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Differential Effects of 1,25-Dihydroxyvitamin D_3-Analogs on Osteoblast-like Cells and on *In Vitro* Bone Resorption

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Although numerous studies have shown potent antiproliferative and differentiation-inducing effects of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and its analogs on cells not directly related to bone metabolism, only few reports focussed on the effects of these analogs on bone. We compared the action of several recently developed analogs with that of 1,25-$(OH)_2D_3$ on human (MG-63) and rat (ROS 17/2.8) osteoblast-like cells and on *in vitro* bone resorption. In MG-63 cells the analogs EB1089 and KH1060 were about 166,000 and 14,000 times more potent than 1,25-$(OH)_2D_3$ in stimulating type I procollagen and 100 and 6,000 times more potent in stimulating osteocalcin production, respectively. Also in ROS 17/2.8 cells EB1089 and KH1060 were most potent in inducing osteocalcin synthesis. *In vitro* bone resorption was 2.3 and 17.5 times more potently stimulated by EB1089 and KH1060, respectively. In MG-63 cells, 1,25-$(OH)_2D_3$ and the analogs inhibited cell proliferation, whereas both 1,25-$(OH)_2D_3$ and the analogs stimulated the growth of ROS 17/2.8 cells. Differences in potency could neither be explained by affinity for the vitamin D receptor nor by a differential involvement of protein kinase C in the action of the analogs. Together, these data show that also in bone the analogs EB1089 and KH1060 are more potent than 1,25-$(OH)_2D_3$ but that the potency of the analogs compared to 1,25-$(OH)_2D_3$ is dependent on the biological response. On the basis of these observations it can be concluded that the reported reduced calcemic effect *in vivo* is not the result of a decreased responsiveness of bone to these analogs. Lastly, in view of eventual clinical application of 1,25-$(OH)_2D_3$-analogs, the observed stimulation of *in vitro* bone resorption and growth of an osteosarcoma cell line warrant *in vivo* studies to further examine these effects.

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INTRODUCTION

The central role of vitamin D in the regulation of calcium and bone metabolism is well established [1]. In addition, in 1981, Abe *et al.* [2] demonstrated that 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) inhibited the proliferation and stimulated the differentiation of mouse myeloid leukemia cells. This finding was promising for the use of 1,25-$(OH)_2D_3$ in the treatment of hyperproliferative diseases and immunological disorders [3]. However, the high doses of 1,25-$(OH)_2D_3$ needed to achieve these non-classical effects may lead to undesirable side-effects on calcium metabolism (hypercalcemia, hypercalciuria). This has prompted the development of 1,25-$(OH)_2D_3$-analogs with potent cell growth regulating properties but relative low calcemic activity [4–8]. Hitherto the *in vitro* effects of these analogs were mostly studied in cells not directly related to bone and calcium metabolism. Therefore, knowledge on their effects on bone and bone cells is limited and mainly restricted to the analogs MC902 and 22-oxa-calcitriol (OCT) [9–13]. In the present study we have analyzed the effects of 1,25-$(OH)_2D_3$ and several of its analogs with side-chain modifications on osteoblast-like cells and on *in vitro* bone resorption and compared their potencies. These analyses may provide insights into whether the *in vivo* reduced calcemic activity observed reflects a decreased responsiveness of the skeleton to these analogs.

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MATERIALS AND METHODS

Reagents

Non-radioactive 1,25-$(OH)_2D_3$ and the 1,25-$(OH)_2D_3$-analogs MC903, CB966, EB1089, KH1049 and KH1060 were generously provided by Dr L. Binderup, Leo Pharmaceuticals, Ballerup, Denmark. OCT was a gift from Dr N. Kubodera, Chugai Pharmaceutical Co., Ltd, Tokyo, Japan. Chemical structures are depicted in Fig. 1. The sterols were dissolved in absolute ethanol and stored at −20°C at a concentration of 10^{-4} M. [23,24-^{3}H]1,25-$(OH)_2D_3$ (120 Ci/mmol) and ^{45}Ca were obtained from Amersham International, Amersham, U.K.. Alpha minimal essential (αMEM) and neutral red were from Sigma Chemical Co., St Louis, MO, U.S.A. The rat osteocalcin antiserum was a generous gift of Professor R. Bouillon, Katholieke Universiteit Leuven, Leuven, Belgium. Penicillin, streptomycin and L-glutamine were from Gibco Life Technologies Ltd., Paisley, Scotland. Fetal calf serum (FCS) was from Sera-Tech, St Salvator, Germany. Biggers Gwatkin-Judah (BGJ) medium was made using reagents from Merck, Darmstadt, Germany. 1-*O*-hexadecyl-2-*O*-methyl-rac-glycerol (AMG) was purchased from Bachem AG, Bubendorf, Switzerland. All other reagents were of the best grade commercially available.

