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Inhibition of Proliferation and Induction of Differentiation of Osteoblastic Cells by a Novel 1,25-Dihydroxyvitamin D_3 Analog With an Extensively Modified Side Chain (CB1093)

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Abstract 1,25-Dihydroxyvitamin D_3 (1,25D) is involved in the regulation of proliferation and differentiation of a variety of cell types including cancer cells. In recent years, numerous new vitamin D_3 analogs have been developed in order to obtain favorable therapeutic properties. The effects of a new 20-epi analog, CB1093 (20-epi-22-ethoxy-23-yne-24a,26a,27a-trihomo-1α,25$(OH)_2D_3$), on the proliferation and differentiation of human MG-63 osteosarcoma cell line were compared here with those of the parent compound 1,25D. Proliferation of the MG-63 cells was inhibited similarly by 22%, 50% and 59% after treatment with 0.1 μM 1,25D or CB1093 for 48 h, 96 h, and 144 h, respectively. In transfection experiments, the compounds were equipotent in stimulating reporter gene activity under the control of human osteocalcin gene promoter. In cell culture experiments, however, CB1093 was more potent than 1,25D at low concentrations and more effective for a longer period of time in activating the osteocalcin gene expression at mRNA and protein levels. Also, a 6-h pretreatment and subsequent culture for up to 120 h without 1,25D or CB1093 yielded higher osteocalcin mRNA and protein levels with analog-treated cells than with 1,25D-treated cells. The electrophoretic mobility shift assay (EMSA) revealed stronger VDR-VDRE binding with analog-treated MG-63 cells than with 1,25D-treated cells. The differences in the DNA binding of 1,25D-bound vs. analog-bound VDR, however, largely disappeared when the binding reactions were performed with recombinant hVDR and hRXRβ proteins. These results demonstrate that the new analog CB1093 was equally or even more effective than 1,25D in regulating all human osteosarcoma cell functions ranging from growth inhibition to marker gene expression and that the differences in effectivity most probably resulted from interactions of the hVDR:hRXRβ-complex with additional nuclear proteins. J. Cell. Biochem. 70:414–424, 1998.

Key words: analog; bone; growth inhibition; differentiation; vitamin D

By binding to the vitamin D receptor (VDR), 1,25-dihydroxyvitamin D_3 (1,25D) directly activates or represses gene expression through vitamin D-responsive elements (VDRE) located in the promoter regions of various target genes [Reichel et al., 1989; Pike, 1991]. The VDR functions as a homodimer (VDR-VDR) but more often heterodimerizes with the retinoid-X-receptor (RXR) [Carlberg et al., 1993; MacDonald et al., 1993]. Via this mechanism, 1,25D acts in vitro as an important modulator of cellular proliferation and differentiation in a number of normal and malignant cells expressing VDR [Bikle, 1992].

Also osteoblasts have receptors for calcitriol [Manolagas et al., 1980]. Osteocalcin is one of the most abundant osteoblast-specific proteins whose levels are postproliferatively upregulated by calcitriol during extracellular matrix mineralization [Suda et al., 1990; Owen et al., 1990]. A vitamin D responsive element (VDRE) has been identified in the promoter region of human osteocalcin gene [Ozono et al., 1990].

Abbreviations: 1,25D, 1,25-dihydroxyvitamin D_3; CB1093, 20-epi-22-ethoxy-23-yne-24a,26a,27a-trihomo-1α,25$(OH)_2D_3$; DBP, vitamin D-binding protein; DMEM, Dulbecco's Modified Eagle Medium; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; OC, osteocalcin; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D responsive element.

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The clinical use of 1,25D has been limited by its hypercalcemic side effects. This has prompted the search for new vitamin D_3 analogs that may uncouple the hypercalcemic activity from those properties inducing differentiation and inhibiting cell growth. Previous in vitro studies have shown that modifications to the side chain of 1,25D often leads to considerably more potent analogs. Especially, vitamin D analogs with the 20-epi configuration have demonstrated to effect potently cancer cell growth and differentition [Binderup et al., 1991], and the immune system [Binderup, 1992].

CB1093 (20-epi-22-ethoxy-23-yne-24a,26a, 27a-trihomo-1α,25$(OH)_2D_3$) is a new synthetic analog of 1,25D with potent activity on differentiation in vitro without inducing hypercalcemia in vivo [Pakkala et al., 1997]. However, little is known about the effects of CB1093 on bone cells. The present study was therefore designed to compare the effects of CB1093 with those of 1,25D in inhibiting proliferation and inducing differentiation of the MG-63 human osteosarcoma cells. A further aim was to investigate the efficacy of the analogs with respect to transcriptional activity and protein-DNA interactions in the vitamin D responsive element of the human osteocalcin gene promoter (VDRE). Also, 3-D modelling by energy minimization of 1,25D and CB1093 was done to gain information on the conformational changes of the compounds as a result of their structural modifications.

MATERIALS AND METHODS

Vitamin D_3 Compounds

1,25-Dihydroxyvitamin D_3 and the side-chain analog CB1093 (Fig. 1) were kindly provided by Dr. Lise Binderup, Leo Pharmaceutical Products Ltd., Denmark. The vitamin D compounds were dissolved in isopropanol at 4 mM concentration and diluted with ethanol.

Cell Culture

MG-63 human osteosarcoma cells (American Type Culture Collection, Rockville, MD) and ROS 17/2.8 rat osteosarcoma cells (kindly provided by Dr. G. Rodan, Merck & Co., Inc. Research Laboratories, West Point, PA) were cultured in DMEM supplemented with 7% (10% for ROS 17/2.8) fetal calf serum (FCS), 2 mM L-glutamine, 0.1 mg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a humidified (95% air: 5% CO^2) incubator.

