Sequential Valproic Acid/All-trans Retinoic Acid Treatment Reprogramms Differentiation in Refractory and High-Risk Acute Myeloid Leukemia

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Abstract
Epigenetic alterations of chromatin due to aberrant histone deacetylase (HDAC) activity and transcriptional silencing of all-trans retinoic acid (ATRA) pathway are events linked to the pathogenesis of acute myeloid leukemia (AML) that can be targeted by specific treatments. A pilot study was carried out in eight refractory or high-risk AML patients not eligible for intensive therapy to assess the biological and therapeutic activities of the HDAC inhibitor valproic acid (VPA) used to remodel chromatin, followed by the addition of ATRA, to activate gene transcription and differentiation in leukemic cells. Hyperacetylation of histones H3 and H4 was detectable at therapeutic VPA serum levels (≥50 μg/mL) in blood mononuclear cells from seven of eight patients. This correlated with myelomonocytic differentiation of leukemic cells as revealed by morphologic, cytochemical, immunophenotypic, and gene expression analyses. Differentiation of the leukemic clone was proven by fluorescence in situ hybridization analysis showing the cytogenetic lesion +8 or 7q– in differentiating cells. Hematologic improvement, according to established criteria for myelodysplastic syndromes, was observed in two cases. Stable disease and disease progression were observed in five and one cases, respectively. In conclusion, VPA-ATRA treatment is well tolerated and induces phenotypic changes of AML blasts through chromatin remodeling. Further studies are needed to evaluate whether VPA-ATRA treatment by reprogramming differentiation of the leukemic clone might improve the response to chemotherapy in leukemia patients. (Cancer Res 2006; 66(17): 8903-11)

Introduction
Epigenetic modifications of chromatin play a critical role in carcinogenesis by causing transcriptional silencing of specific control regions related to cell proliferation/differentiation (1–3). In acute myeloid leukemia (AML), the molecular event underlying the differentiation block and the transforming ability of chromosomal translocation-generated AML fusion proteins [PML/retinoic acid receptor α (RARα), PLZF/RARα, and AML1/ETO] seems strictly dependent on the aberrant recruitment of histone deacetylase (HDAC) activities on genes that are relevant to the transformation process and tumor development (1, 5). By changing nucleosomal packaging of DNA, HDACs remodel chromatin in a gene-specific fashion and consequently affect proper cell function, differentiation, and proliferation. In particular, deacetylation of the N-terminal tails of histones H3 and H4 by HDACs inhibits transcription, whereas histones H3 and H4 acetylation by histone acetyltransferase (HAT) activities results in transcriptional activation by favoring access of DNA-binding proteins and of the transcriptional apparatus (2, 4, 5).

In acute promyelocytic leukemia (APL), the AML-M3 subtype, the formation of RARα-fusion proteins induces an aberrant recruitment of protein complexes containing HDAC and DNA methyltransferase activities on the all-trans retinoic acid (ATRA) target genes, resulting in their transcriptional silencing (6–13). Moreover, the use of ATRA in PML/RARα+ APL represents a paradigmatic example of a highly effective transcriptional/differentiation therapy in leukemias (13, 15). This clinical efficacy of ATRA in APL is due to its ability at pharmacologic doses to release the HDAC repressory complex and to recruit the multisubunit HAT complex on specific ATRA-responsive elements (RARE) present on target gene promoters (6–10). RAREs are present on promoter regions of transcription factors involved in granulocytic myelopoiesis, thus suggesting their crucial role in leukemogenesis (10, 16–18).

In agreement with this hypothesis, we have shown that the ATRA signaling pathway is constitutively repressed through a HDAC-dependent mechanism in non-APL AML subtypes, which are insensitive to retinoids (19, 20). Thus, therapeutic targeting of aberrant HDAC activities might represent a potentially novel treatment strategy in AML.

Several naturally occurring and synthetic HDAC inhibitors (HDACi) have been recently characterized with diverse structures ranging from simple compounds (i.e., butyrates) to more complex agents, such as hydroxamic acids (1, 21). Interestingly, the global chromatin remodeling activity of these inhibitors seems specific, because it affects only few (4-10%) selected genes (1). This specificity and the low in vivo toxicity of these compounds raise the possibility of their clinical use (1, 22).
Valproic acid (VPA) is a well-tolerated and long since used antiepileptic drug. At concentrations equivalent to those used for the treatment of patients with epilepsy (≥50 μg/mL), VPA acts as a powerful HDACi (20). Moreover, VPA is as effective as other HDACi compounds in driving differentiation/apoptosis of AML blasts in vitro and in murine model systems for PML/RARα and AML1/ETO+ AML leukemias (20, 23, 24). In vitro, the effect of VPA on terminal myeloid differentiation is increased by ATRA addition in blasts from newly diagnosed and refractory/relapsed AML patients independently from the presence of specific genetic lesions (20). The clinical efficacy of VPA in combination with ATRA in elderly patients with de novo AML and of VPA monotherapy in patients with myelodysplastic syndromes (MDS) have been also recently reported (25–27).

