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Sensitive Induction of Apoptosis in Breast Cancer Cells by a Novel 1,25-Dihydroxyvitamin D_3 Analogue Shows Relation to Promoter Selectivity

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Abstract The biologically active form of vitamin D_3, the nuclear hormone 1α,25-dihydroxyvitamin D_3 (VD), is an important regulator of cellular growth, differentiation, and death. The hormone mediates its action through the activation of the transcription factor VDR, which is a member of the superfamily of nuclear receptors. In most cases the ligand-activated VDR is found in complex with the retinoid X receptor (RXR) and stimulates gene transcription mainly from VD response elements (VDREs) that are formed by two hexameric core binding motifs and are arranged either as a direct repeat spaced by three nucleotides (DR3) or as an inverted palindrome spaced by nine nucleotides (IP9). The two VD analogues CB1093 and EB1089 are both very potent inhibitors of the proliferation of MCF-7 cultured breast cancer cells displaying approximately 100-fold lower IC_{50} values (0.1 nM) than the natural hormone. In addition, CB1093 is even more potent in vivo than EB1089 in producing regression of experimental mammary tumors. Moreover, both VD analogues induce apoptosis in MCF-7 cells, but CB1093 is effective at concentrations approximately 10-fold lower than EB1089. In accordance, the reduction of Bcl-2 protein expression showed CB1093 to be more potent than EB1089. This suggests that the antiproliferative effect of CB1093 may be related mainly to its apoptosis inducing effect, whereas EB1089 may preferentially have effects on growth arrest. EB1089 is known to result in a selectivity for the activation of IP9-type VDREs, whereas CB1093 shows a preference for the activation of DR3-type VDREs. This promoter selectivity suggests that the effects of VD and its analogues on growth arrest and the induction of apoptosis may be mediated by different primary VD responding genes. In conclusion, CB1093 was found to be a potent inhibitor of rat mammary tumor growth in vivo. CB1093 also displayed a high potency in vitro in the induction of apoptosis, a process that may be linked to a promoter selectivity for DR3-type VDREs. J. Cell. Biochem. 66: 552–562, 1997.

Key words: apoptosis; tumor regression; control of proliferation; vitamin D_3 analogues; breast cancer; vitamin D_3 receptor; regulation of transcription; promoter selectivity

INTRODUCTION

The hormonal form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (VD), is well known for its regulatory role in calcium homeostasis, but it also plays an important role in the modulation of the proliferation and the differentiation of several normal and malignant cell types [Walters, 1992; Bouillon et al., 1995]. Recently, VD was also shown to induce apoptosis (programmed cell death) in human breast cancer and leukemic cell lines [Elstner et al., 1995; 1996; James et al., 1995]. Its cell regulatory function provides VD with a promising therapeutic potential [Jones and Calverley, 1993; Pols et al., 1994] but is limited by unwanted side effects such as hypercalcemia, hypercalciuria and soft tissue calcification [Vieth, 1990]. Modifications of the seco-steroid VD, mainly in the side chain, provided analogues that have been tested for both their calcemic and their antiproliferative effects. During the last decade more than 800 VD analogues have been synthesized in an effort to dissociate the effects on

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proliferation and differentiation from those on calcium homeostasis [Bouillon et al., 1995]. The selective biological activity of some of these VD analogues, such as MC903 (calcipotriol), is mainly related to their rapid metabolic clearance [Masuda et al., 1994]. However, the selectivity in biological functions of some of the more potent VD analogues may be based on the mechanisms of their molecular action.

VD and its analogues are lipophilic molecules that easily pass biological membranes and bind with high affinity to the nuclear receptor VDR [Pike, 1991; Walters, 1992], which is a transcription factor belonging to the superfamily of nuclear receptors [Mangelsdorf et al., 1995]. VDR binds as a dimeric complex with other nuclear receptors to a pair of hexameric core binding motifs that are in a directly repeated or an inverted palindromic arrangement, referred to as simple VDREs [Carlberg, 1995]. The number of spacing nucleotides between the core binding motifs is specific for each nuclear receptor complex. The VDR can form a homodimer [Carlberg et al., 1993; Cheskis and Freedman, 1994] or a heterodimer with the nuclear receptors for all-*trans*-retinoic acid (RA) and thyroid hormone (RAR and T_3R) [Schräder et al., 1994], but the main partner of the VDR is the retinoid X receptor (RXR), which is the nuclear receptor for 9-*cis*-RA [Carlberg, 1996b]. VDR-RXR heterodimers bind to direct repeats separated by three nucleotides (DR3) and to inverted palindromes spaced by nine nucleotides (IP9) [Schräder et al., 1995]. Recently, the VDR was also described to form DNA-binding complexes with other transcription factors like NFAT [Alroy et al., 1995] and CTF/NF-1 [Candeliere et al., 1996]. The functional organization of such complex VDREs is not yet understood.

