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Synthetic analogues of vitamin D_3 with an oxygen atom in the side chain skeleton

A trial of the development of vitamin D compounds which exhibit potent differentiation-inducing activity without inducing hypercalcemia

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Four analogues of vitamin D_3 with an oxygen atom in the side chain skeleton were synthesized to determine whether their differentiation-inducing activity could be separated structurally from their activity to induce hypercalcemia. The order of the in vitro potency to reduce nitroblue tetrazolium in human myeloid leukemia cells (HL-60) was 22-oxa-1α,25-$(OH)_2D_3$ > 1α,25-$(OH)_2D_3$ > 20-oxa-1α,25-$(OH)_2D_3$ ≒ 22-oxa-1α-(OH)D_3 > 1α-(OH)D_3 > 20-oxa-1α-(OH)D_3. 22-Oxa-1α,25-$(OH)_2D_3$ was also about 10-times more potent than 1α,25-$(OH)_2D_3$ in suppressing proliferation and inducing differentiation of mouse myelomonocytic leukemia cells (WEHI-3), but the former was much weaker than the latter in inducing the release of ^{45}Ca from prelabeled fetal mouse calvaria. These results suggest that the differentiation-inducing activity of vitamin D compounds can be separated structurally from their activity to induce hypercalcemia.

Vitamin D; 1α,25-Dihydroxy-22-oxavitamin D_3; Differentiation-inducing activity; Bone-resorbing activity

1. INTRODUCTION

1α,25-Dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$), the active form of vitamin D_3, has been known to regulate serum calcium levels by promoting intestinal calcium transport and bone mineral mobilization. In 1981, Abe et al. [1] first demonstrated that 1α,25-$(OH)_2D_3$ suppressed proliferation of mouse myeloid leukemia cells (M1) and caused them to be differentiated into cells that are morphologically and functionally similar to mature monocyte-macrophages. Administration of 1α-hydroxyvitamin D_2 (1α-(OH)D_3), a synthetic analogue of 1α,25-$(OH)_2D_3$ active in anephric patients, either with or without daunomycin considerably prolonged the survival time of leukemic mice inoculated with M1 cells [2,3]. But the concentration of 1α,25-$(OH)_2D_3$ in vitro required to induce maximal growth suppression and differentiation was 10 nM. This is about 100-times higher than the physiological serum levels of 1α,25-$(OH)_2D_3$ [1]. This prompted organic chemists to synthesize vitamin D analogues which are capable of potent differentiation-inducing activity without inducing hypercalcemia. They have synthesized numerous new derivatives of vitamin D_3, but none of these has achieved the intended purpose [4–7].

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In our laboratory, 4 interesting new derivatives of vitamin D_3 have been synthesized for this purpose [8,9]. One of these is 1α-hydroxy-21-nor-20-oxavitamin D_3 (20-oxa-1α-(OH)D_3). The second one is 1α,25-dihydroxy-21-nor-20-oxavitamin D_3 (20-oxa-1α,25-$(OH)_2D_3$). In these two compounds the carbon atom at position 20 is replaced by oxygen. The third one is 1α-hydroxy-22-oxavitamin D_3 (22-oxa-1α-(OH)D_3) and the fourth is 1α,25-dihydroxy-22-oxavitamin D_3 (22-oxa-1α,25-$(OH)_2D_3$). In these two compounds the carbon atom at position 22 is replaced by oxygen. We report here that 22-oxa-1α,25-$(OH)_2D_3$ is about 10-times more potent than 1α,25-$(OH)_2D_3$ in suppressing growth and inducing differentiation in vitro of human and mouse myeloid leukemia cells, but the former compound is much weaker than the latter in inducing the release of ^{45}Ca from prelabeled fetal mouse calvaria.

