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Biochemical Pharmacology, Vol. 43, No. 10, pp. 2292–2295, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

Effects of 1,25-dihydroxyvitamin D_3 analogue 1,24(OH)2-22-ene-24-cyclopropyl D_3 on proliferation and differentiation of a human megakaryoblastic leukemia cell line

(*Received* 20 *January* 1992; *accepted* 24 *February* 1992)

Abstract—The novel analogue of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2$ D_3), 1,24(OH)2-22-ene-24-cyclopropyl D_3 (calcipotriol, MC903), exhibits similar effects on cell proliferation and cell differentiation in a newly established human megakaryoblastic leukemia cell line (HIMeg). MC903 was found to inhibit cell proliferation and induce cell differentiation in a liquid culture system at concentrations comparable to those of 1,25$(OH)_2$ D_3. Colony formation assay showed that MC903 or 1,25$(OH)_2$ D_3 markedly diminished the colony-forming ability of HIMeg cells at concentrations of 10^{-6} M to 10^{-10} M. Cell cycle analysis demonstrated that, as seen with 1,25$(OH)_2$ D_3, MC903 also altered the cell cycle distribution; the fraction of cells in $G_0 + G_1$ increased while those in S and G_2 + M decreased. It can be concluded from these findings that 1,25$(OH)_2$ D_3 and its analogue MC903 have approximately equipotent effects on cells of megakaryoblastic lineage and are potentially useful in studying the cellular processes that are responsible for megakaryocytopoiesis.

Vitamin D_3 is a secosteroid; sequential hydroxylation in the liver and kidney converts it to 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2$ D_3*] which is the active form. 1,25$(OH)_2$ D_3 has profound effects on the regulation of bone and mineral metabolism [1]. Most, if not all, of its actions are mediated like steroid hormones via binding and activation of high affinity nuclear receptors. The activated receptors then modulate gene expression by binding to hormone response elements of the target genes [2]. In classical target tissues, 1,25$(OH)_2$ D_3 enhances the expression of the calcium-binding protein, alkaline phosphatase, and the 24-hydroxylase [1–3]. Extremely sensitive radioligand receptor binding assays have shown that 1,25$(OH)_2$ D_3 receptors are present not only in the classical target tissues such as bone, intestinal mucosa and kidney, but also in the skin, endocrine glands, thymus, brain, bone marrow, and activated or transformed lymphopoietic cells, among other tissues [4, 5]. The presence of high affinity 1,25$(OH)_2$ D_3

* Abbreviations: MC903, calcipotriol, 1,24(OH)2-22-ene-24-cyclopropyl D_3; 1,25$(OH)_2$ D_3, 1,25-dihydroxyvitamin D_3; HIMeg, human megakaryoblastic leukemia cell line; IMDM, Iscove's modified Dulbecco's medium; FCS, fetal calf serum.

receptors in so many cell types suggests that the biological functions of this hormone may go well beyond calcium homeostasis; indeed, it has been proposed that $1,25(OH)_2$ D_3 may be involved in the regulation of gene expression, cell proliferation and cell differentiation in many diverse systems [5–7].

Previous studies showed that $1,25(OH)_2$ D_3 causes cells from both the murine myeloid leukemia line (M1) and the human promyelocytic leukemia line (HL60) to differentiate to monocyte/macrophage-like cells [4, 8]. Further studies showed that the clonal proliferation of a variety of human acute myelogenous leukemia cell lines is inhibited by $1,25(OH)_2$ D_3 and this inhibition is independent of the ability of the hormone to induce differentiation of these cells [9]. Clonogenic blast cells from patients with acute myelogenous leukemia are also inhibited in their proliferation by $1,25(OH)_2$ D_3 [10]. Studies *in vivo* suggest that $1,25(OH)_2$ D_3 can prolong the survival of mice injected with leukemic cells [8]. The effects of $1,25(OH)_2$ D_3 on the megakaryocytic lineage are unclear. Vitamin D_3 metabolites may thus be of potential interest in the treatment of a number of proliferative diseases, but their useful therapeutic range is limited by their potent effects on calcium metabolism, leading to hypercalcemia, hypercalciuria and calcifications in kidney, heart and blood vessels. These considerations have stimulated interest in the development of new vitamin D_3 derivatives and analogues which retain their regulatory effects on cell proliferation and differentiation but exhibit decreased activity toward calcium metabolism.

