Leukemia (2006) 20, 1117–1122

www.nature.com/leu

ORIGINAL ARTICLE

Comparison between anagrelide and hydroxycarbamide in their activities against haematopoietic progenitor cell growth and differentiation: selectivity of anagrelide for the megakaryocytic lineage

Y Hong[1], G Wang[1], A Gutierrez del Arroyo[1], J Hernandez[1], C Skene[1] and JD Erusalimsky[1,2]

[1]*The Wolfson Institute for Biomedical Research, University College London, London, UK and* [2]*University of Wales Institute, Cardiff, Cardiff School of Health Sciences, Cardiff, UK*

Anagrelide (ANA) and hydroxycarbamide (HC) are two distinct pharmacological agents used to treat thrombocythaemia associated with myeloproliferative disorders. Although both drugs have been in clinical use for a number of years, comparative studies of their selectivity and mode of action are still lacking. Here, we have evaluated the activities of ANA and HC on the growth and differentiation of human haematopoietic progenitor cells in liquid culture. Both drugs inhibited thrombopoietin-induced megakaryocytopoiesis in a dose-dependent manner, but with strikingly different potencies ($IC_{50} = 26$ nM for ANA and 30 μM for HC) and modes of action. Whereas HC inhibited cell proliferation, ANA acted primarily on the differentiation process. At doses that abrogated megakaryocytopoiesis, HC also inhibited the expansion of $CD34^+$ cells stimulated by stem cell factor, interleukin-3 and Flt-3 ligand and also induced apoptosis. Furthermore, HC inhibited erythroid and myelomonocytic cell growth, induced by erythropoietin or granulocyte–macrophage colony-stimulating factor, respectively. In contrast, ANA showed none of these additional effects. Taken together, these results demonstrate that ANA is a potent and selective inhibitor of megakaryocytopoiesis, having no significant activity against haematopoietic progenitor cell expansion or differentiation into other lineages. In contrast, the anti-megakaryocytopoietic activity of HC cannot be dissociated from its more general cytoreductive and cytotoxic actions.
Leukemia (2006) **20,** 1117–1122. doi:10.1038/sj.leu.2404180; published online 23 March 2006
Keywords: megakaryocyte; thrombocythaemia; hydroxyurea; anagrelide; platelet reduction

Introduction

Anagrelide (ANA) and hydroxycarbamide (HC) are two distinct pharmacological agents that inhibit bone marrow megakaryocytopoiesis.[1–3] This effect underlies their effectiveness in reducing the high platelet count associated with myeloproliferative disorders.[4–7] In addition, these agents have other distinctive biological actions (reviewed by Hong and Erusalimsky[8]). Hydroxycarbamide, previously referred to as hydroxyurea, inhibits cell proliferation, is teratogenic in animals and a clastogen in cell culture (reviewed by IARC Working Group[9]). Anagrelide inhibits platelet aggregation, causes vasodilatation and has inotropic effects. Hydroxycarbamide is a simple chemical antimetabolite that inhibits ribonucleoside diphosphate reductase (reviewed by Yarbro[10]). This enzyme catalyses the conversion of ribonucleosides into deoxyribonucleosides, which are the building blocks of DNA synthesis and repair. Inhibition of ribonucleoside diphosphate reductase explains the cytoreductive effects of this compound, the concerns about its mutagenic potential and its platelet-lowering action. Anagrelide, in contrast, is an imidazoquinazoline derivative, whose only known primary target is a Type III phosphodiesterase found in platelets and the myocardium.[11,12] Inhibition of this enzyme accounts for the activity of ANA against platelet aggregation and for its inotropic and vasodilatadory effects,[7] but not for the inhibition of megakaryocyte development.[13]

Because of these different pharmacological profiles, comparison between ANA and HC with regard to their selectivity and mode of action is important to help in the evaluation of their relative risk to benefit ratio. However, surprisingly, comparative studies of this kind are still lacking. In an effort to fill this gap, we have evaluated the activities of ANA and HC on the growth and differentiation of human haematopoietic progenitor cells in liquid culture. Here, we report that ANA is 1000-fold more potent than HC as an inhibitor of megakaryocytopoiesis and that the two compounds have a different mode of action. Whereas HC inhibits cell proliferation, ANA acts primarily on the differentiation component of megakaryocytopoiesis. Furthermore, we show that HC but not ANA, at concentrations that effectively inhibit megakaryocytic cell growth, abrogates the expansion of haematopoietic progenitor cells and induces apoptosis. Finally, we demonstrate that HC, unlike ANA, also inhibits erythroid and myelomonocytic cell growth. These studies suggest that whereas the platelet-lowering effects of ANA are due to selective inhibition of megakaryocytopoiesis, those of HC are part of a more generalized myelosuppressive effect.

