Biochemical Pharmacology, Vol. 51, pp. 887–892, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02242-2

A Novel Vitamin D Analog with Two Double Bonds in its Side Chain

A POTENT INDUCER OF OSTEOBLASTIC CELL DIFFERENTIATION

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ABSTRACT. EB 1089 (1α,25-dihydroxy-22,24-diene-24,26,27-trihomovitamin D_3) is a novel, synthetic analog of calcitriol, characterized by two extra double bonds in its side chain. It is less potent than calcitriol in its calcemic action, but is an order of magnitude more potent in its antiproliferative action. The aim of this study was to determine the ability of EB 1089 to induce the well-known biological effects of calcitriol in MG-63 human osteosarcoma cells (i.e. by inhibiting cell proliferation and by induction of differentiation). Both calcitriol and EB 1089 significantly decreased cell growth after 2 days in culture. At 5 days, however, EB 1089 was more potent than the natural hormone in inhibiting the proliferation of MG-63 cells. Potent effects of EB 1089 on cell differentiation were also seen in the stimulation of alkaline phosphatase activity, cellular vitamin D receptor mRNA levels, and medium osteocalcin synthesis. EB 1089 was clearly more effective than calcitriol in stimulating alkaline phosphatase activity and osteocalcin synthesis. In gel shift assays, the binding of vitamin D receptor to the composite AP-1 plus vitamin-D-responsive promoter region of the human osteocalcin gene after EB 1089 treatment was stronger and longer-lasting than after calcitriol treatment. BIOCHEM PHARMACOL 51;7:887–892, 1996.

KEY WORDS. vitamin D; analog; osteoblast; osteocalcin; alkaline phosphatase; differentiation

The physiologically active metabolite of vitamin D, calcitriol, regulates calcium and phosphate metabolism in the body primarily by its effects on intestine, bone, and kidney. In recent years, calcitriol has been shown to possess many cell-regulating properties in a number of tissues and cells not directly involved in intestinal calcium absorption and bone mineralization [1]. Notably, calcitriol is able to inhibit cell proliferation and induce differentiation in a number of cancer cell lines [2, 3].

These observations suggest that calcitriol may be of interest in cancer therapy. However, the clinical use of calcitriol is often limited by its potent effects on calcium metabolism, with the risk of inducing side effects such as hypercalcaemia and soft tissue calcification. A novel vitamin D analog, EB 1089†, that causes regression of carcinogen-induced mammary tumors in rats [4] and has strong antiproliferative and differentiation-inducing effects on cancer cells *in vitro*, has recently been described [5].

The aim of this study was to determine the ability of EB 1089 to induce the biological effects of calcitriol in MG-63 human osteosarcoma cells by inhibiting cell proliferation and by induction of osteoblastic cell differentiation.

MATERIALS AND METHODS

Chemicals

Calcitriol was from Hoffman-La Roche, Nutley, NJ. EB 1089 was a kind gift from Dr. Lise Binderup, Leo Pharmaceutical Products Ltd., Ballerup, Denmark. Radioimmunoassay kits for osteocalcin were obtained from CIS, Bio International, Gif-Sur-Yvette, France. [α^{32}P]-dCTP (>3000 Ci/mmol) and [γ^{32}P]-ATP (>6000 Ci/mmol) were purchased from Du Pont de Nemours GmbH, Bad Homburg, Germany. Nick-translation and 3′-end labelling kits were from Boehringer Mannheim GmbH, Mannheim, Germany.

Cell Culture

MG-63 human osteosarcoma cells were obtained from the American Type Culture Collection, Rockville, MD. The cells were cultured in DMEM supplemented with 7% irradiated FCS, 2 mM L-glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin in a humidified 95% air/5% CO_2 incubator. The culture medium was replaced 24 hr before each experiment with a medium containing 2% charcoal-treated FCS to minimize the effects of endogenous hormones. Calcitriol and EB 1089 were added to the media in propanol and control cells were treated with the vehicle.