Cells

Human osteoblast-like MG-63 cells were generously provided by Professor R. Bouillon, Katholieke Universiteit Leuven, Leuven, Belgium. The rat osteoblast-like osteosarcoma cell line ROS 17/2.8 and the

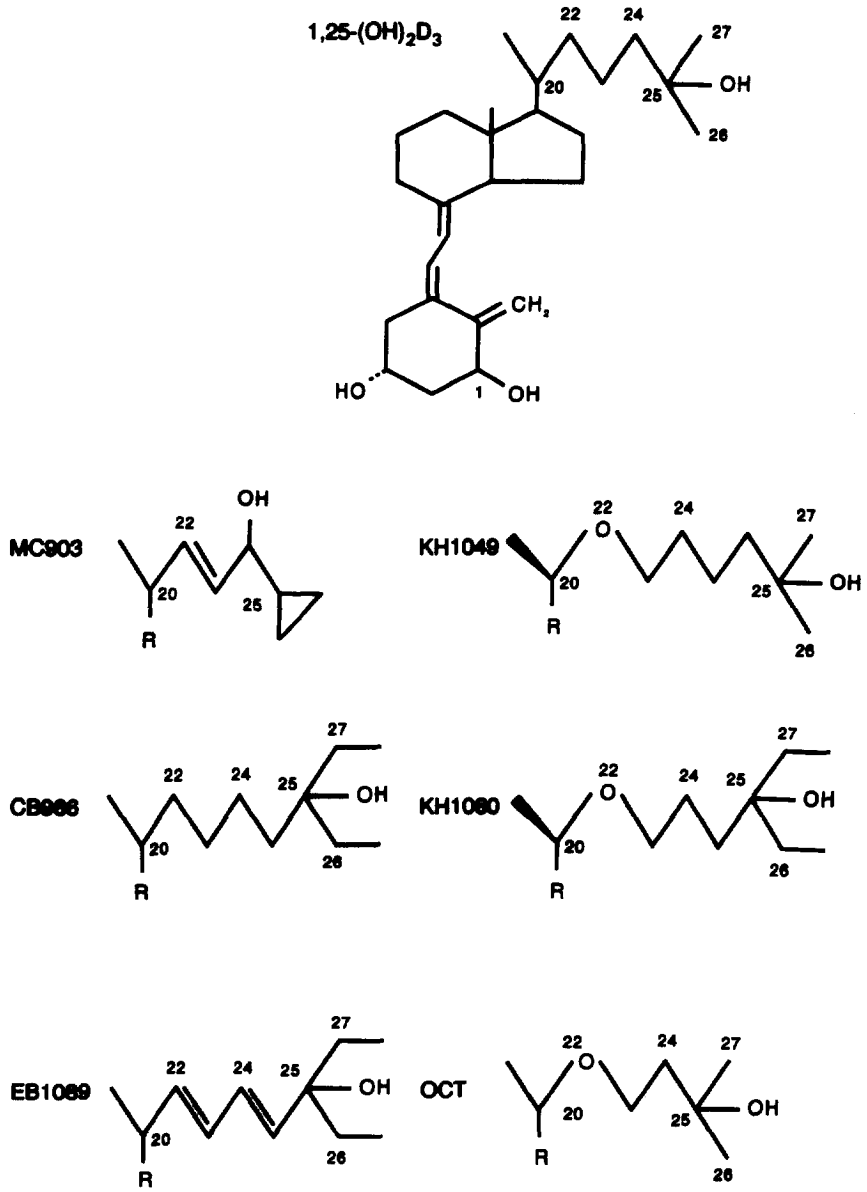

Fig. 1. Chemical structures of 1,25-$(OH)_2D_3$, MC903, CB966, EB1089, KH1049, KH1060 and OCT.

non-osteoblast-like osteosarcoma cell line ROS 25.1 were a gift from Dr S. B. Rodan, Merck, Sharp & Dohme Research Laboratories, West Point, U.S.A. The cells were cultured in αMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1% D-glucose plus the indicated concentration FCS or charcoal-treated FCS (CT-FCS).

Osteocalcin measurements

The cells were seeded in 24-well culture plates at a density of 40,000 cells/cm² and cultured for 24 h (MG-63) or 48 h (ROS 17/2.8) in αMEM plus 10% FCS. Next, the medium was replaced by αMEM with 2% CT-FCS and the cells were cultured in the absence or presence of various concentrations of 1,25-$(OH)_2D_3$ or its analogs. After 24 h medium was collected and osteocalcin content was determined. Osteocalcin measurements in medium of ROS 17/2.8 cells were performed according to the method described by Verhaeghe *et al.* [14]. Osteocalcin measurements in the medium of MG-63 cells were performed by RIA (Incstar, Stillwater, MN, U.S.A.).

Type I procollagen measurements

MG-63 cells in αMEM + 10% FCS were seeded in 24-well culture plates at a density of 40,000 cells/cm². After 24 h medium was replaced by αMEM with 2% CT-FCS and the various concentrations of 1,25-$(OH)_2D_3$ or its analogs. After 24 and 48 h media were collected and analyzed for type I procollagen by RIA (Orion Diagnostica, Espoo, Finland).

Proliferation assay

Cell proliferation was studied using the neutral red assay described by Löwik *et al.* [15]. The absorbance of neutral red is proportional to the number of viable cells. In short, 1000 MG-63 cells/cm² and 3000 ROS 17/2.8 or ROS 25.1 cells/cm² were seeded in a 96 wells plate and cultured for 24 h in αMEM plus 10% FCS. After 24 h the medium was replaced by αMEM with 2% CT-FCS with or without various concentrations of 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$-analogs. After 3 days medium was replaced by new medium with or without the addition of the various analogs. After 6 days 50 μl of a neutral red solution (0.5 mg/ml in 0.9% NaCl) was added to each well for a 90 min incubation. Next, the medium was removed, the wells were washed twice with 100 μl phosphate-buffered saline and the neutral red was extracted from the cells with 100 μl 0.05 M NaH_2PO_4 in 50% ethanol. The absorption was measured at 540 nm (630 nm reference filter) in a microplate reader (Bio-Rad 450). All measurements were performed in quadruplicate and expressed as the percentage of control optical density values.