Materials and methods

Cell culture

$CD34^+$ cells were purified from human umbilical cord blood by magnetic immunoselection with a purity of >95% and then cultured for up to 12 days in Iscove's modified Dulbecco's medium supplemented with 10% human umbilical cord blood plasma, 0.2% bovine serum albumin, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids (Gibco Invitrogen, Paisley, UK), minimal essential medium vitamins (Gibco Invitrogen), 0.1 mM 2-mercaptoethanol, 100 U/ml penicillin, 0.1 mg/ml streptomycin and diverse haematopoietic growth factors, as indicated in the figure legends. Detailed procedures of cell isolation and culture have been previously described.[14,15]

Correspondence: Dr JD Erusalimsky, The Wolfson Institute for Biomedical Research, University College London, Cruciform Building, Gower Street, London WC1E 6BT, UK.
E-mail: j.erusalimsky@ucl.ac.uk
Received 26 July 2005; revised 30 January 2006; accepted 7 February 2006; published online 23 March 2006

Drug treatments

Stock solutions of anagrelide hydrochloride (10 mM) (Cambridge Major Laboratories Inc., Germantown, WI, USA) and HC (0.5 M) (Sigma, Poole, Dorset, UK) were made in DMSO and water, respectively, and then stored in aliquots at −20°C. Drugs were diluted in culture medium immediately before the initiation of treatment. Treatments were commenced 1 or 4 days after the start of the culture period by adding the drug or an equivalent amount of vehicle in a volume of 20 μl to 1 ml cell suspension ($0.15–0.2 \times 10^6$ cells/ml). Following addition of the drug, cells were left to grow undisturbed until the completion of the incubation period. Treatments were carried out in 2–3 replicates.

Cell counting

The final cell density of the cultures was determined using a Sysmex CDA-500 Particle Analyser (Malvern Instruments, Worcs, UK). In some experiments, viable cell counting was carried out in 0.2% Trypan blue using a haemocytometer.

Analysis of lineage differentiation

Phenotypic differentiation was monitored by flow cytometry using the following fluorescein isothiocyanate-conjugated monoclonal antibodies: Y2/51 (DAKO, Ely, UK), which detects the megakaryocytic lineage-specific marker CD61 (glycoprotein IIIa); CLB-409 (Cymbus Biotechnology, Hants, UK), which recognizes the erythroid differentiation marker glycophorin A; and MϕP9 (Becton Dickinson, Oxford, UK), which detects the antigen CD14 on myelomononcytic cells. Cells were stained and analysed as described previously.[14,15] The fraction of antigen-positive cells was established according to the fluorescence distribution of cells stained with an isotype-matched control antibody. The number of differentiated cells was calculated by multiplying the total number of cells in the culture by the fraction of antigen-positive cells.

Detection of apoptosis

Apoptotic cells were detected by flow cytometry using dual staining with annexin V and propidium iodide (Annexin-V-FLUOS Staining Kit, Roche Applied Science, Lewes, UK) as described previously.[16] In addition, the activity of caspase-3 and -7 was examined using the proluminescent substrate DEVD-aminoluciferin (Caspase-Glo™ assay, Promega, Southampton, UK), according to the manufacturer's instructions. Luminescence was measured in a Tecan ULTRA multifunctional plate reader (Tecan, Austria) and luminescence units were normalized to the viable cell number as described previously.[17]

Statistical analysis

Experiments were repeated at least three times, using for each experiment CD34$^+$ cells from a different donor. To compare data from different experiments, results are expressed relative to an untreated sample run in parallel. Results were evaluated by the Student's *t*-test or by analysis of variance followed by the Student–Newman–Keuls *post hoc* test, as appropriate. A value of $P<0.05$ was considered to denote statistical significance.

Results

To compare the effects of ANA and HC on megakaryocytopoiesis, we cultured human CD34$^+$ progenitor cells in the presence of thrombopoietin (TPO) and then monitored the expression of CD61 by flow cytometry, as an index of megakaryocyte development. The selection of drug concentration ranges tested in this study was guided by previously published pharmacokinetic data indicating that, at doses usually used in myeloproliferative diseases, the mean peak plasma concentrations (C_{max}) of ANA and HC lie around the ~20 nM and ~100 μM values, respectively (reviewed by IARC Working Group[9] and Pescatore and Lindley[18]). Figure 1 shows representative flow cytometric profiles of CD61 expression after 12 days of culture. In control cultures, the majority of cells expressed very high levels of this megakaryocytic differentiation marker, whereas in cultures treated with 30 nM ANA, the fraction of CD61^{bright} cells was reduced by ~2.4-fold. In contrast, although addition of 30 μM HC affected the overall number of cells in the culture (see below), in this case a reduction in the fraction of CD61^{bright} cells was not observed (see Figure 1). The dose–responses for the overall effect of these drugs on the total number of megakaryocytes produced are shown in Supplementary Figure S1. These results demonstrate that ANA and HC inhibited TPO-induced megakaryocytopoiesis with strikingly different potencies, ANA being about 1000-fold more potent than HC ($IC_{50}=26$ nM for ANA and 30 μM for HC). Nonetheless, the maximum reduction in megakaryocytes achieved by ANA was 75–80% compared to ~100% with