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† *Abbreviations:* AP-1+VDRE, AP-1 plus vitamin-D-responsive promoter region; calcitriol, 1,25-dihydroxyvitamin D; DMEM, Dulbecco's modified Eagle's medium; EB 1089, 1α,25-dihydroxy-22,24-diene-24,26,27-trihomovitamin D_3; FCS, fetal calf serum; OC, osteocalcin; RXR retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin-D-responsive promoter region.

Received 12 July 1995; accepted 6 November 1995.

Effects on Cell Proliferation

The cells were seeded at 10^4 cells per well onto 8-well plates and incubated in DMEM containing 7% FCS for 24 hr. The medium was replaced by a medium containing 2% charcoal-treated FCS and calcitriol or EB 1089, and the cells were incubated for up to 5 days. Cell numbers, as well as their viability, were determined after each treatment with a hemocytometer.

Measurement of Osteocalcin

For osteocalcin measurements, the cells were seeded onto 90-mm culture plates and cultured into confluency. The medium was replaced by a medium containing 2% charcoal-treated FCS 24 hr before the treatments. The cells were incubated as a function of time and concentration in this medium with EB 1089 or calcitriol for the times indicated. After the treatments, the media were collected for osteocalcin radioimmunoassay and the cells for Northern analyses (see below).

Determination of Alkaline Phosphatase Activity

For determination of alkaline phosphatase activity, MG-63 cells were seeded onto 60-mm culture plates and cultured into confluency. The medium was changed into a medium containing 2% charcoal-treated FCS 24 hr before treatment. The cells were treated for up to 5 days with a medium containing the vehicle, calcitriol, or EB 1089. Alkaline phosphatase activity was determined by measuring the release of p-nitrophenol (pNP) from *p*-nitrophenylphosphate spectrophotometrically (410 nm) at 37°C in a buffered substrate solution containing 10 nM *p*-nitrophenylphosphate, 10 mM diethanolamine, pH 10.5 and 1 mM $MgCl_2$. The results are expressed as μmol pNP $\times$ min^{-1} $\times$ mg $protein^{-1}$. Total protein was measured with a Bio-Rad protein assay kit (Bio Rad Laboratories, Melville, NY).

Northern Analysis

Total RNA was prepared from the cultured cells according to the method of Anderson *et al.* [6]. For Northern analysis, samples were run in a formaldehyde-agarose gel and electrotransferred onto Immobilon P membranes (Millipore, Bedford, MA). The hybridization probe for osteocalcin was a 5′-end-labelled 40-residue oligonucleotide, 5′-CCAACTCGTCACAGTCCGGATTGAGCTCACACACCTCCCT-3′, complementary to human mRNA sequence coding for amino acids 20–32 of the mature osteocalcin [7]. As controls, filters were washed and prehybridized with human γ-actin probe [8]. The probe for human VDR was a cDNA clone (hVDR 1/3) [9].

Gel Mobility Shift Assays

MG-63 cells were seeded onto 90-mm plates and treated with the vehicle, calcitriol, or EB 1089 in a medium containing 2% charcoal-treated FCS. After the treatments, nuclear extracts were prepared from MG-63 cells as described [10]. The binding reaction mixture contained 10 μg nuclear protein with 2 μg poly(dI-dC)poly(dI-dC) (Pharmacia Biotech, Uppsala, Sweden) in 20 mM Hepes, pH 7.6, 4.2% (v/v) glyserol, 70 mM NaCl, 2.25 mM $MgCl_2$, 2.03 mM EDTA, 2.17 mM dithiothreitol, and protease inhibitors 0.08 mM phenylmethylsulfonylfluoride, 0.33 μg/mL trasylol, and 0.33 μg/mL leupeptin. The following oligonucleotides were used in gel mobility shift assays: AP-1 + VDRE (5′-TTGGTGACTCACCGGGTGAACGGGGGCATT-3′) (upper strand) and VDRE (5′-ACCGGGTGAACGGGGGCATTGCG-3′) (upper strand) [11].The 5′-end-labelled double-stranded oligonucleotides complexed with nuclear proteins were separated from the free probes in a 5% polyacrylamide gel run in 0.25 $\times$ TBE (1 $\times$ TBE; 0.1 M Tris-borate, pH 8.3, 2 mM EDTA).