Bone resorption assay

The *in vitro* bone resorption assay was performed with 17-day-old fetal mouse radii/ulnae, using a method based on the fetal rat limb explant assay described by Raisz [16]. By injecting the mother on day 16 of gestation with 30 μCi of ^{45}Ca, the fetal radii/ulnae were labelled *in utero*. After explantation on day 17 the radii/ulnae were precultured for 24 h in 400 μl of BGJ medium with 5% CT-FCS at 37°C, to reduce free exchangeable calcium. Next, the medium was changed to BGJ medium supplemented with 5% CT-FCS with or without various concentrations of 1,25-$(OH)_2D_3$ or analogs. After 3 days the medium was replaced by fresh medium, i.e. with or without 1,25-$(OH)_2D_3$ or the analogs. The ^{45}Ca content of the medium at 3 and 6 days of culture and of the 5% formic acid extracts of the bones were measured by liquid scintillation counting and used to calculate the total ^{45}Ca and the cumulative percentage ^{45}Ca released.

VDR binding studies

Competitive receptor binding assays were performed as previously described [17]. Receptor protein was prepared by cytosolic extraction on ice of MG-63 and ROS 17/2.8 cells with a hypertonic buffer consisting of 10 mM Tris–HCl, 300 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 10 mM sodiummolybdate and 0.1% Triton X-100 (pH 7.4). After centrifugation at 100,000 *g* for 1 h at 4°C, the cytosolic extracts (250 μl ± 1 mg protein/ml) were incubated at 0°C with 0.5 nM [23,24-^3H]1,25-$(OH)_2D_3$ and increasing concentrations of non-radioactive 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$-analogs. After 3 h the bound and free [23,24-^3H]1,25-$(OH)_2D_3$ were separated by charcoal adsorption. Concentrations of 1,25-$(OH)_2D_3$ or the analogs at which half-maximal displacement of [23,24-^3H]1,25-$(OH)_2D_3$ was achieved were calculated.

DNA measurements

DNA measurements were performed according to the method of Karsten and Wollenberger [18].

Statistical analysis

The data shown are the mean of at least two independent experiments each consisting of 2–4 separate cultures. For clarity purposes standard deviations were not depicted in the figures. In all cases standard deviations were smaller than 10%.

RESULTS

Effect of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$-analogs on osteocalcin production by MG-63 and ROS 17/2.8 cells

In both cell lines 1,25-$(OH)_2D_3$ and the analogs stimulated osteocalcin production in a dose-dependent manner (Fig. 2). Besides differences in ED_{50} in MG-63 cells but not in ROS 17/2.8 cells, also a difference in maximal response was detected. The maximal stimulation of osteocalcin production by the compounds with the lowest ED_{50} (KH1060, EB1089, KH1049) was about twice that of 1,25-$(OH)_2D_3$, OCT, and CB966