MC903 [1,24(OH)2-22-ene-24-cyclopropyl D_3, calcipotriol] is a cyclopropyl side chain analogue of $1,25(OH)_2$ D_3 which exhibits similar effects on cell proliferation and differentiation but possesses greatly reduced intestinal calcium absorption and bone calcium mobilization activity [11]. In the present study the comparative effects of MC903 and $1,25(OH)_2$ D_3 on the proliferation and differentiation of a newly established human megakaryoblastic leukemia cell line (HIMeg) were studied. Our results demonstrate that $1,25(OH)_2$ D_3 and its analogue have *in vitro* effects on megakaryocytopoiesis.

Materials and Methods

Materials. MC903 and $1,25(OH)_2$ D_3 were kind gifts from Dr L. Binderup, Leo Pharmaceutical Products (Ballerup, Denmark). Both compounds were dissolved in ethanol and stored at −20°. Iscove's modified Dulbecco's medium (IMDM) powder was from Gibco (Grand Island, NY, U.S.A.). Linbro tissue culture plates (24 well) were obtained from Flow Laboratories (McLean, VA, U.S.A.). All other chemicals and reagents were of analytical grade and obtained commercially.

Cells and cell culture. HIMeg cell line was established from a long term culture of peripheral blood cells of a patient with chronic granuocytic leukemia in chronic phase, and it has megakaryoblastic characteristics with ph' chromosome [12]. MIMeg cells were cultured in IMDM medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin G (100 U/mL) and streptomycin (100 μg/mL) in a humid atmosphere of 5% CO_2 in air at 37°. One half of the medium was replaced every 3 or 4 days.

Treatment of cells with hormones. Cells were plated onto 24-well clusters at cell densities of 1×10^5 cells/well in 1.0 mL of IMDM. The test substance, $1,25(OH)_2$ D_3 or MC903, was added at various concentrations at the beginning of the culture. Equal amounts of ethanol were added to control cultures. The final ethanol concentration was 0.1%. Cells grown under various conditions were harvested and counted and the viability of cells was determined by Trypan blue dye exclusion. Following treatment with MC903 or $1,25(OH)_2$ D_3, the morphological characteristics of the cells were determined by examination of Wright-Giemsa-stained cells and multinucleated cells (cells with two or more nuclei) were counted.

Colony assay. The colony assay was performed according to the methylcellulose culture method [13]. The HIMeg cells were suspended in medium containing 0.8% methylcellulose (Dow Chemicals, Midland, MI, U.S.A.), 20% FCS and various concentrations of MC903 or $1,25(OH)_2$ D_3. Aliquots of culture medium were plated onto 96.well plates at 500 cells/well in 0.1 mL and cultured at 37° in a 5% CO_2 humidified atmosphere. Incubated plates were cultured for 7 days and were counted with an inverted microscope. A colony formed by HIMeg cells was defined as a cell cluster containing more than 50 cells.

Flow cytometry. Cells in log phase of growth were exposed to vitamin D_3 compounds at 10^{-6} M. Cells were removed from culture at 4 days following exposure, washed with ice-cold phosphate-buffered saline and fixed with ice-cold 70% ethanol. Before analysis, cells were treated with

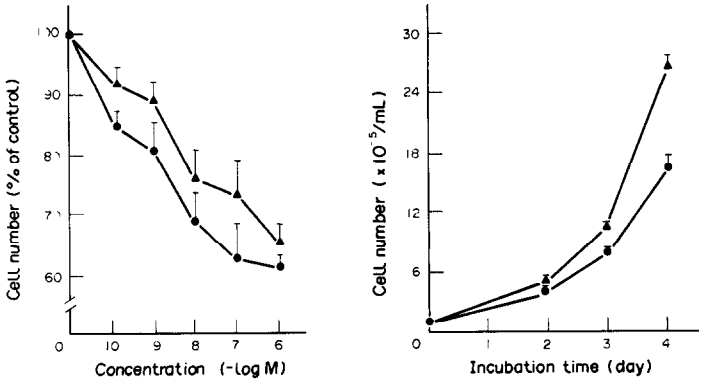

Fig. 1. Effects of MC903 and $1,25(OH)_2$ D_3 on growth of HIMeg cells. HIMeg cells were plated at 1×10^5 cells/mL onto 24-well clusters and incubated in the presence or absence of MC903 (circle) or $1,25(OH)_2$ D_3 (triangle) at the indicated concentrations for 4 days (left). For time-course studies (right), cells were incubated in the absence (triangle) or presence of 10^{-7} M MC903 (circle) for 0–4 days. Viable cells were counted by Trypan blue dye exclusion. Values represent the means ± SEM of four determinations.

RNase A (200 μg/mL) to reduce fluorescence from RNA and then stained with propidium iodide (10 μg/mL). DNA-bound propidium iodide was excited at a wavelength of 488 nm and cellular DNA content was determined by fluorescence at a wavelength of 630 nm. Cell cycle distribution was calculated.

