Effect of histone deacetylase inhibitor valproic acid on progenitor cells of acute myeloid leukemia

Gesine Bug, Kerstin Schwarz, Claudia Schoch, Manuela Kampfmann, Reinhard Henschler, Dieter Hoelzer, Oliver G. Ottmann, Martin Ruthardt

From the Medizinische Klinik II, Abt. Hämatologie/Onkologie, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt, Frankfurt, Germany (GB, KS, MK, DH, OGO, MR); MLL Münchner Leukämielabor GmbH, München, Germany (CS); Institut für Transfusionsmedizin und Immunhämatologie, DRK-Blutspendedienst Baden-Württemberg - Hessen GmbH, Frankfurt, Germany (RH).

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ABSTRACT

Histone deacetylase inhibitor valproic acid (VPA) was recently shown to enhance proliferation and self-renewal of normal hematopoietic stem cells, raising the possibility that VPA may also support growth of leukemic progenitor cells (LPC). Here, VPA maintains a significantly higher proportion of CD34+ LPC and colony forming units compared to control cultures in six AML samples, but selectively reduces leukemic cell numbers in another AML sample with expression of AML1/ETO. Our data suggest a differential effect of VPA on the small population of AML progenitor cells and the bulk of aberrantly differentiated blasts in the majority of AML samples tested.

Key words: histone deacetylase inhibitor, CD34+ leukemic progenitor cells, interphase FISH, AML1/ETO, AML blast colonies

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AML is a clonal disorder involving a hierarchy of leukemic cells that differ in their phenotypic characteristics and proliferation potential. Similar to normal repopulating stem cells, leukemic stem cells from all subtypes of AML reside exclusively in the CD34+38− population. They are characterized by indefinite self-renewal and give rise to a population of extensively proliferating progenitor cells which produce the vast pool of aberrantly differentiated and arrested blasts. Thus, the efficiency of any molecular therapy will ultimately depend on the treatment’s ability to eradicate the leukemic stem and progenitor cell compartment. In acute myeloid leukemia (AML) with the translocation t(8;21), the oncogenic fusion protein AML1/ETO recruits histone deacetylases (HDACs) into DNA-associated corepressor complexes, leading to an inappropriate modulation of chromatin structure by HDACs and repression of AML1 target genes critical for myeloid differentiation, induction of apoptosis, regulation of cell cycle, and interferon signaling. AML1/ETO positive AML has therefore served as a model for the differentiation-inducing effect of HDAC inhibitors such as valproic acid and trichostatin A (TSA), which is attributed to chromatin remodeling. VPA and TSA have been shown to promote in vitro maturation of primary AML blasts independently of the underlying chromosomal aberration and the ability of VPA to induce in vivo differentiation of AML blasts has recently been established. On the other hand, VPA is known to enhance proliferation and self-renewal of normal hematopoietic stem cells. We hereby aimed to study the impact of VPA on AML progenitor cells.

Design and Methods

AML samples

Peripheral blood samples were obtained from AML patients at diagnosis after informed consent and with the approval of the ethics committee of the J.W. Goethe-University of Frankfurt. Baseline morphology, cytogenetics and cell surface antigen analysis were performed as part of the routine clinical evaluation of the patients. Diagnosis and classification of the AML were based on the criteria of the French-American-British (FAB) group. All samples
had a chromosomal marker readily detectable by interphase FISH by commercially available probes given in Table 1 (Abbott, Wiesbaden, Germany).

**Culture and analysis of leukemic progenitor cells**

The CD34+CD38- cell selection was performed using the StemSep Primitive Progenitor Enrichment Cocktail (CellSystems Biotech., St. Katharinen, Germany) and magnetic cell separation technology from Miltenyi Biotec (Bergisch Gladbach, Germany) according to manufacturers instructions. CD34+CD38- cells were maintained in liquid culture supplemented with interleukin-3, thrombopoietin (25 ng/mL each), stem cell factor and FLT3 ligand (50 ng/mL each, R&D, Wiesbaden, Germany) for 14 days and analysed by colony assay, flow cytometry and interphase fluorescence in situ hybridization (FISH) as previously described.13,14

VPA (Orfifil®, Desitin Pharma, Liestal, Switzerland) was added at a concentration of 100 μg/mL based on the results of our clinical trial in which a maximum serum level of free and protein-bound VPA of 87 ± 6 μg/mL (mean ± SEM) was achieved in AML patients and proved to be biologically active.15 For the AML blast colony assay, cells harvested from suspension culture were plated in methylcellulose (Methocult® GF H4434, CellSystems Biotech) ± VPA colonies (> 20 cells) were counted after 12-14 days and cells harvested from the colony assays were analysed by flow cytometry and FISH.14 Data were given as mean ± SEM and compared by the Student t test. p values <0.05 were considered to be significant.