Statistical Analysis

Statistical analysis was performed by the Student's *t*-test for 2 independent variables using a software package (StatWorks™, Cricket Software Inc, Philadelphia, PA).

RESULTS

Calcitriol and EB 1089 significantly decreased cell growth after 2 days in culture (Fig. 1). The doubling time of the cells increased from 26 to 50 hr after treatment with either compound. At 5 days, however, EB 1089 was more potent than calcitriol in inhibiting the proliferation of MG-63 cells. The doubling time was increased to 165 hr with EB

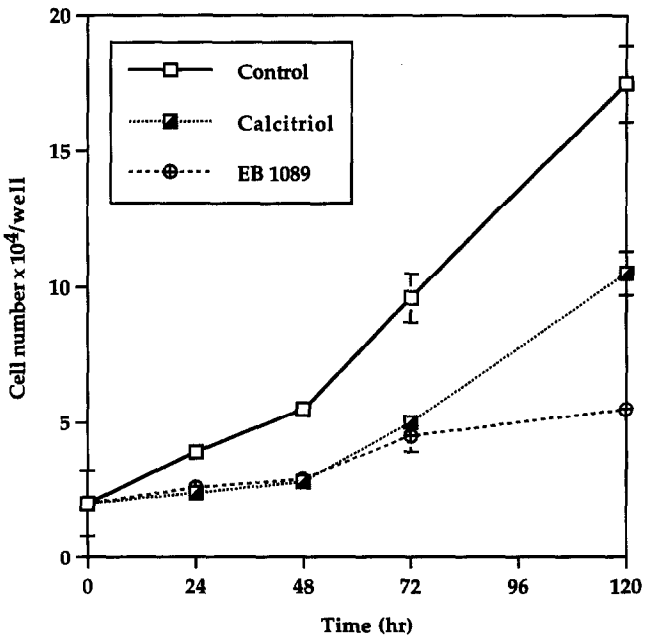

FIG. 1. Effects of calcitriol and EB 1089 on cell proliferation. MG-63 cells were treated for 1 to 5 days with the vehicle, 100 nM calcitriol, or 100 nM EB 1089 before determination of cell numbers. Results represent means ± SE (n = 5).

1089 vs 107 hr with calcitriol. The concentrations resulting in a 50% inhibition (IC_{50}) were 10 nM for calcitriol and 0.2 nM for EB 1089 (a 50-fold difference).

The ability of EB 1089 to regulate the concentration of VDR mRNA was compared with that obtained after treatment with calcitriol. The results in Fig. 2 demonstrate that calcitriol and EB 1089 increase VDR mRNA concentrations almost identically.

Potent stimulatory effects of EB 1089 on cell differentiation were seen in alkaline phosphatase activity and osteocalcin synthesis. MG-63 cells were treated with increasing concentrations of the compounds for 72 hr. Maximal induction of alkaline phosphatase activity occurred at 100 nM calcitriol or EB 1089 concentration (data not shown). Subsequently, a time-course experiment was performed with this concentration (Fig. 3). The increase in alkaline phosphatase activity was approximately 2-fold at 72 hr after the addition of EB 1089 compared with the calcitriol treatment.