Based on the well-established safe profile of VPA and ATRA treatments in humans, we tested the feasibility and activity of a regimen including the sequential association of these two compounds in a group of patients with high-risk and/or very advanced AML. By an extensive multiparametric approach, we also evaluated in vivo the biological changes induced in the leukemic cells during the first 4 weeks of sequential VPA-ATRA treatment.

Materials and Methods

Patients. A pilot study was conducted in eight patients with advanced AML (n = 7) or myeloid blast crisis of chronic myeloid leukemia (CML; n = 1) who received oral VPA and ATRA as compassionate treatment. Written informed consent was obtained before therapy. The diagnosis of AML and the leukemic phenotype was defined according to the French-American-British (FAB) classification and WHO recommendation (28, 29). Eligibility criteria to enter the VPA-ATRA study included (a) performance status ≤2 (according to the WHO scale), (b) adequate renal and hepatic functions (creatinine <1.5 mg%, bilirubin <2 mg/mL, transaminase no more than 3 times normal value), and (c) no clinical evidence of pulmonary leukostasis and/or central nervous system leukemia. Administration of hematopoietic growth factor must have occurred within 3 weeks before protocol entry and was prohibited while on study.

Genetic characterization. At diagnosis and before entry to the study, all patients had cytogenetic analysis carried out on bone marrow cells after 24 hours of unstimulated culture. GTG bands with trypsin were obtained. Karyotypes were reviewed and defined according to the International System for Cytogenetic Nomenclature (30). Fluorescence in situ hybridization (FISH) was carried out before and during VPA-ATRA treatment in bone marrow smears from the two patients whose leukemia blasts carried at diagnosis the +8 and del(7)(q31) aberrations using the method described elsewhere (31). The May-Grünwald-Giemsa-stained smears were analyzed morphologically. Subsequently, FISH analysis was done as described (31). Digitally imaged cells were relocated through the microscope coordinates and documented using the “Easy FISH” system. The cutoff level to exclude false-positive results was calculated on normal bone marrow smears and set at 12% for 7q deletion and 10% for +8 (mean ± 2 SD).

Monitoring of VPA serum levels. VPA serum levels were measured with a fluorescence polarization immunoassay (Abbott, Wiesbaden, Germany) at days 3, 7, 14, 21, and 28 or every time a new drug was introduced in the treatment to evaluate potential pharmacologic interactions. VPA serum concentration was maintained within the therapeutic limits established for the treatment of patients with epilepsy (50–110 μg/mL).

Treatment protocol. The study protocol was approved by the local ethic committees. VPA was given from day 1, at the initial dosage of 30 mg/kg/d p.o. subdivided in three administrations, with dose escalation until optimal serum levels (50–110 μg/mL). ATRA at the dosage of 45 mg/m² p.o./d, divided in two administrations, was added at the time of therapeutic VPA serum levels or at day 14 of treatment. If neither significant side effects nor progression of disease occurred, treatment was continued. Cytoreduction therapy was started with low-dose chemotherapy to control hyperleukocytosis (WBC > 50 × 10⁹/L) and the potentially related occurrence of an ATRA syndrome until the WBC < 10 × 10⁹/L. Chemotherapy was chosen according to that in use in the two centers participating the study. Hydroxyurea was given daily at a dose of 2 g/m² in patient 1 starting from days 17 to 24 and in patient 3 at day 28 of VPA-ATRA treatment. Treatment with aracytin 20 mg/m² twice daily s.c. was initiated in patient 4 at day 24 of VPA-ATRA treatment. Prophylactic RBC were given at hemoglobin levels <8 and 9 g/dL in patients ages <60 and >60 years, respectively. Platelets were transfused below the level of 100 × 10⁹/L.

Response criteria. Therapeutic response was assessed after the first 4 weeks of treatment and graded in line with the criteria standardized by an International Working Group in AML (32). Because these drugs were supposed to induce cell lineage maturation, therapeutic response was also defined according to the International Working Group for Myelodysplastic Syndromes criteria (33). Therefore, hematologic improvement was defined as (a) ≥50% restoration of the deficit in one or more peripheral blood cell lines but insufficient to meet criteria for complete or partial remission and (b) ≥50% decrease in packed RBC or platelet transfusion requirement without a significant change in bone marrow blasts percentage. Progressive disease is worsening of the disease variables evaluated at diagnosis by ≥25% or comparison of new ones; stable disease is no qualification for hematologic improvement or progressive disease.

