2. Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G, et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. Blood 1998; 92: 2322-33.
3. Grimwade D, Walker H, Harrison G, Oliver F, Chatters S, Harrison CJ, et al. The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial. Blood 2001;98:1312-20.
4. Slovak ML, Kopecky KJ, Cassileth PA, Harrington DH, Theil KS, Mohamed A, et al. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. Blood 2000;96:4075-83.
5. Frohling S, Skelin S, Liebisch C, Scholl C, Schlenk RF, Dohner H, et al. Comparison of cytogenetic and molecular cytogenetic detection of chromosome abnormalities in 240 consecutive adult patients with acute myeloid leukemia. J Clin Oncol 2002; 20:2480-5.
6. Mrozek K, Prior TW, Edwards C, Marcucci G, Carroll AJ, Snyder PJ, et al. Comparison of cytogenetic and molecular genetic detection of t(8;21) and inv(16) in a prospective series of adults with de novo acute myeloid leukemia: a Cancer and Leukemia Group B Study. J Clin Oncol 2001;19:2482-92.
7. Olesen LH, Clausen N, Dimitrijevic A, Kerndrup G, Kjeldsen E, Hokland P. Prospective application of a multiplex reverse transcription-polymerase chain reaction assay for the detection of balanced translocations in leukaemia: a single-laboratory study of 390 paediatric and adult patients. Br J Haematol 2004;127:59-66.
8. Rowe D, Cotterill SJ, Ross FM, Bunyan DJ, Vickers SJ, Bryon J, et al. Cytogenetically cryptic AML1-ETO and CBF beta-MYH11 gene rearrangements: incidence in 412 cases of acute myeloid leukaemia. Br J Haematol 2000;111:1051-6.
9. Pallisgaard N, Hokland P, Riishoj DC, Pedersen B, Jorgensen P. Multiplex reverse transcription-polymerase chain reaction for simultaneous screening of 29 translocations and chromosomal aberrations in acute leukemia. Blood 1998;92:574-88.
10. Strehl S, Konig M, Mann G, Haas OA. Multiplex reverse transcriptase-polymerase chain reaction screening in childhood acute myeloblastic leukemia. Blood 2001;97:805-8.

Acute Myeloid Leukemia

Treatment of elderly acute myeloid leukemia with valproic acid and all-trans retinoic acid

Valproic acid (VPA) has been demonstrated to be able to inhibit histone deacetylase activity and to synergize with all-trans retinoic acid (ATRA) in inducing the differentiation of acute myeloid leukemia (AML) cells. A pilot study of the VPA/ATRA combination was performed in 11 elderly patients with *de novo* AML (median age, 82 years). Complete marrow response was observed in 3 patients, including 1 complete remission. Two additional patients had hematologic improvement.

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Acute myeloid leukemia (AML) is characterized by arrested differentiation of leukemic cells at an early stage of maturation. Revival of the maturation process and reversal of the immature phenotype have been obtained with all-*trans* retinoic acid (ATRA) in acute promyelocytic leukemia (APL) cells (AML-M3 in the French-American-British classification). *In vitro*, ATRA is also capable of inducing differentiation of non-M3 AML cells, such as HL60 cells. The differentiation process is dramatically increased in the presence of cyclic adenosine monophosphate (cAMP).[1] However, *in vivo*, ATRA-induced differentiation of AML cells has only been observed in APL patients. Apart from the PML-RARα fusion oncoprotein target, one explanation of this selective effect might be that the ATRA-receptor complex is unable to bind target gene promoters because of epigenetic chromatin modifications, such as hypermethylation of promoter CpG islands, recruitment of co-repressors, and histone amino acid deacetylation. Some studies using sodium phenylbutyrate as a histone deacetylase (HDAC) inhibitor in patients with AML or myelodysplastic syndromes (MDS) have already been reported.[2]