**Results and Discussion**

**VPA does not selectively abrogate the leukemia clone in most AML samples**

Peripheral blood samples from seven patients with newly diagnosed (n=6) or relapsed (n=1) AML with a blast cell content ranging between 11% and 83% were studied. FACS analysis of the blast population revealed that 17-65% of cells expressed CD34 (Table 1). CD34+CD38- cells were enriched to a purity of 75.7 ± 8.2% (Table 2) and cultured in the presence or absence of VPA. As the subpopulation of CD34+CD38- peripheral blood cells of untreated AML patients contains leukemic as well as residual normal hematopoietic stem and progenitor cells,16 the clonal origin of the cells was determined by FISH and evaluable in all but patient sample #6. Irrespective of VPA treatment, > 90% of cells analysed before and after one or two weeks of liquid culture belonged to the leukemic clone in patient samples #2, 3, 4, 5 and 7. In patient sample #1 with t(8;21), VPA led to a progressive loss of leukemic cells, thus confirming previous in vitro results.5 After 7 and 12 days of VPA treatment, 60% and 17% of cells carried the t(8;21) versus 96% and 86% in control cultures. VPA selectively reduced the AML1/ETO positive leukemic cell count to 85% of VPA-free control cultures on day 7 and 20% on day 12. This was associated with a proliferation of normal cells. On the other hand, VPA supported survival of highly enriched leukemic progenitor cells from patient #2 with t(8;21) and additional der(7)(p7;12). Although the aberration on chromosome 7 may contribute to the different VPA responses of those two t(8;21) AMLs, the evidence that VPA may promote leukemic cell growth is consistent with the clinical observation of rapid disease progression during combined VPA and ATRA treatment in an AML patient with t(8;21).15

**VPA maintains a high proportion of CD34+ cells in suspension culture**

In VPA-treated suspension cultures, the median proportion of CD34 expressing progenitor cells was maintained at input levels, but declined significantly in control cultures by day 14 (64.3 ± 11.2% vs. 15.5 ± 6.9%, p=0.003, Table 2). The total number of CD34+ cells recovered per 10×10⁶ CD34+ input cells was higher in VPA-supplemented cultures of all seven patients compared to controls. However, this result did not achieve significance. A distinct response was observed in patient

### Table 1. Patients’ characteristics.

<table>
<thead>
<tr>
<th>Patient sample</th>
<th>Age (years)</th>
<th>Sex</th>
<th>FAB type</th>
<th>WBC (&lt;10⁵/L)</th>
<th>Type of AML</th>
<th>% PB blasts</th>
<th>%CD34+ blasts</th>
<th>BM cytogenetics</th>
<th>FISH probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41</td>
<td>F</td>
<td>2</td>
<td>13.5</td>
<td>de novo</td>
<td>51</td>
<td>65</td>
<td>46,XX.t(8;21)(q22;q22)</td>
<td>AML1-ETO</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>F</td>
<td>3</td>
<td>36.9</td>
<td>de novo</td>
<td>83</td>
<td>78</td>
<td>45,XX.t(8;21)(q22;q22) [8], AML1-ETO</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>F</td>
<td>7</td>
<td>1.1</td>
<td>de novo</td>
<td>8</td>
<td>17</td>
<td>47,XX+8(16)/46,XX [4]</td>
<td>CEPHS</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>F</td>
<td>4</td>
<td>37.2</td>
<td>de novo</td>
<td>33</td>
<td>nd</td>
<td>48,XX+11,14 [23], 49,XX,der(7)(p7;12)q21,q11</td>
<td>CEP11</td>
</tr>
<tr>
<td>5</td>
<td>53</td>
<td>M</td>
<td>nd</td>
<td>15.5</td>
<td>relapse</td>
<td>28</td>
<td>nd</td>
<td>complex1</td>
<td>LSI Sp15.2/5p31</td>
</tr>
<tr>
<td>6</td>
<td>66</td>
<td>M</td>
<td>nd</td>
<td>20.2</td>
<td>sec</td>
<td>77</td>
<td>nd</td>
<td>complex2</td>
<td>LSI Sp15.2/5p31</td>
</tr>
<tr>
<td>7</td>
<td>59</td>
<td>M</td>
<td>nd</td>
<td>15.2</td>
<td>sec</td>
<td>11</td>
<td>21</td>
<td>complex2</td>
<td>CEP7# LSI T7q31</td>
</tr>
</tbody>
</table>

FAB, French-American-British group; WBC, total white blood cell count at diagnosis; BM, bone marrow; nd, not done; sec, secondary AML; PB, peripheral blood; *42-46:XX,der(1)(p12-13q44) [2], add(3)(p11) [15], der(5)(q15p15), del(5)(q14q34), +add(12)(p11)del(12)(p11)q21, -del(12)(p11)q21, +mar[par1] | 44,XX,t(5;5)(p10q10),del(5)(q11q23), t(7,8) | 45,XX,der(7)(p7;12)q21,q11 | 47,XX+8(16)/46,XX [4] | CEP7# LSI T7q31 | 48,XX,der(7)(p7;12)q21,q11 | 49,XX,der(7)(p7;12)q21,q11 | 47,XX+8(16)/46,XX [4] | CEPHS |

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**Note:**

- FAB: French-American-British group.
- WBC: Total white blood cell count at diagnosis.
- BM: Bone marrow.
- ND: Not done.
- Sec: Secondary AML.
- PB: Peripheral blood.
- *: Specific chromosomal abnormalities.