Effects on Cell Growth

The MG-63 cells were seeded at 5×10^4 cells/well onto six-well plates and incubated in DMEM containing 7% fetal calf serum for 24 h. The medium was replaced by a medium containing 2% charcoal-treated fetal calf serum combined with 1,25D or CB1093, and the cells were incubated for 1 to 6 days. The cells were trypsinized and quantified with a Coulter Counter cell counter. All counts were obtained in triplicate at each time point.

Transfection of ROS 17/2.8 Rat Osteosarcoma Cells

A reporter plasmid was constructed by cloning a PCR-fragment into a pXP-1 vector [Nordeen, 1988]. The amplification product contains the upstream portion of the human osteocalcin gene, extending from nucleotides −881 to +29 [Celeste et al., 1986] and containing the func-

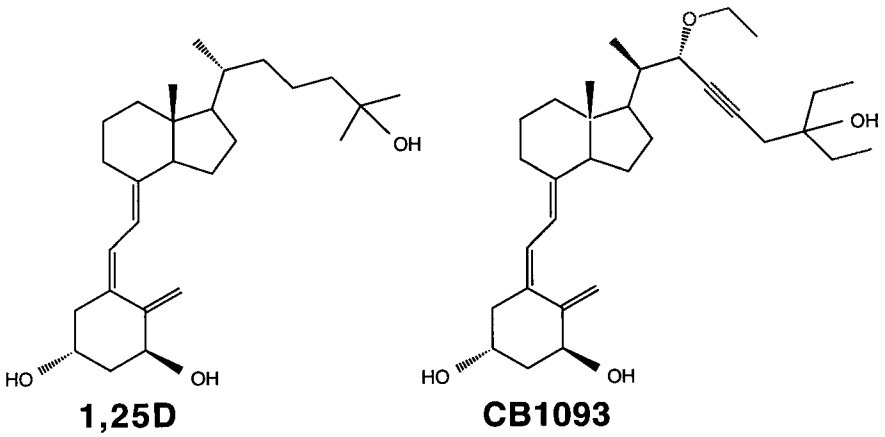

Fig. 1. Chemical structures of 1,25-dihydroxyvitamin D_3 and the analog CB1093.

tional VDRE [Ozono et al., 1990]. DNA isolated from MG-63 cells was used as a template in the amplifications. For DNA sequencing, the amplified PCR product was cloned into the pUC57 vector using a TA Cloning Kit according to instructions provided by the manufacturer (MBI Fermentas, Vilnius, Lithuania). After sequencing, the amplification product was cloned in a coding orientation into the Bam HI-Kpn I site of the pXP-1 vector.

ROS 17/2.8 cells were seeded at 250,000 cells per well onto six-well plates and grown for 24 h in DMEM containing 10% FCS. The medium was replaced by a medium containing 2% charcoal-treated fetal calf serum 4 h before transfections. A reporter plasmid pXP-1/hOC-910 and a control plasmid pCMVβ (Promega, Madison, WI), which provides constitutive expression of β-galactosidase, were introduced into the cells using a lipofectin reagent according to the manufacturer's instructions (Boehringer Mannheim, Germany). After DNA addition, the vitamin D compound at 10 nM concentration (or the vehicle) was added and the cells were incubated for 24 to 48 h, lysed, and the luciferase activity was measured (Bio-Orbit, Finland). The β-galactosidase activity was measured as described in the Protocols and Applications Guide (Promega, Madison, WI, 1991). The luciferase activities were normalized with respect to the β-galactosidase activity, and an induction factor was calculated as a ratio of the luciferase activity of the ligand-stimulated cells to that of the vehicle-treated cells.

Osteocalcin Synthesis and Secretion

The culture plates were treated with 0.1 μM 1,25D or CB1093 either as a function of time (for up to 120 h) or for 72 h with different concentrations (1 pM to 1 μM) without medium change to determine osteocalcin levels. To study the duration of the effect after the withdrawal of the vitamin D compound, the cells were treated for 6 h with either 0.1 μM 1,25D or CB1093. The medium was then replaced with a fresh medium containing 2% charcoal-treated FCS, and the cells were cultured without the vitamin D compounds for the next 96 h. The media were collected for osteocalcin radioimmunoassay (CIS Bio International, Gif-Sur-Yvette, France) and the cells for Northern analysis and EMSA.

Northern Blot Analysis

Total cellular RNA was isolated by the guanidinium thiocyanate method [Chomczynski and Sacchi, 1987]. Denatured RNA samples (10 μg) were fractionated in 1% agarose/formaldehyde gels and transferred onto nylon filters. The filters were prehybridized, hybridized, and washed as previously described [Mahonen et al., 1990]. The ^{32}P-labelled (Du Pont de Nemours, Boston, MA) hybridization probe for osteocalcin was a 1.2-kb SacI fragment of the human osteocalcin gene [Celeste et al., 1986].

EMSA

Nuclear extracts from MG-63 cells were prepared as described by Hurst et al. [1990] without the heat denaturation step. Nuclear extracts (7 μg) or recombinant hVDR and hRXRβ proteins (Affinity BioReagents Inc., Golden, CO) were preincubated for 10 min with p[(dI-dC)(dI-dC)] (Pharmacia, Uppsala, Sweden). A ^{32}P-radiolabelled oligodeoxyribonucleotide from the human osteocalcin gene promoter (VDRE, 5′-accGGGTGAacgGGGGCAttgcg-3′ upper strand [motifs recognized by VDR are indicated by capital letters; Ozono et al., 1990]) was added and incubation was continued for 20 min. When using individual recombinant hVDR and hRXRβ proteins, the vitamin D compounds were added and incubated for 60 min. Separation of the bound and free probes was performed in 7% polyacrylamide gel run in 25 mM Tris/borate, pH 8.3, 0.5 mM EDTA.

