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Growth inhibition and differentiation induction in human monoblastic leukaemia cells by 1α-hydroxyvitamin D derivatives and their enhancement by combination with hydroxyurea

M Makishima, J Okabe-Kado and Y Honma

Department of Chemotherapy, Saitama Cancer Center Research Institute, 818 Komuro, Ina-machi, Kita-adachi, Saitama 362, Japan

Summary The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), is a potent inducer of differentiation in myeloid leukaemia cells, but its clinical use is limited because of its hypercalcaemic activity. We examined the ability of 1,25$(OH)_2D_3$ in combination with several anti-cancer drugs to inhibit the proliferation of, and induce differentiation in, human monoblastic leukaemia U937 cells. Hydroxyurea (HU), cytarabine and camptothecin showed effective synergism with 1,25$(OH)_2D_3$ with regard to growth inhibition, while daunorubicin and etoposide had only modest synergistic effects. HU and cytarabine effectively enhanced nitroblue tetrazolium-reducing activity induced by 1,25$(OH)_2D_3$. HU also enhanced the morphological maturation and expression of CD11b and CD14 in cells treated with 1,25$(OH)_2D_3$. Among the anti-cancer drugs examined, HU had the greatest synergistic effects with 1,25$(OH)_2D_3$ with regard to growth inhibition and differentiation induction in U937 cells. HU also enhanced the differentiation of other myeloid leukaemia HL-60, ML-1, THP-1, P39/TSU, P31/FUJ and NB4 cells induced by 1,25$(OH)_2D_3$ and that of U937 cells induced by 24-*epi*-1,25$(OH)_2D_2$ and 1,25$(OH)_2D_7$. Interestingly, 1α(OH)D derivatives (1α-hydroxyvitamin D_3, D_2, D_4 and D_7) effectively induced the differentiation of monoblastic leukaemia U937, P39/TSU and P31/FUJ cells. HU also enhanced the growth inhibition and differentiation of U937 cells induced by 1α(OH)D derivatives. As 1α(OH)D derivatives preferentially act on monocytic cells, they may be useful in the treatment of acute monocytic leukaemia, both alone and in combination with HU.

Keywords: leukaemia; vitamin D_2; vitamin D_3; vitamin D_4; vitamin D_7; hydroxyurea

The prognosis of acute myeloid leukaemia has recently improved through the application of intensive chemotherapy and bone marrow transplantation. However, intensive chemotherapy is not used in elderly patients or in patients with hypoplastic leukaemia or myelodysplastic syndrome because of severe complications. The incidence of induction death among elderly patients in their initial induction therapy, even when supported with cytokines, is higher than 10% (Schiffer, 1996). Indications for bone marrow transplantation are limited to young patients with HLA-matched donors (Goldman, 1994). Differentiation therapy is one possible approach for surviving patients who cannot be treated with intensive chemotherapy or bone marrow transplantation.

Differentiation therapy has been used successfully to treat acute promyelocytic leukaemia (Degos et al, 1995). All-*trans* retinoic acid induces complete remission in more than 90% of patients with acute promyelocytic leukaemia with a t (15;17) chromosomal translocation. However, the use of all-*trans* retinoic acid is limited to acute promyelocytic leukaemia. Vitamin D is another potential inducer for differentiation therapy. The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), induces differentiation in mouse and in human leukaemia cells (Abe et al, 1981; Miyaura et al, 1981) and prolongs the survival of mice inoculated with myeloid leukaemia cells (Honma et al, 1983). However, clinical trials of 1,25$(OH)_2D_3$ in patients with myelodysplastic syndrome have not been successful because of hypercalcaemia (Koeffler et al, 1985). Several analogues of 1,25$(OH)_2D_3$ that show anti-cancer activity and only weak activity for inducing hypercalcaemia have been developed, but they are not yet available for the clinical treatment of cancer or leukaemia (Abe et al, 1991; Pakkala et al, 1995). To overcome the adverse effects of vitamin D, we are investigating the effects of the combination of 1,25$(OH)_2D_3$ with other drugs (Makishima and Honma, 1996; Makishima et al, 1996). As the combination of all-*trans* retinoic acid with low doses of anti-cancer drugs produced better results than either drug alone for the treatment of acute myeloid leukaemia (Venditti et al, 1995), in this study, we investigated the effects of the combination of 1,25$(OH)_2D_3$ and its analogues with various anti-cancer drugs on growth inhibition and differentiation induction in myelomonocytic leukaemia cells.

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Correspondence to: Y Honma

MATERIALS AND METHODS

Materials

1α,25-Dihydroxyvitamin D_7 (1,25$(OH)_2D_7$), 24-*epi*-1α,25-dihydroxyvitamin D_2 (24-*epi*-1,25$(OH)_2D_2$), 1α-hydroxyvitamin D_3 (1α(OH)D_3), 1α(OH)D_2, 1α(OH)D_4 and 1α(OH)D_7 were synthesized (Figure 1) (Tachibana and Tsuji, 1992) and donated by the Fine Chemical Research Center, Nisshin Flour Milling (Saitama, Japan). Chlorambucil, daunorubicin, actinomycin D, hydroxyurea

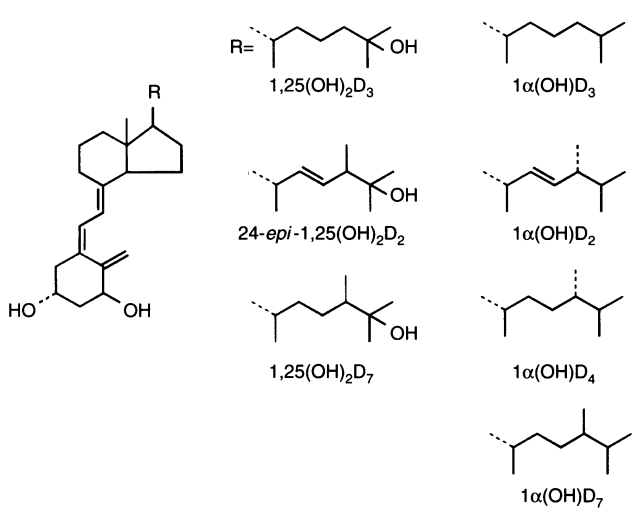

Figure 1 Chemical structures of vitamin D derivatives

(HU), cytarabine (Ara-C) and camptothecin were purchased from Sigma (St Louis, MO, USA), and $1,25(OH)_2D_3$ was from Wako Pure Chemical Industry (Osaka, Japan). Etoposide was obtained from Nippon Kayaku (Tokyo, Japan).