Biological studies. Mononuclear cells were isolated at days 0, 7, 14, 21, and 28 of treatment from the bone marrow and/or peripheral blood of patients by Ficoll-Hypaque and evaluated by the following: (a) Morphology in conventional light-field microscopy of Wright-Giemsa-stained smears or cytoplasm. (b) Cytochemical staining of myeloperoxidase (MPO), chloroacetate esterase (CAE), and α-naphthyl acetate esterase (ANAE) and ANAE inhibition by NaF according to manufacturer’s instructions (Sigma-Aldrich, Milan, Italy). (c) Fluorescence-activated cell sorting analysis of the cell cycle. For every determination, 2 × 10⁶ mononuclear cells from the bone marrow were resuspended in 50% FCS, fixed in 70% ethanol for 24 hours, incubated with 50 μg/mL propidium iodide (Sigma-Aldrich) and 50 units/mL DNase free RNase A (Sigma-Aldrich), and analyzed after 3 hours (10,000 events) using a Epics XL Cytometer (Beckman Coulter, Milan, Italy). Only one cell population was detectable through scatter properties over the time of treatment in the six cases tested. Apoptosis was quantified by evaluating the sub-G₁ fraction of the living cell populations after propidium iodide staining of permeabilized cells. (d) Direct immunofluorescence analysis of cell surface antigens. Two-color flow cytometry analysis was done using FITC-conjugated anti-CD34 and phycocerythrin-conjugated anti-CD117 monoclonal antibodies (Becton Dickinson, Milan, Italy) to determine cell surface markers expressed by immature blasts. Anti-CD15-FITC and anti-CD11b-phycocerythrin monoclonal antibodies (Becton Dickinson) were used for cell surface markers expressed by granulocytic and monocytic cells either alone or combined. A minimum of 50,000 un gated cells for each measurement was acquired by a FACScan flow cytometer (Becton Dickinson) using CellFit software (Becton Dickinson). (e) Immunoblot analysis of acetylated histones H3 and H4 were done on mononuclear cell homogenates (30 μg) by using anti-acetylated histones H3 and H4 antibodies (Upstate, Lake Placid, NY) as described (19). Immunoblots were probed with an anti-histone H3 (Upstate) to ensure an equal loading of the samples. (f) RNA Preparation and quantitative real-time PCR analysis. Total RNA was extracted from Ficoll-Hypaque-isolated bone marrow and peripheral blood cells as described (19) and reverse transcribed with random primers and SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD). The cDNA was used for quantitative real-time PCR (qRT-PCR) experiments carried out in ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Gaithersburg, MD). Taqman oligonucleotides (Assay-on-Demand) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), MPO, colony-stimulating factor 3 (CSF3R or G-CSFR), colony-stimulating factor 2 receptor (CSF2R or GM-CSFR), and GATA-binding protein 1 (GATA-1) were from Applied Biosystems. ΔΔCt values were normalized with those obtained from the amplification of GAPDH. For the SYBR Green dye detection method, primer sequences were designed using the Primer Express software (Applied Biosystems). Primers were monocyte/macrophage serine esterase-1 (MSE) forward 5’-GAACCAACAGAGATGCTGGAGC-3’ and reverse
5'-TCCCCGTGGTCTCCTATCAC-3' (34) and GAPDH forward 5'-ATCAGCAATGCCCCCCGTCAG-3'. Reactions were done in triplicates. (g) Chromatin immunoprecipitation. Cross-linking of histones was done in 2 x 10^6 mononuclear cells from patients and chromatin was immunoprecipitated with an antibody against the acetylated form of histone H4 (Upstate) as described (35, 36). Primers for PCR detection of a genomic region of the RAR

Statistics. This pilot study was designed to investigate the feasibility and activity of the sequential association of VPA and ATRA and the biological changes induced in leukemic cells from high-risk and/or very advanced AML. Therefore, a valuable patient was defined as a patient who had completed the 4 weeks of VPA-ATRA treatment and undergone biological testing at defined time points. Only descriptive statistic reported as mean ± SE was applied given the small number of patients under consideration. Student's t test was used to test the probability of significant differences between the mean fold increase detected in samples from all cases. Differences were considered significant if P < 0.05.

Results

Patients. The main clinical characteristics of the AML patients included in this study are shown in Table 1. Median age was 61.5 years (range, 31-69). Four patients had a history of MDS, three had de novo AMLs (M0-M2) by FAB (28). The remaining patient had a history of previous intensive chemotherapy course (range, 1-3; median, 2). The remaining three patients were not eligible for conventional intensive chemotherapy for the presence of active pulmonary infection, hepatocarcinoma, or a severe impairment of cardiac function (c). NA, not available; NC, not classifiable.

