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Structural Variants of the Vitamin D Analogue EB1089 Reduce Its Ligand Sensitivity and Promoter Selectivity

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Abstract The nuclear hormone 1α,25-dihydroxyvitamin D_3 (VD) has important cell-regulatory functions but also a strong calcemic effect. Therefore, various VD analogues have been synthesized and screened for their biological profile. In order to gain more insight into the molecular basis of the high antiproliferative but low calcemic action of the VD analogue EB1089, we characterized this compound in comparison to five structurally related VD analogues. The activities of the six VD analogues in in vitro assays (limited protease digestion assays for determining interaction with monomeric vitamin D receptor (VDR), ligand-dependent gel shift assays for showing the increase of DNA binding of VDR-retinoid X receptor (RXR) heterodimers, and reporter gene assays on different types of VD response elements for demonstrating the efficacy in nuclear VD signalling) were found to represent their biological potency (antiproliferative effect on different malignant cell lines). In this series, EB1089 proved to be the most potent VD analogue; that is, every structural modification (20-epi configuration, *cis*-configuration at position C24, or changes at the ethyl groups at position C25) appeared to reduce the determined activities mediated through the VDR of these analogues. Moreover, the modifications of EB1089 resulted in a loss of VD response element selectivity, suggesting that this parameter is very critical for the biological profile of this VD analogue. J. Cell. Biochem. 71:340–350, 1998.

Key words: vitamin D analogues; vitamin D receptor; ligand binding; limited protease digestion; ligand-dependent gel shift assay; gene regulation

The secosteroid 1α,25-dihydroxyvitamin D_3 (VD) is the biological active form of vitamin D_3 and has pleiotropic physiological effects, such as regulation of calcium homeostasis [DeLuca et al., 1990], inhibition of cell growth, and induction differentiation in several normal and malignant cell types [Abe et al., 1981; Colston et al., 1982; Frampton et al., 1982]. Moreover, VD has also recently been found to induce apoptosis (programmed cell death) in human breast cancer and leukemic cell lines [Elstner et al., 1995; James et al., 1995; Danielsson et al., 1997]. These cell-regulatory functions of VD suggest a promising therapeutic potential [Jones and Calverley, 1993; Pols et al., 1994], which is, however, limited by side effects such as hypercalcemia, hypercalciuria, and soft tissue calcification [Vieth, 1990]. Therefore, VD analogues with a high antiproliferative but low calcemic effect are of interest. During the last decade, over 800 VD analogues have been synthesized in an effort to dissociate the effects on proliferation and differentiation from those on calcium homeostasis [Bouillon et al., 1995]. As a result of this screening, EB1089 has been identified as a very promising VD analogue [Colston et al., 1992; Mathiasen et al., 1993].

VD and its analogues are lipophilic molecules that easily pass biological membranes and bind with high affinity to the vitamin D receptor

Abbreviations used: DR3, direct repeat spaced by three nucleotides; EC_{50}, half-maximal activation; EB1089, 1(S),3(R)-dihydroxy-20(R)-(5′-ethyl-5′-hydroxy-hepta-1′(E), 3′(E)-dien-1′-yl)-9,10-secopregna-5(Z),7(E),10(19)-triene; IC_{50}, half-maximal inhibition; IP9, inverted palindrome spaced by nine nucleotides; RXR, retinoid X receptor; VD, 1α,25-dihydroxyvitamin D_3; VDR, 1α,25-dihydroxyvitamin D_3 receptor; VDRE, VD response element.

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(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 to a pair of hexameric core binding motifs, referred to as VD response elements (VDREs) [Carlberg, 1995]. The main partner of the VDR is the retinoid X receptor (RXR), which is the nuclear receptor for 9-*cis* retinoid acid [Carlberg, 1996b]. VDR-RXR heterodimers bind preferentially to directly repeated hexameric binding site arrangements with three spacing nucleotides (DR3-type VDREs) or to inverted palindromic structures with nine intervening nucleotides (IP9-type VDREs) [Carlberg, 1996b]. Binding of VD or of a VD analogue to the VDR results in stabilization of a functional conformation of the ligand binding domain, which results in receptor activation [Nayeri and Carlberg, 1997]. According to the model of multiple VD signalling pathways [Carlberg, 1996a], the pleiotropic function of VD is based on a variety of dimeric VDR complexes bound to different types of VDREs. The model assumes that each of these VDR-containing protein-DNA complexes may represent one function of VD (i.e., that such kinds of complexes may preferentially be found in the regulatory region of those genes that mediate the respective function of the hormone). Thus, the model provides the basis for an understanding of a selective action of VD analogues.

The limited protease digestion assay has been shown to be a powerful method for visualizing the interaction of VD analogues with functional VDR conformations [Nayeri and Carlberg, 1997], which is not possible with the traditional competition assays using radiolabelled ligand [Mørk Hansen et al., 1996]. Moreover, reporter gene assays on different types of VDREs have been used to demonstrate promoter selectivity of different VD analogues [Nayeri et al., 1995; Danielsson et al., 1996, 1997; Schräder et al., 1997]. In addition to these two important assay systems, a ligand-dependent gel shift assay will be introduced in this report as a sensitive and informative method for the characterization of VD analogues. In order to get more insight into the sharp biological profile of the VD analogue EB1089, the compound will be characterized in comparison to five other VD analogues that are closely related to EB1089 in their structure.