Cell lines and cell culture

Human myeloid leukaemia U937, HL-60, ML-1, THP-1, P39/TSU, P31/FUJ and NB4 cells (Lanotte et al, 1991) were cultured in suspension in RPMI 1640 medium containing 10% fetal bovine serum and 80 μg ml^{-1} gentamicin at 37°C in a humidified atmosphere of 5% carbon dioxide in air (Makishima et al, 1996).

Cell growth and differentiation

Suspensions of cells were cultured with or without the test compounds in multidishes. The cells were counted in a Model ZM Coulter Counter (Coulter Electronics, Luton, UK). Nitroblue tetrazolium (NBT) reduction was assayed colorimetrically (Makishima et al, 1996). Lysozyme activity in the conditioned medium was determined using a lysoplate (Makishima et al, 1996). One unit is equivalent to 1 μg ml^{-1} egg-white lysozyme. Cell morphology was examined in cell smears stained with May-Grünwald and Giemsa solutions (Merck, Darmstadt, Germany).

Analysis of the effects of combinations of drugs

The interaction of the two compounds was quantified by determining the combination index (CI) according to the classic isobologram equation:

$$CI = D_1/Dx_1 + D_2/Dx_2$$

where Dx is the concentration of one drug alone required to produce an effect and D_1 and D_2 are the doses of compounds 1 and 2, respectively, in combination that produce the same effect (Berenbaum, 1989). Using this analysis, the combined effects of the two drugs can be assessed as being either additive (CI = 1), synergistic (CI < 1) or antagonistic (CI > 1). An isobologram was also used to determine the effect of combinations of drugs (Berenbaum, 1989). Concentration-dependent effects were determined from isoeffective concentrations for each compound and for one compound with fixed concentrations of another. The additive lines were indicated as calculated by mode I and mode II systems (Steel and Peckham, 1979).

Flow cytometry

Expression of the granulocyte- and monocyte-specific antigens CD11b and CD14 on the cell surface was determined using indirect immunofluorescent staining and flow cytometry (Makishima and Honma, 1996). Mouse monoclonal antibodies to CD11b (2LPM19c), CD14 (TÜK4), control mouse IgG1, IgG2a and FITC-conjugated $F(ab')_2$ fragment of goat antimouse IgG were obtained from Dako (Glostrup, Denmark). The stained cells were assayed using a flow cytometer (Epics XL; Coulter Electronics)

Table 1 Effects of the combination of anticancer drugs and $1,25(OH)_2D_3$ on growth inhibition and NBT-reducing activity of human monoblastic leukaemia U937 cells

Drugs	IC_{50} for growth suppression		CI[b]	NBT reduction[c] (A_{560} per 10^7 cells)		Ratio[d]
	$-VD_3$	$+VD_3$[a]		$-VD_3$	$+VD_3$	
Chlorambucil	5.19 μM	3.90 μM	0.90	1.15 ± 0.13	2.14 ± 0.27	1.9
Daunorubicin	2.73 nM	1.53 nM	0.71	0.93 ± 0.07	2.17 ± 0.14	2.3
Actinomycin D	88.1 pM	76.7 pM	1.02	0.65 ± 0.07	2.06 ± 0.06	3.2
HU	50.7 μM	15.8 μM	0.46	1.26 ± 0.25	7.78 ± 0.34[e]	6.2
Ara-C	6.86 nM	2.41 nM	0.50	1.02 ± 0.14	3.56 ± 0.46	3.5
Camptothecin	9.84 nM	4.14 nM	0.57	1.84 ± 0.16	3.70 ± 0.38	2.0
Etoposide	38.3 nM	25.3 nM	0.81	1.20 ± 0.03	2.82 ± 0.10	2.4
None				0.54 ± 0.04	1.42 ± 0.09	2.6

Cells (5×10^4 cells ml^{-1}) were treated with anti-cancer drugs in the absence or presence of $1,25(OH)_2D_3$ (VD_3) for 4 days. IC_{50} values were determined from the means of triplicate data and values of NBT reduction represent the means ± SD of three separate experiments. [a]IC_{50} values for anti-cancer drugs in the presence of 3×10^{-9} M $1,25(OH)_2D_3$. [b]Combination index (CI) at IC_{50} for growth inhibition. CI values at a fixed concentration of $1,25(OH)_2D_3$ (3×10^{-9} M) were calculated as described in the Materials and methods. IC_{50} of $1,25(OH)_2D_3$ was 2.01×10^{-8} M. In this assay, CI = 1 indicates an additive effect, CI <1 indicates synergism and CI >1 indicates antagonism. [c]NBT reduction in the cells treated with anti-cancer drugs at their IC_{50} for growth inhibition in the absence or presence of 3×10^{-9} M $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ at the IC_{50} induced the activity to 4.64 A_{560}. [d]Ratio of the NBT reduction: $+1,25(OH)_2D_3$ / $-1,25(OH)_2D_3$. [e]$P<0.0005$ compared with other anti-cancer drugs plus $1,25(OH)_2D_3$.