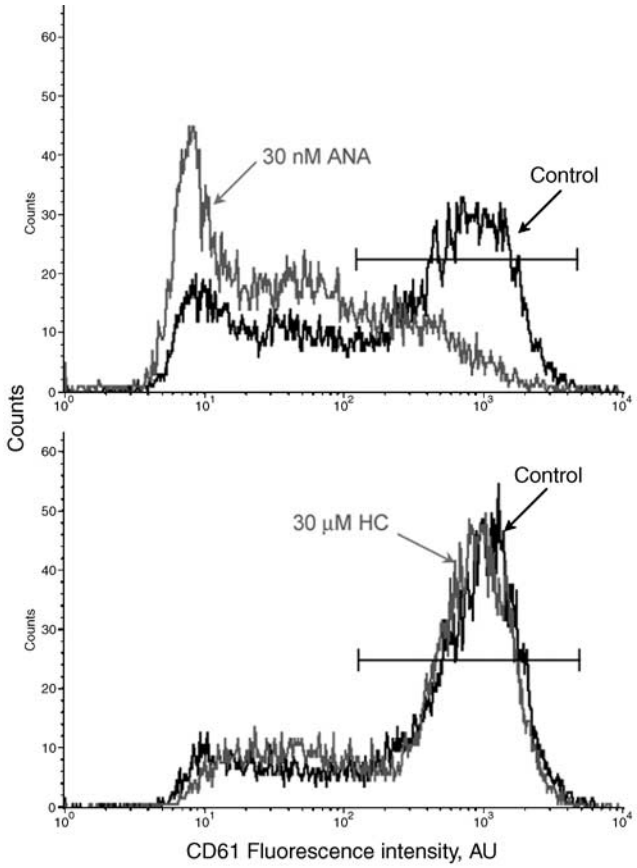

Figure 1 Flow cytometric histograms of CD61 expression in megakaryocyte cultures treated with ANA and HC. CD34$^+$ cells were cultured for 12 days with 40 ng/ml thrombopoietin and then analysed by flow cytometry. Drugs were added after 4 days of culture. The marker encompasses the CD61^{bright} cell fraction. Note that in the ANA-treated cultures the increase in the first peak represents the CD61$^-$ non-megakaryocytic cells.

HC. Thus, although ANA showed higher potency, HC displayed an apparent greater efficacy. Similar results were obtained when drugs were added after 1 or 4 days of culture (data not shown).

To assess whether the inhibitory effects of ANA and HC in these cultures arised from a selective activity against the megakaryocytic lineage, we examined the action of these compounds on the growth of the non-megakaryocytic cells ($CD61^-$). In 12-day control cultures, these cells represent 20–35% of the total population (Figure 1 and data not shown). As depicted in Figure 2, at 30 μM (its IC_{50} for megakaryocyte growth), HC caused only a small (~15%) and not significant reduction in the number of non-megakaryocytic cells, whereas at 100 μM, it inhibited the growth of both megakaryocytic and non-megakaryocytic cells essentially completely. In sharp contrast, at every concentration tested, ANA inhibited only megakaryocytic cell growth; furthermore, ANA appeared to shift cell growth to the non-megakaryocytic compartment, an effect that suggested that it was devoid of cytotoxic activity. To substantiate these findings, we examined the effects of ANA and HC on cell death by annexin V/propidium iodide staining under conditions of TPO-stimulated growth. As summarized in Supplementary Table S1, ANA showed no cytotoxic effects. In contrast, HC, at concentrations that effectively inhibit megakaryocyte growth, reduced cell viability and induced apoptotic cell death. Consistent with these findings, as shown in Table 1, HC also inhibited the expansion of $CD34^+$ cells stimulated by interleukin-3 (IL-3), stem cell factor (SCF) and Flt-3 ligand; furthermore, also under these growth conditions, it induced apoptosis. In contrast, in these experiments, ANA showed no cytotoxic activity, revealing only a marginal cytostatic effect at doses above those required for maximal inhibition of megakaryocytopoiesis (Table 1). To corroborate these results, we also examined an additional marker of apoptosis, namely caspase-3/7 activity. As shown in Figure 3, in agreement with the annexin V/propidium iodide data, an increase in caspase-3/7 activity was detected only in HC-treated cells, thus confirming that ANA did not induce apoptosis.