MATERIALS AND METHODS

Compounds

EB1089 (1(S),3(R)-dihydroxy-20(R)-(5′-ethyl-5′-hydroxy-hepta-1′(E),3′(E)-dien-1′-yl)-9,10-secopregna-5(Z),7(E),10(19)-triene) [Binderup and Calverley, 1989] and its five structural relatives, EB1072, EB1129, EB1133, EB1155, and EB1270 (for their structures see Fig. 1), were synthesized at LEO Pharmaceutical Products (Ballerup, Denmark). The ligands were dissolved in 2-propanol at a stock concentration of 4 mM; dilutions were performed in ethanol.

DNA Constructs

The cDNAs for human VDR and human RXRα were subcloned into pSG5 (Stratagene, Heidelberg, Germany). The fusion of the DR3-type VDRE (core sequence AT**GGGTCA**TAT**GGTTCA**) from the pig osteopontin gene promoter and of the IP9-type VDRE (core sequence **TGCCCT**TCCTTATGG**GGTTCA**) from 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].

Limited Protease Digestion Assay

Linearized cDNA of human VDR was used for in vitro transcription as recommended (Promega, Heidelberg, Germany). Four micrograms of in vitro transcribed RNA was mixed with 70 µl rabbit reticulocyte lysate (Promega), 80 U RNasin, 2 µl [^{35}S]-methionine (1,000 Ci/mmol), and 20 µM amino acid mixture (minus methionine) in a total volume of 100 µl and incubated at 30°C for 2 h. Two microliters of in vitro translated protein, 4.5 µl of 50 mM Tris, pH 7.9, and 1 µl ligand (or 1 µl ethanol as a control) were preincubated for 15 min at room temperature. Then 2.5 µl of trypsin (Promega) (final concentration 25 µg/ml) was added, and the mixtures were further incubated for 10 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). The samples were denatured at 95°C for 5 min and electrophoresed through a 12 % SDS-polyacrylamide gel (acrylamide/*N*,*N*'-methylene-bisacrylamide weight ratio 33:1). The gels were dried and exposed to a Fuji MP2040S imager screen overnight. The individual protease-resistant VDR fragments were detected on

a Fuji BAS1500 reader and compared to full-length VDR input using TINA software (Raytest, Sprockhövel, Germany). Each condition was analyzed in triplicate.

Gel Shift Assays

Human VDR and human RXRα were transcribed and translated in vitro as recommended by the supplier (Promega), and equal amounts of both proteins were mixed. Five microliters of this protein mixture was incubated in each assay with 2 µl graded concentrations of EB1089 and its structural relatives (or 2 µl ethanol as a control) for 15 min at room temperature in a total volume of 20 µl binding buffer (10 mM Hepes (pH 7.9), 80 mM KCl, 1 mM DTT, 0.2 µg/µl poly(dI-C), and 5% glycerol). The mouse osteopontin response element probe was isolated from Hind III/Bam HI restriction of the respective pBLCAT2-derived reporter gene construct [Schräder et al., 1994] and labelled by a fill-in reaction using [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I (Promega). Approximately 1 ng of labelled probe (50,000 cpm) was added to the receptor-ligand mixture, and incubation was continued for 20 min. Protein-DNA complexes were resolved on a 5% nondenaturing polyacrylamide gel (at room temperature) in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA [pH 8.3]). The gels were dried and exposed to a Fuji MP2040S imager screen overnight. The ratio of free probe to protein-probe complexes was quantified on a Fuji BAS1500 reader. Each condition was analyzed at least in triplicate.

Transfection and CAT Assays

Human MCF-7 breast cancer cells were seeded into six-well plates (10^5 cells/ml) and grown overnight in phenol red–free Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% charcoal-treated fetal calf serum (FCS). Liposomes were formed by incubating 2 µg of the reporter plasmid and 1 µg of the reference plasmid pCH110 (Pharmacia, Freiburg, Germany) with 15 µg N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) (Boehringer Mannheim, Mannheim, Germany) for 15 min at room temperature in a total volume of 100 µl. After dilution with 0.9 ml phenol red–free DMEM,