2. MATERIALS AND METHODS

2.1. *Cells and cell culture*

The human myeloid leukemia cell line HL-60 and the murine myelomonocytic leukemia cell line WEHI-3 were kindly provided by Dr H. Hemmi, School of Medicine, and by Dr K. Kumagai, School of Dentistry, respectively, both at Tohoku University, Sendai, Japan. Both cell lines were cultured in RPMI-1640 medium supplemented with L-glutamine (0.29 mg/ml), Kanamycin (0.6 μg/ml) and 5% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2. *Vitamin D_3 derivatives*

1α,25-$(OH)_2D_3$ was purchased from Philips Duphar, Amsterdam, The Netherlands. 1α-(OH)D_3 was synthesized in our laboratory. The new derivatives of vitamin D_3: 20-oxa-1α,25-$(OH)_2D_3$, 20-oxa-1α-(OH)D_3, 22-oxa-1α,25-$(OH)_2D_3$ and 22-oxa-1α-(OH)D_3 (fig.1), were synthesized from dehydroepiandrosterone in our laboratory. The detailed synthesis procedures have been described [8,9]. These compounds were dissolved in absolute ethanol and the stock solutions were stored at −20°C. The final concentration of ethanol in the culture medium was less than 0.1%, which had no effect on the viability of these cells.

2.3. *Assessment of cell growth and differentiation of HL-60 and WEHI-3*

To examine the effect of vitamin D_3 compounds on cell growth, WEHI-3 cells (1×10^5) were cultured for 5 days in 10 ml of culture medium containing vitamin D_3 compounds. On each day, cells were harvested and the cell number was determined.

Expression of phenotypes associated with differentiation was examined with the nitroblue tetrazolium (NBT)-reducing activity in HL-60 cells and with the phagocytic activity in WEHI-3 cells. To measure NBT-reducing activity, HL-60 cells (1×10^4/ml) were incubated for 20 min at 37°C in RPMI-1640 medium containing NBT (1 mg/ml) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 100 ng/ml). The number of cells containing formazan deposits was counted to determine the percentage of reacting cells [10]. To measure phagocytic activity, WEHI-3 cells (1×10^5/ml) were incubated for 4 h at 37°C with 5 μl latex particles (average diameter, 0.81 μm; Difco) in PBS. After washing 3 times with PBS, the percentage of phagocytic cells was determined by counting [10].

2.4. *Measurement of bone-resorbing activity*

The bone-resorbing activity was measured as described [5]. In short, mice (ddy strain), 16 days pregnant, were injected intraperitoneally with 25 μCi $^{45}CaCl_2$ (New England Nuclear, Boston, MA). One day later the calvaria was isolated and divided into paired halves. These were precultured for 1 day at 37°C in BGJb medium (Gibco) containing 0.1% BSA. Then each half-calvarium was cultured for 5 days in fresh medium with (treated) or without (control) assay samples. At the end of the culture period, the ^{45}Ca was counted separately in the medium and in the bone and the ratio of the treated to the control was calculated.

2.5. *Statistical analysis*

Results were expressed as the mean ± SD of at least 4 independent sets of experiments. In each experiment, triplicate assays were performed. The statistical significance of the difference between the control and the experimental group was analysed by Student's *t*-test.

