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Metabolism of the vitamin D_3 analogue EB1089 alters receptor complex formation and reduces promoter selectivity

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1 1α,25-dihydroxyvitamin$_3$ (VD) is a nuclear hormone that has important cell regulatory functions but also a strong calcemic effect. EB1089 is a potent antiproliferative VD analogue, which has a modified side chain resulting in increased metabolic stability and a selective functional profile. Since EB1089 is considered for potential systemic application, it will be investigated to what extent its recently identified metabolites (hydroxylated at positions C26 and C26a) contribute to biological profile of the VD analogue.

2 Limited protease digestion analysis demonstrated that EB1089 is able to stabilize the high affinity ligand binding conformation of the VDR, starting at concentrations of 0.1 nM and affecting up to 80% of all receptor molecules. The metabolites EB1445 and EB1470 showed to be 100 fold less potent than EB1089, whereas the remaining three metabolites (EB1435, EB1436 and EB1446) showed a clearly reduced ability to stabilize the high affinity ligand binding conformation. Interestingly, at pharmacological concentrations all EB1089 metabolites stabilized a second, apparently lower affinity conformation to a much higher extent than EB1089.

3 In reporter gene assays all metabolites showed lower potency than EB1089. Moreover, the preference of EB1089 for activation of VDR binding to sites formed by inverted palindromic arrangements spaced by nine nucleotide (IP9-type VD response elements) appeared to be reduced (with EB1445 and EB1470) or completely lost (with EB1435, EB1436 and EB1446). The ranking of EB1089 and its metabolites that was obtained by limited protease digestion and reporter gene assays was confirmed by an analysis of their antiproliferative effect in breast cancer cells.

4 The potency and selectivity of the EB1089 metabolites in mediating gene regulatory effects was found to be drastically reduced in comparison to the parent compound suggesting that the contribution of the metabolites to the biological effect of EB1089 is minor. However, the compounds showed to be interesting tools for understanding the selective biological profile of EB1089.

Keywords: Vitamin D_3 receptor; vitamin D_3 analogues; limited protease digestion; receptor mutagenesis

Introduction

The nuclear hormone 1α,25-dihydroxyvitamin D_3 (VD) is the physiologically active form of vitamin D_3 and is involved in the regulation of a variety of important biological functions such as calcium homeostasis (DeLuca *et al*., 1990) as well as cellular growth, differentiation and apoptosis (Walters, 1992). These properties provide VD with an interesting therapeutic potential against a variety of diseases such as osteoporosis, cancer and psoriasis (Pols *et al*., 1994), however a more selective biological profile of the hormone would be desired. In the case where VD is used for treatment of hyperproliferative diseases, the calcemic function of the hormone can cause side effects such as hypercalcemia, hypercalciuria and soft tissue calcification (Vieth, 1990). Therefore, VD analogues with a high antiproliferative but low calcemic effects are of interest. A very promising VD analogue is EB1089, which has demonstrated a strong antiproliferative effect combined with a reduced calcemic action *in vitro* and *in vivo* (Colston *et al*., 1992; Mathiasen *et al*., 1993). Therefore, EB1089 has been selected as the pilot VD analogue in clinical test with different types of cancer (Mørk Hansen & Mäenpää, 1997).

The nuclear hormone VD and its analogues easily pass through cellular membranes and enter the nucleus to bind the vitamin D receptor (VDR) (Pike, 1991; Carlberg, 1996a). VDR is a member of a superfamily of structurally-related nuclear receptor transcription factors (Mangelsdorf *et al*., 1995) that binds to specific sequences in the promoter of VD target genes, commonly referred to as VD response elements (VDREs) (Carlberg, 1995). Simple VDREs are formed by two hexameric nuclear receptor binding sites, as the VDR binds to DNA as a homo- or heterodimeric complex. The main partner receptor for the VDR is the retinoid X receptor (RXR), which is the nuclear receptor for 9-*cis* retinoic acid. VDR-RXR heterodimers bind preferentially to directly repeated 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 (LBD), which results in receptor activation (Nayeri & Carlberg, 1997).