To obtain further evidence for the lack of selectivity of HC against the megakaryocytic lineage in comparison to that of ANA, we examined the activity of both drugs against the development of other haematopoietic lineages. As shown in Figure 4 (middle panel), HC inhibited significantly and to similar extents the growth of megakaryocytic, erythroid and myelomonocytic cells induced by TPO, erythropoietin (EPO) and granulocyte–macrophage colony-stimulating factor (GM-CSF), respectively. In these experiments, it is noteworthy that although HC reduced the overall number of cells, irrespective of the culture conditions (Figure 4, left panel), it did not reduce significantly the fraction of differentiated cells within each culture (Figure 4, right panel); that is, it did not affect the process of differentiation proper. In contrast, ANA significantly inhibited only megakaryocytic growth (middle panel), reducing both the overall number of cells (left panel) and the fraction of differentiated $CD61^+$ cells (right panel).

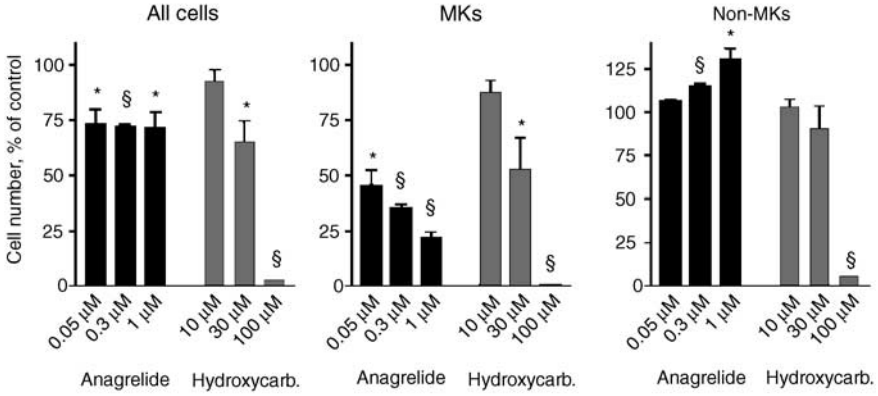

Figure 2 Effect of anagrelide and hydroxycarbamide on the growth of non-megakaryocytic cells. $CD34^+$ cells were cultured for 12 days with 40 ng/ml thrombopoietin. Drugs were added after 1 day of culture. Results show the total number of $CD61^-$ and $CD61^+$ cells relative to untreated samples. Values represent the mean ± s.d. of three experiments. $^*P<0.05$; $^{\S}P<0.01$.

Table 1 Differential effects of anagrelide and hydroxycarbamide on the expansion and survival of $CD34^+$ cells grown with SCF, IL-3 and FL-3 ligand

	Cell expansion		*Early apoptosis*	*Late apoptosis and necrosis*
	Density ($\times 10^6$/ml)	*Fold*	*Annexin V^+ PI^- (% of total)*	*Annexin V^+ PI^+ (% of total)*
Control	2.67 ± 0.10	18.4 ± 0.7	1.5 ± 0.1	1.2 ± 0.1
ANA 0.1 μM	2.64 ± 0.09	18.2 ± 0.6	1.6 ± 0.1	1.4 ± 0.1
ANA 1.0 μM	2.64 ± 0.06	18.0 ± 0.4	1.5 ± 0.1	1.3 ± 0.1
ANA 10.0 μM	2.38 ± 0.20	17.0 ± 1.3	1.6 ± 0.1	1.3 ± 0.1
HC 30.0 μM	0.97 ± 0.13‡	7.1 ± 0.8‡	2.3 ± 0.1‡	2.6 ± 0.2‡
HC 100.0 μM	0.46 ± 0.06‡	3.4 ± 0.4 ‡	10.4 ± 0.3‡	23.4 ± 2.7‡

Abbreviations: ANA, anagrelide; HC, hydroxycarbamide; IL-3, interleukin-3; SCF, stem cell factor.
$CD34^+$ cells were cultured for 4 days with 50 ng/ml SCF, 10 ng/ml IL-3 and 100 ng/ml FL-3 ligand. Drugs were added after 1 day of culture. Results represent the mean ± s.d. of three replicates from one representative experiment. $^‡P<0.01$.

Discussion

Megakaryocytes develop from CD34$^+$ multipotent haematopoietic progenitors through a complex process of proliferation and differentiation, driven primarily by the hormone TPO.[19] This process can be reproduced *ex vivo* by culturing isolated CD34$^+$ cells in liquid media supplemented with TPO. Such culture systems have been used to investigate some of the processes involved in the regulation of platelet production[20] as well as the mechanisms that underlie the platelet-lowering effects of drugs such as ANA[3] and interferon-α.[21] Similar *ex vivo* studies examining the effects of HC on TPO-induced megakaryocyte development have not been reported. Furthermore, comparative studies on the selectivity of platelet-lowering drugs using these systems have not been undertaken. The results reported here demonstrate that ANA and HC inhibit megakaryocytopoiesis by different mechanisms. Hydroxycarbamide acts mainly by inhibiting TPO-induced cell proliferation, whereas ANA acts primarily on the differentiation phase of megakaryocytopoiesis and to a lesser extent on the proliferation phase of this process. In addition, the present study shows that HC induces apoptosis and inhibits a number of additional haematopoietic processes including erythroid cell growth induced by EPO, myelomonocytic cell growth induced by GM-CSF and the expansion of early haematopoietic progenitors induced by a combination of IL-3, SCF and Flt-3 ligand. Anagrelide induces none of these additional effects to a significant degree. Taken together, these findings indicate that inhibition of megakaryocyte development by ANA arises from a potent and selective action on this lineage. In contrast, the anti-megakaryocytopoietic activity of HC results from non-selective cytostatic and cytotoxic actions that emanate from its known effects on DNA synthesis and repair, rather than from an effect on the process of differentiation proper.