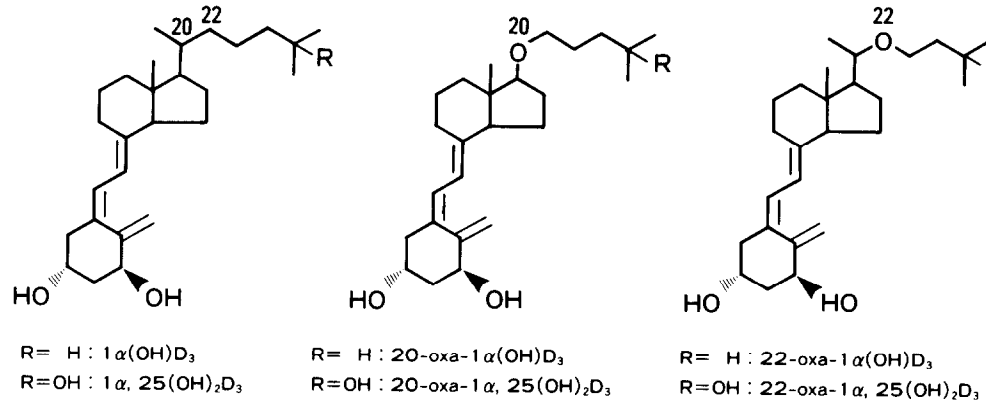

Fig.1. Structures of 1α-(OH)D_3, 1α,25-$(OH)_2D_3$, 20-oxa-1α-(OH)D_3, 20-oxa-1α,25-$(OH)_2D_3$, 22-oxa-1α-(OH)D_3 and 22-oxa-1α,25-$(OH)_2D_3$.

3. RESULTS

First, in an attempt to screen biological activity of the four oxa compounds, their differentiation-inducing activity was compared with that of 1α,25-$(OH)_2D_3$ and 1α-(OH)D_3, using the induction of NBT-reducing activity in HL-60 cells. Fig.2 shows their dose-response curves. 22-Oxa-1α,25-$(OH)_2D_3$ had the highest activity, followed by 1α,25-$(OH)_2D_3$, 20-oxa-1α,25-$(OH)_2D_3$, 22-oxa-1α-(OH)D_3, 1α-(OH)D_3 and 20-oxa-1α-(OH)D_3, in that order. As expected, the vitamin D_3 derivatives with a hydroxyl function at position 25 induced NBT-reducing activity more potently than the corresponding compounds without a hydroxyl function at position 25. It is also clear that the compounds with oxygen substituted at position 22 exhibit a stronger NBT-reducing activity, and the compounds with oxygen substituted at position 20 are weaker than the corresponding original compounds (1α,25-$(OH)_2D_3$ or 1α-(OH)D_3).

To examine further the differentiation-inducing activity of 22-oxa-1α,25-$(OH)_2D_3$, its activity to suppress growth and to induce differentiation was

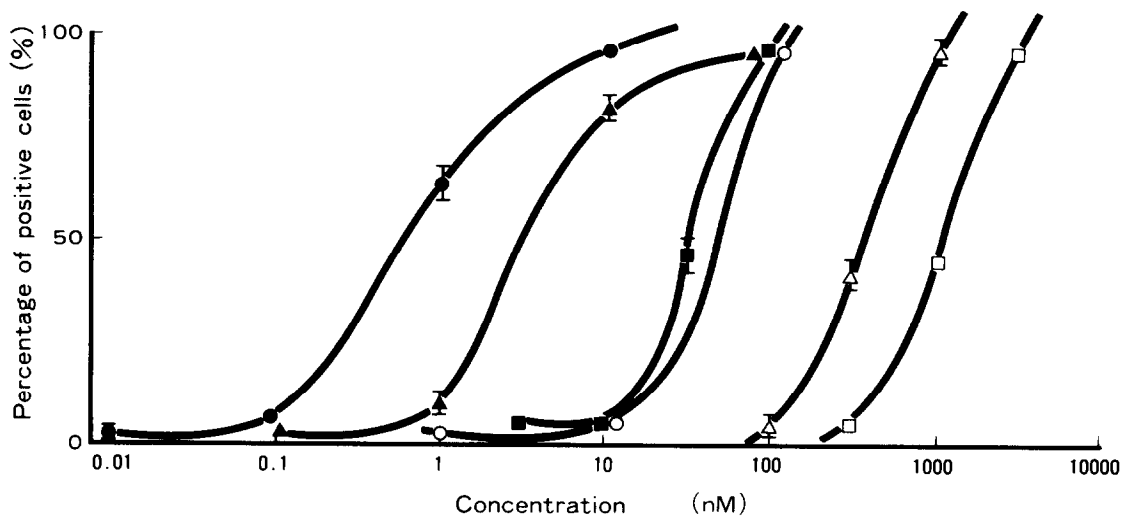

Fig.2. Relative potency of the oxa compounds of vitamin D_3 in inducing NBT-reducing activity in HL-60 cells. 1α-(OH)D_3 (△), 1α,25-$(OH)_2D_3$ (▲), 20-oxa-1α-(OH)D_3 (□), 20-oxa-1α,25-$(OH)_2D_3$ (■), 22-oxa-1α-(OH)D_3 (○), and 22-oxa-1α,25-$(OH)_2D_3$ (●).