3-D Structures of 1,25D and CB1093

The 3-D structures were generated using the program QUANTA (vers.4.1)/CHARMM® (vers.23.r1; Molecular Simulations Inc., San Diego, CA) [Brooks et al., 1983], and the modeling was performed by IBM RISC 6000/365 computer. The visual presentation of the molecules was created using a SGI O2/R5000 workstation. The X-ray structure of calcitriol was retrieved from Cambridge Structural Database (CCDC) at CSC, Finland. The molecules were built with Molecular Editor and the charges were calculated by the Template method. The models were embedded into the water box with dimensions 31.0432 Å, 31.0432 Å, and 31.0432 Å. The nonbonded cut-off value was set to 9 Å and the nonbonded energies were updated at every 20 steps. The nonbonded energies and forces were smoothly truncated using a van der Waals switching function and an electrostatic shifting function [Brooks et al., 1985]. The structures were minimized by Adopted-Basis Newton Raphson (ABNR) method [Levitt and Lifson, 1969; Ermer, 1976]. The first minimization

was done with fixing the coordinates of the solvated molecule and then the molecule was released and the minimization was continued until the energy tolerance gradient of 0.00001 kcal/mol was satisfied. The minimizations in the water box were performed using periodic boundary conditions (IMAGE) [Allen and Tildesley, 1987]. To facilitate comparison of the final results, the 3-D structures of the compounds are presented so that the CD-ring is fixed into an identical position in both cases.

Statistical Analysis

Data were analyzed by Student's *t*-test using Statworks software (Cricket Software Inc., Philadelphia, PA).

RESULTS

Cell Growth Assay

Both vitamin D compounds effectively blocked proliferation of the MG-63 osteosarcoma cells in culture. Inhibition of cell growth was seen with 0.1 μM 1,25D or CB1093 already after the 48-h treatment (Table 1). The degree of inhibition with CB1093 was identical to that with 1,25D at all time points (−22% after 48 h, −50% after 96 h, and −59% after 144 h).

Transfection Assay With ROS 17/2.8 Cells

The potency of the analog CB1093, on transcriptional activity is shown in Table 2. At both time points, CB1093 was as potent as 1,25D in enhancing luciferase activity in ROS 17/2.8 cells transfected with the construct in which expression of the reporter gene is under control of the human osteocalcin promoter. Different concentrations (0.1 nM or 0.1 μM) of 1,25D or the analog showed no significant differences with respect to efficacy in inducing the reporter gene activity (data not shown).

TABLE I. Growth Inhibitory Activity of $1,25(OH)_2D_3$ and CB1093 in MG-63 Cells*

Time (h)	1,25D vs. control	CB1093 vs. control
24	0.97 ± 0.02	0.99 ± 0.03
48	0.78 ± 0.04	0.78 ± 0.01
72	0.63 ± 0.04	0.61 ± 0.01
96	0.50 ± 0.01	0.49 ± 0.01
120	0.44 ± 0.01	0.44 ± 0.01
144	0.42 ± 0.01	0.40 ± 0.01

*The cells were treated for 1 to 6 days with 0.1 μM 1,25D, CB1093 or the vehicle before determination of cell numbers. The results are from three individual experiments. The data expressed as means ± SE (n = 3).

TABLE II. Activation of hOC Gene Promoter by $1,25(OH)_2D_3$ or CB1093*

	Fold induction	
	Time	
Treatment	24 h	48 h
Control (EtOH)	1.00 ± 0.26	1.00 ± 0.12
1,25D	2.93 ± 0.39[a] **	1.62 ± 0.13[a] *
CB1093	2.94 ± 0.18[a] **	1.31 ± 0.31[a] *

*ROS 17/2.8 cells were transfected with pXP-1/hOC-910 (2 μg/well) and the control plasmid pCMVβ (0.5 μg/well) and incubated with the vehicle or 10 nM vitamin D compounds for 24 to 48 h in a medium containing 2% charcoal-treated fetal calf serum. Numbers (mean ± SEM, n = 5) are the ratios of induced to uninduced luciferase activity (fold induction).

[a]Significantly different from control (*$P < 0.05$ and **$P < 0.01$).

Effects of CB1093 on Osteocalcin mRNA and Protein Levels

The ability of different concentrations of 1,25D or CB1093 to induce the synthesis of osteocalcin mRNA and the secretion of osteocalcin in human MG-63 cells was determined after the 72-h treatment (Fig. 2). Osteocalcin mRNA levels increased at a concentration of 0.1 nM 1,25D, reaching a maximal level at 1 μM (Fig. 2A–C). However, osteocalcin mRNA expression was increased with CB1093 already at a concentration of 1 pM with maximum responses being attained already at a 0.1 nM concentration (a 1.8-fold greater induction than with 1,25D). With 1,25D, osteocalcin secretion was induced in a dose-dependent (0.1 nM to 1 μM) manner (Fig. 2D). With CB1093, the osteocalcin secretion was already detected at a 1 pM concentration, with a plateau being reached at a concentration of 0.1 nM. Osteocalcin protein levels were 1.8-fold greater than those obtained with 1,25D at the same concentration.