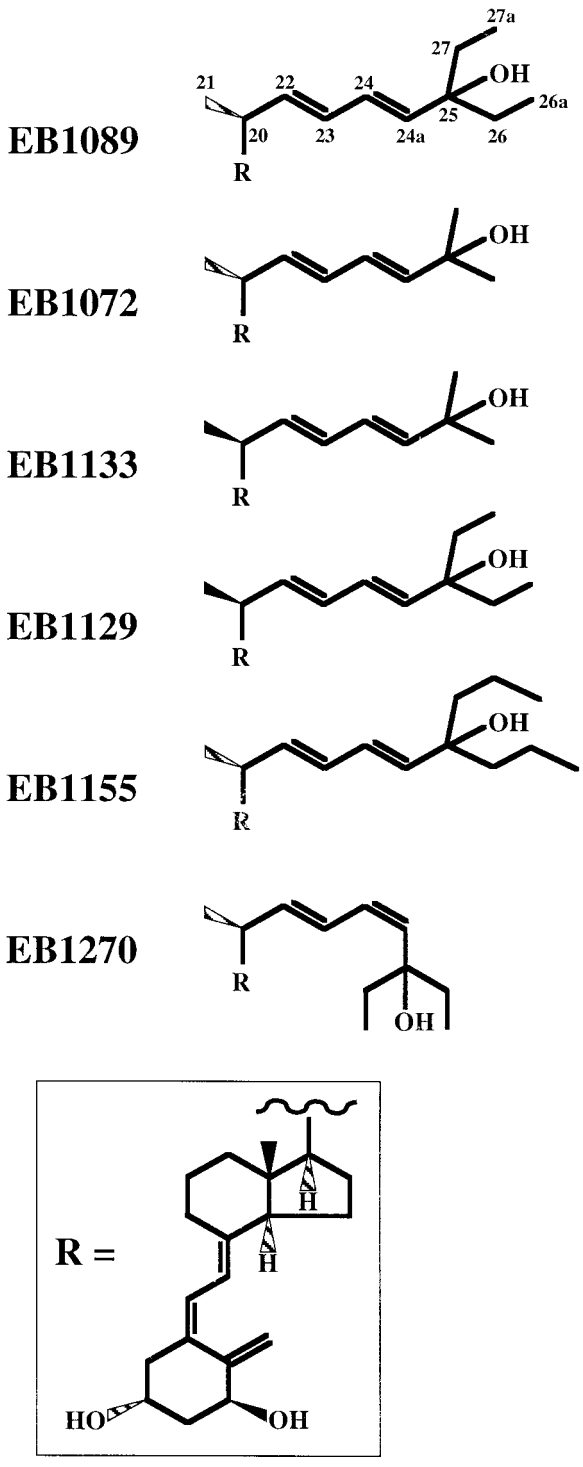

Fig. 1. Structure of EB1089 and its structural relatives. In comparison to the natural hormone VD, the side chain of EB1072 is extended by one carbon atom and contains double bonds at positions C22 and C24 (both in *trans* configuration). EB1133 is the 20-epi stereoisomer of EB1072. Compared to EB1072, both methyl groups at position C25 are replaced by ethyl groups in EB1089. EB1129 is the 20-epi stereoisomer of EB1089, and in EB1270 the second double bond at position 24 is in *cis* configuration. Compared to EB1089, both ethyl groups are substituted by propyl groups in EB1155.

the liposomes were added to the cells. Phenol red–free DMEM supplemented with 15% charcoal-treated FCS (500 µl) was added 4–8 h after transfection. At this time, graded concentrations of EB1089 or its structural relatives were also added. The cells were harvested 40 h after onset of 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. Each condition was analyzed in triplicate.

Cell Proliferation Assay

MCF-7 cells were seeded at a density of 1.5×10^4 cells/ml in 24-well plates and grown in phenol red–free DMEM supplemented with 5% charcoal-treated FCS. Test compounds were added 2 h after seeding, and freshly diluted compounds were added every second or third day when the medium was changed. Control cells received the same concentration of solvent as the cells treated with the test compounds, and the final concentration of ethanol did not exceed 0.0025%. After 5 days, 1 µCi/ml [^{3}H]-thymidine was added to the cells, and incubation was continued for 4 h. Cells were then washed three times with NaCl and 25 µg/ml nonlabelled thymidine and solubilized for 5–10 min in 1 ml 0.5 M NaOH, and incorporated [^{3}H]-thymidine was measured with a β-counter. Two separate experiments, each with four determinations, were performed.

RESULTS

The effects of the VD analogues, EB1089, and its structural relatives, EB1072, EB1129, EB1133, EB1155 and EB1270 (for their structures see Fig. 1), on VD signalling were analyzed on four different levels. Firstly, the six VD analogues were studied for their ability to stabilize functional conformations of the VDR. For this purpose, [^{35}S]-methionine labelled, in vitro translated VDR, graded ligand concentrations, and trypsin were used to perform limited protease digestion assays (Fig. 2). All six compounds were found to stabilize VDR fragments of an approximate size of 33 and 29 kD that represent two functional VDR conformations (named 1 and 3, respectively) [Nayeri et al., 1996; Nayeri and Carlberg, 1997]. The relative amounts of conformation 1 and 3 were quantified individually and plotted over ligand concentration. Interestingly, the ratio of ligand-dependent stabilization of conformations 1 and 3 appears to be characteristic for each VDR ligand. At any ligand concentration, the compounds stabilized a higher proportion of VDR molecules in conformation 1 than in conformation 3. All six VD analogues did not cause stabilization of more than 20% of all VDR molecules in conformation 3 even at high pharmacological concentrations (10 µM). In contrast, at the same concentration EB1089 stabilized 90% of all VDR molecules in conformation 1. The respective values were 59% for EB1155, 52% for EB1072, 36% for EB1270, 23% for EB1129, and 22% for EB1133 (Table 1). The concentration-dependent stabilization of VDR conformation 1 appears to be even more characteristic. The concentration that stabilized half the number of VDR molecules at 10 µM was 10 nM for EB1089, 12 nM for EB1072, 40 nM for EB1270, 500 nM for EB1129, 1 µM for EB1155, and 1.5 µM for EB1133 (Table 1).