Recently, it was observed that the highly antiproliferative VD analogue EB1089 [Mathiasen et al., 1993] activated an IP9-type VDRE at 15-fold lower concentrations than a DR3-type VDRE [Nayeri et al., 1995]. This observation of promoter-selectivity led to the hypothesis that primary VD responding genes that are involved in the control of cellular growth may contain an IP9-type VDRE in their promoter region [Carlberg, 1996a]. This hypothesis also implies the prediction that in a given cell line the half-maximal growth inhibition (IC_{50}) value should correlate with the half-maximal gene activation (EC_{50}) value on an IP9-type VDRE rather than with the EC_{50}-value on a DR3-type VDRE. This idea was recently supported by a study of a small series of structurally related VD analogues [Mørk Hansen et al., 1996a].

In this study, the new VD analogue CB1093 was characterized for its cell- and gene-regulatory properties. The antiproliferative effect of CB1093 on human MCF-7 breast cancer cells and its effects on in vivo regression of rat mammary tumors were found to be superior to those of EB1089, but, most interestingly, CB1093 was found to induce apoptosis at a 10-fold lower concentration than EB1089. Moreover, in contrast to EB1089, CB1093 shows a clear preference for the induction of DR3-type VDREs. The induction of apoptosis may be an explanation for the higher potency seen with CB1093 compared to EB1089, in inducing in vivo regression of mammary tumors. Due to the difference in the promoter selectivity of CB1093 and EB1089, the selectivity for DR3-type VDREs seen with CB1093 may be linked to the potent induction of apoptosis.

MATERIALS AND METHODS

Compounds

EB1089 (22,24-diene-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3) and CB1093 (20-epi-22(S)-ethoxy-23yne-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3) are analogues of VD (for the structure of their side chain see Fig. 1). They were synthesized in the Department of Chemical Research (LEO Pharmaceutical Products, Denmark) and dissolved in 2-propanol at 4 mM for in vitro use and in propylene glycol at 0.1 mg/ml for in vivo use.

Effects on Calcium Homeostasis

LEW/MOL female rats (130–170 g) were placed in metabolism cages for 7 days. Test compounds were administered orally each day for 7 days. Control rats received propylene glycol. Each group consisted of three rats. Urine was collected daily, while blood was collected by cardiac puncture on day 7. Calcium levels in urine and serum were determined by complex formation with o-cresolphthalein (Boehringer Mannheim). The urinary excretion of calcium was calculated for days 3–7 (steady-state conditions); serum calcium was measured at day 7. Statistical analysis was carried out using Student's t-test. Mean urine calcium values caused by three different VD analogue concentrations and the vehicle (Table I) provided a dose-

TABLE I. Calcemic Effects of VD, EB1089, and CB1093*

	Compound dose (μg/kg/day) p.o. 7×	Mean of urine calcium (mmol/day) ± SD	Mean of serum calcium(mM) ± SD	Approached calcemic activity relative to VD (%)
Vehicle	0	16 (3)	2.76 (0.12)	
CB1093	0.1	20 (5) $P < 0.01$	2.67 (0.09) n.s.	27
CB1093	1.0	45 (22) $P < 0.001$	2.79 (0.08) n.s.	
CB1093	5.0	246 (63) $P < 0.001$	3.11 (0.09) $P < 0.01$	
VD	0.5	51 (15) $P < 0.001$	2.85 (0.10) n.s.	
Vehicle	0	21 (13)	2.76 (0.09)	
EB1089	0.5	71 (27) $P < 0.001$	2.88 (0.03) n.s.	50
EB1089	1.0	136 (39) $P < 0.001$	2.86 (0.10) n.s.	
EB1089	2.5	255 (75) $P < 0.001$	2.98 (0.11) n.s.	
VD	0.5	121 (40) $P < 0.001$	2.86 (0.08) n.s.	

*Calcium levels in urine and serum in rats after p.o. administration of VD, EB1089, and CB1093 for 7 days.
SD, standard deviation; n.s., nonsignificant.

response curve, in which the analogue concentration was determined that would cause the same calcemic effect than 0.5 μg/kg/day VD; the ratio of 0.5 μg/kg/day and this concentration provided the relative calcemic effect.

Treatment of Tumor-Bearing Rats

An inbred strain of virgin female Ludwig/Wistar/Olac rats bearing mammary tumors induced by nitrosomethylurea (NMU) were maintained as described [Wilkinson et al., 1986]. Rats bearing at least one assessible tumor (>10 mm in diameter) were randomly assigned to treated or control groups. CB1093 was given at a dose of 1 μg/kg body weight, whereas control animals received propylene glycol vehicle alone. The rats were treated each day orally by gavage for 28 days and tumor volume was measured weekly. The tumor volume was determined by measuring the two largest diameters at right angles, using Vernier calipers. From these values, the total tumor for each animal was calculated using the formula $0.17\pi(D_1 \times D_2)^{1.5}$, where D_1 and D_2 are the two diameters. The percentage change in the total tumor volume was compared with the tumor volume at the start of treatment. Animals whose tumors showed signs of ulceration or in which the tumor burden became excessive (>25-mm diameter) were culled. The percentage change in the total tumor volume at each week of the study was compared between groups using the nonparametric Mann-Whitney U-test. Comparison of serum calcium measurements used the unpaired Student's t-test.

Cell Culture and Proliferation Assay

MCF-7 human breast cancer cells were grown in phenol red-free DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5% charcoal-treated fetal calf serum (FCS) for all in vitro studies. The cells were treated for a period of 5 days with graded concentrations of VD, EB1089 or CB1093. Dilutions were performed in ethanol or 2-propanol (the maximum concentration never exceeded 0.1% in the medium). The compounds were administered to the cells after plating and fresh dilutions added every second day when the medium was changed. Control cells were treated with the diluent.

