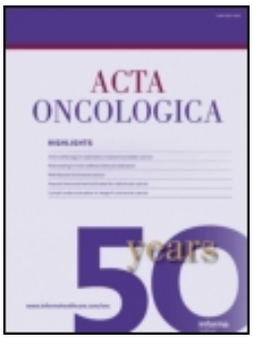

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LETTER TO THE EDITOR

Treatment with valproic acid, all-trans retinoic acid (ATRA) and theophyllamine for 9 days caused a persistent increase in peripheral blood platelet counts for a patient with acute myelogenous leukemia

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To the Editor

New therapeutic strategies are now considered in acute myelogenous leukemia (AML), including the use of histone deacetylase (HDAC) inhibitors, all-trans retinoic acid (ATRA) and the cyclic AMP increasing agent theophyllamine [1–5]. Firstly, the presently used and relatively unspecific HDAC inhibitors would be expected to alter acetylation of many intracellular proteins and not only histones [6], and modulation of gene expression through decreased histone acetylation is probably one of several pharmacological effects. Valproic acid is a short chain fatty acid that is used as an anticonvulsant but can also function as an HDAC inhibitor and induce differentiation and apoptosis of AML cells in vitro [1,2]. Secondly, ATRA is regarded as mandatory in the treatment of acute promyelocytic leukemia (APL), but its use in non-APL disease has not improved long-term AML-free survival even though in vivo effects on AML blasts have been detected for a relatively large fraction of treated patients [7]. Finally, increasing the intracellular levels of the second messenger cyclic AMP (e.g. by theophyllamine) is another therapeutic possibility [8]. For our present AML patient we combined these three therapeutic strategies.

Increased peripheral blood platelet counts have been described after treatment with several HDAC inhibitors (i.e. valproic acid, butyrate derivatives, depsipeptide) for a subset of AML and myelodysplastic syndrome (MDS) patients [1–5]. Most of these studies investigated continuous treatment with HDAC inhibitor plus ATRA. Even though hematological remission was very rare and was not observed in most studies, increased platelet counts may be an important effect for these patients who often experience severe thrombocytopenia. In the present report we present evidence that increased thrombopoiesis can occur early during treatment with valproic acid +ATRA +theophyllamine, and the platelet levels may continue to increase and persist at high levels for several weeks following short-term treatment.

Case history

The patients investigated represent the first six patients included in a clinical study. The investigation was approved by the local Ethic's Committee and patients included after informed consent.

The patient (female, born 1924) was treated for duodenal ulcer many years ago and had also received antihypertensive treatment for many years. In May 2003 carcinoma of the urinary bladder was diagnosed. This tumor was treated with transurethral resection. She did not receive cytotoxic drugs and later there was no evidence of local relapse or metastatic disease.

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In February 2004 she received the diagnosis Primary myelodysplastic syndrome (MDS). According to the WHO criteria the disease was subclassified as refractory cytopenia (anemia, neutropenia, thrombocytopenia) with multilineage dysplasia. She received only supportive therapy. AML FAB-type M1 was diagnosed in October the same year. Microscopy of bone marrow smears showed 50% myeloblasts and trilineage dysplasia. The AML cells had a normal karyotype, no genetic Flt3 abnormalities and membrane molecule phenotype $CD11C^+CD13^+CD14^-CD15^-CD33^+CD34^+CD117^+$.

The patient was treated with a combination of valproic acid (day 3 – 9), ATRA (day 1 – 9) and theophyllamine (day 3 – 9) (Table I). The treatment was stopped after nine days due to serious fatigue and gastrointestinal side effects, and later she received only supportive therapy. On day 77 a paraneoplastic dermatosis was diagnosed, biopsy showed only inflammation without evidence of leukemia cell infiltration. These symptoms resolved during 28 days of prednisolone therapy (initially 25 mg daily, thereafter gradual reduction). Her AML was regarded as stable until a high peripheral blood blast count was detected on day 120, and she died 25 days later.

We investigated the peripheral blood levels of total as well as reticulated platelets for the patient during and following therapy and these results were compared with five other patients receiving the same treatment. The percentage of reticulated platelets was then determined by flow cytometry. 1-Methyl-4-[(3-methyl-2(3*H*)-benzothiazolylidene)methyl] quinolinium*p*-tosylate (Thiazole orange, TO) (Sigma-Aldrich, St. Louis, MO) was dissolved in methanol at 1 mg/ml and stored dark at $-20^{\circ}C$ until diluted to 1 μg/ml with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) at the day of analysis. Methanol-free paraformaldehyde (Sigma-Aldrich) was diluted to 1% with PBS. Phycoerythrin (PE)-conjugated anti-GPIb (CD42b-PE) (Becton Dickinson, San Jose, CA) was used for platelet identification. Five μl EDTA-anticoagulated whole blood samples were incubated with 5 μl anti-CD42b and 50 μl TO-working solution in the dark at room temperature for 15 minutes, 1 ml 1% paraformaldehyde was thereafter added and samples analyzed by flow cytometry within 30 minutes. For each measurement 10 000 events were collected using a standard FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer equipped with an Argon laser (488 nm) and Cell Quest Lysis II software. Fluorescensce from TO and anti-CD42b-PE was detected through the 530/30 nm and 585/42 nm bandpass filters, respectively. A cytogram based on anti-CD42b and TO was made to define a threshold discriminating platelets from erythrocytes and TO-bright leukocytes. The platelets were gated and transferred to another cytogram (side scatter versus TO) for estimation of TO-positive platelets. In pilot studies of healthy individuals a region was defined to include 1% TO-positive platelets and exclude interference based on aggregate formation; patients were analyzed using the same regions. The mean percentage of TO-positive platelets was calculated from duplicate samples [9].