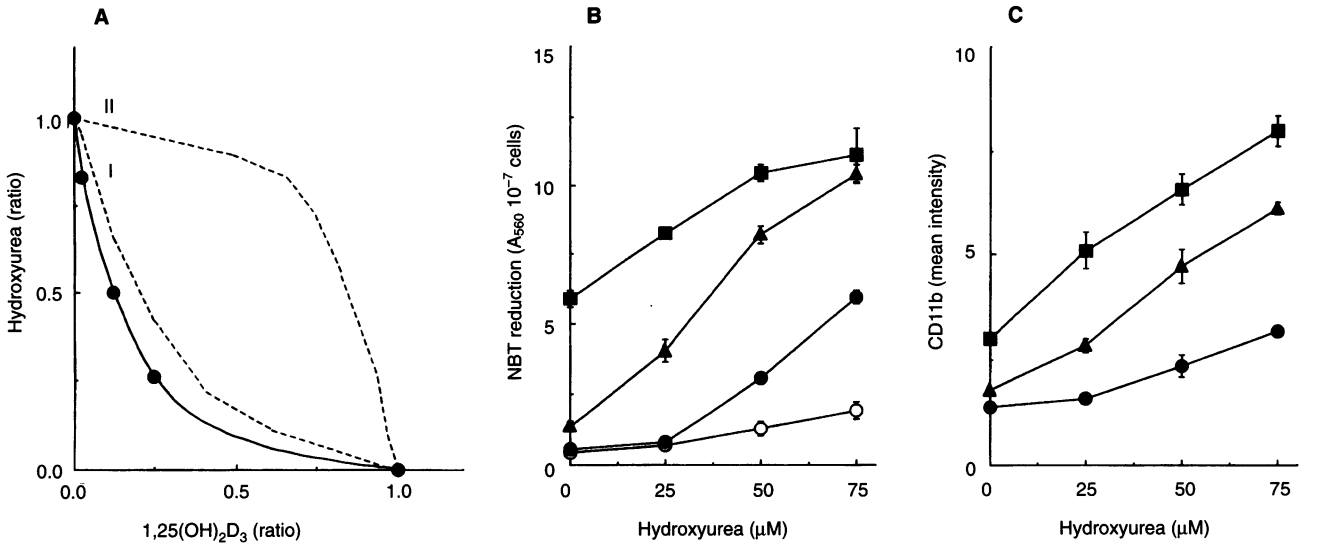

Figure 2 Effects of the combination of HU and $1,25(OH)_2D_3$ with regard to growth inhibition and differentiation induction in human monoblastic leukaemia U937 cells. (**A**) Isobologram for $1,25(OH)_2D_3$ and HU at the IC_{50} for growth inhibition. The dashed lines I and II indicate additive interaction calculated by mode I and II systems respectively (Steel and Peckham, 1979). In mode II, when the dose of HU is chosen, an isoeffect curve is calculated by taking the dose increment of $1,25(OH)_2D_3$ that gives the required contribution to IC_{50}, estimating how much HU reduces the requirement of $1,25(OH)_2D_3$. (**B**) NBT-reducing activities of cells treated with a combination of HU and 0 (○), 3×10^{-10} M (●), 3×10^{-9} M (▲) or 3×10^{-8} M (■) $1,25(OH)_2D_3$. (**C**) CD11b expression by cells treated with a combination of HU and 0 (●), 3×10^{-10} M (▲) or 3×10^{-9} M (■) $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ at 3×10^{-6} M induced the expression to 6.41 units. Cells (5×10^4 cells ml^{-1}) were cultured with test compounds for 4 days. Values represent the means (± SD) of three separate experiments

and the mean fluorescence intensity of fluorescence-positive cells was calculated using the Immuno-4 histogram analysis program (Coulter), with mouse immunoglobulin of the same isotype as a negative control. The Immuno-4 program subtracts a control histogram from a test histogram to calculate the mean fluorescence intensity in the test histogram (Overton, 1988).

Statistical evaluation

Statistical analyses were performed using an unpaired two-tailed Student's *t*-test.

RESULTS

Effects of the combination of anti-cancer drugs with $1,25(OH)_2D_3$ on the growth and differentiation of human monoblastic leukaemia U937 cells

We examined several anti-cancer drugs in combination with $1,25(OH)_2D_3$ to determine the effects on growth inhibition in human monoblastic leukaemia U937 cells. Chlorambucil is an alkylating agent; daunorubicin and actinomycin D are antibiotics; HU and Ara-C are inhibitors of nucleotide metabolism; and camptothecin and etoposide are inhibitors of topoisomerases. These drugs all inhibited the proliferation of U937 cells concentration dependently; their IC_{50} values are indicated in Table 1. The effects of the combination of anti-cancer drugs and $1,25(OH)_2D_3$ were determined using the CI calculated from the IC_{50} values of anti-cancer drugs in the presence of 3×10^{-9} M $1,25(OH)_2D_3$. HU inhibited the proliferation of U937 cells at an IC_{50} of 50.7 μM in the absence of $1,25(OH)_2D_3$ and at an IC_{50} of 15.8 μM in its presence (CI = 0.46, indicating synergism). The confidence intervals (CIs) for Ara-C, camptothecin, daunorubicin and etoposide were 0.50, 0.57, 0.71 and 0.81, respectively, also indicating synergism. The combinations of chlorambucil and actinomycin D with $1,25(OH)_2D_3$ were additive.

We examined the effects of anti-cancer drugs in combination with $1,25(OH)_2D_3$ on NBT-reducing activity, a typical marker of myelomonocytic differentiation, in U937 cells. The anti-cancer drugs showed only weak activity for inducing NBT reduction (Table 1). Next, the NBT-reducing activity induced by anti-cancer drugs in combination with 3×10^{-9} M $1,25(OH)_2D_3$ was examined. HU plus $1,25(OH)_2D_3$ effectively increased the activity 6.2-fold from HU alone and 5.5-fold from $1,25(OH)_2D_3$ alone (Table 1). Camptothecin and Ara-C in combination with $1,25(OH)_2D_3$ modestly induced this activity. Among the anti-cancer drugs we examined, HU had the greatest synergistic effect with $1,25(OH)_2D_3$ for growth inhibition and induction of NBT-reducing activity in U937 cells.