The time-dependency of osteocalcin mRNA and protein synthesis was studied using treatments for 120 h with 1,25D or CB1093 (Fig. 3). At a constant concentration of 0.1 μM, the maximum responses in osteocalcin mRNA levels were reached within 24 h (Fig. 3A–C). At this point, however, increases in osteocalcin mRNA levels obtained with the 20-epi analog were already 1.3-fold greater than those obtained with 1,25D. With 1,25D, the levels of mRNA decreased quickly thereafter, reaching the control level at 120 h. With CB1093, however, osteocalcin mRNA was stimulated even at 72 h to 4.8-fold

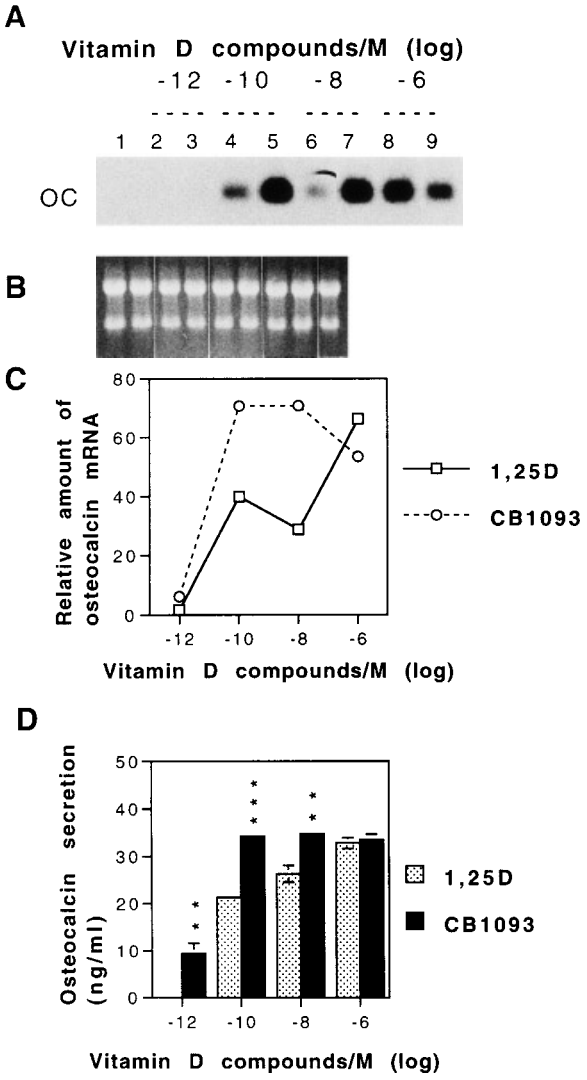

Fig. 2. Dose-dependent expression of the human osteocalcin gene in MG-63 cells by treatment with 1,25-dihydroxyvitamin D_3 or CB1093. **A**: The levels of osteocalcin mRNA in control **(lane 1),** 1,25D- **(lanes 2, 4, 6, 8)**, and CB1093-treated **(lanes 3, 5, 7, 9)** cells after the 72-h treatment. **B**: Ethidium bromide-stained loading controls. **C**: Osteocalcin mRNA levels quantified by densitometric scanning of the autoradiograms. **D**: Osteocalcin secretion into the medium by the cells. The columns represent means ± SEM (n = 3). OC, osteocalcin. Statistical significance between 1,25D- vs. CB1093-treatment (**$P < 0.01$ and ***$P < 0.001$).

levels compared with 1,25D; after the 120-h treatment, the mRNA levels still remained at about 60% of the maximum levels. Both 1,25D and CB1093 showed identical levels of osteocalcin secretion into the medium during the 24-h treatment; whereas, at 120 h, the levels obtained with the analog were about 1.4-fold greater than those obtained with 1,25D (Fig. 3D).

The duration of the effects of the 6-h preincubation with 0.1 μM 1,25D or CB1093 was followed for the next 96 h by measuring osteocalcin mRNA levels and the secretion of osteocalcin into the culture medium (Fig. 4). With 1,25D, the levels of osteocalcin mRNA peaked at around 24 h after withdrawal of the hormone and decreased to the control level at 72 h (Fig. 4A–C). Analog CB1093 increased osteocalcin mRNA levels more efficiently than 1,25D (2.1-fold