The patient's total peripheral blood platelet count showed a minor decrease during the nine days of therapy, whereas a marked increase in the level of reticulated platelets was detected at the end of the treatment period (Table I). The levels of reticulated platelets later declined. In contrast, the platelet count showed a marked increase that persisted for several weeks until disease progression even though the bone marrow blast percentage was not reduced during this period. For comparison, five other patients receiving the same therapy showed a similar decrease in peripheral blood platelets during treatment (10–60% decrease). A small increase in reticulated platelets was observed for two of these patients, but a marked increase was only observed for one additional patient (from 8.4 to 15.3%) who died from septicemia after 12 days. Furthermore, serum TPO levels (Quantikine ELISA kits; R&D Systems, Abingdon, UK) were also determined for all six patients. Pretherapy TPO and platelet levels and therapy-induced alterations in these levels showed no correlations (data not shown).

Discussion

Previous studies have demonstrated that increased peripheral blood platelet counts can be observed in AML patients after continuous, long-term treatment with valproic acid in combination with ATRA, whereas complete hematological remission seems to be very rare [1–5]. Common side effects that may require dose reduction or discontinuation of therapy are severe thrombocytopenia and neurological toxicity. However, side effects may become tolerable if short-term treatment regimens can be used.

Reticulated platelets represent the most recently released platelets and their circulating level is regarded to reflect thrombopoiesis [10]. In our present publication we describe an AML patient who developed an initial increase in reticulated platelets and later increased peripheral blood platelet counts following nine days therapy with valproic

Table I. Treatment of an AML patient with valproic acid, ATRA and theophyllamine.

		Peripheral blood cell counts				
Treatment period	Bone marrow microscopy	Day[1]	Total platelet counts ($\times 10^9$/L; normal level 140–400 $\times 10^9$/L)	Reticulated platelets (%; healthy individuals <2.6%)	Peripheral blood AML blast counts ($\times 10^9$/L)	TPO serum level (pg/ml)
Antileukemic therapy days 1–9:	Before start of therapy:	1	358	5, 28	<2.0	<31
– ATRA 22.5 mg/m^2 twice daily, days 1–9	Increased cellularity, 50%	3	↓271	6, 12	<2.0	<31
– Valproic acid days 3-8, serum levels 237–432 μM (therapeutic level 300–600)	myeloblasts and dysplastic erythropoiesis, megakaryopoiesis and granulopoiesis.	8	↓246	5, 9	<2.0	<31
– Theophyllamine days 3-9, serum levels 61–118 μM (therapeutic level 55–110)	Blast membrane molecule phenotype $CD11b^+CD13^+CD14^-CD15^-CD33^+CD34^+CD117^+$					
Stable AML with supportive therapy, days 10–125	Day 28: Increased cellularity with 50% myeloblasts. No change in morphology or immunophenotype	12	350	↑13, 41	<2.0	<31
		28	↑952	↑8, 85	<2.0	nt[2]
		49	↑639	3. 5	<2.0	nt
		77	↑445	nt	<2.0	nt
		98	↑442	nt	2.5	<31
Disease progression, days 120–145		120	235	nt	107	
		144	18	nt	165	
Death		145				

[1]Days after start of therapy.
[2]nt, not tested.

acid + ATRA + theophyllamine. Minor increases in reticulated platelets were also observed during the first week of treatment for other patients who received the same therapy. This increase probably reflects an early increase in thrombopoiesis that is commonly observed but less often sufficient to increase peripheral blood platelet counts. A possible molecular mechanism behind this effect could be valproic acid-induced alteration of acetylation of hematopoietic transcription factors with stimulation of remaining normal thrombopoiesis [11,12]. Prednisolone could not contribute to the platelet increase because the dermatosis occurred several weeks after the maximal platelet value.

Our present results demonstrate that a relatively long-lasting platelet response can be achieved in AML patients even after a single treatment period of nine days. This increase was preceeded by an early increase in the levels of circulating reticulated platelets. We therefore conclude that (i) intermittent treatment with HDAC inhibitors combined with ATRA and eventually theophyllamine should be further explored as supportive treatment in AML and possibly MDS; and (ii) an early increase in reticulated platelets may then be used to identify patients who will benefit from this treatment.

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