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

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Funding: this work was supported by the Alfred und Angelika Gutermuth-Stiftung to GB and the Deutsche José Carreras Leukämie-Stiftung (R04/05) to MR and RH.

Manuscript received December 30, 2006.
Manuscript accepted February 14, 2007.

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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

Haematologica 2007; 92:542-545

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.[1,2] 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.[3,4] 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.[5-9] 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 remodelling.[6,7] VPA and TSA have been shown to promote *in vitro* maturation of primary AML blasts independently of the underlying chromosomal aberration[6,7] and the ability of VPA to induce *in vivo* differentiation of AML blasts has recently been established.[10] On the other hand, VPA is known to enhance proliferation and self-renewal of normal hematopoietic stem cells.[11,12] 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 (Orfiril®, 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 leukemic 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 73.7±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.[6,7] 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)t(7;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 10×10^5 $CD34^+$ 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.

Patient sample	Age (years)	Sex	FAB type	WBC ($\times10^9$/L)	Type of AML	% PB blasts	%$CD34^+$ blasts	BM cytogenetics	FISH probe
1	41	F	2	13.5	*de novo*	51	65	46,XX,t(8;21)(q22;q22)	AML1-ETO
2	23	F	4	36.9	*de novo*	83	78	45,X,-X,t(8;21),(q22;q22) [8], 45,idem,der(7)t(7;12)(p21;q11)	AML1-ETO
3	23	F	7	1.1	*de novo*	8	17	47,XX,+8 [16]/46,XX [4]	CEP#8
4	68	F	4	37.2	*de novo*	33	nd	48,XX,+11,+14 [23], 49,idem,+8 [2]	CEP#11
5	53	M	nd	15.5	relapse	28	nd	complex[1]	LSI 5p15.2/5q31
6	66	M	nd	20.2	sec	77	nd	complex[2]	LSI 5p15.2/5q31
7	59	M	nd	15.2	sec	11	21	complex[3]	CEP#7 LSI 7q31

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; [1]42-46,XY,r(1)(p?32-33q44) [2], add(3)(p11) [5], der(5)t(5;17)(q12;q11) [10], add(6)(p24) [7], -6 5,del(7)(q21) [10], ?-hsr(8)(q21) [3], ?hsr(11)(q23) [6], -11 [3], -16 [5], -17 [10], +mar [1] cp17 [2]41-47, X,-Y,der(5)t(5;7)(p15;p15), del(5)(q14q34),-7,add(12)(p11).dic(12;18)(p11;p11)del(12)(q13q23),-18,-18,+1-2r,+1-3mar [cp21] [3]44,XY,t(3;5)(p10;q10),del(5)(q11q23),-7,der(11)dup(11)(q25q14),add(11)(q14),del(13)(q?21),-15,-18,add(18)(p11),del(21)(q21q22),+r,+mar.

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

Patient sample	FISH probe	D0 %CD34⁺38⁻ cells	D14 % of CD34⁺ cells control	VPA	% of CD11b⁺ cells control	VPA
1	AML1-ETO	82.0	1.7	9.8	22.3	16.0
2	AML1-ETO	84.8	52.6	91.8	23.2	8.0
3	CEP#8	85.8	8.8	87.2	34.5	27.1
4	CEP#11	69.8	19.7	85.1	41.7	15.9
5	LSI 5p15.2/5q31	81.9	19.7	65.4	7.1	3.7
6	LSI 5p15.2/5q31	85.6	01	70.4	01	63.1
7	CEP#7 LSI 7q31	26.2	5.8	40.7	22.0	22.9
Mean		73.7	15.5	64.3	21.5	22.4
P			0.003		ns	

ns; not significant; [1]no cells detectable.

sample #3 for whom VPA stimulated proliferation of leukemic 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 10^4 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 leukemic 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 leukemic stem and progenitor cells. The poor responses of AML patients reported in previous clinical studies using VPA is consistent with these data.[10,15,17,18,19] Furthermore, our findings have clinical implications for the use of HDAC