assessed in WEHI-3 cells (fig.3). 22-Oxa-1α,25-$(OH)_2D_3$ was about 10-times more potent than 1α,25-$(OH)_2D_3$ in suppressing proliferation (A) and in inducing phagocytic activity (B). The oxa compound induced both growth inhibition and appearance of phagocytic activity as fast as, or even faster than, 1α,25-$(OH)_2D_3$ did (C and D).

In contrast, 22-oxa-1α,25-$(OH)_2D_3$ first induced the release of ^{45}Ca from prelabeled fetal bone at as high as 4.8 nM, whereas 1α,25-$(OH)_2D_3$ induced a significant bone-resorbing activity at as low as 0.12 nM (table 1). The in vitro bone-resorbing activity of 22-oxa-1α,25-$(OH)_2D_3$ was estimated to be only 1/50–1/100 that of 1α,25-$(OH)_2D_3$.

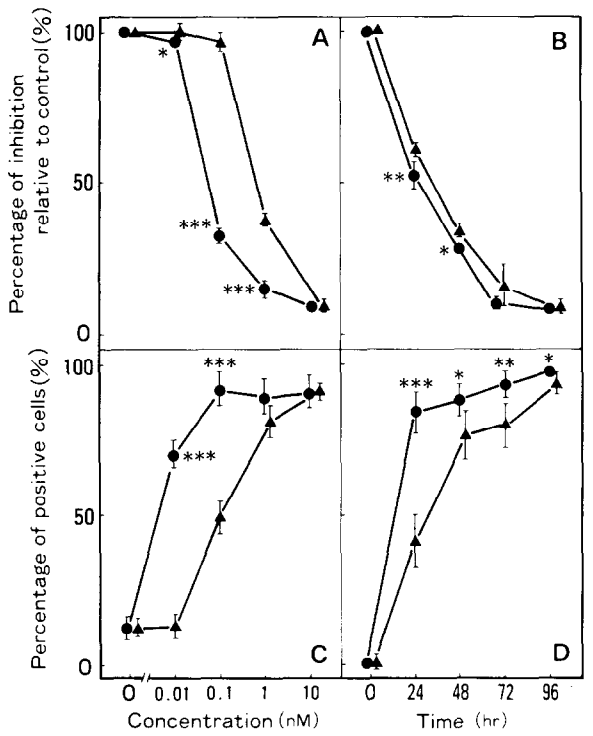

Fig.3. Comparison of the growth-inhibiting activity and the differentiation-inducing activity of 22-oxa-1α,25-$(OH)_2D_3$ (●) and 1α,25-$(OH)_2D_3$ (▲). (A) Dose-response curves in growth inhibition and (B) their time course change. (C) Dose-response curves in the appearance of phagocytic activity and (D) their time course change. The dose-response effect was measured on day 3, and the time course effect was measured at 10 nM. Data are the mean ± SD of 6 independent sets of experiments. Significantly different from the 1α,25-$(OH)_2D_3$ group on each concentration or each time point (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Table 1

Effect of 22-oxa-1α,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_3$ on the release of ^{45}Ca from prelabeled fetal mouse calvaria

Concentration of vitamin D_3 compounds (nM)	^{45}Ca release (T/C ratio)	
	22-oxa-1α,25-$(OH)_2D_3$	1α,25-$(OH)_2D_3$
0.0	0.94 ± 0.14 (11)	
0.12	1.10 ± 0.36 (4)	1.50 ± 0.29* (10)
0.60	–	2.02 ± 0.61* (4)
1.20	1.22 ± 0.11 (11)	2.52 ± 0.33** (11)
4.80	1.37 ± 0.10* (4)	–
12.00	1.75 ± 0.26* (11)	–