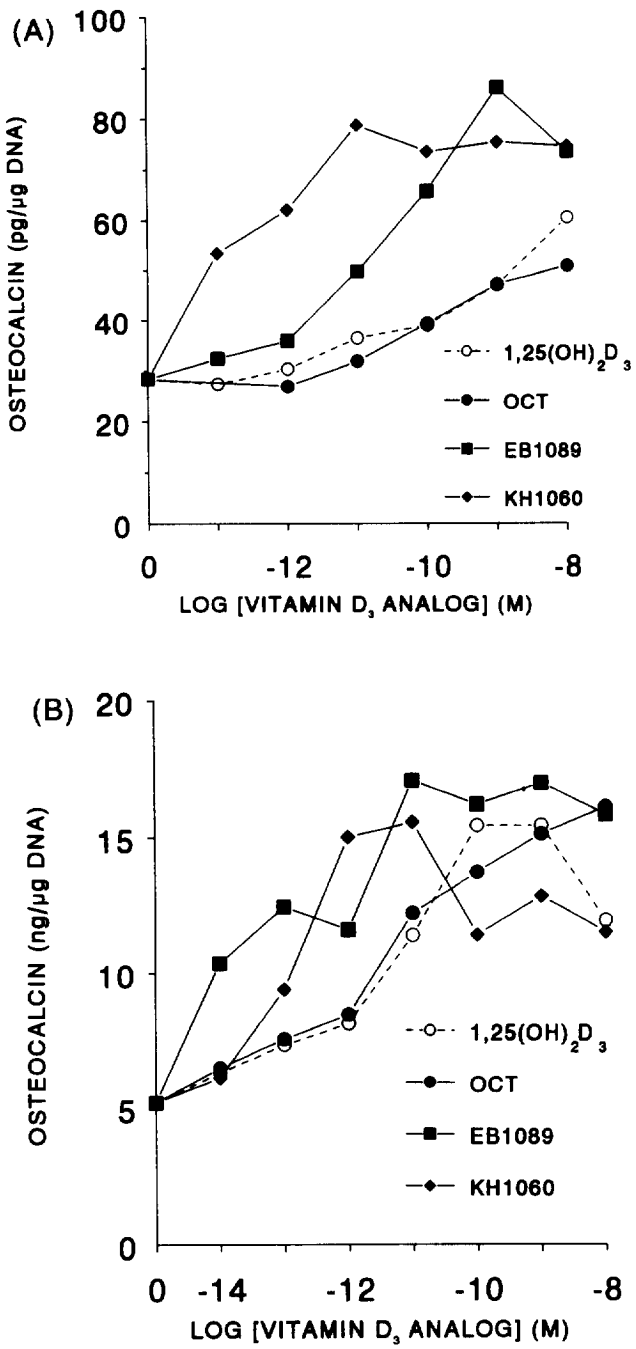

Fig. 2. Effect of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$-analogs on osteocalcin production in MG-63 (A) and ROS 17/2.8 cells (B). The cells were stimulated with 1,25-$(OH)_2D_3$ or the 1,25-$(OH)_2D_3$-analogs for 24 h and osteocalcin was measured as described in Materials and Methods.

[Fig. 2(A): 1,25-$(OH)_2D_3$, OCT, EB1089, KH1060]. Except for KH1060 and KH1049, the ROS 17/2.8 cells appeared to be more sensitive for 1,25-$(OH)_2D_3$ and the analogs than the MG-63 cells (Table 1).

Effect of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$-analogs on type I procollagen production by MG-63 cells

The effect on type I collagen synthesis was assessed by measuring the concentration of carboxyterminal propeptide of type I procollagen [19] in the medium of MG-63 cells after 24 and 48 h treatment with 1,25-$(OH)_2D_3$ or its analogs. Figure 3 shows the dose–response curves after 48 h of incubation with 1,25-$(OH)_2D_3$ and the most potent analogs. KH1060 and EB1089 were about 14,000 and 166,000 times, respectively, more potent than 1,25-$(OH)_2D_3$. The ED_{50}'s were 7×10^{-13} M and 6×10^{-14} M for KH1060 and EB1089, respectively, and 1×10^{-8} M for 1,25-$(OH)_2D_3$. Only a slight effect of the sterols could be detected after 24 h of culture (data not shown).

Effect on the proliferation of MG-63 cells and ROS 17/2.8 cells

The proliferation of the human osteoblast-like cell line MG-63 was inhibited by 1,25-$(OH)_2D_3$ and the analogs tested. No significant difference in maximum inhibitory effect (approx. 45%) between 1,25-$(OH)_2D_3$ and the analogs was found. Figure 4(A) shows dose–response curves of 1,25-$(OH)_2D_3$, OCT, EB1089 and KH1060 after 7 days of culture. The ED_{50}'s and the potencies relative to 1,25-$(OH)_2D_3$ (at ED_{50}) for these and the other analogs are summarized in Table 1. KH1060 was the most effective analog (ED_{50} was 4×10^{-14} M compared to 1×10^{-8} M for 1,25-$(OH)_2D_3$). KH1049 and EB1089 were also more potent than 1,25-$(OH)_2D_3$; 25 and 200 times respectively, whereas the other analogs were equipotent or only slightly more potent (Table 1).

In contrast to MG-63 cells, the growth of the rat osteoblast-like cells ROS 17/2.8 was not inhibited but stimulated by 1,25-$(OH)_2D_3$ and the analogs tested. The proliferation was stimulated in a dose-dependent manner and no significant difference in maximum effect between 1,25-$(OH)_2D_3$ and the analogs was observed [Fig. 4(B)]. EB1089 and KH1060 were the most potent, reaching 50% growth stimulation at 1×10^{-14} M and 1×10^{-12} M, respectively, compared to 2×10^{-10} M for 1,25-$(OH)_2D_3$ (Table 1). Changing culture conditions (serum free and serum containing medium) and seeding density did not change the effect of 1,25-$(OH)_2D_3$ and the analogs (data not shown). The observed stimulation of the proliferation by 1,25-$(OH)_2D_3$ and the analogs was confirmed by cell counts using a Coulter counter (Sysmex-Toa, model F-300, Kobe, Japan).

Effect of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$-analogs on in vitro bone resorption

1,25-$(OH)_2D_3$ and all analogs tested stimulated *in vitro* bone resorption in a dose-dependent manner, both after 3 and 6 days of culture. Figure 5 shows the *in vitro* bone resorption activity of 1,25-$(OH)_2D_3$ and the analogs OCT, EB1089, KH1049 and KH1060 after 6 days of culture. In Table 2 the concentrations of the sterols leading to a half-maximal ^{45}Ca release after 6 days of culture and the relative potency compared to the ED_{50} of 1,25-$(OH)_2D_3$ are presented. There was no difference in ED_{50}-values between 3 and 6 days of

Table 1. Effects of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$-analogs on osteocalcin production and cell proliferation and their affinity for the VDR