At the next level of analysis, the modulation of the DNA binding affinity of VDR-RXR heterodimers by ligands was studied. For this purpose, in vitro translated VDR and RXR proteins were incubated with pharmacological concentrations of EB1089 (10 µM), its five structural relatives, and ethanol (as a control), and gel shift assays were performed with the DR3-type VDRE of the mouse osteopontin gene [Noda et al., 1990]. All six VD analogues enhanced the amount of VDR-RXR heterodimers that bind to DNA compared to the ethanol-treated control (Fig. 3A). This increase of heterodimer binding was then quantified in response to graded concentrations of all six VD analogues (Fig. 3B). As visualized in Figure 3A, quantitative analysis confirmed that at a high saturating concentration all six VD analogues enhanced the amount of VDR-RXR heterodimers that were bound to a DR3-type VDRE by a factor of approximately 3. However, the six compounds were found to differ in the concentration that allowed a half-maximal increase of heterodimer binding. These concentrations were 0.06 nM for EB1089, 0.16 nM for EB1072, 0.18 nM for EB1270, 1.3 nM for EB1129, 1.4 nM for EB1155, and 1.8 nM for EB1133 (Table 1).

Human MCF-7 breast cancer cells were transiently transfected with plasmid constructs containing a CAT reporter gene under the control of either a DR3-type or an IP9-type VDRE. The cells were then stimulated with graded concentrations of EB1089 and its structural relatives.