Results

It has been reported that $1,25(OH)_2$ D_3 can inhibit the proliferation of leukemia cell lines such as HL-60 and U_{937} [4–11]. Therefore, the proliferation and differentiation response of the newly established HIMeg to $1,25(OH)_2$ D_3 and MC903 treatment was determined. HIMeg cells were treated with increasing concentrations of MC903 or $1,25(OH)_2$ D_3 for 4 days and the total viable cell number was determined. Figure 1 shows that treatment of HIMeg cells with vitamin D_3 compounds inhibited HIMeg cell proliferation in a dose- and time-dependent manner.

During treatment of HIMeg cells with MC903 or $1,25(OH)_2$ D_3, the morphology of the cells changed significantly from the second day: the cytoplasmic membranes became ruffled, sending out pseudopod-like processes. Multinucleated cells increased in number 4 days after exposure to MC903 or $1,25(OH)_2$ D_3 in a dose-dependent manner (Fig. 2). The correlation between these morphological changes and the induction of differentiated characteristics in these cells has been determined [14].

The effects of MC903 or $1,25(OH)_2$ D_3 on colony formation by HIMeg cells were investigated with the methylcellulose culture method. As shown in Fig. 3, almost no colony formation was observed in the presence of MC903 or $1,25(OH)_2$ D_3 at high concentrations (10^{-6}–10^{-9} M); low concentrations of the vitamin D_3 compounds could also markedly diminish the colony-forming ability of HIMeg cells.

Cell-cycle profiles were determined on HIMeg cells following exposure to MC903 or $1,25(OH)_2$ D_3. As demonstrated in Fig. 4, significant alterations in cell-cycle distribution occurred 4 days after treatment, i.e. there was a significant increase in the percentage of cells in $G_0 + G_1$ phase while the percentage of cells in both S and $G_2 + M$ phases decreased.

Discussion

A human megakaryoblastic cell line, HIMeg, was established from a patient with chronic granulocytic

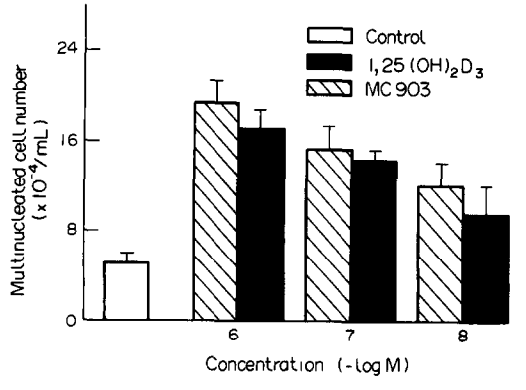

Fig. 2. Effects of MC903 and $1,25(OH)_2$ D_3 on the formation of multinucleated cells. HIMeg cells were incubated in the presence of absence of vitamin D_3 compounds at the indicated concentrations for 4 days and multinucleated cells were counted. Results shown were means ± SEM of three determinations. Control denotes untreated cultures. All changes were significantly different from controls ($P < 0.01$).

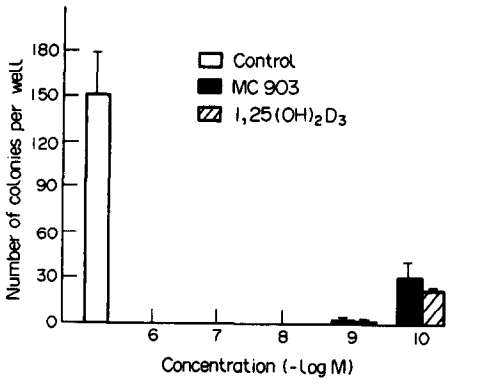

Fig. 3. Effects of MC903 and $1,25(OH)_2$ D_3 on colony formation by HIMeg cells. HIMeg cells were plated at 5000 cells/well onto 96-well plates with various concentrations of vitamin D_3 compounds. After 7 days of culture, colonies were scored under an inverted microscope. Values are means ± SEM of six determinations and all changes were significantly different from controls ($P < 0.001$).