A salient finding of the present study is that the concentrations of ANA required to reduce megakaryocyte development by 50% were 1000-fold lower than those of HC. Importantly, the observed IC_{50} values are in the range of the respective concentrations of ANA and HC reached in plasma at doses usually employed in anti-thrombocythaemic therapy.[9,18] One pharmacokinetic report on HC indicates that the C_{max} after a 0.5 g dose, given twice per day, was ~100 μM,[22] a concentration that, in the present study, caused complete inhibition of both megakaryocytic and non-megakaryocytic cell growth (Supplementary Figures S1 and S2), and also showed profound cytotoxic effects (Supplementary Tables S1 and Table 1). By contrast, the C_{max} after administration of a 1 mg dose of ANA was reported to be ~20 nM (5 ng/ml),[23] whereas in another pharmacokinetic study, a 2 mg dose (the highest recommended single dose of ANA) gave a C_{max} of ~50 nM (12 ng/ml) (R Franklin, personal communication). These plasma concentrations of ANA are well within the range that, in the present study, was shown to be effective in reducing megakaryocyte growth in

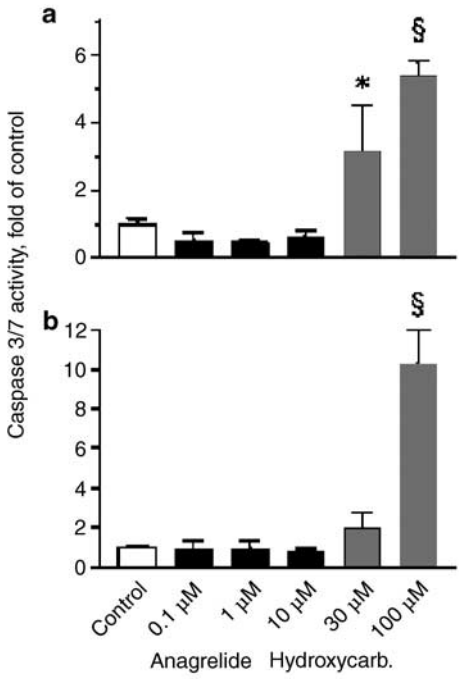

Figure 3 Differential effects of anagrelide and hydroxycarbamide on caspase-3/7 activity in cultures of CD34$^+$ cells. Cells were grown for 5 days with 40 ng/ml thrombopoietin (**a**) or for 3 days with 50 ng/ml stem cell factor, 10 ng/ml interleukin-3 and 100 ng/ml FL-3 ligand (**b**). Drugs were added after the first day of culture. Caspase activity is expressed relative to untreated samples. Results represent the mean ± s.d. of three (top) or four (bottom) replicates from one representative experiment. *$P<0.05$; §$P<0.01$.

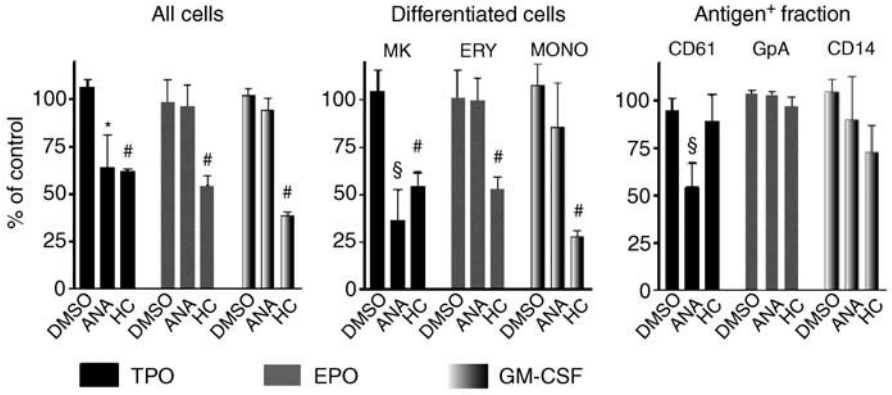

Figure 4 Comparison between ANA and HC for their effects on haematopoietic lineage differentiation. CD34$^+$ cells were cultured for 12 days with either 40 ng/ml TPO, 8 U/ml EPO or 20 ng/ml GM-CSF. HC (30 μM), ANA (0.8 μM) or DMSO was added after 1 day of culture. Values represent the mean ± s.d. of 3–4 experiments and are expressed relative to the corresponding untreated samples. *$P<0.05$ or §$P<0.01$ vs DMSO; #$P<0.01$ vs control. MK, ERY and MONO denote megakaryocytic, erythroid and myelomonocytic cells, respectively.