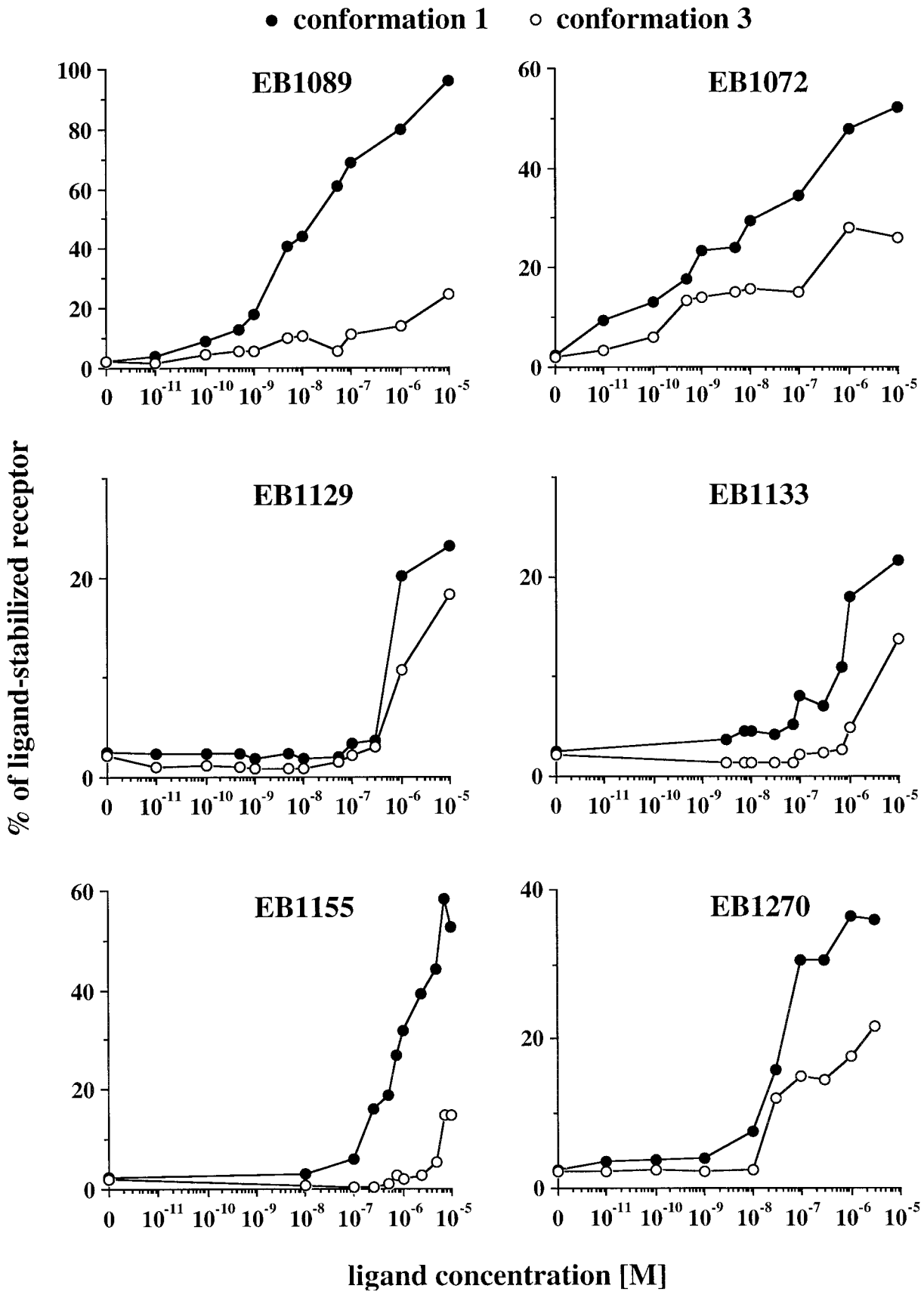

Fig. 2. Concentration-dependent stabilization of functional VDR conformations is characteristic for EB1089 and its structural relatives. Two microliters of in vitro synthesized [^{35}S]-methionine-labelled VDR was preincubated with the indicated concentrations of EB1089 and its structural relatives. Trypsin was added at a final concentration of 25 µg/ml, and the mixtures were further incubated for 10 min at room temperature. Samples were electrophoresed through a 12% SDS-polyacrylamide gel, dried, and exposed to a Bioimager screen. The relative amount of ligand-stabilized VDR in conformations 1 (solid circles) and 3 (open circles) was determined as the mean of at least three independent experiments.

TABLE I. Summary of the Characterization of EB1089 and Its Structural Relatives

	EB1089	EB1072	EB1129	EB1133	EB1155	EB1270
Stabilization of VDR conformation 1 at 10 μM (%)	90	52	23	22	59	36
Concentration of half-maximal stabilization of VDR conformation 1 (nM)	10	12	500	1,500	1,000	40
Concentration of half-maximal increase of VDR-RXR heterodimer binding (nM)	0.06	0.16	1.3	1.8	1.4	0.18
EC_{50} value for activation of a DR3-type VDRE (nM)	2.2	3.0	5.0	8.7	12.4	5.6
EC_{50} value for activation of an IP9-type VDRE (nM)	0.06	1.1	8.0	6.7	12.5	3.2
IC_{50} value for inhibition of growth of MCF-7 cells	0.27	2.0	4.6	11	23	3.8
Relative antiproliferative potency compared to VD (IC_{50} value of VD was 16 nM)	60×	8×	3.5×	1.5×	0.7×	4.2×

The induction of CAT gene activity was determined in comparison to ethanol-stimulated controls (Fig. 4). Comparison of all six VD analogues showed that the induction factors varied between three- and 4.5-fold, but for a given compound the variation between the maximal activation of the DR3- and the IP9-type VDRE was less than 20%. However, the most evident characteristic was the difference in the ligand concentration that was necessary for obtaining half-maximal activation (EC_{50} value) of DR3- and IP9-type VDRE-driven genes. In accordance with previous reports [Nayeri et al., 1995; Schräder et al., 1997], EB1089 demonstrated a clear promoter selectivity, with EC_{50} values of approximately 0.06 nM on the IP9-type VDRE and approximately 2.2 nM on the DR3-type VDRE (Table 1). Faint IP9-type VDRE selectivity was found only with EB1072, resulting in EC_{50} values of 1.1 nM on the IP9-type VDRE and 3.0 nM on the DR3-type VDRE. A significant promoter selectivity could not be observed with the remaining four VD analogues; their respective EC_{50} values were found to be in the order of 4 nM for EB1270, 6 nM for EB1129, 7 nM for EB1133, and 13 nM for EB1155 on both types of VDREs (Table 1).

Finally, the antiproliferative effect of the VDR ligands on MCF-7 cell growth was assessed. Cells were grown in the presence of graded concentrations of the six VD analogues, the natural hormone VD (as reference), and ethanol (as control). DNA synthesis was measured after 5 days in culture and compared to that of control cells (Fig. 5). At high concentrations (100 nM), all seven compounds reduced the proliferation level of the MCF-7 cells down to 10–20% of that of control cells. Moreover, EB1089 appeared to be the most potent analogue, being 60 times (IC_{50} value 0.27 nM) more potent than its parent compound VD in this assay series (Table 1). EB1072 was demonstrated to be eight times, EB1270 4.2 times, EB1129 3.5 times, and EB1133 1.5 times more potent than VD, whereas EB1155 appeared to be 1.5 times less potent than the natural hormone (Table 1). Further proliferation assays that were performed with human U937 histiocytic lymphoma cells, and human HaCaT immortalized keratinocyte cells showed the same ranking in the potency of the VD analogues (data not shown).