De novo AMLs (M0-M2) by FAB (28) were allowed (36). These two patients presented MDS-AML and cytogenetic analysis revealed a trisomy of chromosome 8 in patient 1 and a del(7)(q31) deletion in patient 4 (Table 1). Before initiation of VPA-ATRA treatment, both patients had a disease resistant to one and three chemotherapeutic courses, respectively (Table 1). Interestingly, both patients developed a marked increase of the WBC count without signs of encephalitis and/or pulmonary leukostasis. WBC reached ≥50 x 10^9/L at days 17 and 24, respectively (Table 2). This leukocytosis features ~40% maturing myeloid cells and did not involved immature blasts that indeed were decreased (Table 3). In patient 1, hyperleukocytosis was controlled by addition of hydroxyurea (2 g/m^2/d) from days 17 to 24, whereas patient 4 received low doses of aracitin (20 mg/m^2/twice daily) starting at day 24 for 15 days. Patients experienced a rapid reduction of leukocytes maintaining the hematologic improvement in the neutrophil count. In patient 1, VPA-ATRA treatment was withdrawn at day 28 due to a marked increase of the direct bilirubin level to 7.8 mg/dL and resumed at day 43 after normalization of the bilirubin values. Interestingly, a new episode of hyperleukocytosis occurred 14 days after the restarting of treatment that again was characterized by maturing cells with an increased number of neutrophils (data not shown). This patient died at day 70 of septicemia. In patient 4, treatment was continued and reappearance of blasts and disease progression occurred 4 months later. Patient 4 died of his disease at day 184 from the start of the VPA-ATRA protocol.

Five patients (patients 2, 3, 5, 7, and 8) showed stable disease for a period ranging from 60 to 180 days. Patient 3 experienced hyperleukocytosis at day 22 composed by 40% of monocytic cells that did not reach the morphologic pattern of mature monocytes (Table 3; Fig. 1B). Therefore, this case did not fit the criteria for hematologic improvement. The CML-blast crisis patient (patient 6) achieved a hematologic improvement as defined by the International Working Group in AML (32). Therefore, two of eight patients (patients 1 and 4 of Table 2) achieved a hematologic improvement as defined by the International Working Group for Myelodysplastic Syndromes criteria (33).

Table 1. Clinical characteristics of patients at the onset of VPA-ATRA regimen

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex/Age</th>
<th>Diagnosis (WHO/FAB)</th>
<th>Cytogenetic</th>
<th>Previous treatment cycles (no.)</th>
<th>Response to induction treatment</th>
<th>Performance status (WHO)</th>
<th>WBC/10^9/L</th>
<th>Hemoglobin (g/dL)</th>
<th>Platelet × 10^9/L</th>
<th>Bone marrow blasts (%)</th>
<th>Peripheral blood blasts (%)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>M/54</td>
<td>MDS-AML-M2</td>
<td>47.XX,+8</td>
<td>1 (a)</td>
<td>Resistant</td>
<td>2</td>
<td>7.0/0.5</td>
<td>8.3</td>
<td>17</td>
<td>95</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>M/61</td>
<td>De novo AML-M2</td>
<td>46.XY</td>
<td>2</td>
<td>Resistant</td>
<td>0</td>
<td>7.5/1.6</td>
<td>8.2</td>
<td>30</td>
<td>85</td>
<td>62</td>
</tr>
<tr>
<td>3</td>
<td>M/31</td>
<td>De novo AML-M3</td>
<td>46.XY,del(12); t(12;?)q22(?)</td>
<td>2</td>
<td>Resistant</td>
<td>0</td>
<td>1.4/0.2</td>
<td>10.8</td>
<td>253</td>
<td>80</td>
<td>95</td>
</tr>
<tr>
<td>4</td>
<td>M/54</td>
<td>MDS-AML-M1</td>
<td>del(7)(q31)</td>
<td>3</td>
<td>Resistant</td>
<td>0</td>
<td>1.5/0.6</td>
<td>7.1</td>
<td>252</td>
<td>61</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>M/62</td>
<td>De novo AML-M1</td>
<td>NA</td>
<td>0 (b)</td>
<td>At onset</td>
<td>2</td>
<td>1.2/0.1</td>
<td>7.5</td>
<td>15</td>
<td>90</td>
<td>12</td>
</tr>
<tr>
<td>6</td>
<td>F/69</td>
<td>CML-blast crisis</td>
<td>45.XXt(9;22),-7</td>
<td>0 (c)</td>
<td>At onset</td>
<td>2</td>
<td>9.9/1.5</td>
<td>12.8</td>
<td>17</td>
<td>83</td>
<td>48</td>
</tr>
<tr>
<td>7</td>
<td>M/64</td>
<td>MDS-AML-NC</td>
<td>NA</td>
<td>1</td>
<td>Resistant</td>
<td>0</td>
<td>0.7/0.2</td>
<td>11.2</td>
<td>19</td>
<td>49</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>M/66</td>
<td>MDS-AML-NC</td>
<td>NA</td>
<td>0 (a)</td>
<td>At onset</td>
<td>1</td>
<td>1.5/0.2</td>
<td>8</td>
<td>20</td>
<td>22</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE: Patients not eligible to conventional intensive chemotherapy for the presence of pulmonary fungal infection (a), hepatocarcinoma (b), or relevant decrease of cardiac function (c). NA, not available; NC, not classifiable.
underwent progressive disease (for the persistence of ≥70% of blasts in the bone marrow and the increase of the absolute number of leukemic cells in the peripheral blood), which however was preceded by an increase in the absolute number of monocytes (Table 3), and died at day 148 (Table 2). The hemoglobin levels of patients 1, 3, and 6 remained unchanged compared with the pretreatment values and transfusional support was not required during treatment. As concern to platelet counts, thrombocytopenia consequent to VPA treatment was not observed. Patients 3 and 4 entered the study with a platelet count within the reference range that remained unchanged in both cases. In the other six cases, the VPA-ATRA did not modify their thrombocytopenic condition.