Expression of the osteocalcin gene was increased efficiently. The secretion of osteocalcin was induced dose-dependently with both compounds (from 1 pM to 1 μM concentration) (Fig. 4A). The secretion was first detected at 0.1 nM concentration and was maximal at 0.1 μM concentration. The EB 1089 analog was clearly more potent in stimulating osteocalcin secretion than calcitriol. The stimulatory effect of EB 1089 on osteocalcin synthesis was also

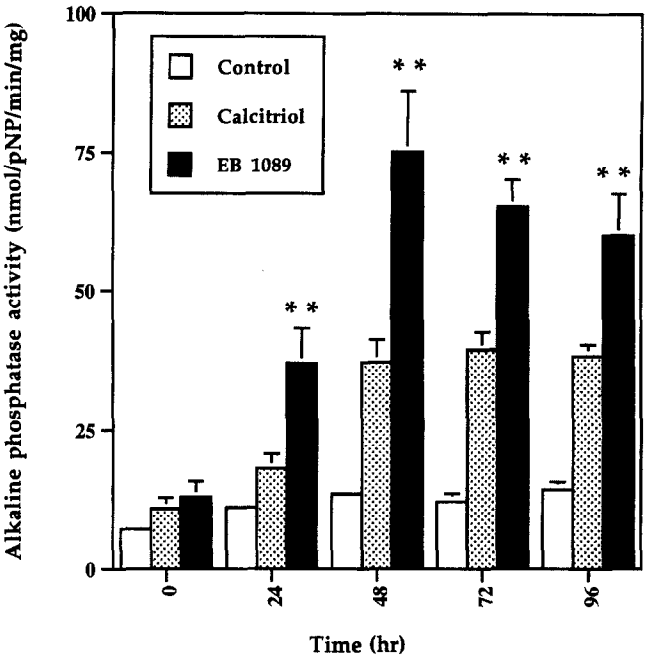

FIG. 3. Effects of calcitriol and EB 1089 on alkaline phosphatase activity. The cells were treated with the vehicle, calcitriol (100 nM), or EB 1089 (100 nM) for up to 5 days. Alkaline phosphatase activity was determined as described in Materials and Methods. Results represent means ± SE of 3 similar experiments. Different from calcitriol: **$P < 0.01$.

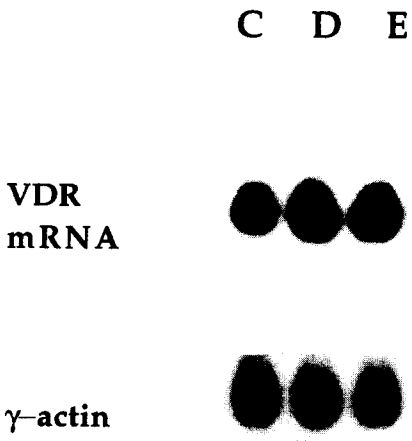

FIG. 2. Effects of calcitriol and EB 1089 on VDR mRNA concentrations. MG-63 cells were treated with the vehicle, 100 nM EB 1089, or 100 nM calcitriol for 48 hr. The levels of VDR mRNA in control (C), calcitriol-treated (D), and EB 1089-treated (E) cells.

demonstrated in osteocalcin mRNA levels using the specific 40-mer oligonucleotide probe (Fig. 4B). The results indicated an increase in osteocalcin mRNA levels analogous to those seen in osteocalcin levels.

At a constant concentration (100 nM), the stimulation of osteocalcin synthesis was first observed at 12 hr and continued for up to 72 hr (Fig. 5A). At 48 hr, the secretion of osteocalcin into the culture medium was increased by 50% with EB 1089 treatment compared with calcitriol treatment. In calcitriol or EB 1089 withdrawal experiments (not shown), the stimulatory effect of the analog on osteocalcin secretion was observed for up to 60 hr after withdrawal, whereas the effect of calcitriol disappeared within 42 hr of withdrawal.

In the experiment described in Fig. 5A, osteocalcin mRNA was found to be increased maximally at 24 hr after treatment (Fig. 5B). With EB 1089, the message concentration was approximately 2.5-fold compared with calcitriol. Subsequently, osteocalcin mRNA concentrations remained elevated for up to 96 hr in the EB 1089-treated cells, but declined much faster in calcitriol-treated cells (i.e. below the detection limit at 96 hr).