According to recently solved crystal structures of RXRα (Bourguet *et al*., 1995) and retinoid acid receptor γ LBDs (Renaud *et al*., 1995), the binding of ligand mainly results in changing the position of the most carboxy-terminal α-helix that contains the so-called AF-2 domain (Danielian *et al*., 1992; Durand *et al*., 1994). The amino acids L417, V418, E420 and F422 of the AF-2 domain of the VDR were found to stabilize to the high affinity ligand binding conformation of the ligand binding domain (Nayeri & Carlberg, 1997) and also

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provide an interface for interaction with co-activator proteins that mediate contacts to the basal transcriptional machinery (Jurutka *et al*., 1997; Masuyama *et al*., 1997).

According to the model of multiple VD signalling pathways, the pleiotropic function of VD is based on a variety of dimeric VDR complexes bound to different types of VDREs (Carlberg, 1996a). The model assumes that each of these VDR-containing protein-DNA complexes may represent one function of VD, i.e. that such kind of complexes may preferentially be found in the regulatory region of those genes that mediate the respective function of the hormone. Moreover, it is assumed that in each of these different protein-DNA complexes the VDR is in a different functional conformation that can specifically be recognized and stabilized by appropriate selective VD analogues. This suggests that the variety of VDR conformations and their interaction with ligand are of central importance in nuclear VD signalling. In support of this model, some VD analogues (e.g. EB1089) have shown the tendency to preferentially activate VDR-RXR heterodimers that are bound to IP9-type VDREs (Nayeri *et al*., 1995), whereas other analogues seem to prefer DR3-type VDRE-bound VDR complexes (Danielsson *et al*., 1997). This indication of promoter selectivity may be correlated with the observation that IP9-type VDREs have been found in some genes that are involved in the regulation of the cell cycle (Schräder *et al*., 1997).

The model of multiple VD signalling pathways provides the basis for an understanding of a selective action of VD analogues, which can be assessed by reporter gene assays on different types of VDREs and by limited protease digestion assays visualizing functional VDR conformations. However, in addition to such an *in vitro* characterization, for an assessment of the efficacy of a VD analogue *in vivo*, its metabolism has to be taken into consideration. Recently, five metabolites of EB1089 have been identified *in vivo* and *in vitro*, which are all hydroxylated at positions C26 and C26a (Kissmeyer *et al*, 1997; Shankar *et al*., 1997) (Figure 1).

In this report, EB1089 was characterized in comparison with its metabolites for the ability to stabilize functional conformations of wild type and mutant VDR proteins. Moreover, the compounds were assessed for their promoter selectivity and antiproliferative effect in MCF-7 breast cancer cells.

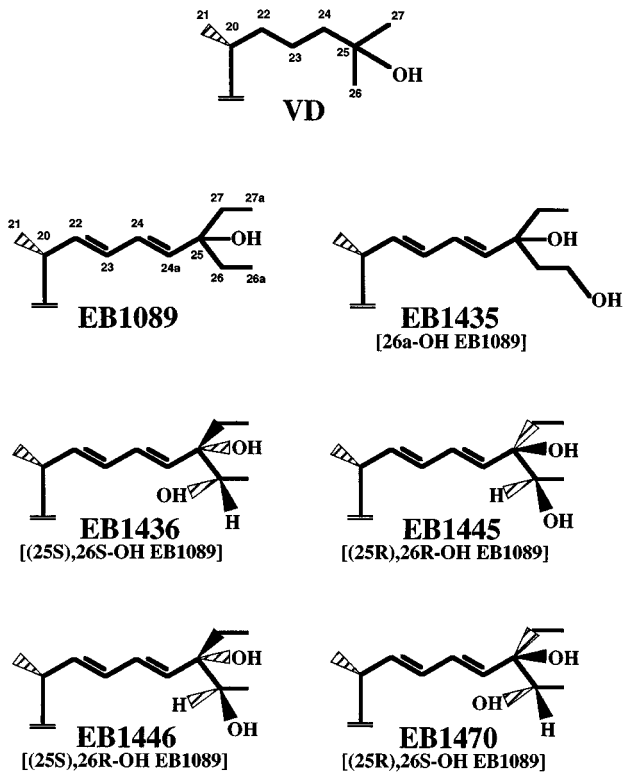

Figure 1 The side chain structure of VD, EB1089 and EB1089 metabolites. In comparison to VD, the side chain of EB1089 is extended by one carbon atom and contains double bonds at positions C22 and C24. The five metabolites of EB1089 are hydroxylated at positions C26a and C26, respectively.