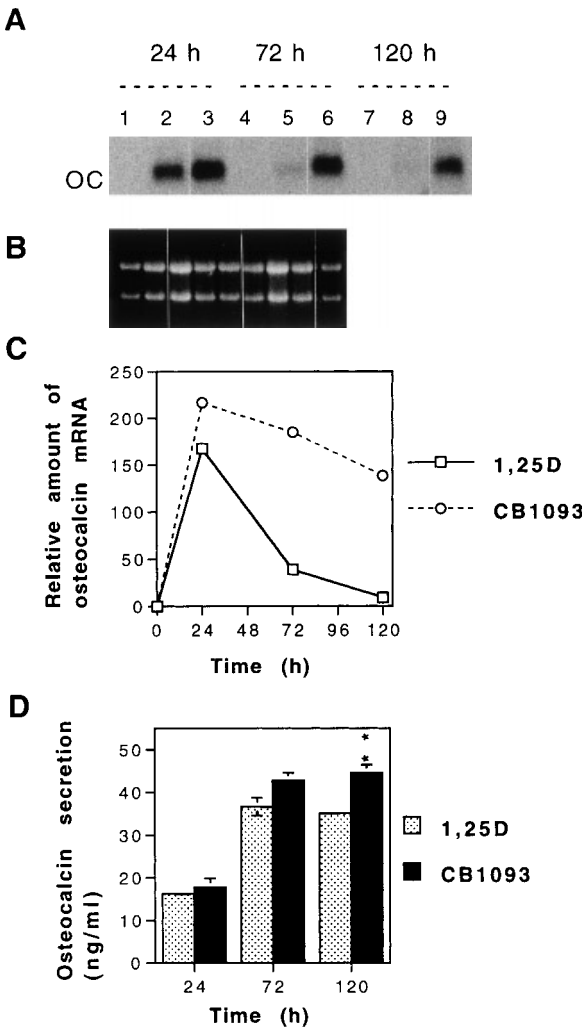

Fig. 3. Time course of osteocalcin gene expression in MG-63 cells by treatment with 1,25-dihydroxyvitamin D_3 or CB1093. **A**: The cells were treated with 0.1 μM 1,25D **(lanes 2, 5, 8)**, or CB1093 **(lanes 3, 6, 9)** without medium change as indicated. The levels of osteocalcin mRNA in control cells are shown in **lanes 1, 4, and 7**. **B**: Ethidium bromide-stained loading controls. **C**: Osteocalcin mRNA levels quantified by densitometric scanning of the autoradiograms. **D**: Osteocalcin secretion into the medium by the cells. The columns represent means ± SEM (n = 3). OC, osteocalcin. Statistical significance between 1,25D- vs. CB1093-treatment (**$P < 0.01$).

greater induction after 24 h, 11.6-fold after 72 h, and 12.5-fold after the 96 h incubation). At the protein level, maximal osteocalcin secretion after the 1,25D-treatment was reached at 48 h after the withdrawal of the hormone (Fig. 4D). In CB1093 withdrawal experiments, the osteocalcin secretion was increased for up to 72 h.

Effect of Vitamin D Compounds on VDR-VDRE Binding

Binding of VDR to the osteocalcin VDRE was followed for up to 96 h after the 6-h pretreatment of the MG-63 cells with 1,25D or CB1093 (Fig. 5). Previously, two VDR specific (fast and slow mobility) interactions have been identified with the human osteocalcin gene VDRE oligonucleotide and MG-63 nuclear extracts in EMSA [Jääskeläinen et al., 1994]. VDR-VDRE binding was observed even in the absence of 1,25D or the analog, but immediately after the 6-h pretreatment with either 1,25D or the analog, the magnitude of the VDR-DNA interaction was increased. At all time points, VDR binding was stimulated with CB1093 to higher levels than with 1,25D. When performing the binding reactions with recombinant hVDR and hRXRβ proteins in the absence of the nuclear extract, the differences in the intensities of the VDR-VDRE complexes previously obtained with nuclear extracts from 1,25D- or analog-treated MG-63 cells largely disappeared (Fig. 6). In addition, it was found that with increasing concentrations of the ligand in the reaction mixture, the binding of the VDR-RXRβ complex to the OC-VDRE weakened.

Optimized 3-D Structures of 1,25D and CB1093

The optimized 3-D structures of 1,25D and CB1093 differed from each other markedly (Fig. 7). In the analog CB1093, the triple bond and the ethoxy group causes an altered conforma-