We used gel mobility shift assays to compare the effects of calcitriol and the EB 1089 analog on osteocalcin synthesis. The binding of AP-1 and VDR to their complex cognate response element was determined as a function of time as previously described [12]. Two VDR specific (fast and slow mobility) interactions were observed (Fig. 6A). The binding of VDR was significantly increased during the

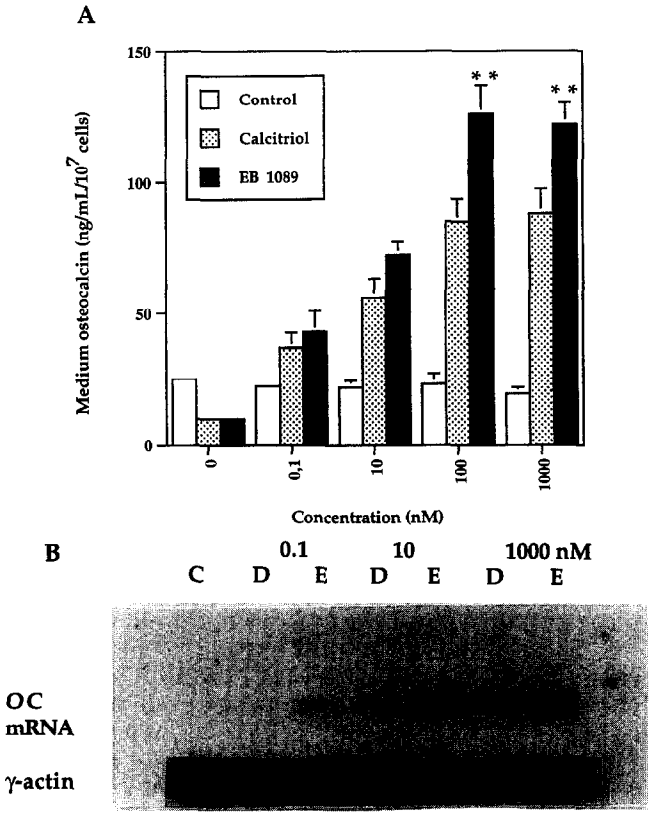

FIG. 4. Effects of calcitriol and EB 1089 on osteocalcin (OC) synthesis as a function of concentration. (A). MG-63 cells were treated with increasing concentrations of the compounds for 48 hr. After the treatments, the media were collected and assayed for osteocalcin. Each bar represents the mean ± SE of 3 independent experiments. (B). Cytoplasmic RNA was isolated from the MG-63 cells shown in (A) and used for Northern analysis as described in Materials and Methods. Abbreviations as in Fig. 2. Different from calcitriol: **$P < 0.01$.

24-hr treatment, being slightly stronger after EB 1089 treatment. The difference was more pronounced at 72 hr.

In the hormone withdrawal experiment, VDR binding to the VDRE was almost identical during pretreatment for 6 hr with calcitriol or EB 1089 (Fig. 6B). After a 16-hr withdrawal, the VDR binding was significantly stronger in cells treated with EB 1089 than in calcitriol-treated cells. At 40 hr, the binding of VDR was only observed in EB 1089-treated cells. At 100 hr, all binding had returned to the control level.

DISCUSSION

Calcitriol has well-known potent effects on bone and calcium regulation. It has become apparent that calcitriol has other effects on cellular proliferation, as well, that could potentially be developed for therapy of human malignancies. Because the hypercalcaemic effect of calcitriol has limited its use in humans, novel nonhypercalcaemic analogs of calcitriol have been synthesized. Alterations of the side chain structure of the calcitriol molecule have yielded important information about the structural features required for biological activity [13].