Effects of HU plus $1,25(OH)_2D_3$ on growth inhibition and differentiation induction in human myelomonocytic leukaemia cells

The concentration-dependent effects of the combination of HU with $1,25(OH)_2D_3$ on U937 cells were examined. Isoboles for growth inhibition show that their combination is synergistic and the presence of HU markedly reduced effective concentrations of $1,25(OH)_2D_3$ (Figure 2A). HU up to 75 μM induced NBT-reducing activity of U937 cells only slightly (Figure 2B). While $1,25(OH)_2D_3$ at 3×10^{-10} M did not induce NBT-reducing activity of U937 cells, in the presence of 75 μM HU, it effectively induced this activity to 5.96 A_{560}, which is similar to the value (5.90 A_{560}) with a 100-fold greater concentration (3×10^{-8} M) of $1,25(OH)_2D_3$ (Figure 2B). Morphologically, monoblastic U937 cells were induced to differentiate into monocytic cells by $1,25(OH)_2D_3$ and became more mature, having abundant and grey cytoplasm and a chromatin-condensed nucleus with the addition of HU (data not shown). HU also effectively enhanced the expression of CD11b in U937 cells induced by low concentrations of $1,25(OH)_2D_3$ (Figure

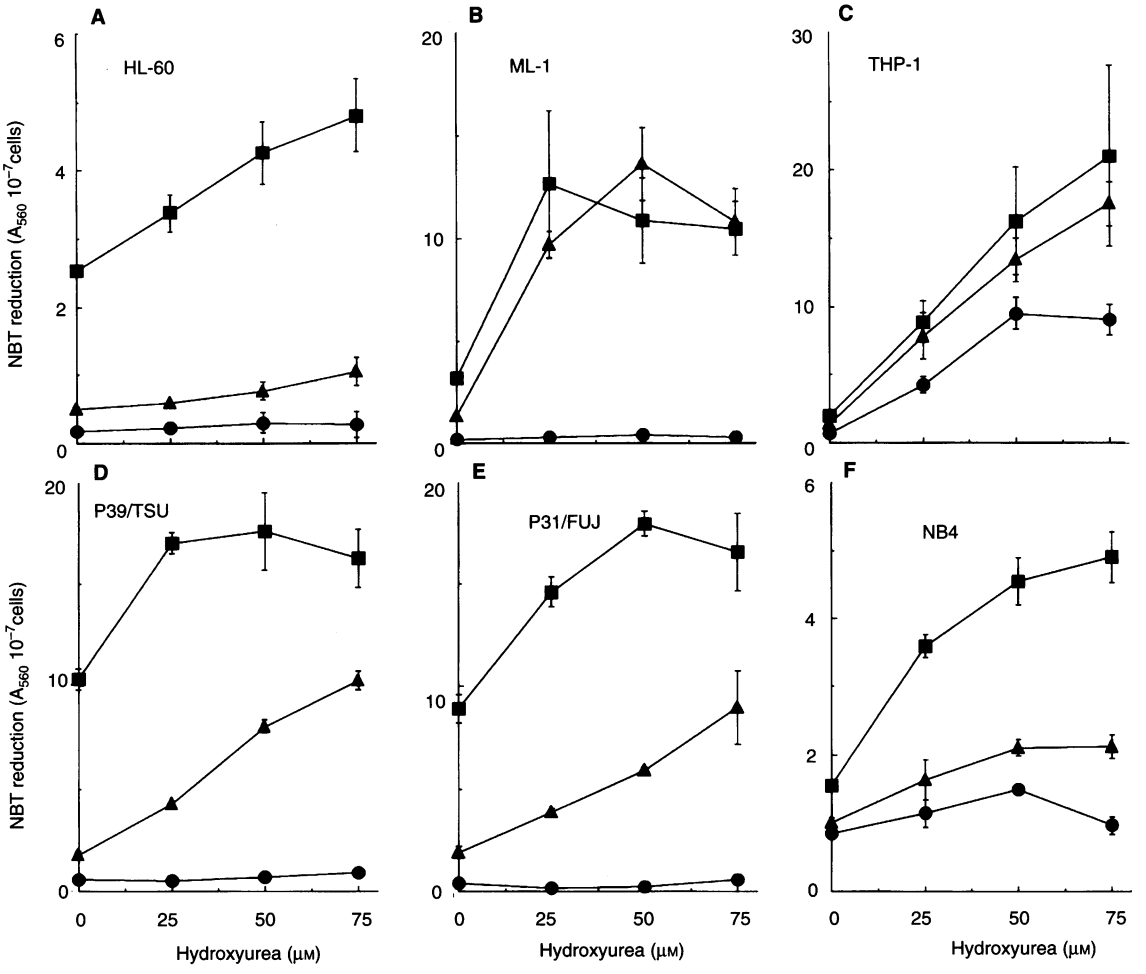

Figure 3 Effects of the combination of HU and $1,25(OH)_2D_3$ with regard to the NBT-reducing activity of human myeloid leukaemia HL-60 (**A**), ML-1 (**B**), THP-1 (**C**), P39/TSU (**D**), P31/FUJ (**E**) and NB4 cells (**F**). Cells (5×10^4 cells ml^{-1}) were cultured with HU in combination with 0 (●), 3×10^{-9} M (▲) or 3×10^{-8} M (■) $1,25(OH)_2D_3$ for 4 days. Values represent the means ± SD of three separate experiments

2C). HU at 75 μM plus $1,25(OH)_2D_3$ at 3×10^{-9} M increased this intensity to 8.00 units, which is greater than 6.41 units with 3×10^{-6} M $1,25(OH)_2D_3$ alone. The enhancing effect of HU on CD14 expression induced by $1,25(OH)_2D_3$ in U937 cells was weak (data not shown). Thus, HU effectively enhanced several differentiation markers in U937 cells treated with $1,25(OH)_2D_3$.