a selective manner and without any apparent cytotoxic effect. Interestingly, although there are substantial concordances between the present *in vitro* findings and the reported *in vivo* effects of these drugs, some differences are noteworthy. In our study, ANA did not inhibit EPO-induced erythroid or GM-CSF-induced myelomonocytic cell growth. These findings are in agreement with previous *in vitro* studies showing that ANA does not affect erythroid or granulocytic–macrophage colony formation in semi-solid cultures.[24] In contrast, *in vivo* ANA has been reported to induce mild anaemia.[5,25–27] Although the origin of this side effect remains to be fully elucidated, the present study suggests that it is unrelated to inhibition of erythroid differentiation. This interpretation is consistent with findings from a previous clinical study showing that anaemia is detected at too early a time for it to be the result of bone marrow inhibition.[26] Furthermore, our results lend further support to previously made suggestions that the anaemia is a reflection of haemodilution secondary to the vasodilatory effects of ANA.[26,27] On the other hand, our study does not rule out entirely the possibility that *in vivo* ANA may affect erythropoiesis in a manner that is not detectable using this culture system.

In the case of HC, its anti-megakaryocytic activity could not be dissociated from its pleiotropic cytoreductive effects. Consistent with these findings, administration of this drug to patients with polycythaemia vera (PV) or essential thrombocythaemia (ET) has been shown to reduce significantly the number of circulating $CD34^+$ cells, as well as the erythroid and CFU-GM colonies grown from their peripheral blood; in the case of ET, this reduction reached levels below those found in normal individuals.[28] Nevertheless, it is important to emphasize that at the doses used to treat myeloproliferative disorders, HC rarely shows acute toxicity and generally has been reported to be well tolerated.[29,30] This low toxicity *in vivo* does not rule out the possibility that more subtle effects associated with progenitor cell depletion or disruption of tissue repair mechanisms, in which bone marrow stem cells are now known to be involved,[31] may occur in the longer term.

For a number of years, HC has been the standard therapeutic treatment to reduce platelet counts in ET and PV for patients at high risk of thrombosis.[6,29] More recently, mainly because of concern about the leukemogenic potential of HC,[32] ANA has also become widely used.[25,26,33–35] However, the choice between the two drugs is often uncertain. Results from a recent clinical trial comparing HC and ANA in high-risk ET patients showed an increased frequency of vascular events and more cases progressing to myelofibrosis in the ANA group.[36] Thus, the findings from this trial raise the possibility that beyond its effects on megakaryocytopoiesis, the broader cytoreductive activity of HC may in fact be beneficial for certain patients. In this regard, considering the alleged involvement of activated monocytes in myelofibrosis,[37] a reduction in the number of these cells could explain the lower number of cases progressing to this condition in the HC-treated group. Similarly, it has been suggested that a reduction in white blood cells, given their pro-coagulant role, could result in a lower number of thrombotic events.[36] However, this possibility should be taken cautiously because in the HC-treated group, there was in fact an increase in the incidence of deep vein thrombosis, whereas in ANA-treated patients, of all the thrombotic complications assessed, only transient ischaemic attacks were significantly increased. The reasons for these seemingly contradictory findings are presently unclear, although the possibility that some of them could be due to the relative small number of events or to heterogeneity in the study groups has been raised.[38]

In the present study, inhibition of megakaryocytopoiesis by ANA was associated with an increase in non-megakaryocytic cells. It could be argued that the increase in this fraction might be related to some of the adverse events observed in the above-mentioned clinical trial. However, the possibility that *in vivo* ANA might promote proliferation of non-megakaryocytic cells seems unlikely given that this has never been reported and that in the clinical trial an increase in transformation to acute myelogenous leukaemia was not observed.[36] Furthermore, it is also unlikely that the increase in the non-megakaryocytic fraction is related to the higher incidence of myelofibrosis referred to above, at least in so far as these cells were non-fibroblastic in nature (data not shown), and ANA did not cause an increase in monocytic cells (Figure 4). Thus, in our study, the increase in the non-megakaryocytic fraction is most probably related to the growth characteristics of the culture system, in which proliferation and differentiation forces are in dynamic equilibrium.