For the proliferation assay cells were seeded at a density of 10^4 cells/well in multidishes. VDR ligand treatment was initiated 2 h after seeding the subconfluent cells. After 5 days of treatment, 1 μCi/ml [^{3}H]thymidine was added to the cells and the dishes were incubated for 4 hours. The cells were then washed three times with NaCl and 25 μg/ml nonlabeled thymidine, solubilized for 20–30 min in 1 ml 0.5 M NaOH. Incorporated [^{3}H]thymidine was measured with a β-counter. Three separate experiments were performed each in quadruplicate. Inhibition of proliferation was expressed as the percentage of mean [^{3}H]thymidine incorporation in relation to mean [^{3}H]thymidine incorporation of solvent-treated control cells.

Apoptosis Assays

MCF-7 cells were seeded at a density of 10^4 cells/ml in 6-well plates (for the Cell Death

Detection ELISA™) or as 2×10^4 cells/ml grown on coverslips (for ApopTag™). The cells were treated with graded concentrations of VD, EB1089 or CB1093 for 5 days. Apoptosis was measured both by the Cell Death Detection ELISA™ (Boehringer Mannheim) and by ApopTag™ In Situ Apoptosis Detection (Oncor) according to the instructions of the supplier. The Cell Death Detection ELISA™ determines photometrically cytoplasmic histone-associated DNA fragments, whereas the ApopTag™ detects directly apoptotic cells by endlabelling fragments of genomic DNA. The results of the Cell Death Detection ELISA™ were normalized by directly counting trypan blue excluding cells that had been grown and treated in parallel. The results of each method were expressed in relation to the solvent control. At least two separate experiments, each with at least three determinations, were performed.

Western Blot Analysis of Bcl-2 Expression

MCF-7 cells were treated for a period of 5 days with graded concentrations of VD, EB1089 and CB1093. Cells were harvested and lysed as described previously [James et al., 1994]. Protein extracts (10 µg) were electrophoresed through 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and electrotransferred to nitrocellulose filters. Equivalent loading was confirmed by Coomassie staining of replicate gels. Preblocked filters were incubated with a monoclonal antibody against Bcl-2 (Boehringer Mannheim). The Bcl-2-specific 26-kD band was detected by using horseradish peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence (Amersham, Little Chatfont, UK). Densitometry was performed on a MicroTek Scanmaker flat bed scanner and Bcl-2 expression was expressed as percentage of control. Three separate experiments with each concentration in duplicate were performed.

Limited Protease Digestion Assay

Linearized cDNA for human VDR was used for in vitro transcription as recommended by the supplier (Promega, Madison, WI). Ten µl of in vitro transcribed VDR RNA were mixed with 175 µl rabbit reticulocyte lysate (Promega), 100 U RNasin, 20 µl [^{35}S]methionine (1,000 Ci/mmol), and 20 µM amino acid mixture (minus methionine) in a total volume of 250 µl and incubated at 30°C for 1 hr. For the determination of the functional dissociation constant (K_{df}), 1 µl of this in vitro-translated VDR was preincubated for 30 min with graded concentrations of CB1093 in a total volume of 7.5 µl. Then 2.5 µl of trypsin (Promega) was added to a final concentration of 27 µg/ml, and the mixtures were incubated for 30 min at room temperature. The digestion reactions were stopped by adding 10 µl protein gel loading buffer (0.25 M Tris, pH 6.8, 20% glycerol, 5% mercaptoethanol, 2% SDS, 0.025% bromophenol blue), and the samples were denatured at 95°C for 5 min, electrophoresed through a 15% SDS-polyacrylamide gel, electrotransferred to a nitrocellulose filter, air-dried, and autoradiographed overnight. The protease-sensitive VDR fragment was localized, excised from the filter and radioactivity was measured in a scintillation counter. This activity was normalized by subtraction of the background activity found in the diluent control.

DNA Constructs, Transfection, and CAT Assays

Fusion of the DR3-type VDRE of the pig osteopontin gene promoter and of the IP9-type VDRE of the human calbindin D_{9k} promoter with the thymidine kinase (*tk*) promoter in front of the chloramphenicol acetyl transferase (CAT) reporter gene has been described previously [Nayeri et al., 1995; Schräder et al., 1995]. The core sequences of the elements are given in Figure 6. MCF-7 cells were seeded into 6-well plates (10^5 cells/ml) and grown overnight in phenol red-free DMEM supplemented with 5% charcoal-treated FCS. Liposomes were formed by incubating 2 µg of the receptor plasmid and 1 µg of the reference plasmid pCH110 (Pharmacia) with 15 µg N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP, Boehringer Mannheim) for 15 min at room temperature in a total volume of 100 µl. After dilution with 0.9 ml phenol red-free DMEM, the liposomes were added to the cells. 500 µl phenol red-free DMEM supplemented with 15% charcoal-treated FCS were added 4–8 h after transfection. At this time graded concentrations of CB1093 were also added. The cells were harvested 40 hours after onset of the stimulation and CAT-assays were performed as described [Pothier et al., 1992]. The CAT activities were normalized in proportion to β-galactosidase activity, and induction factors were calculated as the ratio of CAT activity of ligand-stimulated cells to that of solvent controls.

