxicity profile observed in this trial for this group of patients with advanced age and poor prognosis are encouraging. The responses observed in our trial are robust. With a median follow up of 21 weeks, the median survival in responding patients has not been reached. The median relapse free survival was 26 weeks. Clinical activity was observed in patients with poor risk cytogenetics as 62% of them achieved a response. Four patients had high risk MDS. All of them were over 60 years (range 65-83), 2 CRs and 2 BM responses were noted which support the activity of this combination in high risk MDS. That said, it should be noted that a fraction
of these responses (13%) included complete bone marrow responses, in particular in patients with relapsed/refractory disease. The clinical significance of that type of response assessment is unclear at the present time but may benefit patients being prepared for instance for allogeneic stem cell transplantation.

Patients age 2 and older were allowed to be enrolled in our study. The reason for the age restriction was the reported hepatotoxicity of VPA when used in patients less than 2 years old. In our study the combination of 5-AZA, VPA and ATRA was safe in the 3 pediatric patients with relapsed/ refractory AML, and one bone marrow response was observed. These findings are similar to the results with the combination of VPA and decitabine. Combinations of VPA with 5-AZA or decitabine, and ATRA need to be further explored in specific pediatric phase I studies in leukemia.

In this study, we evaluated four potential biomarkers: VPA levels, histone acetylation, induction of global DNA hypomethylation, and induction of p21 and p15 mRNA expression. First, higher VPA levels were demonstrated in patients who responded versus those that did not. This is consistent with our in vitro data that indicate that the antileukemic effect of VPA is directly related to its concentration, and with our previous study of decitabine and VPA, in which untreated patients with higher VPA levels had a higher response rate. The implications of these findings are important in that replacement of VPA with a more potent HDAC inhibitor with a better toxicity profile may improve the activity and tolerability of this type of combination. Another potential implication is the specific targeting of plasma levels of VPA in future clinical trials, instead of using doses based on weight. Second, HDAC inhibition resulted in histone acetylation. Consistent with the in vitro HDAC inhibitory effect, we observed that 54% of
the patients had evidence of histone acetylation. Induction of histone acetylation was not associated with response. A similar phenomenon has been observed in patients treated with single agent vorinostat\textsuperscript{25} and with the combination of 5-AZA and phenylbutyrate\textsuperscript{26} and other studies\textsuperscript{16}. The lack of association between histone acetylation and response could have different explanations. One explanation may relate to the sensitivity of the assays used to measure acetylation. It is also possible that clinical responses are related to other molecular effects unrelated or downstream of HDAC inhibition. It should be noted that VPA levels were not associated with histone acetylation. Third, similarly to the experience of Gore et al\textsuperscript{27}, we confirmed that 5-AZA had in vivo hypomethylating activity. We have tested this using the LINE bisulfite pyrosequencing assay. As with decitabine, we have documented that global methylation is a transient phenomenon that peaks shortly after 5-AZA exposure to return gradually to baseline. As with decitabine, there was no correlation between response and induction of hypomethylation. Because LINE methylation occurs in both normal and neoplastic cells, the analysis of global methylation dynamics could be considered as a marker of the biological activity of the drug but not necessarily related to its clinical activity. It is possible that specific gene methylation studies may identify markers of response. Those studies are ongoing. It is also possible that the clinical activities of both 5-AZA and VPA are only partially related to the induction of DNA methylation or histone acetylation. Finally, the combination therapy used here resulted in modest induction of p21 and p15 mRNA expression, a phenomenon not associated with clinical response.
While the regimen was safe and active, we did not prove that the combination is superior to single agent 5-AZA as this study was not designed to test that hypothesis. This should be tested in a randomized study. Our results suggest that the combination may be superior. An analysis by Silverman et al\textsuperscript{3}, reported a complete response rate of 10-17% and a median number of 3 courses to response in patients with AML treated in different CALGB studies. Similarly, Sudan et al\textsuperscript{2} reported a complete response in 4 of 20 (20%) patients older than 55 years with AML, also with a median time to response of 3 months (range 2-5 months). Because of the heterogeneous characteristics of the study groups, it is difficult to compare our results with those reported by Silverman et al\textsuperscript{3} and Sudan et al\textsuperscript{2}. Acknowledging this limitation, the response rate in patients over 60 years was 52%, the complete response rate was 33%, and the median number of courses to response was 1 (1-3) which may support the contribution of VPA and ATRA to the single agent activity of 5-AZA. Furthermore, VPA levels were higher in patients achieving a response.

VPA has been used as a single agent or in combination with ATRA in patients with MDS\textsuperscript{14,28} and AML\textsuperscript{15,28,29}. Kuendgen et al\textsuperscript{15} reported 58 patients with AML treated with single agent VPA or in combination with ATRA who were not considered candidates for intensive chemotherapy. The response rate was 5%. In that study, VPA levels of 50 to 100 µg/ml were targeted. HDAC inhibition is observed at concentrations of VPA greater than 1 mM \textsuperscript{9}. This is greater than the concentration achieved with targeted VPA levels of 50 to 100 µg/ml used in the treatment of seizures. These low levels of VPA may explain the low number of responses observed in that study\textsuperscript{15}. The higher levels of VPA
achieved in our study, particularly in responders, support a role of VPA in this combination.