Patients 2-5 and 8 continued the VPA-ATRA treatment for 50, 102, 60, 94, and 88 days, respectively. Patients 6 and 7 were withdrawn from the study at day 28 for progressive disease and refusal to remain in such experimental approach, respectively. Patients received daunorubicin at the dose of 45 mg/m² for 3 days. Afterward, all patients died with their disease within 60 to 184 days (median, 119 days).

Adverse effects. Side effects were mild. Only one patient discontinued treatment for a grade III hepatic toxicity characterized by marked increase of the direct bilirubin level to 7.8 mg/dL that normalized at day 43. Hyperleukocytosis was observed in three cases. One patient, who reached serum VPA level up to 100 µg/mL, presented vertigo and tremor that disappeared at the reduction of the VPA dosage.

VPA serum level. Therapeutic VPA serum levels ≥50 µg/mL were reached between 5 and 26 days (median, 8 days) from treatment initiation (Table 2). Seven patients reached this value between 5 and 14 days with a daily VPA doses of 15 to 30 mg/kg (median, 20 mg/kg), whereas in the remaining case (patient 8) the VPA serum level peaked to 60.4 µg/mL later at day 26 using a daily VPA dose of 42 mg/kg (Table 2).

Changes in cell proliferation and differentiation. The biological activity of VPA-ATRA was evaluated in ex vivo samples collected from bone marrow and/or peripheral blood of the eight patients during the first 4 weeks of treatment. Cell cycle analysis was done at days 0, 7, 14, and 21 in bone marrow samples from six of eight patients (patients 5 and 6 were not studied). A consistent increase of the S-G2-M phase and a reduction of the G0-G1 phase was measurable in bone marrow cells from patients in which therapeutic VPA serum levels were reached but not in the "VPA-resistant" patient 8 (Table 2; Supplementary Table S1). Moreover, the frequency of apoptotic cells present in the bone marrow of patient 4 before treatment were greatly reduced by 7 days of VPA treatment. These results were consistent with the hyperleukocytosis detected in three of these patients (patients 1, 3, and 4) and with the absence of myelosuppressive properties of this regimen in the other cases (Table 3). VPA-ATRA treatment induced a gradual decrease in the percentage of bone marrow and peripheral blood blasts and a concomitant increase of cells exhibiting morphologic features of myeloid maturation, including chromatin condensation with nuclear segmentation, decreased nuclear/cytoplasmic ratio, changing in cytosolic basophilia, and appearance of paranuclear Golgi region and specific granules (Fig. 1 for three separate cases). Morphologic changes of bone marrow and peripheral blood cells related to “myelomonocytic differentiation” were observable in patient 1 by day 14 of VPA treatment, 7 days after the inclusion of ATRA (Table 3; Fig. 1A). Cytochemical staining that allows a functional characterization of myeloid-monocytic cells showed that MPO and CAE reactions and staining for NaF-inhibited ANAE activities were increased ~2- to 3-fold in bone marrow cells over the 28 days of VPA-ATRA treatment (Fig. 1A). CAE and MPO are enzymes restricted to the primary granules of granulocytes, whereas ANAE activity is inhibited by NaF only in monocytes.

VPA-ATRA induced cell differentiation toward the “monocytic lineage” in patients 3 and 5 to 7 within 14 to 21 days of treatment as indicated by the specific morphologic changes occurring in bone marrow and peripheral blood cells and the appearance of bone marrow cells in which the ANAE staining was inhibited by NaF (Table 3; Fig. 1B).