In the present study, we administered ATRA in combination with another HDAC inhibitor, namely valproic acid (VPA),[3] in elderly patients with AML. Of interest, VPA has been demonstrated to be able to synergize with ATRA in inducing the differentiation of non-M3 AML cells and more recently to be capable, as a single agent, of inducing clinical responses in patients with MDS or secondary AML.[4,5] All consecutive patients aged 70 years or more with *de novo* non-M3 AML unsuitable for intensive chemotherapy were eligible after informed consent had been obtained. Between November 2001 and August 2003, 11 patients (median age, 82 years) were treated (Table 1). Two eligible patients diagnosed during the same period were not included because of white blood cell counts (WBC) > 30×10^9/mm^3. Oral VPA was administered daily at the dosage required to reach therapeutic serum concentrations for treatment of seizures (between 50 and 100 μg/mL). ATRA was given daily at the dosage of 45 mg/m^2/day, beginning a week after the initiation of VPA. In order to increase intracellular cAMP levels, patients also received theophylline orally at the dosage required to reach therapeutic serum concentrations for treatment of asthma (between 10 and 15 μg/mL), at least when well tolerated. Patients with WBC > 10×10^9/mm^3 (patients nos. 1, 2, 5, 7, and 9) initially received low-dose hydroxyurea

Table 1. Patients' characteristics, response to therapy, and outcome.

Pt. N.	Age (years)	FAB subtype	BM blast percentage	Cytogenetics	Treatment duration	Response		Time to response	Response duration	Survival
						AML criteria[7]	MDS criteria[8]			
1	82	M0	80%	Complex	2 months	Progression	Progression	NA	NA	3 months
2	70	M2	70%	Normal	26 months	CR	CR	4 months	22 months	28 months
3	81	M2	80%	Normal	19 months	CRi	Stable	3 months	16 months	28 months
4	84	M2	42%	Complex	5 months	CRi	Stable	2 months	3 months	6 months
5	82	M2	53%	Normal	4 months	No response	MaR-N, MiR-E	2 months	2 months	4 months
6	83	M2	44%	Normal	2 months	No response	Stable	NA	NA	6 months
7	84	M2	40%	ND	1.5 months	Progression	Progression	NA	NA	2 months
8	79	M4	30%	Normal	6 months	No response	MaR-N	2 months	4 months	6 months
9	83	M5	80%	Complex	1 month	Progression	Progression	NA	NA	1 month
10	70	M6	75%	Complex	2 months	Progression	Progression	NA	NA	7 months
11	85	M6	52%	Normal	6 months	No response	Stable	NA	NA	8 months

ND: not done; NA: not applicable; BM: bone marrow; CR: complete remission; CRi: morphologic CR with incomplete blood count recovery; MaR-N: major neutrophil response; MiR-E: minor erythroid response.

Table 2. Results of non-intensive approaches in very old patients with AML treated in our institution.

	Present VPA/ATRA study	Low-dose cytarabine*	Best supportive care*
Patients (N.)	11	14	13
Median age (range)	82 y (70-85)	83 y (80-89)	82 y (80-89)
Performance status >2 (N.)	4	3	7
White blood cell count > 30×10^9/L (N.)	0	1	2
CR/CRi/PR (N)	1/2/0	2/1/2	0/0/0
Median survival	24 weeks	12 weeks	3 weeks
Alive at 12 months (N.)	2	3	0

**historical data; CR: complete remission; CRi: morphologic CR with incomplete blood count recovery; PR: partial remission.*

(500 mg twice a day for a maximum of 7 days). All patients were treated, as far as possible, as out-patients. They all received at least one month of the VPA/ATRA combination therapy. The median duration of VPA/ATRA therapy was 3 months (range, 1 to 26). Overall, the treatment was quite well tolerated. The main side effects were tremor, mental confusion (leading to treatment interruption in 2 patients), and theophylline-related palpitations. Target VPA serum concentrations were rapidly obtained in all patients. These concentrations have been shown to be high enough to induce HDAC inhibition *in vitro*, as well as *in vivo*.[3,4,6] Conversely, the target theophyylline serum concentrations were reached in only 4 patients (patients nos. 2, 3, 5, and 6), because of side effects.