RESULTS

VD Analogues and Their Calcemic Effect

Compared to VD the side chain of the VD analogue EB 1089 is elongated by three carbon atoms and contains in addition two double-bonds [Colston et al., 1992]. Compared to EB 1089 the VD analogue CB 1093 has altered stereochemistry at C-20 (20-epi analogue), has an ethoxy substituent at C-22 and contains a triple bond at C-23–C-24, instead of the two double bonds of EB 1089 [Calverley et al., 1995] (Fig. 1). In vivo studies investigating the effects of VD, EB1089 and CB1093 on the calcium homeostasis in rats treated orally with the compounds for 7 days have shown that EB1089 and CB1093 displayed only 50% and 27%, respectively, of the calcemic effect of VD (Table I).

Breast Cancer Growth In Vivo

VD and EB1089 have previously been assessed for their effectiveness in causing regression of experimental rat mammary tumors [Colston et al., 1992]. The same experimental system was now used to study CB1093. A dose of 1 µg CB1093/kg body weight/day for 28 days caused a significant reduction in tumor size (in average

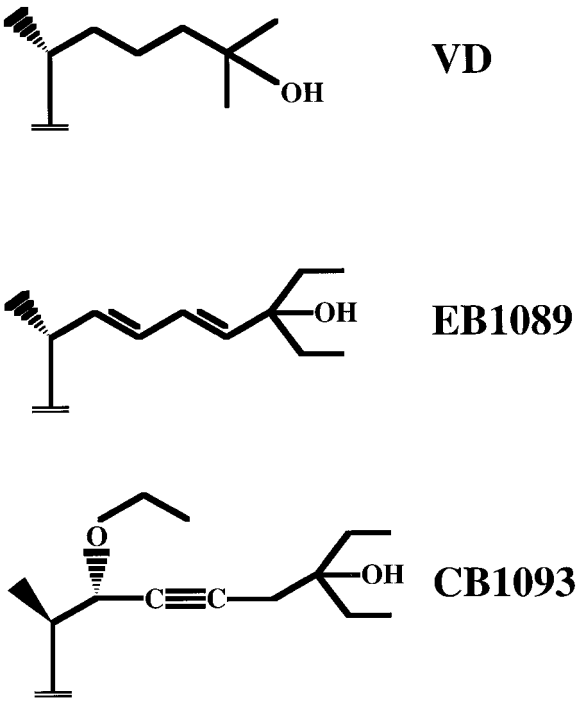

Fig. 1. Structure of the side chain of the VD analogues EB1089 and CB1093 in comparison with VD. The two analogues EB1089 and CB1093 were obtained by modifying the side chain of the VD molecule.

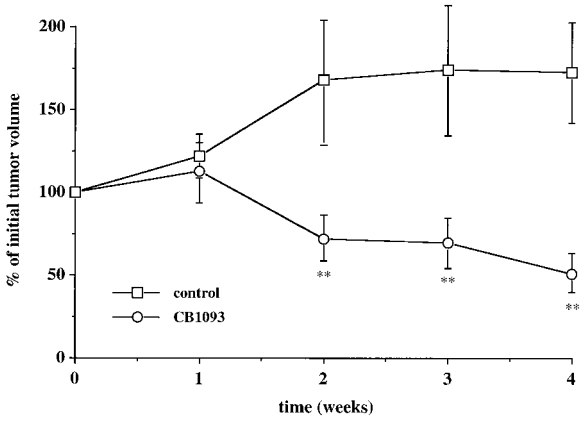

Fig. 2. Effect of CB1093 in NMU-induced rat mammary tumors. CB1093 was given each day orally for 28 days at a dose of 1 µg/kg body weight to rats bearing NMU-induced mammary tumors. Control animals received propylene glycol vehicle alone. Tumor volume was assessed once per week and the results are expressed as the percentage change compared to day 0 (Mann-Whitney U-test). Mean serum calcium concentration was 2.98 ± 0.07 mM in treated and 2.67 ± 0.02 mM in controls. $^{**}P < 0.005$ (unpaired Student's t-test).

49% of the initial tumor volume) (Fig. 2). By contrast, control animals showed tumor progression. CB1093 produced at this dose a slight rise in serum calcium concentrations comparable to that seen with the same dose of EB1089 in a previous study [Colston et al., 1992]. However, induction of hypercalcemia was not observed in the calcium metabolism experiments (Table I), where rats were treated with test compounds for 7 days.

Breast Cancer Growth In Vitro

The sensitivity of the MCF-7 human breast cancer cells to EB1089 and CB1093 has been tested in comparison to the natural hormone VD. The cells were incubated for 5 days with graded concentrations of VD, EB1089, and CB1093 before assessment of cell proliferation by [^{3}H]thymidine incorporation. All three compounds exhibited a dose-dependent inhibition of cell growth down to 10–20% of control (Fig. 3), but showed a clear difference in their potency. The IC_{50} value found for CB1093 (0.10 nM) was about 160-fold lower than the IC_{50} value obtained with VD (16 nM). Also EB1089 displayed a higher potency than VD with an IC_{50} value of 0.27 nM, which is about 59-fold lower than that of VD.