Using U937 human histiocytic lymphoma cell line and MCF-7 breast cancer cells, the EB 1089 analog has been shown to be 50 to 100 times more active in inhibiting cell proliferation and inducing cell differentiation than calcitriol [5] and having a 3-fold lower calcemic activity *in vivo* [14]. The serum half-life of EB 1089 after *in vivo* administration to rats is 2.8 hr compared to 2.4 hr for calcitriol [14]. In MCF-7 cells, the binding affinity of EB 1089 for the VDR is not well correlated with the biological potency of the compound, since the affinity of EB 1089 for the VDR was approximately 40 times lower than that of calcitriol [5]. The binding affinity may vary, however, in different cell lines, since a recent report indicates that EB 1089 binds to

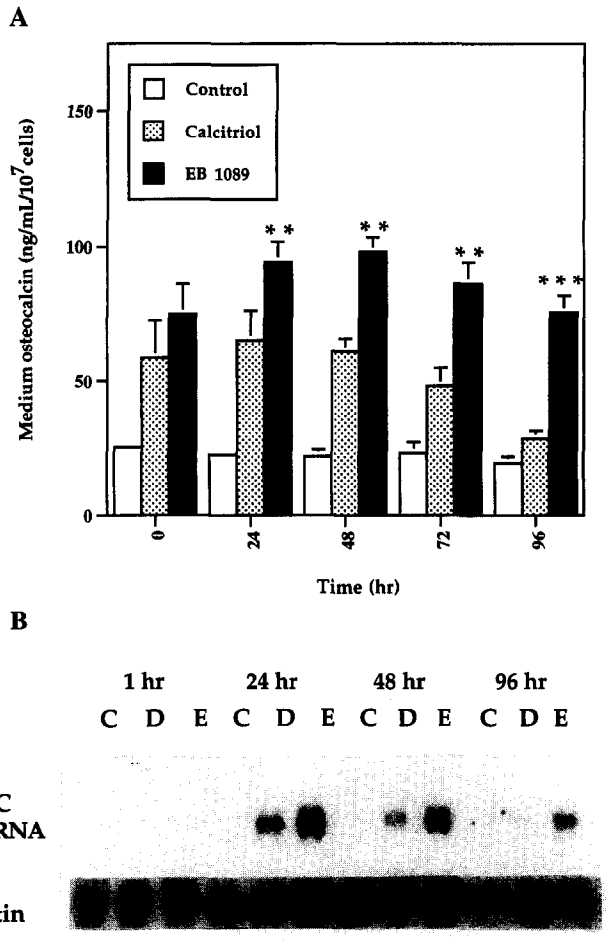

FIG. 5. Effects of calcitriol and EB 1089 on osteocalcin synthesis and osteocalcin mRNA concentrations as a function of time at a constant concentration (100 nM). (A). Medium osteocalcin concentrations were determined as described in Materials and Methods. Each point represents the mean ± SE of 3 independent experiments. (B). Osteocalcin mRNA concentrations. After the treatments, the cells were assayed for osteocalcin mRNA concentrations by Northern analysis as in Fig. 4. Different from calcitriol: **$P < 0.01$, ***$P < 0.001$.