Next, we examined the combination of HU and $1,25(OH)_2D_3$ on the differentiation of other myelomonocytic leukaemia cells. HU did not induce NBT-reducing activity in promyelocytic HL-60 and myeloblastic ML-1 cells, but enhanced the activity induced by $1,25(OH)_2D_3$ (Figure 3A and B). HU alone induced NBT-reducing activity in monoblastic THP-1 cells but not in other monoblastic P39/TSU or P31/FUJ cells. It also effectively enhanced the differentiation of these cells induced by $1,25(OH)_2D_3$ (Figure 3C–E). NB4 cells are promyelocytic leukaemia cells with a t(15;17) chromosomal translocation and have been reported to be resistant to $1,25(OH)_2D_3$ (Testa et al, 1994). HU also induced the NBT-reducing activity in NB4 cells in combination with $1,25(OH)_2D_3$ (Figure 3F). Thus, HU plus $1,25(OH)_2D_3$ effectively induces the differentiation of myelomonocytic leukaemia cells.

Effects of HU in combination with vitamin D derivatives on growth inhibition and differentiation induction in U937 cells

24-*Epi*-$1,25(OH)_2D_2$ and $1,25(OH)_2D_7$ have been reported to exhibit less hypercalcaemic activity than $1,25(OH)_2D_3$ and to be able to induce the differentiation of HL-60 cells (Sato et al, 1991). They also induced the NBT-reducing activity of U937 cells, and HU effectively enhanced the activities induced by their suboptimal concentrations (Figure 4A). 24-*Epi*-$1,25(OH)_2D_2$ and $1,25(OH)_2D_7$ inhibited the proliferation of U937 cells concentration dependently, with IC_{50} values of 2.25×10^{-7} M and 2.28×10^{-7} M respectively (data not shown). At a low concentration of 9×10^{-9} M, they slightly inhibited the proliferation of U937 cells and augmented the growth inhibition in combination with 50 μM HU (Table 2).

We have previously reported that $1\alpha(OH)D_3$ induces the differentiation of monoblastic leukaemia cells as well as $1,25(OH)_2D_3$ and is less toxic than $1,25(OH)_2D_3$ (Honma et al, 1983; Okabe-Kado et al, 1992). We examined the effects of several 1α(OH)D derivatives on

Table 2 Growth inhibition in human monoblastic leukaemia U937 cells by vitamin D derivatives in combination with HU

Compounds	Growth (% of control)		
	– HU	+ HU	Ratio[a]
None	100	54 ± 3	100
$1,25(OH)_2D_3$ (3×10^{-9} M)	74 ± 1	25 ± 3	46
24-*Epi*-$1,25(OH)_2D_2$ (9×10^{-9} M)	87 ± 2	33 ± 1	61
$1,25(OH)_2D_7$ (9×10^{-9} M)	89 ± 2	32 ± 1	59
$1\alpha(OH)D_3$ (3×10^{-8} M)	72 ± 2	27 ± 1	50
$1\alpha(OH)D_2$ (6×10^{-8} M)	91 ± 2	31 ± 1	57
$1\alpha(OH)D_4$ (6×10^{-8} M)	89 ± 1	28 ± 3	52
$1\alpha(OH)D_7$ (6×10^{-8} M)	100 ± 4	27 ± 2	50

Cells (5×10^4 cells ml^{-1}) were cultured with vitamin D derivatives in the absence or presence of 50 μM HU for 4 days. [a]Ratio (%) represents the growth of cells in combination with HU compared with that of cells treated with 50 μM HU alone.

growth inhibition and differentiation induction in U937 cells in combination with HU. $1\alpha(OH)D_3$ inhibited proliferation with an IC_{50} value of 0.67×10^{-7} M and induced myelomonocytic differentiation markers, such as the NBT-reducing and lysozyme activities of U937 cells (Figure 4B, data not shown). HU effectively enhanced the NBT-reducing activity in U937 cells induced by $1\alpha(OH)D_3$ (Figure 4B). For example, 1.2×10^{-8} M $1\alpha(OH)D_3$ plus 50 μM HU induced this activity to 9.02 A_{560}, while this activity was 8.54 A_{560} with 1.2×10^{-6} M $1\alpha(OH)D_3$ alone, indicating that $1\alpha(OH)D_3$ was more than 100 times as active in the presence of HU. $1\alpha(OH)D_3$ at 3×10^{-8} M slightly inhibited the proliferation of U937 cells, but augmented the inhibition in combination with HU (Table 2). $1\alpha(OH)D_2$, $1\alpha(OH)D_4$ and $1\alpha(OH)D_7$ also inhibited the proliferation with IC_{50} values of 1.35×10^{-7}, 1.47×10^{-7} and 3.25×10^{-7} M, respectively (data not shown), and induced the differentiation of monoblastic U937 (Figure 4C), P39/TSU and P31/FUJ cells, but not of promyelocytic HL-60 cells (data not shown). Among these four $1\alpha(OH)D$ derivatives, $1\alpha(OH)D_3$ was the most effective in inhibiting the proliferation of U937 cells ($P<0.005$, compared at IC_{50}). At the IC_{50} values for growth inhibition, $1\alpha(OH)D_3$, $1\alpha(OH)D_2$, $1\alpha(OH)D_4$ and $1\alpha(OH)D_7$ induced NBT-reducing activity of U937 cells from 0.83 A_{560} to 5.80, 5.50, 6.08 and 5.84 A_{560}, respectively, and lysozyme activity from 1.99 units to 5.03, 5.11, 6.34 and 6.03 units, respectively (data not shown), indicating that $1\alpha(OH)D_4$ was slightly more effective for inducing these activities than the others. The NBT-reducing activity induced by these $1\alpha(OH)D$ derivatives in U937 cells was also effectively enhanced by HU (Figure 4C). HU enhanced the induction of NBT reduction by $1\alpha(OH)D_4$ slightly more effectively than that by $1\alpha(OH)D_2$ and $1\alpha(OH)D_7$. At a concentration of 6×10^{-8} M, $1\alpha(OH)D_2$ and $1\alpha(OH)D_4$ inhibited the proliferation of U937 cells only slightly, while $1\alpha(OH)D_7$ had no such effect, but they augmented the growth-inhibitory activity with HU (Table 2). Thus, the combination of HU with $1\alpha(OH)D$ derivatives is effective for inhibiting the proliferation and inducing the differentiation of U937 cells.