Induction of Apoptosis

Apoptosis-associated DNA fragmentation can be detected sensitively either by antibodies,

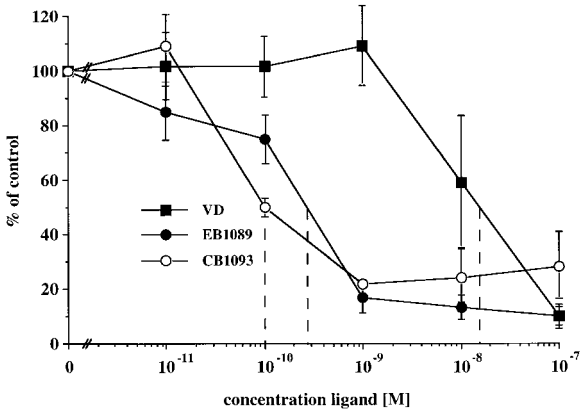

Fig. 3. Antiproliferative effects of VD, EB1089, and CB1093 on MCF-7 cells. Proliferation was assessed by [^{3}H]thymidine incorporation after 5 days in culture with graded concentrations of VD, EB1089 or CB1093. Results are expressed as the percentage mean of control and are based on 8–36 determinations. Dashed lines indicate IC_{50} values.

which are specific for histone-associated DNA fragments, or by labeling of newly generated DNA ends. Both methods were used for the analysis of MCF-7 cells that had been treated for 5 days with graded concentrations of VD, EB1089, and CB1093. Using the Cell Death Detection ELISA™ (Fig. 4A), CB1093 showed at saturating ligand concentration (100 nM) a 5.9-fold higher apoptosis signal than control cells, whereas the increase with the same concentration of EB1089 and VD was 5.0- and 4.4-fold, respectively. Half-maximal induction of apoptosis (EC_{50}) was achieved with CB1093 at 0.14 nM, with EB1089 at 0.72 nM and with VD at 5.4 nM. In addition, CB1093 was always found to be most effective in comparison to EB1089 and VD, when tested in the ApopTag™ In Situ Apoptosis Detection system, although with this assay the difference between CB1093 and EB1089 could not be shown to be statistically significant (Fig. 4B). The percentage of apoptotic cells, when cells were treated with CB1093 (100 nM) was 3.0-fold higher compared to the induction of apoptosis observed in the control, whereas with EB1089 and VD a 2.8- and 1.7-fold increase, respectively, was observed. By presenting the data as curves the EC_{50}-values were determined as 0.07 nM for CB1093, 0.28 nM for EB1089, and 3 nM for VD. The different apoptosis-inducing potential of CB1093, EB1089 and VD was confirmed by Western blot analysis of the cell survival factor Bcl-2 in MCF-7 cells.

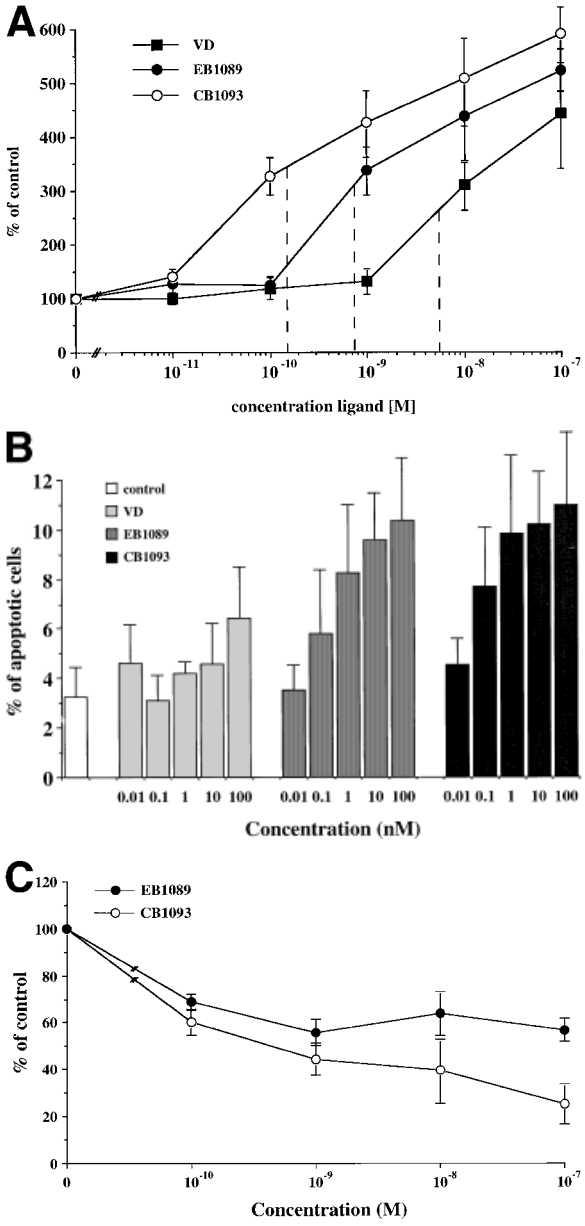

Fig. 4. Apoptosis-inducing potential of VD, EB1089, and CB1093. MCF-7 cells were stimulated for 5 days with graded concentrations of VD, EB1089, and CB1093. The induction of apoptosis was quantified by the Cell Death Detection ELISA™ (Boehringer Mannheim) normalized by cell number (**A**) and by ApopTag™ In Situ Apoptosis Detection (Oncor) (**B**). The dashed lines in A indicate EC_{50} values. Bcl-2 expression was assessed by Western blot analysis and quantified by scanning (**C**). Each data point represents the mean of 6–12 determinations and the bars indicate standard deviation.