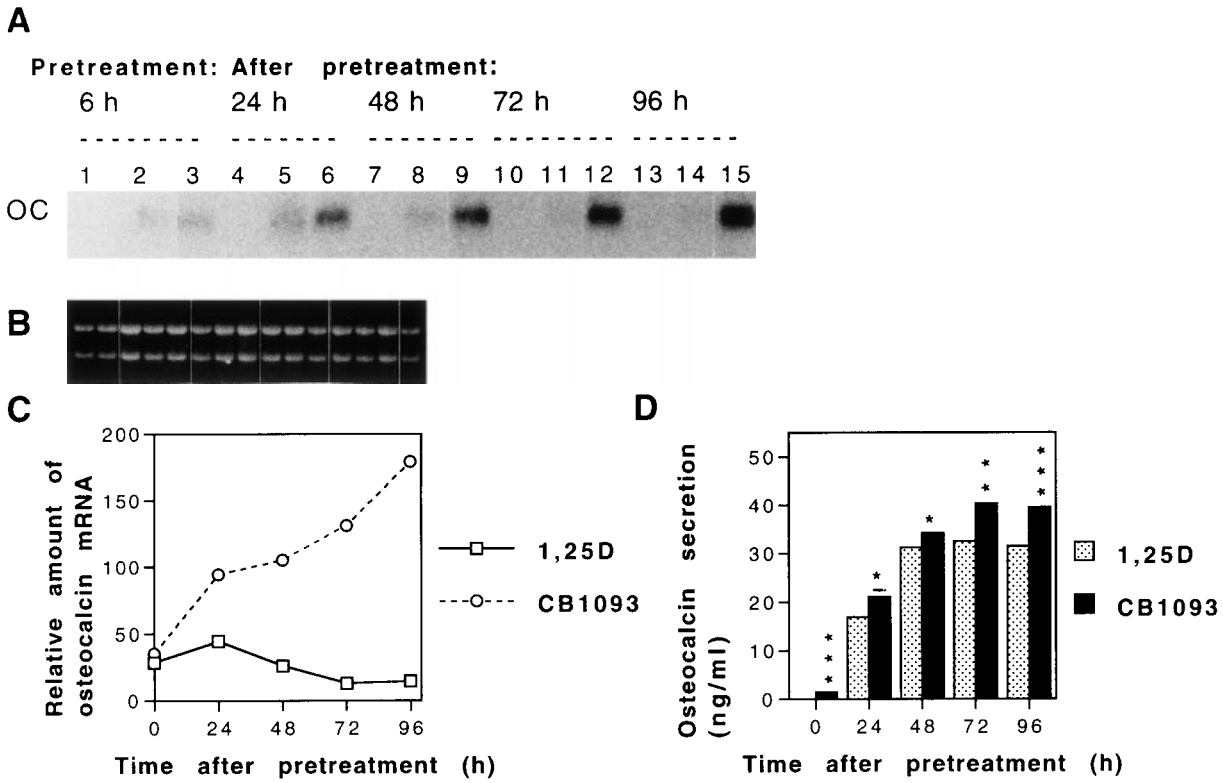

Fig. 4. Maintenance of osteocalcin gene expression in MG-63 cells after pretreatment with 1,25-dihydroxyvitamin D_3 or CB1093. The medium was replaced after the 6-h pretreatment period and the incubation was continued with a fresh medium without 1,25D or CB1093 for up to 96 h. **A**: The cells were treated with 0.1 μM 1,25D **(lanes 2, 5, 8, 11, 14)**, or CB1093 **(lanes 3, 6, 9, 12, 15)**. The levels of osteocalcin mRNA in the control cells are shown in lanes 1, 4, 7, 10, and 13. **B**: Ethidium bromide-stained loading controls. **C**: Osteocalcin mRNA levels quantified by densitometric scanning of the autoradiograms. **D**: Osteocalcin secretion into the medium by the cells. The columns represent means ± SEM (n = 3). OC, osteocalcin. Statistical significance between 1,25D- vs. CB1093-treatment (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

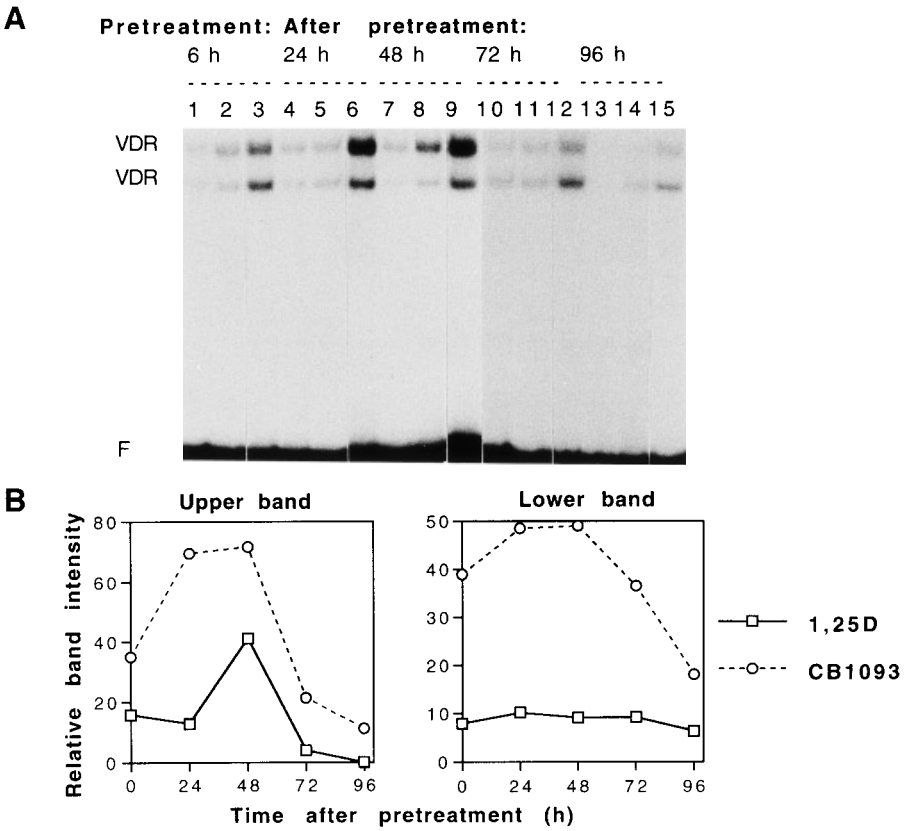

Fig. 5. Protein-DNA interactions at the VDRE region of the human osteocalcin gene promoter after pretreatment of MG-63 cells with 1,25-dihydroxyvitamin D_3 or CB1093. The medium was replaced after the 6-h pretreatment and the incubation was continued for up to 96 h without 1,25D or CB1093. **A**: Nuclear extracts were prepared from control **(lanes 1, 4, 7, 10, 13)**, 1,25D- **(lanes 2, 5, 8, 11, 14)**, or CB 1093-treated **(lanes 3, 6, 9, 12, 15)** cells. **B**: The scanning values for the upper (slow mobility) and lower (fast mobility) bands. F, free probe.

tion in the side chain compared with the natural compound 1,25D (Fig. 7B).

DISCUSSION

A great number of new vitamin D_3 analogs have recently been synthesized in an effort to obtain compounds that would retain the ability of 1,25D to inhibit proliferation and stimulate differentiation, yet have little calcemic effects. Thus far the side chain analogs with either 20-epimerization and/or 22-oxa substitution, in combination with multiple double bonds, have proved to be the most potent vitamin D analogs [Bouillon et al., 1995; Mørk Hansen and Mäenpää, 1997]. We have now studied CB1093, a 20-epi analog with an extensively modified side chain, and found out that it was equal in its antiproliferative activity but more effective than 1,25D in its prodifferentiating potency in the MG-63 human osteoblastic osteosarcoma cell line.