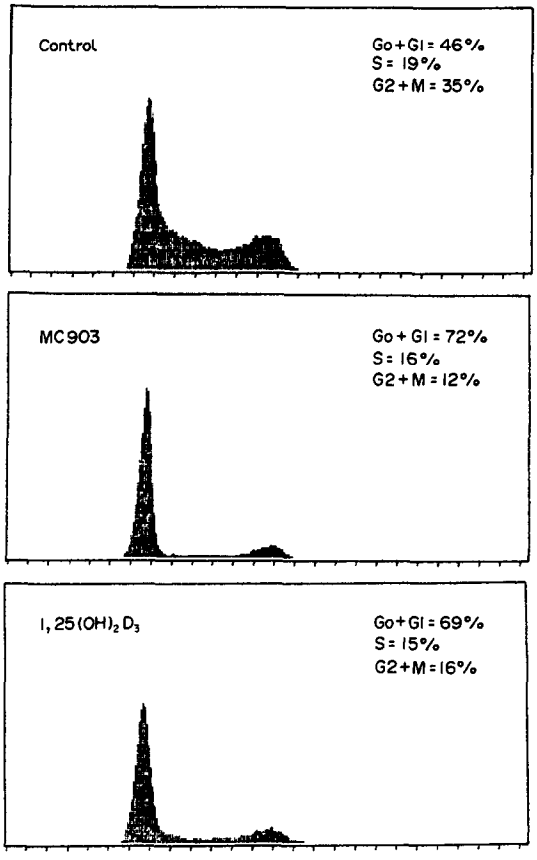

Fig. 4. Cell cycle profiles following exposure to vitamin D_3 compounds at 10^{-6} M for 4 days. DNA content of HIMeg cells was determined by fluorescence of propidium bound to DNA. The left-hand peak in the figure represents $G_0 + G_1$ cells (2 N DNA), the right-hand peak represents $G_2 + M$ cells (4 N DNA) and the area between the two peaks represents cells in S phase (between 2 and 4 N DNA). The percentage of cells in these cell-cycle phases is shown in the right part of the figure.

leukemia. HIMeg cells were identified as megakyoblasts for the following reasons: (a) the platelet peroxidase reaction; (b) the expression of glycoprotein IIb/IIIa (GP IIb/IIIa); and (c) the lack of specific markers for myeloid, monocytic and erythroid lineages [14]. In this report, the comparative effects of $1,25(OH)_2$ D_3 and MC903 on the proliferation and differentiation of HIMeg cells were studied. The results show that both $1,25(OH)_2$ D_3 and MC903 could significantly inhibit the proliferation and induce the differentiation of HIMeg cells. In a liquid culture system, exposure to MC903 or $1,25(OH)_2$ D_3 led to a significant decrease in cell growth rate, and dramatic changes in morphological appearance which were characteristic of differentiation. MC903 and $1,25(OH)_2$ D_3 almost completely abolished the colony-forming ability of HIMeg cells at high concentrations (10^{-6}–10^{-9} M) and markedly diminished the ability at low concentrations (10^{-10} M). MC903 and $1,25(OH)_2$ D_3 were approximately equipotent.

Cell-cycle analysis showed that MC903 and $1,25(OH)_2$ D_3 induced significant alterations in cell-cycle distribution. Exposure of HIMeg cells to vitamin D_3 at 10^{-6} M for 4 days resulted in the accumulation of cells in $G_0 + G_1$ phase while the percentage of cells in S and G_2 + M phases was decreased. This experiment suggests that MC903 and $1,25(OH)_2$ D_3 inhibited proliferation of HIMeg cells by slowing transition of the cells from the $G_0 + G_1$ to the S phase, resulting in induction of the $G_0 + G_1$-arrested cells that differentiate into mature megakaryocytes.

$1,25(OH)_2 D_3$ is the physiologically important secosteroid that not only mediates calcium reabsorption of bone and intestinal calcium absorption, but also has effects on hematopoiesis [1]. $1,25(OH)_2$ D_3 induces terminal differentiation of a variety of myeloid leukemic cells from patients and cell lines as well as normal committed myeloid stem cells [4, 10, 15]. The results of the present study further demonstrate that $1,25(OH)_2$ D_3 could also play a role in megakaryocytopoiesis; and that MC903, a cyclopropyl side chain analogue of $1,25(OH)_2$ D_3, is as effective as $1,25(OH)_2$ D_3 in inhibiting proliferation and stimulating differentiation of HIMeg cells. These effects of $1,25(OH)_2$ D_3 are probably mediated directly or indirectly by initial ligand binding to an intracellular $1,25(OH)_2$ D_3 receptor, which is a member of the steroid receptor superfamily. Further studies are needed to determine whether there is a functional $1,25(OH)_2$ D_3 receptor in HIMeg cells. It will be also necessary to assess the *in vivo* potency of $1,25(OH)_2$ D_3 and its derivatives in blocking neoplastic growth.

Acknowledgements—We wish to thank Dr L. Binderup for the kind gifts of MC903 and $1,25(OH)_2$ D_3. The authors also express their thanks to Prof. R. B. Xu for her critical reading of this manuscript. This work was supported in part by the National Natural Science Foundation of China.

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