DISCUSSION

Among the anti-cancer drugs we examined, HU showed the greatest synergistic effect with $1,25(OH)_2D_3$ with regard to growth inhibition and differentiation induction in U937 cells. Ara-C and camptothecin showed modest synergism with regard to growth inhibition, and Ara-C enhanced the differentiation induced by $1,25(OH)_2D_3$ second only to HU. In another study, treatment with

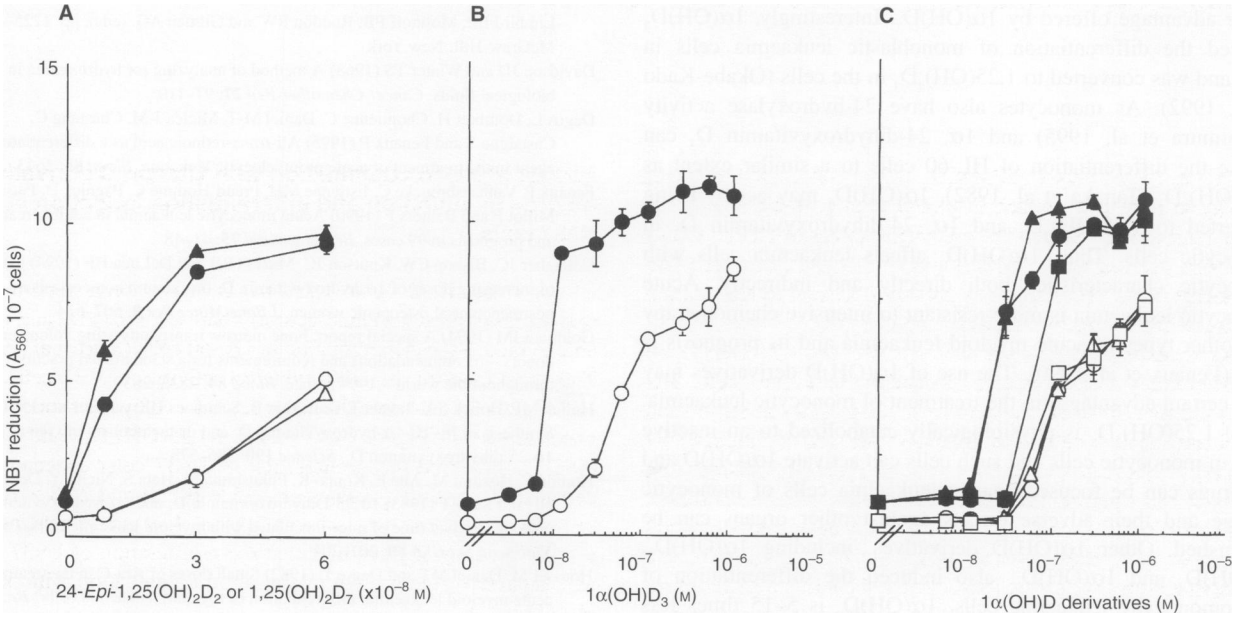

Figure 4 Effects of vitamin D derivatives in combination with HU with regard to the induction of NBT-reducing activity in human monoblastic leukaemia U937 cells. (**A**) NBT-reducing activities of cells treated with 24-*epi*-$1,25(OH)_2D_2$ (○, ●) or $1,25(OH)_2D_7$ (△, ▲) in the absence (○, △) or presence (●, ▲) of 50 μM HU. (**B**) NBT-reducing activities of cells treated with $1\alpha(OH)D_3$ in the absence (○) or presence (●) of 50 μM HU. (c) NBT-reducing activities of cells treated with $1\alpha(OH)D_2$ (○, ●), $1\alpha(OH)D_4$ (△, ▲) or $1\alpha(OH)D_7$ (□, ■) in the absence (○, △, □) or presence (●, ▲, ■) of 50 μM HU. Cells (5×10^4 cells ml^{-1}) were cultured with the test compounds for 4 days. Values represent the means ± SD of three separate experiments

$1,25(OH)_2D_3$ increased the cytotoxicity of Ara-C and HU against HL-60 cells (Studzinski et al, 1986). HU inhibits nucleotide metabolism by inhibiting ribonucleotide reductase and Ara-C also inhibits nucleotide synthesis (Calabresi and Chabner, 1996). HU enhances the differentiation of HL-60 cells induced by all-*trans* retinoic acid (Yen et al, 1987). Ara-C induces the differentiation of some myeloid leukaemia cells and low doses of Ara-C have been used to treat acute myeloid leukaemia (Housset et al, 1982). Other inhibitors of nucleotide metabolism also induce the differentiation of myeloid leukaemia cells (Bodner et al, 1981; Ishiguro and Sartorelli, 1985). These findings indicate that some inhibitors of nucleotide metabolism may induce leukaemia cells to differentiate and to enhance differentiation induced by other compounds more effectively than other types of anti-cancer drugs.

HU is useful for treating chronic myelogenous leukaemia and, when administered orally at daily doses from 500 to 3000 mg, for controlling blood cell counts within desirable ranges (Athens, 1993). Its major adverse effect is bone marrow suppression, but the bone marrow recovers promptly if the drug is discontinued for a few days. Thus, HU can be used safely in elderly patients. Pharmacokinetic studies have shown that serum concentrations of HU after a single oral administration of 1000 mg reach 20–30 μg ml^{-1} (263–394 μM) in 1–3 h, then gradually decrease and remain higher than 5 μg ml^{-1} (66 μM) for at least 10 h (Davidson and Winter, 1963; Bolton et al, 1965). These findings indicate that the concentrations of HU needed to enhance the anti-leukaemic activity of vitamin D derivatives can be achieved clinically.