	MG-63 cells			ROS 17/2.8 cells		
Compound	Osteocalcin production ED_{50} (M)*	Growth inhibition ED_{50} (M)	Relative affinity for the VDR	Osteocalcin production ED_{50} (M)	Growth stimulation ED_{50} (M)	Relative affinity for the VDR
1,25-$(OH)_2D_3$	6×10^{-10} (1)†	1×10^{-8} (1)	1‡	4×10^{-12} (1)	2×10^{-10} (1)	1
OCT	6×10^{-10} (1)	3×10^{-9} (3)	0.15	5×10^{-12} (0.8)	6×10^{-10} (0.3)	0.08
MC903	ND	1×10^{-8} (1)	0.21	4×10^{-12} (1)	ND	0.33
CB966	2×10^{-10} (3)	2×10^{-9} (5)	0.70	2×10^{-12} (2)	ND	0.80
EB1089	6×10^{-12} (100)	5×10^{-11} (200)	0.71	1×10^{-14} (400)	1×10^{-14} (20,000)	0.80
KH1049	2×10^{-12} (300)	6×10^{-12} (1660)	0.22	1×10^{-12} (4)	ND	0.19
KH1060	1×10^{-13} (6000)	4×10^{-14} (250,000)	0.29	1×10^{-13} (40)	1×10^{-12} (200)	0.20

The effect of 1,25-$(OH)_2D_3$ and the analogs on the production of osteocalcin was measured by RIA. Cell proliferation was determined by the neutral red assay after 6 days of culture in the presence of 1,25-$(OH)_2D_3$ or the analogs. Binding to the VDR was measured in cytosolic extracts by displacement of [23,24-^{3}H]-1,25-$(OH)_2D_3$. *The concentration needed to achieve the half-maximal effect of 1,25-$(OH)_2D_3$ is designated as ED_{50}. †The potency of the analogs (at ED_{50}) was calculated in relation to that of 1,25-$(OH)_2D_3$. ‡Results are expressed as relative affinity (at half-maximal displacement) in comparison with 1,25-$(OH)_2D_3$. The data are the mean of three independent experiments. ND, not determined.

culture (data not shown). KH1049, KH1060 and EB1089, analogs with a far more potent effect on extracellular matrix synthesis and cell proliferation, were also more potent than 1,25-$(OH)_2D_3$ in inducing *in vitro* bone resorption (35, 17.5 and 2.3 times more potent, respectively), whereas CB966 and OCT were equipotent to 1,25-$(OH)_2D_3$.

Ability of the 1,25-$(OH)_2D_3$-analogs to compete with 1,25-$(OH)_2D_3$ for binding to the VDR

In view of the potent effects of some analogs, the ability to bind to the VDR from MG-63 and ROS 17/2.8 cells was examined. Displacement studies performed with cytosolic extracts demonstrated that all analogs exhibited lower affinity for the VDR compared to 1,25-$(OH)_2D_3$ (Fig. 6). In both cell types similar results were obtained (Table 1).

Effect of 1,25-$(OH)_2D_3$ and KH1060 on the proliferation of ROS 25.1 cells

Regarding the very potent effects of some analogs and their reduced affinity for the VDR we further examined the role of the VDR. The ROS 25.1 cell line is a non-osteoblast-like rat osteosarcoma cell line with no detectable VDR expression. The growth of ROS 25.1 cells was not affected by neither 1,25-$(OH)_2D_3$ nor by the potent regulator of proliferation in both MG-63 and ROS 17/2.8 cells, KH1060. In addition, as expected, no effects on osteocalcin production by 1,25-$(OH)_2D_3$ or the analogs OCT, KH1049, EB1089 and KH1060 were observed (data not shown).

Role of protein kinase C in 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$-analogs stimulated osteocalcin production

Considering the dissociation between VDR binding and biological activity and the fact that protein kinase C (PKC) has been shown to play a role in 1,25-$(OH)_2D_3$ action in bone [20] an attempt was made to investigate a differential involvement of PKC in the action of 1,25-$(OH)_2D_3$ and the analogs. To examine the role of PKC in the stimulation of osteocalcin production we cultured the ROS 17/2.8 cells with various concentrations of 1,25-$(OH)_2D_3$ or analogs, in combination with AMG (25 μM), a specific inhibitor of PKC. In both the 1,25-$(OH)_2D_3$ and analogs incubated cells AMG decreased the maximal osteocalcin production to

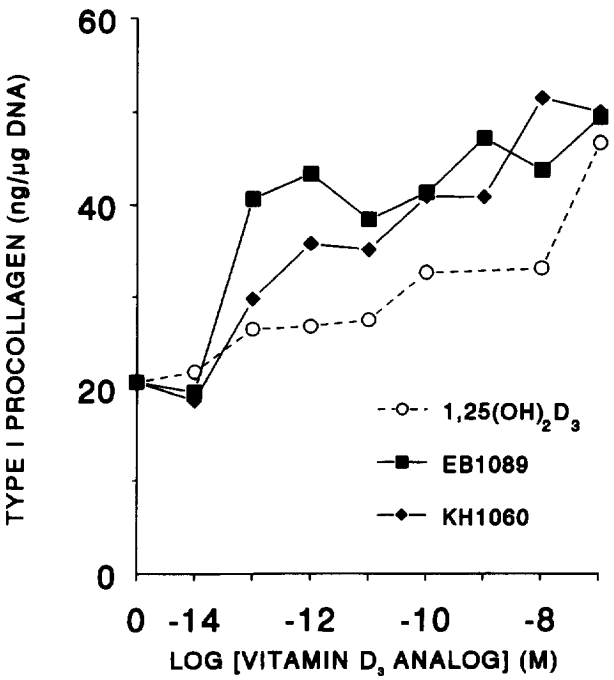

Fig. 3. Effect of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$-analogs on the production of type I procollagen by MG-63 cells after 48 h of incubation. Medium was evaluated for type I procollagen content by RIA as described in Materials and Methods.