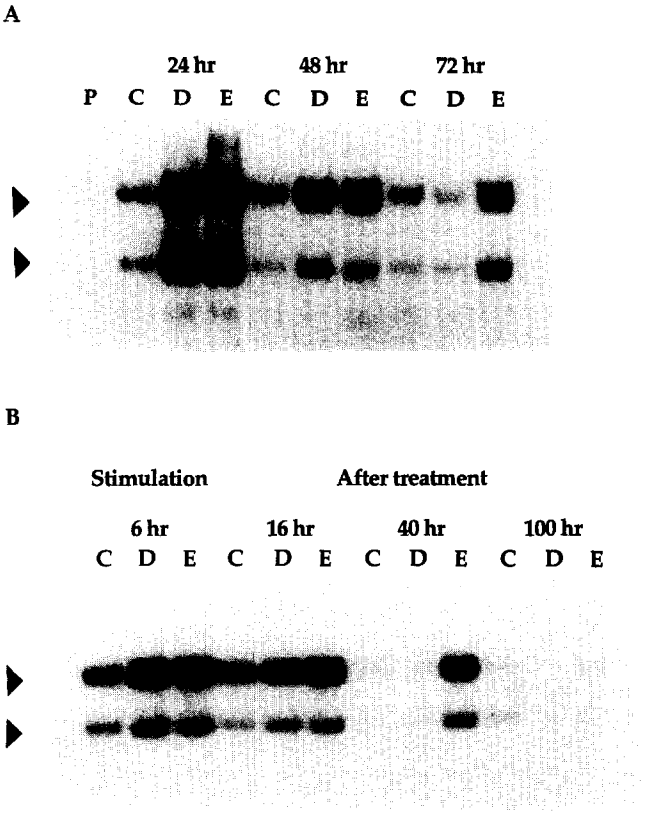

FIG. 6. Gel mobility shift assays of AP-1 and VDR present in nuclear extracts of MG-63 cells. The reactions were performed in the presence of excess probe, but only the complexes are shown. (A). The AP-1+VDRE response element was used as a probe in the assays with 10 μg nuclear protein from control, calcitriol, and EB 1089-treated (100 nM) cells (same as in Fig. 5). Two VDR specific interactions were observed. (B). The VDRE response element was used as a probe in the assays and the decrease in the two VDR-specific interactions after withdrawal of calcitriol or EB 1089 after the 6-hr pretreatment was measured.

VDR from the human prostate carcinoma cell line, LNCaP, with twice the potency of calcitriol [15].

In MG-63 osteosarcoma cells, the cellular response to calcitriol is consistent with the predicted action of the hormone in modulating the levels of its own receptor and in stimulating alkaline phosphatase activity and the synthesis of osteocalcin, as well as type I collagen [16, 17]. The present results indicated, however, that although EB 1089 and calcitriol were equipotent in upregulating the level of VDR mRNA, the analog was much more potent than calcitriol in stimulating osteocalcin gene expression and alkaline phosphatase activity, two well-known markers of the differentiated osteoblastic phenotype. In transfection studies using MCF-7 cells, it has recently been demonstrated that approximately 20-fold higher concentrations of EB 1089 than calcitriol are needed to activate the DR3- and DR6-type response elements [18]. In the present study, however, no significant concentration differences between the analog and calcitriol were observed in the activation of medium osteocalcin synthesis on the natural osteocalcin promoter.

A recent report indicates that, in MG-63 cells, the VDR complex formed on the VDRE is different (faster mobility on gel electrophoresis) when EB 1089 is a ligand, and more resistant to specific competition with the ligand than the corresponding receptor complex formed with calcitriol [19]. Our results did not indicate a size difference between the 2 VDR:DNA containing complexes (fast and slow mobility) when EB 1089 or calcitriol were used. However, the complexes with EB 1089 were more intensive than those with calcitriol and were formed for a longer period of time after the withdrawal of calcitriol or EB 1089. This may indicate a difference in the ability of the analog to form complexes with auxiliary factors (e.g. the retinoid X receptor (RXR) or with DNA). A recent report indicates that a number of 20-epi analogs of vitamin D augment the transcriptional activity of VDR more than calcitriol, in spite of their relatively similar affinities for the nuclear VDR, by inducing conformational changes in the VDR that enhance dimerization of VDR with RXR [20]. Moreover, there is a possibility that tight binding to VDR may retard the catabolism of the vitamin D analog. Further studies are clearly required to find out the exact mechanism of the more potent action of EB 1089 in regulating specific gene expression.

To conclude, EB 1089 inhibited the proliferation of human MG-63 osteosarcoma cells more potently than the natural hormone. Second, the analog was also a potent inducer of osteoblastic differentiation as studied by marker gene expression. Third, EB 1089 treatment resulted in a stronger and longer-lasting binding of VDR to the osteocalcin promoter VDRE complex.

This work was supported in part by grants from the Academy of Finland. We thank Dr. Lise Binderup, Leo Pharmaceutical Products, Ltd., for kindly providing EB 1089.

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