In this study, we observed the differentiation-inducing activities of 1α(OH)D derivatives. After the administration of $1\alpha(OH)D_3$, it is converted to an active form, $1,25(OH)_2D_3$, by liver 25-hydroxylase (Holick et al, 1975). $1\alpha(OH)D_3$ was more potent than $1,25(OH)_2D_3$ in increasing the survival time of mice inoculated with mouse myeloid leukaemia M1 cells (Honma et al, 1983). The relatively stable concentrations of $1,25(OH)_2D_3$ after the administration of $1\alpha(OH)D_3$ compared with $1,25(OH)_2D_3$ may contribute to the advantage offered by $1\alpha(OH)D_3$. Interestingly, $1\alpha(OH)D_3$ induced the differentiation of monoblastic leukaemia cells in vitro and was converted to $1,25(OH)_2D_3$ in the cells (Okabe-Kado et al, 1992). As monocytes also have 24-hydroxylase activity (Kamimura et al, 1995) and 1α, 24-dihydroxyvitamin D_3 can induce the differentiation of HL-60 cells to a similar extent as $1,25(OH)_2D_3$ (Tanaka et al, 1982), $1\alpha(OH)D_3$ may act by being converted to $1,25(OH)_2D_3$ and 1α, 24-dihydroxyvitamin D_3 in monocytic cells. Thus, $1\alpha(OH)D_3$ affects leukaemia cells with monocytic characteristics both directly and indirectly. Acute monocytic leukaemia is more resistant to intensive chemotherapy than other types of acute myeloid leukaemia and its prognosis is poor (Fenaux et al, 1990). The use of 1α(OH)D derivatives may offer certain advantages in the treatment of monocytic leukaemia, as (a) $1,25(OH)_2D_3$ is physiologically catabolized to an inactive form in monocytic cells and such cells can activate 1α(OH)D and (b) drugs can be focused against leukaemia cells of monocytic lineage and their adverse effects against other organs can be diminished. Other 1α(OH)D derivatives, including $1\alpha(OH)D_2$, $1\alpha(OH)D_4$ and $1\alpha(OH)D_5$, also induced the differentiation of myelomonocytic leukaemia cells. $1\alpha(OH)D_2$ is 5–15 times less toxic than $1\alpha(OH)D_3$ in rats (Sjöden et al, 1985). A clinical study in post-menopausal osteopenic patients showed that $1\alpha(OH)D_2$ at daily doses of less than 5.0 μg did not induce hypercalcaemia, whereas $1\alpha(OH)D_3$ at daily doses above 1.0 μg had toxic effects (Gallagher et al, 1994). An active form of $1\alpha(OH)D_2$, $1,25(OH)_2D_2$, has less hypercalcaemic activity (Sato et al, 1991). Therefore, the 1α(OH)D derivatives may be useful for treating monocytic leukaemia. Pharmacokinetics for serum concentrations of 1α(OH)D derivatives and their metabolites after administration should be further investigated. HU also effectively enhanced the differentiation induced by the 1α(OH)D derivatives. The combination of 1α(OH)D derivatives with HU may be a promising candidate for 'chemo-differentiation therapy' of acute monocytic leukaemia.

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REFERENCES

Abe E, Miyaura C, Sakagami H, Takeda M, Konno K, Yamazaki T, Yoshiki S and Suda T (1981) Differentiation of mouse myeloid leukemia cells induced by 1α,25-dihydroxyvitamin D_3. *Proc Natl Acad Sci USA* **78**: 4990–4994

Abe J, Nakano T, Nishii Y, Matsumoto T, Ogata E and Ikeda K (1991) A novel vitamin D_3 analog, 22-oxa-1,25-dihydroxyvitamin D_3, inhibits the growth of human breast cancer *in vitro* and *in vivo* without causing hypercalcemia. *Endocrinology* **129**: 832–837

Athens JW (1993) Chronic myeloid leukaemia. In *Wintrobe's Clinical Hematology*, Lee GR, Bithell TC, Foerster J, Athens JW and Lukens JN. (eds), pp. 1969–1998. Lea & Febger: Philadelphia

Berenbaum MC (1989) What is synergy? *Pharmacol Rev* **41**: 93–141

Bolton BH, Woods LA, Kaung DT and Lawton RL (1965) A simple method of colorimetric analysis for hydroxyurea (NSV-32065). *Cancer Treat Rep* **46**: 1–5

Bodner AJ, Ting RC and Gallo RC (1981) Induction of differentiation of human promyelocytic leukemia cells (HL-60) by nucleosides and methotrexate. *J Natl Cancer Inst* **67**: 1025–1030

Calabresi P, Chabner BA (1996) Chemotherapy of neoplastic diseases. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th edn, Hardman JG, Limbird LE, Molinoff PB, Ruddon RW and Gilman AG. (eds), pp. 1225–1287. McGraw-Hill: New York

Davidson JD and Winter TS (1963) A method of analyzing for hydroxyurea in biological fluids. *Cancer Chemother Rep* **27**: 97–110

Degos L, Dombret H, Chomienne C, Daniel M-T, Miclea J-M, Chastang C, Castaigne S and Fenaux P (1995) All-*trans*-retinoic acid as a differentiating agent in the treatment of acute promyelocytic leukemia. *Blood* **85**: 2643–2653

Fenaux P, Vanhaesbroucke C, Estienne MH, Preud'Homme C, Pagniez D, Facon T, Millot F and Bauters F (1990) Acute monocytic leukaemia in adults: treatment and prognosis in 99 cases. *Br J Haematol* **75**: 41–48

Gallagher JC, Bishop CW, Knutson JC, Mazess RB and DeLuca HF (1994) Effects of increasing doses of 1α-hydroxyvitamin D_2 on calcium homeostasis in postmenopausal osteopenic women. *J Bone Miner Res* **9**: 607–614

Goldman JM (1994) A special report: bone marrow transplants using volunteer donors – recommendations and requirements for a standardized practice throughout the world – 1994 update. *Blood* **84**: 2833–2839