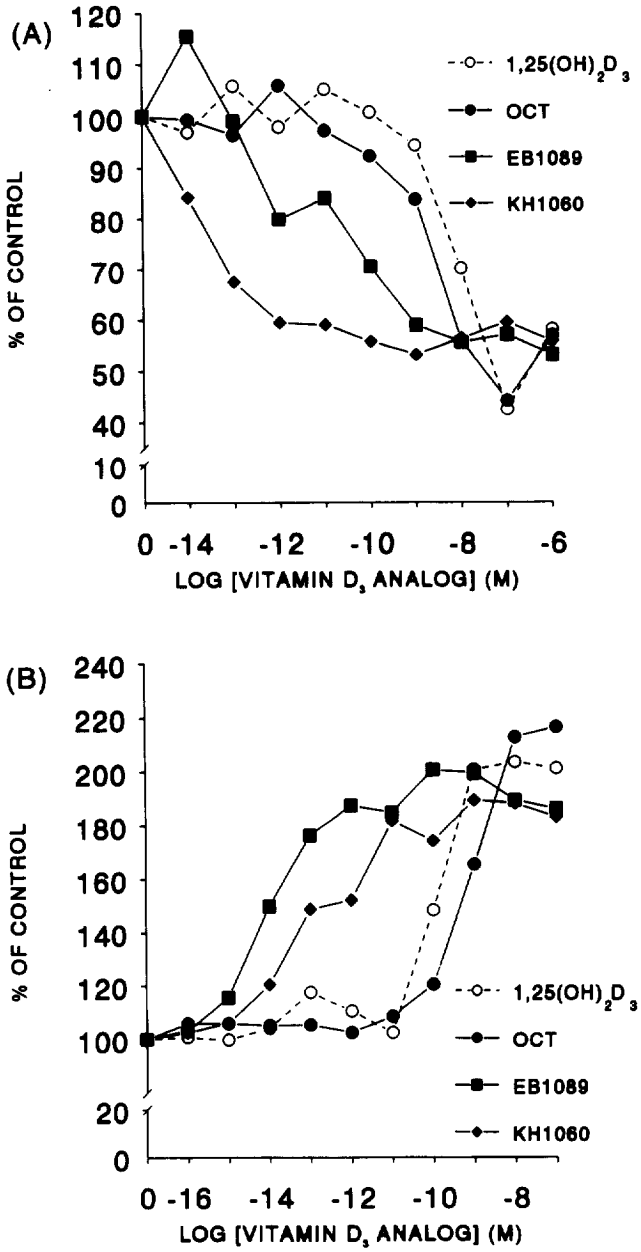

Fig. 4. Effect of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$-analogs on the proliferation of MG-63 (A) and ROS 17/2.8 cells (B). The effects on cell proliferation were studied using the neutral red assay. See Materials and Methods. The data represent the mean percentage of control optical density values.

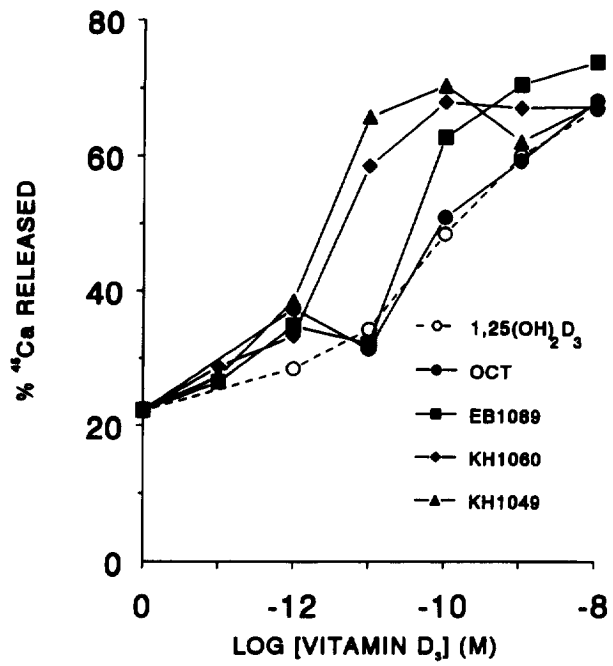

Fig. 5. Effect of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$-analogs on ^{45}Ca release from fetal mouse radii and ulnae after 6 days of culture. For details see Materials and Methods.

a similar extent (±40% inhibition). AMG did not cause a shift in ED_{50} values (data not shown).

DISCUSSION

The present study demonstrates that in bone the same analogs are more potent compared to 1,25-$(OH)_2D_3$ than, as has been reported before, in cells not directly related to bone and calcium metabolism. However, the potency of the analogs compared to 1,25-$(OH)_2D_3$ is dependent on the biological response. For example, the analogs KH1060 and EB1089 are far more potent stimulators of type I procollagen and osteocalcin synthesis than 1,25-$(OH)_2D_3$, whereas the differences in ED_{50} for the stimulation of bone resorption are less marked. This observation indicates that, at least in an *in vitro* situation, for these analogs the balance between stimulation of bone matrix proteins synthesis and bone resorption is in favor of bone formation (Table 3). One may hypothesize that both analogs may direct the osteoblast to a more mature phenotype with higher bone formation capabilities without affecting bone resorption in a similar way. The shift in the balance between bone resorption and bone formation in favor of bone formation is an interesting characteristic for a possible application in metabolic bone diseases. Although the use of 1,25-$(OH)_2D_3$/derivatives is

Table 2. Stimulation of in vitro bone resorption by 1,25-$(OH)_2D_3$ and the analogs after 6 days of culture

Compound	ED_{50} (M)	Potency relative to 1,25-$(OH)_2D_3$
1,25-$(OH)_2D_3$	7×10^{-11}	1
CB966	7×10^{-11}	1
OCT	6×10^{-11}	1.2
EB1089	3×10^{-11}	2.3
KH1060	4×10^{-12}	17.5
KH1049	2×10^{-12}	35

The *in vitro* bone resorptive activity of 1,25-$OH)_2D_3$ and the analogs was determined by measuring ^{45}Ca-release from pre-labelled radii/ulnae from fetal mice.