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**References:**

1. Abbott, Wiesbaden, Germany.
2. StemSep Primitive Progenitor Enrichment Cocktail (CellSystems Biotech.).
3. Orfifil®, Desitin Pharma, Liestal, Switzerland.
5. VPA (Orfifil®).
6. t(8;21).
7. AML1-ETO.
8. AML1-ETO.
sample #3 for whom VPA stimulated proliferation of leukemia CD34+ cells to 210% on day 14 compared to a reduction to 14% in control cultures. The proportion and number of CD11b+ monocytic and granulocytic cells harvested from liquid cultures was not significantly altered by VPA (Table 2).

**VPA enhanced growth of AML blast colonies**

Five out of seven patient samples tested displayed clonogenic growth. VPA-treated colony assays yielded a significantly higher number of colonies per 10^6 cells plated and colonies appeared much larger compared to untreated controls. In the presence of VPA, most colonies were composed of small, uniform blast cells typical of AML colony-forming units. FISH analysis was performed on cells harvested from colony assays of samples 4-7 confirming the leukemia origin of > 95% of analysed cells. The impact of VPA was also demonstrated by FACS analysis. Colonies grown in VPA-supplemented methylcellulose consisted of a higher proportion of immature CD34+ progenitor cells than control cultures (15.2±7.4% vs. 0.7±0.6%, mean ± SEM of five patient samples, p = 0.086). Results of a representative patient are depicted in Figure 1.

To summarize, we provide the first data indicating that the HDAC inhibitor VPA may enhance maintenance and clonogenic capacity of CD34+ AML progenitor cells and that this effect does not appear to be associated with a specific cytogenetic subtype of AML. These results were not anticipated, because the clinical use of HDAC inhibitors is based on the premise that their epigenetic effects result in selectively overcoming the differentiation block and induction of apoptosis in AML blasts. A possible explanation for our current finding that VPA has a different effect on the bulk of AML blasts and the small subsets of leukemia stem and progenitor cells. The poor responses of AML patients reported in previous clinical studies using VPA is consistent with these data. Furthermore, our findings have clinical implications for the use of HDAC inhibitors in the treatment of AML.

### Table 2. Impact of VPA on the proportion of CD34+ and CD11b+ cells in liquid culture.

<table>
<thead>
<tr>
<th>Patient sample</th>
<th>FISH probe</th>
<th>D0</th>
<th>%CD34+ cells</th>
<th>% of CD34+ cells control</th>
<th>% of CD34+ cells VPA</th>
<th>D14</th>
<th>% of CD11b+ cells control</th>
<th>% of CD11b+ cells VPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AML1-ETO</td>
<td>82.0</td>
<td>1.7</td>
<td>9.8</td>
<td>22.3</td>
<td>16.0</td>
<td></td>
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</tr>
<tr>
<td>2</td>
<td>AML1-ETO</td>
<td>84.8</td>
<td>52.6</td>
<td>91.8</td>
<td>23.2</td>
<td>8.0</td>
<td></td>
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<tr>
<td>3</td>
<td>CEP#6</td>
<td>85.8</td>
<td>8.8</td>
<td>87.2</td>
<td>34.5</td>
<td>27.1</td>
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<tr>
<td>4</td>
<td>CEP#11</td>
<td>69.8</td>
<td>19.7</td>
<td>85.1</td>
<td>41.7</td>
<td>15.9</td>
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<tr>
<td>5</td>
<td>CEP#11</td>
<td>81.9</td>
<td>19.7</td>
<td>65.4</td>
<td>7.1</td>
<td>3.7</td>
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</tr>
<tr>
<td>6</td>
<td>CEP#11</td>
<td>85.6</td>
<td>0.1</td>
<td>70.4</td>
<td>0.1</td>
<td>63.1</td>
<td></td>
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</tr>
<tr>
<td>7</td>
<td>CEP#7 LSI 7q31</td>
<td>26.2</td>
<td>5.8</td>
<td>40.7</td>
<td>22.0</td>
<td>22.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>73.7</td>
<td>15.5</td>
<td>64.3</td>
<td>21.5</td>
<td>22.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P = 0.003

*ns; not significant; * no cells detectable.
inhibitors in the treatment of AML as they raise the possibility that these compounds may stimulate leukemic progression.

**Author Contributions**

GB performed experiments, contributed to the design and development of the study as well as interpretation of the data and wrote the manuscript; KS performed experiments, contributed to the design and development of the study as well as interpretation of the data and wrote the manuscript; CS performed experiments, contributed to the design and development of the study as well as interpretation of the data; MR contributed to the design and development of the study as well as interpretation of the data and wrote the manuscript; MK performed experiments and contributed to the design and development of the study as well as interpretation of the data; OGO contributed to the design and development of the study as well as interpretation of the data and wrote the manuscript.

**Conflict of Interest**

The authors reported no potential conflicts of interest.

**References**