Holick MF, Holick SA, Tavela T, Gallagher B, Schonoes HK and DeLuca HF (1975) Synthesis of [6-^{3}H]-1α-hydroxyvitamin D_3 and its metabolism in vivo to [^{3}H]-1α,25-dihydroxyvitamin D_3. *Science* **190**: 576–578

Honma Y, Hozumi M, Abe E, Konno K, Fukushima M, Hata S, Nichii Y, DeLuca HF and Suda T (1983) 1α,25-Dihydroxyvitamin D_3 and 1α-hydroxyvitamin D_3 prolong survival time of mice inoculated with myeloid leukemia cells. *Proc Natl Acad Sci USA* **80**: 201–204

Housset M, Daniel MT and Degos L (1982) Small doses of Ara-C in the treatment of acute myeloid leukaemia: differentiation of myeloid leukaemia cells? *Br J Haematol* **51**: 125–129

Ishiguro K and Sartorelli AC (1985) Enhancement of the differentiation-inducing properties of 6-thioguanine by hypoxanthine and its nucleosides in HL-60 promyelocytic leukemia cells. *Cancer Res* **45**: 91–95

Kamimura S, Gallieni M, Zhong M, Beron W, Slatopolsky E and Dusso A (1995) Microtubules mediate cellular 25-hydroxyvitamin D_3 trafficking and the

genomic response to 1,25-dihydroxyvitamin D_3 in normal human monocytes. *J Biol Chem* **270**: 22160–22166

Koeffler HP, Hirji K, Itri L, The Southern California Leukemia Group (1985) 1,25-Dihydroxyvitamin D_3: in vivo and in vitro effects on human preleukemic and leukemic cells. *Cancer Treat Rep* **69**: 1399–1407

Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F and Berger R (1991) NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). *Blood* **77**: 1080–1086

Makishima M and Honma Y (1996) Ethacrynic acid and 1α,25-dihydroxyvitamin D_3 cooperatively inhibit proliferation and induce differentiation of human myeloid leukemia cells. *Leuk Res* **20**: 781–789

Makishima M, Kanatani Y, Yamamoto-Yamaguchi Y and Honma Y (1996) Enhancement of activity of 1α,25-dihydroxyvitamin D_3 for growth inhibition and differentiation induction of human myelomonocytic leukemia cells by tretinoin tocoferil, an α-tocopherol ester of all-*trans* retinoic acid. *Blood* **87**: 3384–3394

Miyaura C, Abe E, Kuribayashi T, Tanaka H, Konno K, Nishii Y and Suda T (1981) 1α,25-Dihydroxyvitamin D_3 induces differentiation of human myeloid leukemia cells. *Biochem Biophys Res Commun* **102**: 937–943

Okabe-Kado J, Honma Y, Kasukabe T and Hozumi M (1992) Synthesis of active metabolite(s) from 1α-hydroxyvitamin D_3 by human monocytic leukemia cells. *FEBS Lett* **309**: 399–401

Overton WR (1988) Modified histogram subtraction technique for analysis of flow cytometry data. *Cytometry* **9**: 619–626

Pakkala S, De Vos S, Elstner E, Rude RK, Uskokovic M, Binderup L and Koeffler HP (1995) Vitamin D_3 analogs: effect on leukemic clonal growth and differentiation, and on serum calcium levels. *Leuk Res* **19**: 65–72

Sato F, Okamoto Y, Ouchi Y, Kaneki M, Nakamura T, Ikekawa N and Orimo H (1991) Biological activity of 1α,25-dihydroxyvitamin D derivatives – 24-*epi*-1α,25-dihydroxyvitamin D-2 and 1α,25-dihydroxyvitamin D-7. *Biochim Biophys Acta* **1091**: 188–192

Schiffer CA (1996) Hematopoietic growth factors as adjuncts to the treatment of acute myeloid leukemia. *Blood* **88**: 3675–3685

Sjöden G, Smith C, Lindgren U and DeLuca HF (1985) 1α-hydroxyvitamin D_3 is less toxic than 1α-hydroxyvitamin D_3 in the rat. *Proc Soc Exp Biol Med* **178**: 432–436

Steel GG and Peckham MJ (1979) Exploitable mechanisms in combined radiotherapy–chemotherapy: the concept of additivity. *Int J Radiat Oncol Biol Phys* **5**: 85–91

Studzinski GP, Bhandal AK and Brelvi ZS (1986) Potentiation by 1-α,25-dihydroxyvitamin D_3 of cytotoxicity to HL-60 cells produced by cytarabine and hydroxyurea. *J Natl Cancer Inst* **76**: 641–648

Tachibana Y and Tsuji M (1992) Synthetic studies on active forms of vitamin D and their analogs. In *Studies in Natural Products Chemistry*, Vol. 11, Rahman AU. (ed.), pp. 379–408. Elsevier: Amsterdam

Tanaka H, Abe E, Miyaura C, Kuribayashi T, Konno K, Nishii Y and Suda T (1982) 1α,25-Dihydroxycholecalciferol and a human myeloid leukaemia cell line (HL-60). The presence of a cytosol receptor and induction of differentiation. *Biochem J* **204**: 713–719

Testa U, Grignani F, Barberi T, Fagioli M, Masciulli R, Ferrucci PF, Seripa D, Camagna A, Alcalay M, Pelicci PG and Peschle C (1994) PML/RARα$^+$ U937 mutant and NB4 cell lines: retinoic acid restores the monocytic differentiation response to vitamin D_3. *Cancer Res* **54**: 4508–4515

Venditti A, Stasi R, Del Poeta G, Buccisano F, Aronica G, Bruno A, Pisani F, Caravita T, Masi M, Tribalto M, Simone MD, Avvisati G, Amadori S and Papa G (1995) All-*trans* retinoic acid and low-dose cytosine arabinoside for the treatment of 'poor prognosis' acute myeloid leukemia. *Leukemia* **9**: 1121–1125

Yen A, Freeman L and Fishbaugh J (1987) Hydroxyurea induces precommitment during retinoic induced HL-60 terminal myeloid differentiation: possible involvement of gene amplification. *Leuk Res* **11**: 63–71