Morphologic changes of bone marrow cells relating to “granulocytic differentiation” were present in patients 2 and 4 (Table 3; Fig. 1C). By day 14 of treatment, the percentage of myeloid precursors and neutrophils in the bone marrow or peripheral blood from these two patients were increased ~2- to 3-fold compared with pretreatment values (Table 3). In patient 4 immature bone marrow cells, the MPO reactivity was also induced ~4-fold by day 14 compared with day 0 (Fig. 1D). In this case, the percentage of bone marrow cells positive for the myeloid antigens CD15 and

<table>
<thead>
<tr>
<th>Patients</th>
<th>Days to VPA serum level &gt;50 µg/mL</th>
<th>Dose (mg/kg) to reach VPA serum level &gt;50 µg/mL</th>
<th>Days to ATRA addition</th>
<th>Therapeutic response</th>
<th>Days to bone marrow blasts ≤5%</th>
<th>Hyperleukocytosis &gt;50 × 10⁹/L (d)</th>
<th>Survival from VPA-ATRA (d)</th>
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<tr>
<td>1</td>
<td>5</td>
<td>15</td>
<td>7</td>
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<td>Yes (+17)</td>
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<tr>
<td>2</td>
<td>14</td>
<td>30</td>
<td>14</td>
<td>SD</td>
<td>—</td>
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<td>3</td>
<td>10</td>
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<td>10</td>
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<tr>
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<td>15</td>
<td>14</td>
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<td>42</td>
<td>20</td>
<td>SD</td>
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<td>180</td>
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NOTE: No significant decrease in hemoglobin levels (a) or platelet count (b) compared with the pretreatment values. Abbreviations: HI-N, hematologic improvement in the neutrophil count; SD, stable disease; PD, progressive disease.
CD11b increased from 41% and 42% to 70% and 85%, respectively. Immature CD34+/CD117+ bone marrow cells decreased from 41% to 12% as measured at days 0 and 28 of treatment, respectively (Fig. 1E).

Cell differentiation remained unchanged in blood samples from the "VPA-resistant" patient 8 who indeed showed ~2-fold increase of CD34+/CD117+ immature cells at day 28 of VPA-ATRA treatment compared with day 0 (Table 3; data not shown).

**Induction of histone hyperacetylation in blood cells.** Figure 2 shows that at VPA serum levels ≥50 μg/mL, hyperacetylation of histones H3 and H4 was measurable in bone marrow and/or peripheral blood mononuclear cells from seven of eight patients by immunoblot analysis using specific antibodies. At therapeutic VPA serum levels, histone hyperacetylation was also maintained over the time of treatment. Indeed, in patient 1, a drop in VPA serum concentration resulted in a parallel decrease of histone acetylation.

### Table 3. Effect of VPA-ATRA treatment on marrow cellularity, WBC counts and morphology of bone marrow and peripheral blood cells

<table>
<thead>
<tr>
<th>Patients</th>
<th>Day of treatment</th>
<th>Cellularity</th>
<th>WBC ×10^9/L</th>
<th>Blast (%)</th>
<th>Erythroid (%)</th>
<th>Myeloid precursor (%)</th>
<th>Neutrophil (%)</th>
<th>Eosinophil (%)</th>
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<td>VPA ATRA BM PB</td>
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<tr>
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NOTE: The bone marrow cellularity is defined as low (L), normal (N), and high (H). WBC indicates the total peripheral blood cell count. The percentage of cells with morphologic features of blasts, erythroid cells, myeloid precursors (promyelocytes, myelocytes, and metamyelocytes); neutrophils (stab cells and segmented neutrophils), eosinophils, and monocytic (monoblasts and monocytes) was evaluated in both bone marrow (BM) and peripheral blood (PB).
levels in mononuclear peripheral blood cells (Fig. 2A). These
evidence and the unchanged acetylation status of histones H3 and
H4 in blood cells from patient 8 (data not shown), in which
therapeutic VPA serum levels were not reached before day 26 of
VPA-ATRA treatment. Reported in the table are the changes in
the percentage of positive cells for MPO, CAE, and ANAE with or
without NaF. Stainings were done as described in Materials and Methods. Note that immature
blasts, which were predominant before treatment, were induced to differentiate
toward the myelomonocytic lineage (patient 1; A), monocytic lineage (patient 3; B), or granulocytic lineage (patient 4; C) by VPA-ATRA treatment as also
detailed in Table 3. Arrow, presence of Auer rods in patient 3 maturing cells.
E, immunophenotypic changes related to the granulocytic differentiation of
leukemic cells (increased expression of CD15 and CD11b, which paralleled the
decrease of CD34+/CD117+ cells) were measured in bone marrow cells from
patient 4 by flow cytometry at the indicated times of treatment.