After 5 days of treatment with graded concentrations of EB1089 and CB1093 the Bcl-2 expression was shown to be reduced with similar potencies as seen for the induction of apoptosis (Fig. 4C).

Functional VDR Affinity

The interesting cell regulatory properties of CB1093 suggested the investigation of its gene regulatory parameters such as the VDR interaction and the VDR-mediated activation of gene transcription through different VDREs. The limited protease digestion method enables the direct visualization and quantification of VDR-VD analogue interactions [Nayeri et al., 1995, 1996] and therefore has a great advantage compared to the traditional displacement binding studies. The latter are not very illustrative for the biological activity of a compound [Mørk Hansen et al., 1996b] and are indicating a very low potency for CB 1093. The principle of the limited protease digestion assay is that the digestion of a VDR–ligand complex with a limited amount of an endoprotease results in a protein fragment that is resistant to digestion in contrast to a receptor without a ligand. The in vitro translated [^{35}S]methionine-labeled VDR was treated with trypsin after a preincubation with graded concentrations of CB1093. The relative amount of resistant protein fragment is directly proportional to the occupation of the receptor with ligand. The ligand concentration that provides 50% of protease-resistant VDR fragment at saturating ligand concentration is defined as K_{df} value. Scatchard plots of the data are shown in Figure 5 and provide a K_{df} value for CB1093 of 0.82 nM. This is comparable with the K_{df} value for VD, which has previously been determined as 0.9 nM, and clearly lower than that of EB1089 (3.4 nM) [Nayeri et al., 1995]. However, it should be noted that the limited protease digestion assay allows only the determination K_{df} value for functional conformational changes of monomeric VDR, whereas bound to DNA the VDR is mainly found as a heterodimeric complex with RXR.

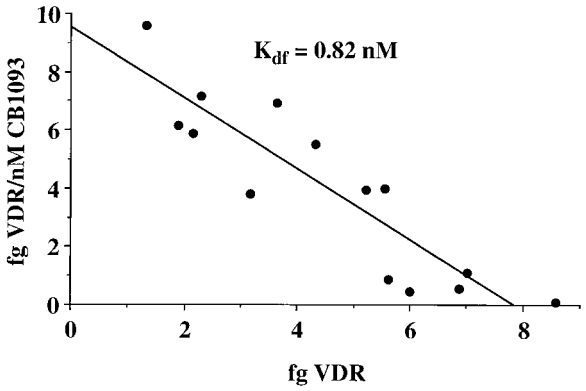

Fig. 5. Binding of CB1093 to in vitro-translated VDR. In vitro-synthesized [^{35}S]methionine-labeled VDR was preincubated with graded concentrations of CB1093. Trypsin was added to a final concentration of 27 μg/ml, and the mixtures were incubated for 30 min at room temperature. Samples were electrophoresed through a 15% SDS-polyacrylamide gel, electrotransferred to a nitrocellulose filter, air-dried, and autoradiographed. The respective protease-resistant VDR fragments (size 30–34 kD) were localized, cut out from the filter, and their radioactivity measured in a scintillation counter. From this, the amount of bound VDR was calculated. Scatchard plots of a representative experiment are shown. The error in the determination of the K_{df} value was estimated to be below 30%.

Transcriptional Regulation

The potency of CB1093 in gene activation was tested with heterologous promoter/reporter gene constructs, which were formed by either the DR3-type VDRE of the pig osteopontin gene [Zhang et al., 1992] or the IP9-type VDRE of the human calbindin D_{9k} gene [Schräder et al., 1995] fused with the *tk* minimal promoter driving the CAT reporter gene. These plasmid constructs were transfected into MCF-7 cells, which were then stimulated with graded concentrations of CB1093. The plot of CAT reporter gene activity over ligand concentration provided typical sigmodial shaped dose response curves (Fig. 6), from which a EC_{50} value of 0.31 nM on the DR3-type VDRE and of 9.2 nM on the IP9-type VDRE were obtained. In the same experimental system the EC_{50} values for EB1089 and VD have already been determined as 2.4 and 0.35 nM, respectively, on the DR3-type VDRE and 0.16 and 0.53 nM on the IP9-type VDREs [Nayeri et al., 1995]. The efficacy of reporter gene activity at saturating ligand concentration did not show remarkable variation, being a 4- to 6-fold induction on both types of VDREs and with both analogues as well as with VD [Nayeri et al., 1995].