DISCUSSION

EB1089 is known to be one of the most potent VD analogues and has been selected as the pilot VD analogue in clinical tests with different

Fig. 3. Ligand- and concentration-dependent increase of VDR-RXR heterodimer formation on DNA by EB1089 and its structural relatives. In vitro translated VDR and RXR protein were preincubated with EB1089 (10 μM), its structural relatives, or ethanol (as control), and gel shift assays were performed using a [^{32}P]-labelled DR3-type VDRE probe (**A**). VDR-RXR heterodimers were separated from free probes. A representative gel is shown. Gel shift experiments were then performed with graded concentrations of the indicated ligands, and the relative amount of the DR3-type VDRE probe that was upshifted by VDR-RXR heterodimers was quantified on a Bioimager (**B**). The increase of VDR-RXR heterodimer binding in comparison to ethanol-treated controls is presented over ligand concentration. Each data point represents the mean of triplicates.

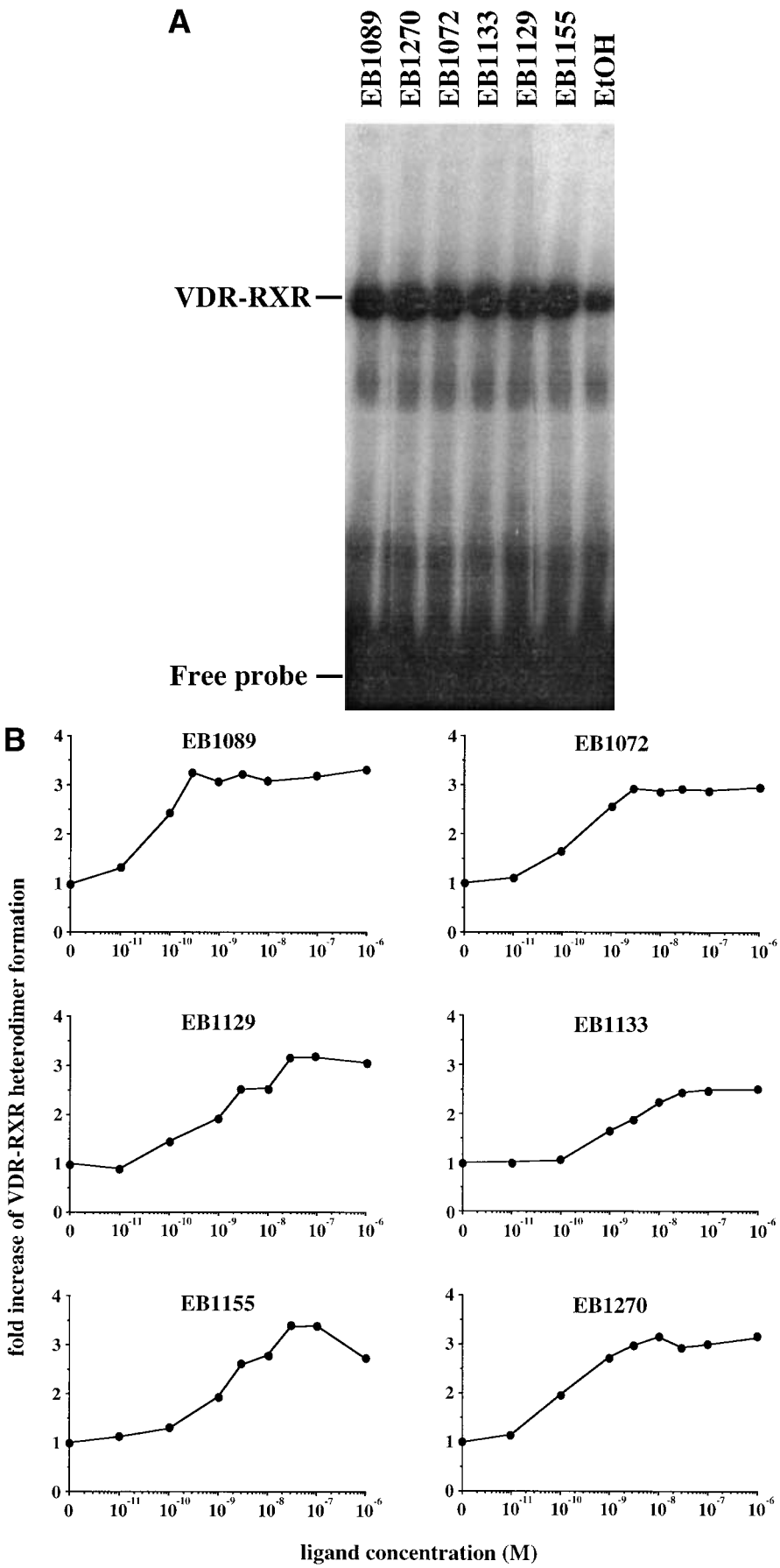
A
EB1089
EB1270
EB1072
EB1133
EB1129
EB1155
EtOH
VDR-RXR
Free probe
B
EB1089
EB1072
EB1129
EB1133
EB1155
EB1270
fold increase of VDR-RXR heterodimer formation
ligand concentration (M)

Figure 3.