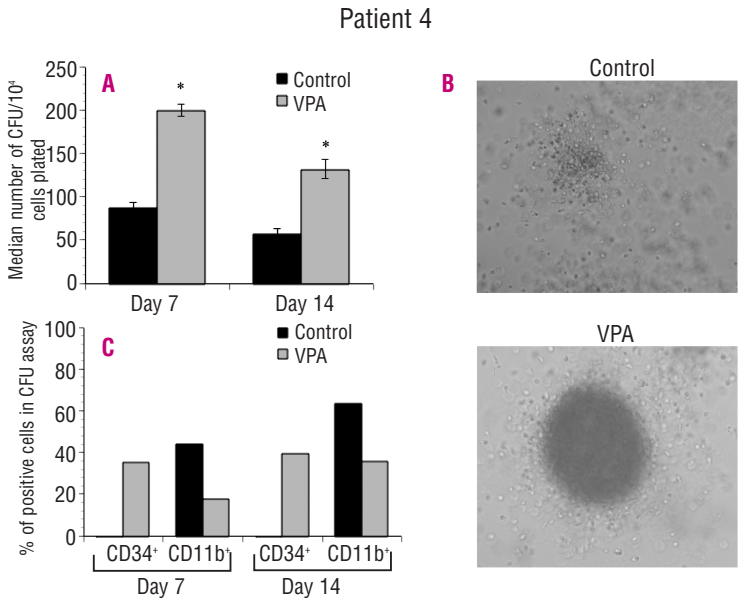

Figure 1. Impact of VPA on AML blast colony growth as shown representatively for patient sample #4. Colony assays were initiated using the total cell output after 7 and 14 days of liquid culture and incubated in the presence or absence of VPA (100 μg/mL). A. VPA-treated methylcellulose yielded a significantly higher number of CFUs (mean of triplicates, asterisk indicates p<0.05). B. Colonies grown in the presence of VPA appeared larger and consisted of many more cells (25-times magnified). C. VPA reduced differentiation of early progenitor cells and maintained a high proportion of CD34⁺ cells in colony assays plated after 7 and 14 days of liquid culture. In the absence of VPA, no CD34⁺ cells were detected.

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

Conflict of Interest

The authors reported no potential conflicts of interest.

References

1. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997;3:730-7.
2. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature 1994;367:645-8.
3. Passegue E, Jamieson CH, Ailles LE, Weissman IL. Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? Proc Natl Acad Sci USA 2003;100 Suppl 1: 11842-9.
4. Jordan CT, Guzman ML. Mechanisms controlling pathogenesis and survival of leukemic stem cells. Oncogene 2004;23:7178-87.
5. Melnick A, Carlile GW, McConnell MJ, Polinger A, Hiebert SW, Licht JD. AML-1/ETO fusion protein is a dominant negative inhibitor of transcriptional repression by the promyelocytic leukemia zinc finger protein. Blood 2000;96:3939-47.
6. Göttlicher M, Minucci S, Zhu J, Krämer O, Schimpf A, Giavara S, et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 2001;20:6969-7 8.
7. Ferrara F, Fazi F, Bianchini A, Padula F, Gelmetti V, Minucci S, et al. Histone deacytylase-targeted treatment restores retinoic acid signaling and differentiation in acute myeloid leukemia. Cancer Res 2001;61:2-7.
8. Gelmetti V, Zhang J, Fanelli M, Minucci S, Pelicci P, Lazar M. Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. Mol Cell Biol 1998;18:7185-92.
9. Wang J, Hoshino T, Redner R, Kajigaya S, Liu J. ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin/HDAC1 complex. Proc Natl Acad Sci USA 1998;95:10860-5.
10. Cimino G, Lo-Coco F, Fenu S, Travaglini L, Finolezzi E, Mancini M, et al. Sequential valproic acid/All-trans Retinoic acid treatment reprograms differentiation in refractory and high-risk acute myeloid leukemia. Cancer Res 2006;66:8903-11.
11. Bug G, Gul H, Schwarz K, Pfeifer H, Kampfmann M, Zheng X, et al. Valproic acid stimulates proliferation and self-renewal of hematopoietic stem cells. Cancer Res 2005;65:2537-41.
12. De Felice L, Tatarelli C, Mascolo MG, Gregorj C, Agostini F, Fiorini R, et al. Histone deacetylase inhibitor valproic acid enhances the cytokine-induced expansion of human hematopoietic stem cells. Cancer Res 2005;65:1505-13.
13. Rossmanith T, Schroder B, Bug G, Muller P, Klenner T, Knaus R, et al. Interleukin 3 improves the ex vivo expansion of primitive human cord blood progenitor cells and maintains the engraftment potential of scid repopulating cells. Stem Cells 2001; 19:313-20.
14. Buhmann R, Kurzeder C, Rehklau J, Westhaus D, Bursch S, Hiddemann W, et al. CD40L stimulation enhances the ability of conventional metaphase cytogenetics to detect chromosome aberrations in B-cell chronic lymphocytic leukaemia cells. Br J Haematol 2002;118:968-75.
15. Bug G, Ritter M, Wassmann B, Schoch C, Heinzel T, Schwarz K, et al. Clinical trial of valproic acid and all-trans retinoic acid in patients with poor-risk acute myeloid leukemia. Cancer 2005;104:2717-25.
16. Ailles LE, Gerhard B, Kawagoe H, Hogge DE. Growth characteristics of acute myelogenous leukemia progenitors that initiate malignant hematopoiesis in nonobese diabetic/severe combined immunodeficient mice. Blood 1999;94:1761-72.
17. Kuendgen A, Schmid M, Schlenk R, Knipp S, Hildebrandt B, Steidl C, et al. The histone deacetylase (HDAC) inhibitor valproic acid as monotherapy or in combination with all-trans retinoic acid in patients with acute myeloid leukemia. Cancer 2006; 106:112-9.
18. Pilatrino C, Cilloni D, Messa E, Morotti A, Giugliano E, Pautasso M, et al. Increase in platelet count in older, poor-risk patients with acute myeloid leukemia or myelodysplastic syndrome treated with valproic acid and all-trans retinoic acid. Cancer 2005;104:101-9.
19. Raffoux E, Chaibi P, Dombret H, Degos L. Valproic acid and all-trans retinoic acid for the treatment of elderly patients with acute myeloid leukemia. Haematologica 2005; 90: 986-8.