Responses are presented in Table 1. According to AML criteria,[7] 3 patients (patients nos. 2, 3, and 4) responded. We observed one complete remission (CR) and two morphologic CR with incomplete blood count recovery (CRi) (Table 1). According to MDS criteria,[8] two additional patients (patients nos. 5 and 8) had hematologic improvement (Table 1). In these 5 responders, the median time to response was 2 months. Finally, all patients died within 1 to 28 months after initiation of treatment, all CR and CRi patients having AML recurrence before death. Because of the low numbers of patients, prognostic factors cannot be readily determined, but it was noteworthy that 4 responders had a normal karyotype. It should also be stressed that 3 responders had reached a therapeutic theophylline serum concentration. As we did not document ATRA-induced differentiation *in vivo*, we cannot confirm the mechanism through which VPA may have induced responses. It remains possible that VPA had direct differentiating and/or pro-apoptotic effects on AML cells.[9,10] Of note, responses have been observed using VPA alone in the Düsseldorf MDS study.[5]

In conclusion, VPA appears to be a safe, orally applicable HDAC inhibitor to be tested in combination with differentiating or demethylating agents, such as 5-azacytidine or decitabine, in elderly patients with AML. Even if hampered by obvious patient selection biases, a comparison of the results of the present study with recent unpublished historical data from AML patients aged 80 years or more from our institution not considered fit for intensive chemotherapy and treated with low-dose cytarabine or best supportive care is shown in Table 2.

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Key words: valproic acid, ATRA, elderly, acute myeloid leukemia, combination therapy.

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References

1. Duprez E, Lillehaug JR, Naoe T, Lanotte M. cAMP signalling is decisive for recovery of nuclear bodies (PODs) during maturation of RA-resistant t(15;17) promyelocytic leukemia NB4 cells expressing PML-RARα. Oncogene 1996;12:2451-9.
2. Gore SD, Weng LJ, Figg WD, Zhai S, Donehower RC, Dover G, et al. Impact of prolonged infusions of the putative differentiating agent sodium phenylbutyrate on myelodysplastic syndromes and acute myeloid leukaemia. Clin Cancer Res 2002;8:963-70.
3. Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J Biol Chem 2001;276:36734-41.
4. Göttlicher M, Minucci S, Zhu P, Krämer OH, Schimpf A, Giavara S, et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 2001;20:6969-78.
5. Kuendgen A, Strupp C, Aivado M, Bernhardt A, Hildebrandt B, Haas R, et al. Treatment of myelodysplastic syndromes with valproic acid alone or in combination with all-trans retinoic acid. Blood 2004;104:1266-9.
6. Kieslich M, Schwabe D, Cinatl J, Driever PH. Increase of fetal hemoglobin synthesis indicating differentiation induction in children receiving valproic acid. Pediatr Hematol Oncol 2003; 20:15-22.
7. Cheson BD, Bennett JM, Kopecky KJ, Büchner T, Willman CL, Estey EH, et al. Revised recommendations of the international working group for diagnosis, standardization of response criteria, treatment outcomes, and reporting standards for therapeutic trials in acute myeloid leukemia. J Clin Oncol 2003; 21:4642-9.
8. Cheson B, Bennett J, Kantarjian H, Pinto A, Schiffer CA, Nimer SD, et al. Report of an international working group to standardize response criteria for myelodysplastic syndromes. Blood 2000;96:3671-4.
9. Ichiyama T, Okada K, Lipton JM, Matsubara T, Hayashi T, Furukawa S. Sodium valproate inhibits production of TNF-α and IL-6 and activation of NF-κB. Brain Res 2000; 857:246-51.
10. Tang R, Faussat AM, Majdak P, Perrot JY, Chaoui D, Legrand O, et al. Valproic acid inhibits proliferation and induces apoptosis in acute myeloid leukemia cells expressing P-gp and MRP1. Leukemia 2004;18:1246-51.