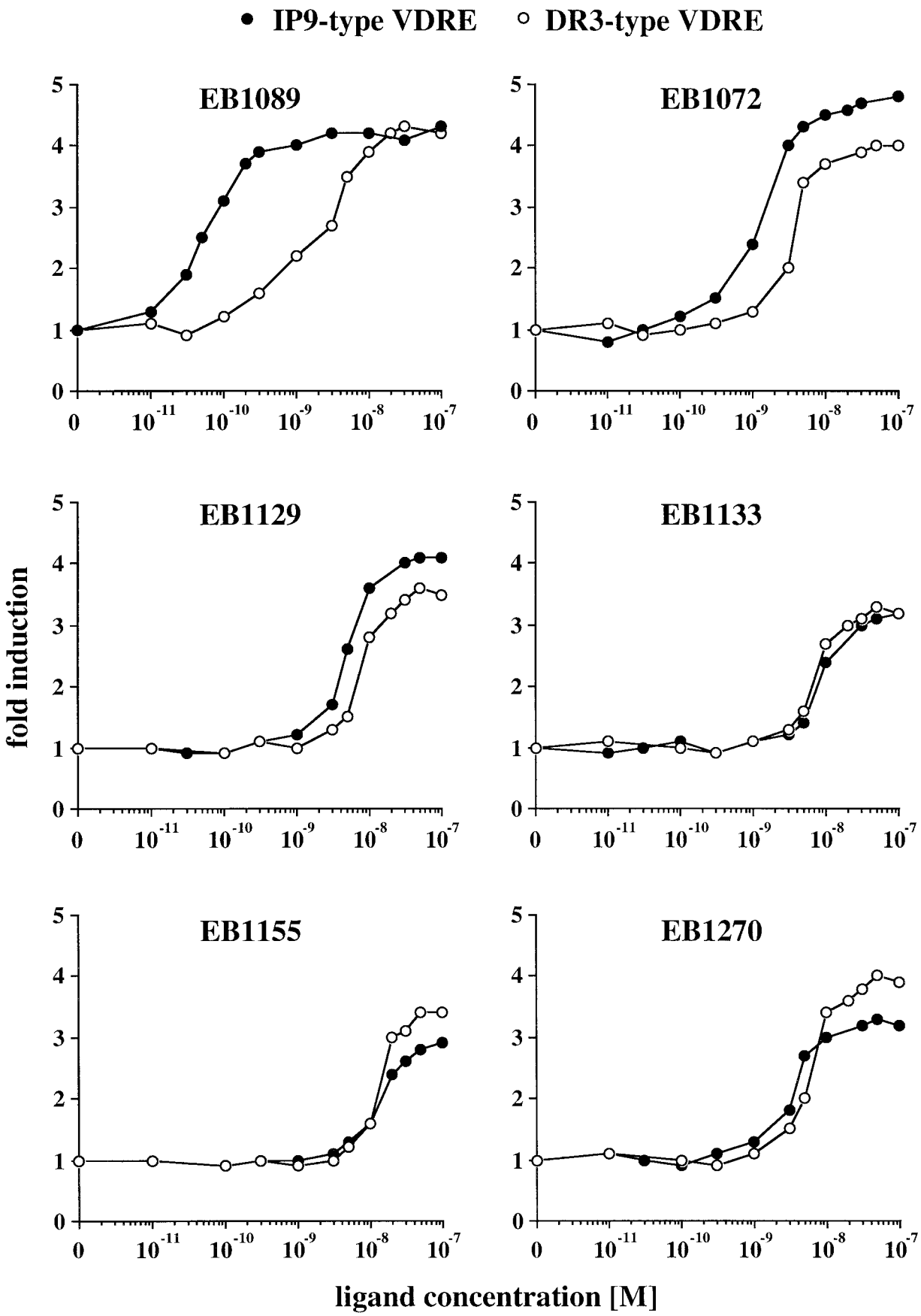

Fig. 4. Selectivity of EB1089 and its structural relatives for gene activation from DR3- and IP9-type VDREs. MCF-7 cells were transfected with *tk* promoter/CAT reporter gene constructs containing either the DR3-type VDRE from the pig osteocalcin gene (open circles) or the IP9-type VDRE from the human calbindin D_{9k} gene (solid circles). Cells were treated for 40 h with graded concentrations of EB1089 and its structural relatives. Stimulation of CAT activity was calculated in comparison to solvent-induced controls. Each data point represents the mean of triplicates.