CB1093 was found to be a very potent inhibitor of MG-63 cell growth. Elstner et al. [1997] have recently shown that CB1093 is a powerful inhibitor of clonal growth in acute promyelocytic leukemia cells (NB 4), while 1,25D has little or no effect. In inducing the death of glioma cells, however, CB1093 was comparable to 1,25D [Baudet et al., 1996]. Inhibition of tumor cell growth by 1,25D or its structural analogs has been shown to often involve activation of apoptosis [Elstner et al., 1995; Simboli-Campbell et al., 1996], an important goal in cancer therapy.

1,25D and CB1093 had similar potency in inhibiting osteosarcoma cell growth and in enhancing reporter gene activity, although CB1093 was more efficient than 1,25D in osteocalcin mRNA and protein expression. Unexpectedly, transcription of the reporter gene containing the osteocalcin VDRE did not differ after treatments with 1,25D or CB1093, suggesting that the transfection experiment represents an unphysiological regulatory system, or that these compounds may also influence posttranscrip-

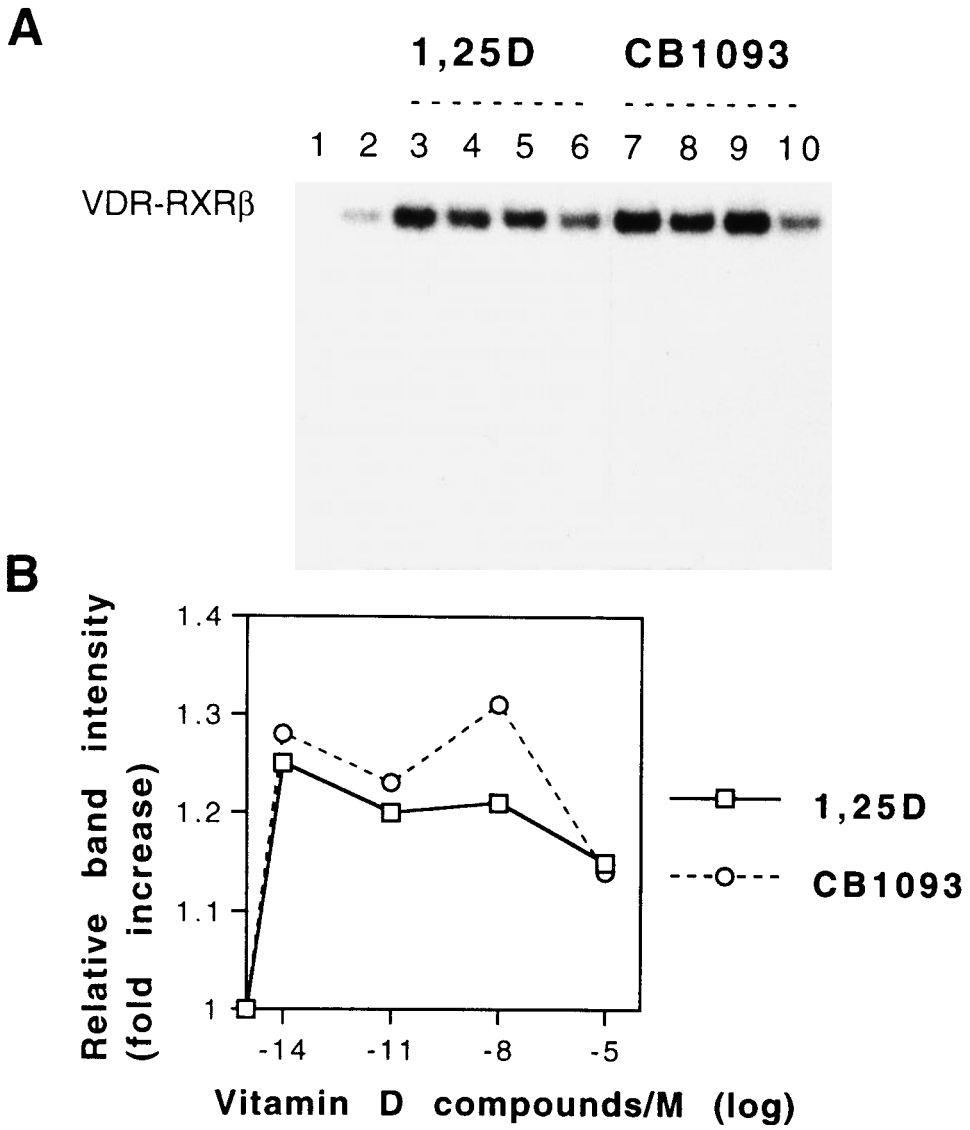

Fig. 6. Formation of the hVDR-hRXRβ-VDRE complex after treatment with different concentrations of 1,25-dihydroxyvitamin D_3 or CB1093. **A**: Recombinant hVDR and hRXRβ proteins were incubated in the absence of nuclear extracts with labelled VDRE **(lane 2)** followed by different concentrations (10 fM, 10 pM, 10 nM, 10 μM) of 1,25D (**lanes 3-6,** respectively), or CB1093 (**lanes 7-10,** respectively). Lane 1 shows the probe only. **B**: Scanning intensities of the bound bands calculated for each treatment against the vehicle treatment (**lane 2,** in A).

tional events in osteocalcin synthesis. We have previously reported that changes in the vitamin D ligand structure have significant effects on target gene expression in these cells [Ryhänen et al., 1996]. Possible explanations for the enhanced osteocalcin expression after the analog treatment include more efficient cellular uptake, which is influenced by the binding affinity of the ligand to the vitamin D binding protein (DBP). A low analog binding to DBP results in higher free concentration of ligand and thereby, possibly, in higher intracellular concentrations of the ligand. Indeed, the 20-epi analogs have been reported to have low DBP binding affinity [Kissmayer et al., 1995]. Another possible explanation is that the rate of catabolism of the 20-epi analogs is slower, thus resulting in stronger and prolonged activity toward target genes [Dilworth et al., 1994].