DISCUSSION

Anticancer agents may induce tumor regression through inhibition of proliferation and/or induction of apoptosis. For this reason, both inhibition of proliferation and induction of apoptosis are relevant parameters to include, when evaluating VD analogues for potential antitumor effects. VD and some of its analogues have been demonstrated to display antiproliferative effects on breast cancer both in vitro and in vivo [Frampton et al., 1983; Colston et al., 1989]. One of these analogues, EB1089, exerts potent

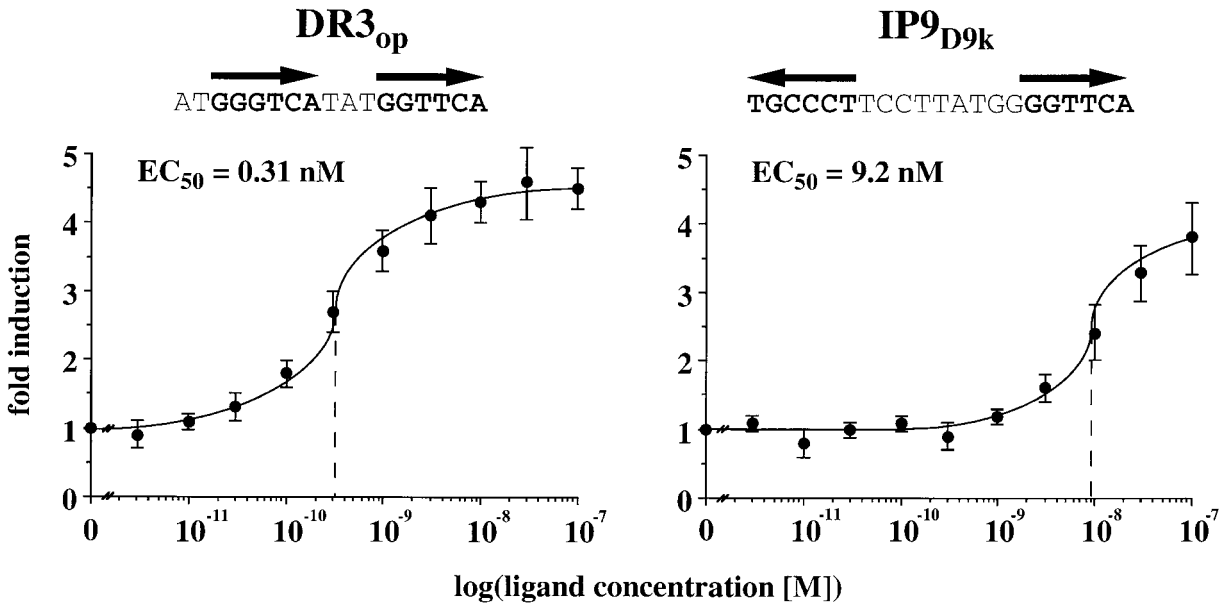

Fig. 6. Selectivity of CB1093 for gene activation from DR3- and IP9-type VDREs. MCF-7 cells were transfected with *tk* promoter/CAT reporter gene constructs containing either the DR3-type VDRE of pig osteopontin or the IP9-type VDRE of human calbindin D_{9k} (core sequences indicated above). The cells were treated for 40 h with graded concentrations of CB1093. Stimulation of CAT activity was calculated in comparison to solvent-induced controls. Each data point represents the mean of triplicates. Bars indicate standard deviation. Dashed lines indicate EC_{50} values.

effects in vitro and in vivo, but also has been demonstrated to induce apoptosis in MCF-7 cells in vitro [Welsh, 1994; Elstner et al., 1995; James et al., 1995; Welsh et al., 1995]. The novel VD analogue CB1093 has been shown to be a very potent antiproliferative drug both in vitro in MCF-7 cells and in vivo on NMU-induced rat mammary tumors. In the present investigation, CB1093 was tested for its effects on induction of apoptosis, as well as on signal transduction at the molecular level. The two VD analogues were analyzed in parallel and were compared to the parent compound.

Apoptosis induced by VD, EB1089 and CB1093 was quantified dose-dependently, for the first time allowing a differentiated view on the antiproliferative effect of these compounds. The induction of apoptosis in MCF-7 cells by CB1093 could be detected at concentrations approximately 10-fold lower concentrations than EB1089. This difference between the two VD analogues indicates that the growth inhibitory effect seen with the two compounds may be caused by different mechanisms. EB1089 and CB1093 are known to be among the most potent antiproliferative VD analogues in vitro. CB1093 has been shown to be even more potent in vivo than EB1089, and in vitro it is slightly more potent. The higher potency seen with CB1093 on the inhibition of proliferation in vitro can explain some of the improved effects seen on in vivo antitumor effects in rats. However, the most interesting difference lies in its ability to induce apoptosis with high potency, suggesting that the effects seen in vivo may be related to induction of apoptosis. An antiproliferative effect, indicated by a reduced DNA synthesis, is the net result of growth arrest and a reduced cell number, and tumor regression occurs, when the rate of cell death is greater than the rate of cell proliferation. This has led to the speculation that in the case of CB1093 both the antiproliferative effect in vitro and the tumor regression in vivo could be mediated mainly by the induction of apoptosis. However, it remains to be verified whether the CB1093 treated rats show a higher induction of apoptosis in tumor sections from the regressing tumors compared to the tumors from EB1089-treated animals.