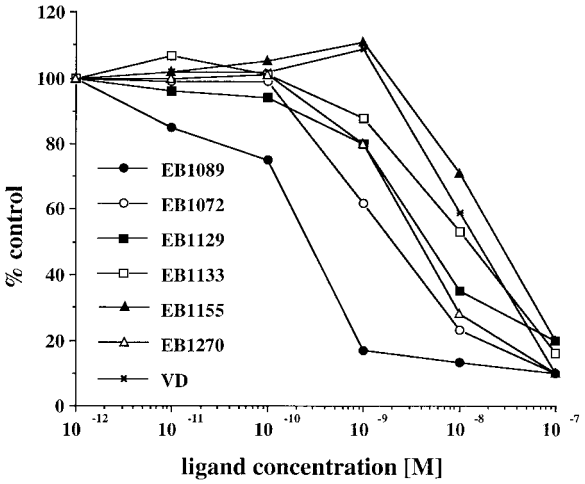

Fig. 5. Antiproliferative effects of EB1089 and its structural relatives on MCF-7 cells. Proliferation was assessed by [^{3}H]-thymidine incorporation after 5 days in culture with graded concentrations of VD, EB1089, and its structural relatives. Results are expressed as the % mean of control and are based on four determinations from one representative experiments out of two. The IC_{50} values were calculated and are compared to that of VD (see Table 1).

types of cancer [Mørk Hansen and Mäenpää, 1997]. In various malignant cell lines, the IC_{50} value of EB1089 is 100-fold lower than that of the natural hormone on average, whereas the calcemic effect of EB1089 in vivo is only 50% of that of VD. EB1089 is a metabolically stable analogue showing a half-life in rats that is comparable with [Kissmeyer et al., 1995] and in cell culture even higher than [Shankar et al., 1997] that of VD. Therefore, the superior selective functional profile of EB1089 compared to VD is most likely to be based on differential activation of VDR-regulated target genes. EB1089 appears to preferentially activate genes that are involved in regulating of the cell cycle, whereas VD activates all genes that contain a functional VDRE in their regulatory region. The molecular basis of this functional selectivity appears to be due to differential interaction with the protein-DNA complexes that contain the VDR. In this report, the interactions of the VDR with EB1089 and five of its close structural relatives have been studied with three different methods.

Analysis of the interaction of VDR monomers with six VD analogues indicated that the ligands stabilized the same two VDR conformations as the natural hormone [Nayeri et al., 1995; Nayeri and Carlberg, 1997]. Conformation 1 is known to be the high affinity ligand binding conformation of the VDR and appears to be most important for the function of the VDR at low physiological concentrations [Nayeri et al., 1996]. In contrast, conformation 3 appears to play a role only at high pharmacological ligand concentrations [Nayeri et al., 1996; Nayeri and Carlberg, 1997]. Therefore, the ability to stabilize conformation 1 (see Table 1) seems to be the most critical parameter. In this respect, the five structural relatives were not found to be as potent as EB1089 (90% at more than 10 nM). However, EB1072 was found to be second in rank (52% at more than 12 nM), whereas both 20-epi stereoisomers of EB1089 and EB1072, EB1129 and EB1133, were found to be lowest in potency (stabilization of only 22–23% at more than 500 nM). In contrast, EB1155 stabilized a reasonable amount (59%) of VDR molecules in conformation 1 but only at very high concentrations (over 1 μM), whereas EB1270 stabilized conformation 1 at lower concentrations (40 nM) but only to a lower percentage of the VDR molecules (36%). For achieving effects on the complete VDR-RXR complex, clearly lower ligand concentrations were necessary than with the VDR monomers. In this assay, EB1089 also appeared to be most potent (0.06 nM), and EB1072 and EB1270 were found to be second and third in rank (0.16 and 0.18 nM). The three remaining VD analogues demonstrated approximately tenfold lower potency (1.3–1.8 nM). The range of concentrations of the six VD analogues that were necessary for activating DR3-type VDREs varied only between 2.2 nM for EB1089 and 12.4 nM for EB1155, whereas the respective range for the activation of IP9-type VDREs was between 0.06 nM for EB1089 and 12.5 nM for EB1155. In this respect, the EC_{50} values for the activation of IP9-type VDREs appear to be more representative for the range of IC_{50} values than the EC_{50} values for the activation of DR3-type VDREs. Based on these data, the high antiproliferative potential of EB1089 appears to be related to its high sensitivity in activating IP9-type VDREs. Since IP9-type VDREs have been found in the promoter regions of the mouse c-*fos* gene [Schräder et al., 1997] and the human and mouse $p21^{WAF1/CIP1}$ gene [unpublished results], these and other currently unknown, IP9-type VDRE carrying genes that have a crucial role in the regulation of the cell cycle could be the key in understanding the growth-regulatory effects of potent antiproliferative VD analogues. More-

over, the selectivity of EB1089 for IP9-type VDREs seems to be a central mechanism that explains the highly selective functional profile of this VD analogue compared to closely related analogues with much less or even no promoter selectivity.

Taken together, the analysis of the molecular effects of EB1089 and its close structural relatives demonstrated that a ranking of VD analogues that is based on their antiproliferative potency is very similar to a ranking based on in vitro assays such as stabilization of protease-resistant VDR conformations, ligand-induced increase of VDR-RXR heterodimer binding, and selective promoter activation. These assays are rather simple and accurate to perform and allow deeper insight into a selective functional profile of a VD analogue.

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