Changes in myeloid gene expression. The expression levels of
genes associated to myelomonocytic differentiation (MPO, GM-
CSFr, G-CSFr, and MSE) were also investigated by qRT-PCR over
the time of VPA-ATRA treatment in bone marrow and peripheral
blood samples from all these patients. Albeit with differences in
potency and kinetics among patients, these gene expressions
changed in cells from the seven patients in which VPA therapeutic levels were reached and histone hyperacetylation was detectable (Fig. 2; data not shown). Modification in gene expressions also related to the phenotypic cell differentiation
into the myelomonocytic (patient 1), granulocytic (patients 2 and 4), and monocytic (patients 3 and 5-7) lineages (Table 3). Compared
with pretreatment values (day 0), a strong up-regulation of MPO
gene and G-CSFr and GM-CSFr mRNA (~2- to 4-fold) were measurable by day 14 of VPA treatment, 7 days after the
addition of ATRA in patient 1 bone marrow and peripheral
blood cells (Fig. 2A). In patients 2 and 4, maturation changes of
bone marrow cells toward the granulocytic lineage related to the
induction in gene transcripts for GM-CSFr, which preceded the
increase of MPO mRNA (Fig. 2B). In four patients, monocytic
differentiation was consistently associated to the induction of the
MSE gene transcripts, which increased ~12-, 23-, 4-, and
17-fold in bone marrow or peripheral blood samples from
patients 3 and 5 to 7, respectively, within 14 to 21 days of
 treatment. In three of these patients, an increased expression of
GM-CSFr gene was also measurable over the time of VPA-ATRA
treatment (Fig. 2C). GATA-1, which is present either in
hematopoietic stem cells or in erythroid and megakaryocytic
progenitors was induced ~2- to 4-fold in bone marrow samples
from patients 1, 3, and 6 by 14 to 28 days of VPA-ATRA compared
with day 0 (Fig. 2).

Therapy-related differentiation of leukemic elements. By
parallel analysis of cytology and cytogenetics, we investigated the
presence of the trisomy 8 and the del(7)(q31) in mature bone
marrow cells from patients 1 and 4, respectively (Fig. 3A and B).
Reported in the table are the bone marrow cell differentials over
time of treatment and respective FISH distribution. Before
treatment, both the +8 chromosomal anomaly and the 7q− were
mainly present in immature blasts (94% and 51% of the cells
from patients 1 and 4, respectively). In both cases, VPA-ATRA
treatment decreased the fraction of blasts positive for these
cytogenetic anomalies, whereas the percentage of maturing
myeloid elements and segmented cells presenting the same
anomalies increased. In patient 4, a gradual maturation of the
leukemic clone was fully detectable at days 14 and 21 of VPA-
ATRA treatment before the initiation of cytoreduction therapy
with aracitcin to control hyperleukocytosis (day 24). Moreover,
morphologic evidence suggesting the ability of VPA-ATRA to
induce differentiation of leukemic cells was provided by the
appearance of Auer rods in the cytosol of cells from the AML-Mo
patient 3 (Fig. 1B, arrow).

In vivo changes of the chromatin state at RARα gene
promoter. We investigated whether VPA treatment, by inhibiting
HDAC activities, affected the chromatin state at ATRA target gene
promoters by chromatin immunoprecipitation assay. Chromatin
fragments isolated from mononuclear cells of two patients
(patients 4 and 6) undergoing treatment were immunoprecipitated
with an anti-acetylated H4 antibody. DNA was amplified by PCR
using primers located on a promoter region encompassing the
RARE of RARα2, the ATRA-inducible isoform of RARα related to
myelomonocytic differentiation (17, 18, 37). The acetylation levels
of histone H4 at this RARα regulatory site were increased on VPA
treatment and further augmented by ATRA addition compared
with those measurable in chromatin samples from these two
patients at day 0 (Fig. 3C). qRT-PCR done in blood samples from all
the eight patients showed a significant increase of RARα mRNA
expression at day 21 of treatment (n = 8; mean ± SD, 1.4 ± 0.13;
P = 0.002) when ATRA was added to VPA in all cases (Fig. 3D).
Of note, RARα mRNA expression was not changed at any
treatment time in bone marrow samples from the “VPA-resistant”
patient 8 (Fig. 3D).
Discussion

We report the results of a pilot study initiated in eight patients with high-risk and/or very advanced AML not eligible for additional intensive chemotherapy in which the HDACi VPA was given to remodel chromatin, followed by the addition of ATRA, to activate gene transcription and differentiation in leukemic cells.