At the molecular level, CB1093 shows 4-fold higher functional affinity for the VDR than EB1089, and a similar affinity to that of VD. Surprisingly, the receptor-mediated signal measured by activation of transcription showed a difference between the two analogues: CB1093 activated DR3-type VDREs at about 30-fold lower concentrations than IP9-type VDREs, whereas EB1089 displayed the opposite prefer-

TABLE II. Summary of Biological Properties of CB1093 and EB1089 in Comparison to VD

	CB1093	EB1089	VD
Relative calcemic activity in normal female rats[a]	27%	50%	100%
Tumor volume after 4 weeks treatment[b,c]	−49% (1 μg)	−26%[f] (1 μg)	+80%[f] (0.5 μg)
Antiproliferative potency on MCF-7 cells (IC_{50} value)	0.10 nM	0.27 nM	16 nM
Apoptosis-inducing potency on MCF-7 cells (EC_{50} value)	0.14 nM[d]	0.72 nM[d]	5.4 nM[d]
	0.07 nM[e]	0.28 nM[e]	3 nM[e]
Relative functional affinity to VDR (K_{df} value)	0.82 nM	3.4 nM[g]	0.9 nM[g]
Potency on DR3-type VDRE activation (EC_{50} value)	0.31 nM	2.4 nM[g]	0.35 nM[g]
Potency on IP9-type VDRE activation (EC_{50} value)	9.2 nM	0.16 nM[g]	0.53 nM[g]

[a]Based on calcium in the urine.
[b]In parentheses, dose per kg body weight.
[c]Negative values indicate tumor regression.
[d]Determined by Cell Death Detection ELISA™.
[e]Determined by ApopTag™ In Situ Apoptosis Detection.
[f]Colston et al. [1992].
[g]Nayeri et al. [1995].

ence (Table II). As in the case of EB1089 (Table II), the IC_{50} value of the majority of VD analogues that had been assessed so far correlated much better with the respective EC_{50} value for the activation of IP9-type VDREs than with the corresponding EC_{50} value for the activation of DR3-type VDREs [Mørk Hansen et al., 1996a]. But, as can be seen from the results presented here, this correlation does not hold true for CB1093. In the case of VD it is important to remember that the compound binds to the vitamin D binding protein (DBP) in the serum used in all biological assays. Therefore, care must be taken to draw any conclusion between the experiments at the molecular level and biological assays.

Primary VD responding genes, such as $p21^{WAF1/CIP1}$ and c-*fos* [Candeliere et al., 1996; Liu et al., 1996], that are known to be mediators of growth arrest, were found to contain putative IP9-type VDREs in their regulatory regions that can be selectively activated by EB1089 [Schräder et al., 1997] (M. Schräder and C. Carlberg, unpublished results). Recently, the existence of a DR3-type VDRE in the human $p21^{WAF1/CIP1}$ promoter has also been suggested [Liu et al., 1996]. With the experimental system used in the present study for the analysis of transcriptional activity, this DR3-type VDRE was found not to be functional [M. Schräder and C. Carlberg, unpublished results). Therefore, the selectivity of CB1093 for the activation of DR3-type VDREs will not necessarily be reflected in the level of $p21^{WAF1/CIP1}$ expression, although $p21^{WAF1/CIP1}$ has been described to be involved in not only growth arrest but also in the induction of apoptosis [Sheikh et al., 1995]. However, CB1093 shows a correlation between its selectivity for inducing apoptosis and its selectivity in activating DR3-type VDREs. Interestingly, with the VD analogue KH1230, which has previously been reported to show a preference for the activation of DR3-type VDREs [Nayeri et al., 1995], a similar correlation had been observed (data not shown). This suggests that primary VD responding genes involved in the induction of apoptosis may be regulated via DR3-type VDREs.

So far, only a minority of all primary VD responding genes has been identified and none of these genes has yet been associated with the induction of apoptosis. It is therefore necessary to investigate whether the observed in vitro apoptosis-induced effects of VD and its analogues are mediated by genes that stimulate apoptosis or by genes that repress survival factors. Potential candidates are the genes of the tumor necrosis factor-α (TNF-α) and of its type I receptor (TNF-αRI). It is known that the TNF-α expression is VD sensitive, but it remains to be verified whether the TNF-α gene is a primary VD responding gene. There is evidence that the retinoid stimulation of the TNFαRI gene is modulated by VD (P. Polly, C. Carlberg, J.A. Eisman, and N.A. Morrison, unpublished results). These and other candidate genes are currently under investigation and it will be interesting to see whether they contain DR3-type VDREs in their regulatory regions as predicted in this report. Moreover, it has to be investigated through which pathway VD and its analogues mediate apoptosis. At least 5 days

of continuous incubation with VD compounds are necessary to observe apoptosis (data not shown), indicating that probably a VD sensitive gene product has to accumulate to a threshold level before the apoptosis process can proceed.

In conclusion, a high potency for induction of apoptosis, as shown here for the novel VD analogue CB1093 appears to be an important property for an effective tumor regressing potential in the rat mammary tumor model. The apparent link of the apoptosis-inducing potential to selectivity to DR3-type VDREs provides an approach for the understanding of the molecular basis of the VD-mediated onset of apoptosis and thereby an easily applicable screening system for new VD compounds.

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