Malignant Lymphomas

An enzyme-linked immunosorbent assay to screen for inhibitors of the oncogenic anaplastic lymphoma kinase

The discovery of novel anti-cancer drugs targeting anaplastic lymphoma kinase (ALK), an oncogenic tyrosine kinase, raises the need for *in vitro* assays suitable for screening compounds for ALK inhibition. To this aim we have developed and optimized an ALK-specific enzyme-linked immunosorbent assay that employs a novel ALK peptide substrate and purified ALK kinase domain.

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The anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase normally expressed in the developing nervous system.[1] However, chromosomal translocations involving the *ALK* gene (2p23) lead to the expression of constitutively activated ALK fusion proteins such as NPM/ALK, in tissues outside the nervous system.[2,3] ALK fusion proteins stimulate mitogenic and anti-apoptotic signaling pathways, leading to malignant transformation in cancers such as anaplastic large cell lymphoma (ALCL).[4-6] Therefore, ALK represents a valid target for pharmaceutical intervention. In the present study, we describe the development of an enzyme-linked immunosorbent assay (ELISA) that can be used to rapidly screen compounds for their ability to inhibit ALK *in vitro*.

His-tagged recombinant ALK (rALK) protein containing the predicted ALK kinase domain spanning amino acid residues 1116-1392 (NCBI Accession Code: Q9UM73), was expressed in Sf9 cells using a baculovirus expression system. rALK protein was purified to apparent homogeneity as assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining and was shown to maintain autophosphorylation activity throughout the purification procedure, indicating the correct folding of the purified protein (Figure 1A). Using purified rALK we identified a specific peptide substrate of ALK (peptide#1: ARDIYRASFFRKGGCAMLPVK) resembling the ALK activation loop (ARDIYRASYYRKGGCAMLPVK), which displayed a phosphorylation rate 3-fold higher than that of the general substrate, polyGlu4Tyr (Figure 1B). In the ALK-ELISA, peptide#1 was immobilized on an ELISA plate and phosphorylated by purified rALK in the presence of ATP and co-factors. The immobilized phosphorylated substrate was detected using anti-phosphotyrosine and horse-radish peroxidase (HRP)-conjugated antibodies. Addition of HRP substrate stimulated a colorimetric reaction which was detected by spectrophotometry. Assay conditions were optimized for measuring ALK inhibition. A saturating amount of peptide#1 (2.5 µg/well) and an amount of rALK within the linear range of the colorimetric response (15 – 150 ng protein/well) were used. Plotting phosphorylation of peptide#1 by rALK against time indicated that there was a linear increase in absorbance up to 16 minutes (Figure 1C). No increase in absorbance was observed in the absence of peptide or with a negative control peptide, indicating that background noise was negligible. A reaction time within the linear range (10 min) was chosen for maximum sensitivity in measuring ALK inhibition.

The reliability of the ALK-ELISA in measuring ALK inhibition was assessed using the general kinase inhibitor, staurosporine. In a conventional in vitro radioactive kinase assay, performed in the presence of 30 µM ATP, staurosporine inhibited phosphorylation of peptide#1 by rALK with an IC_{50} of 123 nM (Figure 2A). In the ALK-ELISA performed under the same conditions, the IC_{50} value for staurosporine was identical to that obtained in the radioactive assay (150 nM) (Figure 2B). In the presence of 300 µM ATP an IC_{50} value of approximately 700 nM was obtained in the ALK-ELISA. This increase in IC_{50} at higher concentrations of ATP reflects the competition between staurosporine and ATP for binding the ALK kinase domain. As the majority of kinase inhibitors developed so far are ATP competitors,[7] and considering the high concentrations of ATP observed in cells (>1 mM), it is desirable that in vitro screening assays for ALK inhibitors are performed in the presence of high concentrations of ATP. Unlike the radioactive assay, which is restricted by specific activity considerations, the ALK-ELISA can be performed at ATP concentrations approaching physiological concentrations, therefore should generate results closer to those observed in cells. Indeed, the IC_{50} value obtained in the presence of 300 µM ATP (700 nM) in the ALK-ELISA is closer to the