*The concentration needed to achieve the half-maximal effect of 1,25-$(OH)_2D_3$ is designated as ED_{50}. Te ED_{50} values are the mean of three independent experiments.

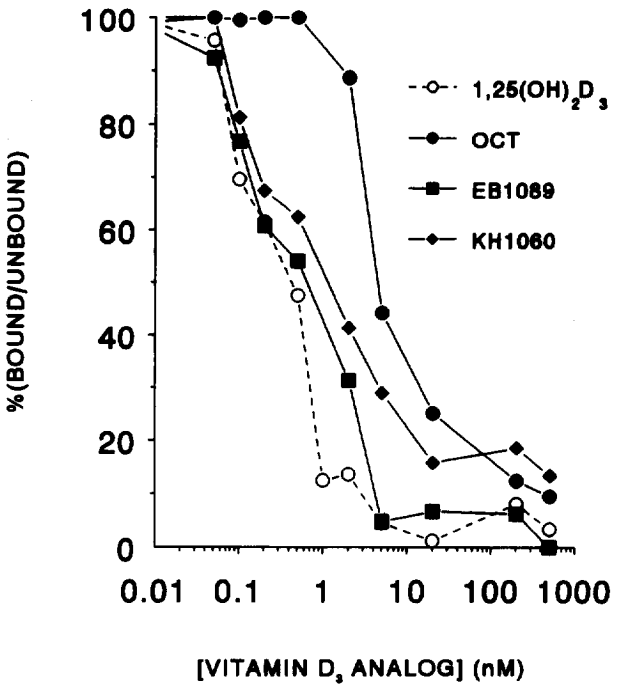

Fig. 6. VDR affinity of 1,25-$(OH)_2D_3$ and the 1,25-$(OH)_2D_3$-analogs. High salt cytosolic extracts of MG-63 cells were incubated with 0.5 nM [^{3}H]1,25-$(OH)_2D_3$ and increasing concentrations of 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$-analogs as described in Materials and Methods.

still controversial [21–23], several studies have shown positive effects of vitamin D metabolites, e.g. 1,25-$(OH)_2D_3$, in the treatment of metabolic bone diseases [24, 25]. Treatment with vitamin D compounds has been shown to reduce bone mineral loss [26, 27] and to reduce vertebral fracture rates [28].

In vivo studies have demonstrated that some of these analogs have a reduced calcemic activity [4–8, 29]. However, despite a favorable balance between bone resorption and bone formation, both EB1089 and KH1060 still exert an increased *in vitro* bone resorptive activity compared to 1,25-$(OH)_2D_3$. Therefore, the present data show that the reduced calcemic activity of the analogs *in vivo* is not the result of a decreased responsiveness of bone to these analogs. Considering *in vivo* application of 1,25-$(OH)_2D_3$ analogs to inhibit tumor cell growth the present observations are important in view of the relationship between stimulated bone resorption and increased risk for bone metastases [30]. Also, in patients with malignant tumors and active Paget's disease the first metastases were found in the pagetic bone lesions with high bone resorption and formation [31, 32].

In relation to the use of 1,25-$(OH)_2D_3$-analogs as antitumor agents the observed stimulation of ROS 17/2.8 cell growth by 1,25-$(OH)_2D_3$ and the analogs is also significant. The more so, since Yamaoka *et al.* [33] reported that 1,25-$(OH)_2D_3$ promoted the growth of tumors arising from intracutaneous inoculations of athymic nude mice with ROS 17/2.8 cells. Furthermore, 1,25-$(OH)_2D_3$ also stimulated the formation of skin tumors in mice treated chronically with 7,12-dimethylbenz[a]anthracene [34]. With low concentrations of 1,25-$(OH)_2D_3$, stimulation of *in vitro* cell proliferation has been described before [35–38]. Although at higher 1,25-$(OH)_2D_3$ concentrations growth inhibition is usually observed, some studies also reported at these concentrations a stimulation of cell proliferation [9, 39, 40]. It has been argued that the observation of growth stimulation is due to culture conditions or cell density [41, 42]. However, in our hands, experiments performed in serum free conditions and in 2% CT-FCS containing medium resulted in similar effects and no relationship between seeding density and the 1,25-$(OH)_2D_3$ effects on proliferation could be demonstrated. Another explanation for the differential actions of 1,25-$(OH)_2D_3$ on cell proliferation might be the large heterogeneity within the ROS 17/2.8 cell line [34, 43–45]. In contrast to ROS 17/2.8 cells, with MG-63 cells a growth inhibition was observed. Interestingly, in both cells the same analogs were, although opposite, the most potent growth regulators. This different growth regulation does not reflect a general difference between these cell lines because in both cell lines 1,25-$(OH)_2D_3$ and the analogs stimulated the osteocalcin production.