To our knowledge, the present data are the first in vivo evidence showing that achievement of VPA therapeutic serum levels (≥50 μg/mL) correlates with global hyperacetylation of histones H3 and H4 in leukemic blasts and clinicobiological response in AML patients. Moreover, in the only case (patient 8), in which therapeutic VPA serum levels were reached at later times of treatment (day 26), histones remained deacetylated and no response was obtained at the clinical or biological level. By contrast, in the other seven cases, the histone hyperacetylation state was found associated to differentiation of the leukemic clone, albeit to a different extent and with a variable spectrum of phenotypes between patients, as shown by (a) changes in morphology of the blasts, (b) progressive decrease in the percentage of immature cells and concomitant increase of cells presenting specific enzymatic activities or markers of mature granulomonocytic cells, and (c) increased expression of genes related to myelomonocytic differentiation. Thus, the detection of hyperacetylated histones H3 and H4 can be a useful marker to monitor the VPA inhibitory activity on HDACs in bone marrow or peripheral blood cells.

Although a reduction of the peripheral blood and bone marrow blasts was measurable in seven patients within the first 4 weeks of treatment, an objective clinical response to VPA-ATRA was observed in two of eight patients that achieved a hematologic improvement in the neutrophil count. We remark that all our patients were affected by very high risk AML or were chemoresistant and heavily pretreated.

These results compare favorably with those reported by Gore et al. (38) where 2 of 23 pretreated AML patients responded to the structurally related HDACi phenylbutyrate. Compared with phenylbutyrate, VPA is an extremely safe, absorbable, and well tolerated drug. Recently, studies by Kuendgen et al. (26) showed the ability of VPA to induce therapeutic responses in a sizable portion of MDS patients and in one of three patients with AML secondary to MDS. In MDS patients, the simultaneous administration of VPA and ATRA was not superior to VPA alone, whereas addition of ATRA to VPA reinduced a clinical response in two of four MDS patients. A further study by the same group done in 75 patients with MDS and relapsed or refractory AML confirmed these results (27). These latter clinical observations and our findings suggest that the sequential administration of ATRA to VPA synergize AML blast responsiveness.

Although we lack a direct proof for an "epigenetic priming" effect of VPA on gene transcription, in the two cases tested, VPA augmented the histone acetylation status on chromatin regions surrounding the RARE of RARα, the ATRA receptor gene involved in normal and pathologic myelopoiesis (16). Thus, VPA alone affected the chromatin status at specific gene target sites that might relate with its ability to induce a certain degree of differentiation in AML blasts in vivo. However, histone acetylation levels at ATRA regulatory sites were further increased by ATRA addition to VPA in samples from these two cases. In agreement with the ligand inducibility of RARα gene, RARα mRNA transcripts were found significantly induced after the addition of ATRA to VPA.

In agreement with the ligand inducibility of RARα gene, RARα mRNA transcripts were found significantly induced after the addition of ATRA to VPA.

![Figure 2](https://www.aacrjournals.org/8909) Changes of VPA serum levels, acetylation status of histones H3 and H4, and expression of genes related to myeloid differentiation over the time of VPA-ATRA treatment. Top, changes in gene expression measured before (day 0) or at the indicated days of treatment in bone marrow and/or peripheral blood samples from patients induced by VPA-ATRA to differentiate toward the myelomonocytic lineage (patient 1; A), granulocytic lineage (patients 2 and 4; B), and monocytic lineage (patient 3 and 5-7; C). Expression levels of genes related to myeloid differentiation (MPO, G-CSFR, GM-CSFR, MSE, and GATA-1) were measured by qRT-PCR analysis on total RNA as detailed in Materials and Methods. Bottom, VPA serum levels (dashed lines) and acetylation status of H3 and H4 histones measured by immunoblot analysis using specific antibodies. Expression of histone H3 was used as a control for histone acetylation levels and sample loading. Arrows, day of ATRA addition to VPA treatment.
total input chromatin was included in the PCR analysis. Cell signaling data were further analyzed by Student’s t test (n = 8). Points were evaluated by Student’s t test.

Materials and Methods. Amplification of GAPDH was used to detect nonrelevant gene expression (17). September 1, 2006 8910 What was the purpose of the VPA-ATRA treatment in bone marrow smears from patient 4? The purpose of the VPA-ATRA treatment was to induce differentiation of leukemic blasts in bone marrow smears from patient 4. May–Grunwald–Giemsa staining was done in 242 non-M3 elderly AML patients recently showed that the addition of ATRA to chemotherapy significantly improved the clinical outcome compared with chemotherapy alone (44).

Acknowledgments

Received 8/2/2005; revised 5/23/2006; accepted 6/19/2006.

Grant support: Associazione Italiana per Ricerca sul Cancro, Italian Ministry of Health, National Program on Stem Cells, and Italian Ministry of University and Research (COFIN and FIFB), Fondazione Italiana per la Ricerca sul Cancro fellowship (L. Travaglini). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank all the patients and clinicians at the University of Rome "La Sapienza" and "Tor Vergata" for making this study possible and Prof. Giuseppe Avvisati and Francesco Grignani for critical reading of the article and helpful advice.

References


Cancer Res 2006; 66: (17). September 1, 2006 8910 www.aacrjournals.org