When considering the clinical significance of our findings, it should also be emphasised that comparisons between the two drugs were made using cells from normal donors, which require cultivation in the presence of growth factors. In contrast, in myeloproliferative disorders, the haematopoietic precursors often show autonomous growth characteristics. In this respect, it was recently found that a significant proportion of patients with ET and the majority of those with PV carry an activating somatic mutation in the protein tyrosine kinase JAK2 (reviewed by Kaushansky[39]). Because JAK2 activation is an early signal transduction event shared by different haematopoietic growth factors, this mutation is likely to have pleiotropic effects. Our results suggest that ANA would only oppose the effect of the mutation on platelet production, whereas HC would counteract the effects of the mutation in all myeloid lineages. Thus, although our findings suggest that *in vivo*, ANA may lower platelet count with less deleterious haematological consequences, they also indicate that for certain patients, non-specific myelosupression with HC may lead to more favourable outcomes. Conversely, the present study does not rule out entirely the possibility that therapeutically HC could show some degree of selectivity in those cases in which the aetiology of the disorder is such that only the megakaryocytic lineage is affected. Finally, the findings that ANA and HC have different mode of actions call for further investigations to assess whether combination therapy with the two drugs may have clinical advantages for some patients.

Acknowledgements

We thank Dr Richard Franklin and Dr Maggi Moss for their valuable advice during the course of this work.

References

1 Jerushalmy Z, Payta M, Pinkhas J, De Vries A. Alteration of rat bone marrow megakaryocytes following administration of cytosine arabinoside, daunomycin and hydroxyurea. *Haemostasis* 1977; **6**: 110–117.
2 Mazur EM, Rosmarin AG, Sohl PA, Newton JL, Narendran A. Analysis of the mechanism of anagrelide-induced thrombocytopenia in humans. *Blood* 1992; **79**: 1931–1937.
3 Solberg Jr LA, Tefferi A, Oles KJ, Tarach JS, Petitt RM, Forstrom LA *et al*. The effects of anagrelide on human megakaryocytopoiesis. *Br J Haematol* 1997; **99**: 174–180.