All analogs examined have a lower affinity for the VDR in comparison with the natural ligand. Despite a decreased VDR affinity, the analogs were equipotent or far more potent in their biological responses. Other studies also reported a dissociation between receptor

Table 3. Ratio of the ED_{50}s for stimulation of in vitro bone resorption and stimulation of extracellular matrix synthesis

Compound	Bone resorption/ osteocalcin MG-63 cells	Bone resorption/ osteocalcin ROS 17/2.8 cells	Bone resorption/ Type I procollagen MG-63 cells
1,25-$(OH)_2D_3$	0.1	17.5	0.007
OCT	0.1	12.1	ND
EB1089	5	3000	500
KH1060	40	40	5.7

A ratio >1 indicates stimulation of the synthesis of the extracellular matrix proteins at lower concentrations than stimulation of bone resorption whereas a ratio <1 indicates the opposite situation. ND, not determined.

affinity and biological activity [8, 12, 46–49]. Therefore, it can be concluded that VDR affinity is not predictive for the biological activity of the 1,25-$(OH)_2D_3$-analogs. Posner *et al.* [47] suggested that the biological action of the analogs they tested is not regulated via binding to the VDR. However, the findings of others [33, 38, 50] and our own results obtained with VDR deficient ROS 25.1 cells underline that presence of the VDR is essential for biological activity of 1,25-$(OH)_2D_3$ and the analogs. In addition, Carlberg *et al.* [51] recently demonstrated that the analogs studied in the present paper are able to activate gene reporter systems both via the VDR homodimer—and the VDR-retinoid X receptor heterodimer pathway. In this respect it is tempting to speculate about a 1,25-$(OH)_2D_3$- or analog-specific induction of homo- or heterodimer formation. A possible higher sensitivity of the VDR target genes for one of these signalling pathways might then explain the observed differences in potency between 1,25-$(OH)_2D_3$ and the analogs.

Although the exact role of vitamin D binding protein (DBP) is not clear, its presence can affect 1,25-$(OH)_2D_3$ action. On the one hand it is known that DBP decreases the free concentration of 1,25-$(OH)_2D_3$ in the circulation. In both *in vivo* [52, 53] and *in vitro* studies [54, 55] DBP was shown to limit the access of 1,25-$(OH)_2D_3$ to the target cells. Therefore, analogs with low affinity for DBP, and thus a high free concentration, may have more potent cellular effects. On the other hand, the unbound sterol is less protected against degradation and cleared more rapidly. For OCT the low DBP affinity has been put forward as an explanation for the decreased calcemic effect *in vivo* [56]. However, the differences in potency between 1,25-$(OH)_2D_3$ and the analogs we observed cannot solely be explained by a lower affinity for DBP, since both equipotent (OCT) and much more potent analogs (EB1089 and KH1060) exhibit diminished affinity for DBP compared to 1,25-$(OH)_2D_3$ [51, 56]. Also, studies performed with different cellular conditions, e.g. in the absence or presence of serum, resulted in similar differences in potency between 1,25-$(OH)_2D_3$ and the analogs (our unpublished observations).

Since the observed increased potency in biological responses could not be explained by a stronger affinity for the VDR and probably not by a lower affinity for DBP, other non-classical mechanisms of action might play a role. As we have reported earlier [20], PKC is involved in the action of 1,25-$(OH)_2D_3$ in bone and bone cells. AMG, a specific inhibitor of PKC, inhibited the 1,25-$(OH)_2D_3$ stimulated osteocalcin production in ROS 17/2.8 cells and in non-transformed isolated fetal rat osteoblasts. In the current study we investigated the role of PKC in the action of 1,25-$(OH)_2D_3$-analogs on osteocalcin production in ROS 17/2.8 cells. The finding that AMG inhibited the 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$-analogs stimulated osteocalcin production to the same extent without a shift in ED_{50}, suggests that the PKC signalling pathway is not differently regulated by 1,25-$(OH)_2D_3$ and the analogs. In other words, activation of PKC by the 1,25-$(OH)_2D_3$-analogs cannot account for the large difference in potency compared to 1,25-$(OH)_2D_3$ in the stimulation of osteocalcin production.

Although the mechanism(s) underlying the differential changes in biological potencies remains unknown, the present data show that modifications in the side-chain of the 1,25-$(OH)_2D_3$ molecule can lead to analogs with enhanced potential in osteoblast-like cells and on *in vitro* bone resorption. Lengthening of the side-chain, especially in combination with the introduction of double bonds (EB1089) or an altered stereochemistry at position C-20 and substitution of an oxygen atom at C-22 (KH1060, KH1049) results in 1,25-$(OH)_2D_3$-analogs with enhanced biological activity within osteoblast-like cells. Furthermore, the analogs EB1089 and KH1060 exhibit characteristics that might be promising for the application of 1,25-$(OH)_2D_3$-analogs in case of metabolic bone diseases. The increase in bone resorption induced by these analogs and the observed stimulation of the proliferation of an osteosarcoma cell line by 1,25-$(OH)_2D_3$ and the analogs urges caution in the systemic application of these compounds in the treatment of hyperproliferative disorders like cancer and points out that further investigations on long term *in vivo* and *in vitro* responses are required.

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