Our earlier experiments with two other 20-epi analogs (KH1060 and MC1288) [Ryhänen et al., 1996] and the results of the present study with CB1093 show that these analogs cause a long-lasting binding of VDR complexes to the functional VDRE. This might be due to a better ability of the analogs to up-regulate VDR expres-

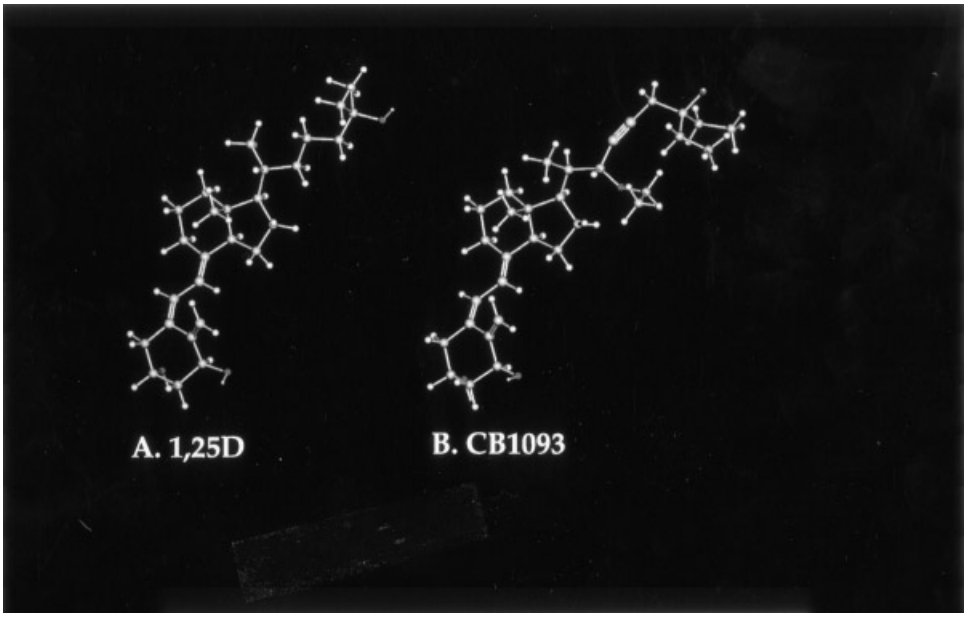

Fig. 7. Optimized 3-D structures of 1,25-dihydroxyvitamin D_3 and CB1093. The structures were optimized by energy minimization as described in Materials and Methods. The structures are oriented so that their CD-rings are in a similar orientation.

sion in the cells compared with 1,25D. Also, differences in the conformation of the VDR-ligand complexes resulting in differences in their interactions with RXR and other proteins present in the complexes might be the reason for the different biological activities observed between the vitamin D_3 compounds. It has been suggested that increased stabilization of the VDR-analog complex could explain the increased cell regulatory properties of some novel analogs [van den Bemd et al., 1996]. However, studies describing binding affinities of the 20-epi analogs to VDR are controversal, as Peleg et al. [1995] have reported lower binding affinity than 1,25D and Nayeri et al. [1996] stronger binding affinity to VDR. Recently, Peleg et al. [1996] have provided evidence that the 20-epi configuration of the side chain restores the antiproliferative activity of an A-ring modified 1,25D analog without increasing its affinity to the vitamin D receptor.

CB1093 or 1,25D in the binding reactions resulted in equally strong in vitro formation of VDR-RXRβ-VDRE complexes, suggesting that the structural differences of the ligands may have no significant effect on the heterodimerization of VDR with RXRβ or on the binding of these dimers to the VDRE. In vivo, the effects of these agents on VDR/RXR expression and on the formation of multiprotein complexes on VDRE might explain the higher levels of VDR-VDRE present after treating the MG-63 cells with the analogs compared with 1,25D. Liu et al. [1997] have recently suggested that VDR has different, multiple contact sites for 1,25D and the 20-epi analogs, resulting in VDR-ligand complexes with different half-lives and transcriptional activities.

The optimized 3-D modeling shows how side chain modifications in analog CB1093 altered its conformation compared with the parent compound 1,25D. This change in the ligand structure may bring about a conformational change in the ligand-receptor complex which realigns helix 12 of the receptor over the ligand binding pocket and may further influence, e.g., the rate of degradation of this complex [Masuyama et al., 1997].

In summary, CB1093 and 1,25D inhibited the growth of human osteoblastic osteosarcoma cells strongly and equally well. Furthermore, the compounds were similar in their ability to enhance human osteocalcin gene promoter driven reporter gene expression in a rat cell line. However, CB1093 was clearly more potent than the parent compound in stimulating osteocalcin synthesis and secretion, even at lower concentrations and for a longer period of time. Our findings on the antiproliferative and prodifferentiating effects of the analog CB1093 together

with its previously detected benefical effects on calcium metabolism [Pakkala et al., 1997] suggest that CB1093 is a promising novel compound for future studies of anticancer activity and/or bone diseases. The markedly different effects of these ligands on protein-DNA binding when using nuclear extracts from ligand-treated MG-63 cells or recombinant hVDR and RXRβ proteins alone suggest that the structures of the ligands have no marked effects on the VDR-RXRb interactions and/or binding of this complex to VDRE but may influence binding of additional nuclear factors to this complex. Further studies are required to reveal the mechanisms of the enhanced biological activity of the 20-epi analogs.

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