There are several limitations in our study. First, 5-AZA was used at 75 mg/m² subcutaneously daily for 7 days, although this is the same 5-AZA dose used in the CALGB studies in patients with high risk MDS¹,³; it is unknown if this is the most effective dose and it is possible that other schedules and doses may be better. Second, VPA, the HDAC inhibitor used in this study, is one of the less effective HDAC inhibitors and its use at a high concentration is associated with potential serious toxicities. The combination of 5-AZA with more effective and less toxic HDAC inhibitors may improve the results observed in this trial. A study combining 5-AZA with MGCD0103, an oral isotype selective HDAC inhibitor is currently ongoing with promising interim results³⁰. Third, so far we have not found a molecular marker predictive of response as both global DNA methylation and histone acetylation were not different in responders. Further ongoing molecular analysis of gene specific methylation, gene expression and effects downstream of histone acetylation may unravel such molecular predictors. Fourth, the role of ATRA in this combination has not been defined. We had based the decision to add it to the combination based on data from Kuendgen et al described above¹⁴, and by the observation that the addition of 5-aza-2’-deoxycytidine to ATRA could result in apoptosis in ATRA-refractory leukemia cell lines³¹. Also, ATRA can induce cell differentiation and has clinical activity in APL¹⁷ but not in non-APL leukemias¹⁸. In ATRA-resistant APL variants, the use of a HDACI like VPA can restore sensitivity to ATRA¹⁹. ATRA can induce hypomethylation and re-expression of the RAR-β2 gene²⁰. This activity is enhanced by 5-AZA²⁰. VPA and 5-AZA have been proposed
to sensitize AML cells to the effects of ATRA\textsuperscript{20,32,33}. Based on this, and on the
experience of Kuendgen et al\textsuperscript{14,15} with VPA in combination with ATRA in MDS and AML,
we added ATRA to the combination of 5-AZA and VPA. That said, in this study we have
not determined the contribution of ATRA in this combination. Fifth, in this study we have
not systematically studied the effects of therapy on cell differentiation. In vitro, the
combination had no effect on cell differentiation\textsuperscript{9}, nor have we observed any case of
ATRA-syndrome or morphological evidence of differentiation. That said, detailed flow
cytometry studies could clarify this issue.

Finally, one remaining issue pertains to the schedule used here. We elected to
administer these agents concomitantly. This was based on our prior experience with 5-aza-2'-deoxycytidine and valproic acid\textsuperscript{16} and in vitro models that indicated no advantage
to a sequential administration\textsuperscript{9}. That said, in vitro data looking at gene reactivation have
suggested a benefit to the sequential administration of these agents\textsuperscript{34}. Therefore it is
possible that other schedules may result in enhance clinical activity.

In summary, this study demonstrated that the combination of 5-AZA, VPA and ATRA is
safe and active. This therapy was associated with significant DNA hypomethylation and
induction of histone acetylation. Despite of clinical activity observed in particular in the
older untreated patients, the number of patients treated was small and this combination
should be considered investigational and should not be recommended outside the
setting of a clinical trial.
Acknowledgements:

This work was supported by Pharmion and by the University of Texas M. D. Anderson Cancer Center's Physician-Scientist Program funded by the Commonwealth Cancer Foundation for Research (both to G G-M). We are grateful to Jonathan Clavell for technical assistance and to Mark Brandt for data analysis. The authors declare there is no conflict of interest.

Author contribution:

A. O. Soriano and H. Kantarjian performed research, analyzed data and wrote manuscript. H. Yang, S. Faderl, Z. Estrov, F. Giles, F. Ravandi, J. Cortes, W. G. Wierda, S. Ouzonian, A. Quezada, J.P. Issa performed research. S. Pierce and E. Estey analyzed data. G. Garcia-Manero designed the study, analyzed data, performed research, wrote manuscript and funded the studies.
References


Table 1. Patient characteristics (N=53)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (N=53) (%) or [range]</th>
<th>Untreated (N=33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age</td>
<td>69 [5-84]</td>
<td>74 [61-84]</td>
</tr>
<tr>
<td>Age ≥ 60 years</td>
<td>44 (83)</td>
<td>33 (100)</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>49 (92)</td>
<td>30 (90)</td>
</tr>
<tr>
<td>MDS</td>
<td>4 (8)</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Median number of prior therapies</td>
<td>0 [0-6]</td>
<td>0</td>
</tr>
<tr>
<td>Untreated</td>
<td>33 (62)</td>
<td>33 (100)</td>
</tr>
<tr>
<td>1st salvage</td>
<td>9 (17)</td>
<td>0</td>
</tr>
<tr>
<td>&gt;1st salvage</td>
<td>11 (21)</td>
<td>0</td>
</tr>
<tr>
<td>Cytogenetics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid</td>
<td>18 (34)</td>
<td>14 (42)</td>
</tr>
<tr>
<td>CBF</td>
<td>1 (2)</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>34 (64)</td>
<td>19 (57)</td>
</tr>
<tr>
<td>Median WBC</td>
<td>2.6 [0.5-116]</td>
<td>4.3 [0.7-116]</td>
</tr>
</tbody>
</table>

AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome; CBF, core binding factor; WBC, white blood cell.
Table 2. Frequency of non-hematological toxicities (Drug-related, First course)

<table>
<thead>
<tr>
<th>VPA dose (mg/kg daily x7)</th>
<th>N</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>6</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>62.5</td>
<td>7</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>75</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Phase II (50 mg/kg)</td>
<td>34</td>
<td>5</td>
<td>14</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

VPA, valproic acid; N, number.
Table 3. Most common non-hematological side-effects (53 patients; 166 courses)

<table>
<thead>
<tr>
<th>Toxicity (Grade 3/4)</th>
<th>Course 1</th>
<th>&gt;Course 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confusion</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Somnolence</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Nausea/Vomiting</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Fatigue</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Hepatic</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 4. Clinical Responses

<table>
<thead>
<tr>
<th>VPA (mg/kg) dose</th>
<th>N</th>
<th>CR</th>
<th>CRp</th>
<th>BM response</th>
<th>Overall N response (%)</th>
<th>N courses to response</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>40</td>
<td>10</td>
<td>3</td>
<td>6</td>
<td>19 (47)</td>
<td>1 (1-3)</td>
</tr>
<tr>
<td>62.5</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1 (14)</td>
<td>2</td>
</tr>
<tr>
<td>75</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2 (33)</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>12</td>
<td>3</td>
<td>7</td>
<td>22 (42)</td>
<td>1 (1-3)</td>
</tr>
<tr>
<td>Previously</td>
<td>33</td>
<td>11</td>
<td>3</td>
<td>3</td>
<td>17 (52)</td>
<td>1 (1-3)</td>
</tr>
<tr>
<td>untreated</td>
<td>20</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>5 (25)</td>
<td>1 (1-2)</td>
</tr>
</tbody>
</table>

N, number; CR, complete remission; CRp, complete remission with incomplete platelet recovery; BM resp, bone marrow response
Table 5. Pretreatment cytogenetics and clinical responses

<table>
<thead>
<tr>
<th>Cytogenetic characteristic</th>
<th>N (%)</th>
<th>Response N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+8</td>
<td>1 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>-5/-7</td>
<td>8 (15)</td>
<td>5 (62)</td>
</tr>
<tr>
<td>Diploid</td>
<td>18 (34)</td>
<td>7 (39)</td>
</tr>
<tr>
<td>Insufficient Metaphases</td>
<td>2 (4)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>21 (40)</td>
<td>7 (33)</td>
</tr>
<tr>
<td>T(8;21)</td>
<td>1 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>-Y</td>
<td>2 (4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cytogenetic Response*</td>
<td>11</td>
<td>4 (36)</td>
</tr>
</tbody>
</table>

*(patients with evaluable metaphases); N, number.
### Table 6. Valproic acid levels and response

<table>
<thead>
<tr>
<th>Response</th>
<th>FVPA (mg/L) Day 5</th>
<th>FVPA (mg/L) Day 7</th>
<th>BVPA (µg/mL) Day 5</th>
<th>BVPA (µg/mL) Day 7</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responders</td>
<td>36.8 (29.4-61.6)</td>
<td>35.1 (17.6-96.4)</td>
<td>132 (69-202)</td>
<td>124 (63-177)</td>
<td>0.005</td>
</tr>
<tr>
<td>Non responders</td>
<td>21.6 (4.2-46.4)</td>
<td>25.05 (7-56.8)</td>
<td>104 (26-165)</td>
<td>106.5 (15-161)</td>
<td></td>
</tr>
</tbody>
</table>

FVPA, free valproic acid; BVPA, bound valproic acid
FIGURE LEGENDS

Figure 1. Survival overall and by response

Figure 2. Relapse free survival

Figure 3. Frequency of histone acetylation by valproic acid dose. Histone acetylation was measured using Western blots. Bars represent the percentage of patients with histone acetylation, N the number of patients evaluated.

Figure 4. Dynamics of global DNA methylation with 5-azacitidine therapy. Global DNA methylation was measured using the LINE bisulfite pyrosequencing assay. It was measured pretreatment and on days 2, 7, 21 of first course and prior to initiation of course 2. There was a significant difference in terms of DNA methylation comparing day 0 with day 7. No differences were observed between responders and non-responders. C, course; D, day.

Figure 5. Induction of p21 and p15 mRNA expression. P21 and p15 mRNA expression was measured using real-time PCR on days 0, 7 and 21.
Figure 1.
Figure 2.

Remission Duration Probability

N=15; Fail=5; Median 26 Weeks

Weeks
Figure 3.

% of patients with acetylation

Valproic acid dose

50 mg/kg  62.5 mg/kg  75 mg/kg
Figure 4.
Figure 5

**p15**

Relative Expression vs. Days

**p21**

Relative Expression vs. Days

**p = 0.02**

**p = 0.005**
Safety and clinical activity of the combination of 5-azacytidine, valproic acid and all-trans retinoic acid in acute myeloid leukemia and myelodysplastic syndrome