Data are the mean ± SD (number of experiments). Significantly different from control (* $p < 0.05$; ** $p < 0.01$)

4. DISCUSSION

The present study indicates that the differentiation-inducing activity of vitamin D compounds could be separated structurally from their bone-resorbing activity, at least in vitro. The differentiation-inducing activity includes suppression of proliferation and induction of differentiation of several hematopoietic cells, whereas the bone-resorbing activity is induced by promoting osteoclast formation from their progenitors and by somehow activating resting osteoclasts [11].

It is possible to postulate several reasons why 22-oxa-1α,25-$(OH)_2D_3$ exhibited an activity more potent than 1α,25-$(OH)_2D_3$ in suppressing growth of WEHI-3 cells and in inducing differentiation of both HL-60 and WEHI-3 cells without exhibiting significant bone-resorbing activity. It is known that the physicochemical properties of 22-oxa-1α,25-$(OH)_2D_3$ are different from those of 1α,25-$(OH)_2D_3$. For instance, the distance of the side chain (between C_{20} and C_{25}) of the two compounds is different: 5.15 Å in 1α,25-$(OH)_2D_3$ and 4.96 Å in 22-oxa-1α,25-$(OH)_2D_3$. The rotation of the side chain is more strictly limited in 22-oxa-1α,25-$(OH)_2D_3$ than in 1α,25-$(OH)_2D_3$, because of the presence of the oxygen atom at position 22 in the former. Furthermore, 22-oxa-1α,25-$(OH)_2D_3$ was more polar than 1α,25-$(OHI)_2D_3$ (not shown). These differences in the physicochemical properties appear to be involved in the separation of the

differentiation-inducing activity from the bone-resorbing activity of vitamin D compounds. Wovkulich et al. [6] and Ostrem et al. [7] have also independently pointed out the importance of the modification of the side chain structure, particularly at positions C_{22}–C_{24}, of vitamin D compounds in separating the two activities.

It is well known that vitamin D compounds exhibit their biological activities by a receptor-mediated mechanism. Very recently, McDonnell et al. [12] succeeded in the molecular cloning of complementary DNA encoding the avian intestinal receptor for 1α,25-$(OH)_2D_3$. The molecular structure of the 1α,25-$(OH)_2D_3$ receptors in tissues other than intestine, however, has not been identified. There may be a qualitative tissue difference in the 1α,25-$(OH)_2D_3$ receptors between myeloid leukemia cells and bone cells. If so, it may become possible to separate the differentiation-inducing activity of vitamin D compounds from their hypercalcemic action.

Another important point is that the binding affinity of vitamin D compounds for 1α,25-$(OH)_2D_3$ receptors is not necessarily correlated with the potency of their biological activity. The binding affinity of 22-oxa-1α,25-$(OH)_2D_3$ for the 1α,25-$(OH)_2D_3$ receptor in HL-60 cells was weaker than that of 1α,25-$(OH)_2D_3$ (not shown), whereas the former compound exhibited a more potent activity than the latter in inducing NBT-reducing activity in HL-60 cells (fig.2). This suggests that factors other than the binding affinity for the receptor, such as stability, cellular uptake and intracellular metabolism of vitamin D compounds, are also important in separating the differentiation-inducing activity from the hypercalcemic action.

It is particularly important to determine whether 22-oxa-1α,25-$(OH)_2D_3$ exhibits potent differentiation-inducing activity in vivo without inducing hypercalcemia. To evaluate this, the in vivo differences in the differentiation-inducing activity and hypercalcemic action of 22-oxa-1α,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_3$, and their metabolism and tissue distribution have to be examined. These are currently under investigation in our laboratories.

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