4 Lofvenberg E, Wahlin A. Management of polycythaemia vera, essential thrombocythaemia and myelofibrosis with hydroxyurea. *Eur J Haematol* 1988; **41**: 375–381.
5 Anagrelide Study Group. Anagrelide, a therapy for thrombocythemic states: experience in 577 patients. *Am J Med* 1992; **92**: 69–76.
6 Cortelazzo S, Finazzi G, Ruggeri M, Vestri O, Galli M, Rodeghiero F *et al.* Hydroxyurea for patients with essential thrombocythemia and a high risk of thrombosis. *N Engl J Med* 1995; **332**: 1132–1136.
7 Tefferi A, Silverstein MN, Petitt RM, Mesa RA, Solberg Jr LA. Anagrelide as a new platelet-lowering agent in essential thrombocythemia: mechanism of actin, efficacy, toxicity, current indications. *Semin Thromb Hemost* 1997; **23**: 379–383.
8 Hong Y, Erusalimsky JD. Comparison of the pharmacological mechanisms involved in the platelet lowering actions of anagrelide and hydroxyurea: a review. *Platelets* 2002; **13**: 381–386.
9 IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Hydroxyurea. *IARC Monog* 2000; **76**: 347–386.
10 Yarbro JW. Mechanism of action of hydroxyurea. *Semin Oncol* 1992; **19**: 1–10.
11 Gillespie E. Anagrelide: a potent and selective inhibitor of platelet cyclic AMP phosphodiesterase enzyme activity. *Biochem Pharmacol* 1988; **37**: 2866–2868.
12 Beavo JA. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol Rev* 1995; **75**: 725–748.
13 Wang G, Franklin R, Hong Y, Erusalimsky JD. Comparison of the biological activities of anagrelide and its major metabolites in haematopoietic cell cultures. *Br J Pharmacol* 2005; **146**: 324–332.
14 Bobik R, Hong Y, Breier G, Martin JF, Erusalimsky JD. Thrombopoietin stimulates VEGF release from c-Mpl-expressing cell lines and haematopoietic progenitors. *FEBS Lett* 1998; **423**: 10–14.
15 Mathur A, Hong Y, Wang G, Erusalimsky JD. Assays of megakaryocyte development: surface antigen expression, ploidy, and size. *Methods Mol Biol* 2004; **272**: 309–322.
16 Beltran B, Mathur A, Duchen MR, Erusalimsky JD, Moncada S. The effect of nitric oxide on cell respiration: a key to understanding its role in cell survival or death. *Proc Natl Acad Sci USA* 2000; **97**: 14602–14607.
17 Wesierska-Gadek J, Gueorguieva M, Ranftler C, Zerza-Schnitzhofer G. A new multiplex assay allowing simultaneous detection of the inhibition of cell proliferation and induction of cell death. *J Cell Biochem* 2005; **96**: 1–7.
18 Pescatore SL, Lindley C. Anagrelide: a novel agent for the treatment of myeloproliferative disorders. *Expert Opin Pharmacother* 2000; **1**: 537–546.
19 Kaushansky K. Thrombopoietin: the primary regulator of platelet production. *Blood* 1995; **86**: 419–431.
20 Kaushansky K. Thrombopoietin: understanding and manipulating platelet production. *Annu Rev Med* 1997; **48**: 1–11.
21 Wang Q, Miyakawa Y, Fox N, Kaushansky K. Interferon-alpha directly represses megakaryopoiesis by inhibiting thrombopoietin-induced signaling through induction of SOCS-1. *Blood* 2000; **96**: 2093–2099.
22 Villani P, Maserati R, Regazzi MB, Giacchino R, Lori F. Pharmacokinetics of hydroxyurea in patients infected with human immunodeficiency virus type I. *J Clin Pharmacol* 1996; **36**: 117–121.
23 Gaver RC, Deeb G, Pittman KA, Smyth RD. Disposition of anagrelide, an inhibitor of platelet aggregation. *Clin Pharmacol Ther* 1981; **29**: 381–386.
24 Silverstein MN, Petitt RM, Solberg Jr LA, Fleming JS, Knight RC, Schacter LP. Anagrelide: a new drug for treating thrombocytosis. *N Engl J Med* 1988; **318**: 1292–1294.
25 Kornblihtt LI, Vassallu PS, Heller P, Molinas FC. Treatment of essential thrombocythemia with anagrelide: a ten-year experience. *Medicina (B Aires)* 2002; **62**: 231–236.
26 Birgegard G, Bjorkholm M, Kutti J, Larfars G, Lofvenberg E, Markevarn B *et al.* Adverse effects and benefits of two years of anagrelide treatment for thrombocythemia in chronic myeloproliferative disorders. *Haematologica* 2004; **89**: 520–527.
27 Storen EC, Tefferi A. Long-term use of anagrelide in young patients with essential thrombocythemia. *Blood* 2001; **97**: 863–866.
28 Andreasson B, Swolin B, Kutti J. Hydroxyurea treatment reduces haematopoietic progenitor growth and CD34 positive cells in polycythaemia vera and essential thrombocythaemia. *Eur J Haematol* 2000; **64**: 188–193.
29 Fruchtman SM, Mack K, Kaplan ME, Peterson P, Berk PD, Wasserman LR. From efficacy to safety: a Polycythemia Vera Study group report on hydroxyurea in patients with polycythemia vera. *Semin Hematol* 1997; **34**: 17–23.
30 Finazzi G, Ruggeri M, Rodeghiero F, Barbui T. Efficacy and safety of long-term use of hydroxyurea in young patients with essential thrombocythemia and a high risk of thrombosis. *Blood* 2003; **101**: 3749.
31 Rafii S, Meeus S, Dias S, Hattori K, Heissig B, Shmelkov S *et al.* Contribution of marrow-derived progenitors to vascular and cardiac regeneration. *Semin Cell Dev Biol* 2002; **13**: 61–67.
32 Sterkers Y, Preudhomme C, Lai JL, Demory JL, Caulier MT, Wattel E *et al.* Acute myeloid leukemia and myelodysplastic syndromes following essential thrombocythemia treated with hydroxyurea: high proportion of cases with 17p deletion. *Blood* 1998; **91**: 616–622.
33 Penninga E, Jensen BA, Hansen PB, Clausen NT, Mourits-Andersen T, Nielsen OJ *et al.* Anagrelide treatment in 52 patients with chronic myeloproliferative diseases. *Clin Lab Haematol* 2004; **26**: 335–340.
34 Steurer M, Gastl G, Jedrzejczak WW, Pytlik R, Lin W, Schlogl E *et al.* Anagrelide for thrombocytosis in myeloproliferative disorders: a prospective study to assess efficacy and adverse event profile. *Cancer* 2004; **101**: 2239–2246.
35 Petrides PE. Anagrelide: a decade of clinical experience with its use for the treatment of primary thrombocythaemia. *Expert Opin Pharmacother* 2004; **5**: 1781–1798.
36 Harrison CN, Campbell PJ, Buck G, Wheatley K, East CL, Bareford D *et al.* Hydroxyurea compared with anagrelide in high-risk essential thrombocythemia. *N Engl J Med* 2005; **353**: 33–45.
37 Rameshwar P, Narayanan R, Qian J, Denny TN, Colon C, Gascon P. NF-κB as a central mediator in the induction of TGF-β in monocytes from patients with idiopathic myelofibrosis: an inflammatory response beyond the realm of homeostasis. *J Immunol* 2000; **165**: 2271–2277.
38 Wagstaff AJ, Keating GM. Anagrelide: a review of its use in the management of essential thrombocythaemia. *Drugs* 2006; **66**: 111–131.
39 Kaushansky K. On the molecular origins of the chronic myeloproliferative disorders: it all makes sense. *Blood* 2005; **105**: 4187–4190.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)