Methods

Compounds

VD, EB1089 [1(S),3(R)-dihydroxy-20(R)-(5′-ethyl-5′-hydroxy-hepta-l′(E),3′(E)-dien-l′yl)-9,10-secopregna-5(Z),7(E),10(19)-triene] and the five EB1089 metabolites EB1435 (mixture of the 25R and the 25S hydroxy-isomers), EB1436, EB1445, EB1446 and EB1470 (for their structures see Figure 1) were synthesized at LEO Pharmaceutical Products (Ballerup, Denmark). The ligands were dissolved in 2-propanol at 4 mM; dilutions were performed in ethanol.

DNA constructs

The cDNA for human VDR was subcloned into the expression vector pSG5 (Stratagene) (Carlberg *et al*., 1993) and was used as template for a linear PCR reaction using *Pfu* DNA polymerase (Stratagene) with a profile of 0.5 min at 94°C, 1 min at 55°C and 11 min at 68°C for 16 cycles. The following primer pairs were used for the L417A, V418A, E420A and F422A point mutations (L = leucine, A = alanine, V = valine, E = glutamic acid and F = phenylalanine):

L417A+ GAAGCTAACGCCCGCTGTGCTCGAAGTGT and
L417A− ACACTTCGAGCACAGCGGGCGTTAGCTTC
V418A+ GCTAACGCCCCTTGCGCTCGA AGTGTTTG and
V418A− CAAACACTTCGAGCGCAAGGGGCGTTAGC,
E420A+ GCCCCTTGTGCTCGCAGTGTTTGGCAATG and
E420A− CATTGCCAAACACTGCGAGCACAAGGGGC,
F422A+ TTGTGCTCGAAGTGGCTGGCAATGAGAT and
F422A− ATCTCATTGCCAGCCACTTCGAGCACAA,

Methylated template DNA was then digested selectively with Dpn I and supercompetent *Epicurian Coli* XL-1 (Stratagene) were transformed with non-digested, PCR-generated plasmid DNA. The respective point mutations were confirmed by sequencing. The fusion of the DR3-type VDRE (core sequence AG**AGGTCA**TGA**AGGACA**) of the rat ANF gene promoter and of the IP9-type VDRE (core sequence **TGACC**CTGG-GAACCG**GGTCCA**) of the mouse c-*fos* promoter with the thymidine kinase (*tk*) promoter in front of the chloramphenicol acetyl transferase (CAT) reporter gene have been described previously (Kahlen & Carlberg, 1996; Schräder *et al*., 1997).

Limited protease digestion assay

Linearized cDNA of wild type VDR and the four point mutations were used for *in vitro* transcription as recommended (Promega). Four μg of *in vitro* transcribed VDR RNA was mixed with 70 μl rabbit reticulocyte lysate (Promega), 80 U RNasin, 4 μl [^{35}S]-methionine (1000 Cimmol^{-1}) and 20 μM amino acid mixture (minus methionine) in a total volume of 100 μl and incubated at 30°C for 2 h. Two μl 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 peincubated for 15 min at room temperature. Then 2.5 μl of trypsin (Promega, final concentration 25 $\mu g\,ml^{-1}$) 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% (v/v) glycerol, 5% (v/v) mercaptoethanol, 2% (w/v) SDS, 0.025% (w/v) bromophenol blue). The samples were denatured at 95°C for 5 min and electrophoresed through a 12% (w/v) 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-sensitive VDR fragments were detected on a Fuji BAS1500 reader and compared to full length VDR input using TINA software (Raytest).

Transfection and CAT assays

Human MCF-7 breast cancer cells were seeded into 6-well plates (10^5 cells/ml) and grown overnight in phenol red-free DMEM supplemented with 5% (w/v) charcoal-treated FCS. Liposomes were formed by incubating 2 μg of the reporter plasmid and 1 μg of the reference plasmid pCH110 (Pharmacia) with 15 μg N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate (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. Phenol red-free DMEM supplemented with 15% (w/v) charcoal-treated FCS (500 μl) was added 4–8 h after transfection. At this time, graded concentrations of EB1089 or its metabolites were also added. The cells were harvested 40 h after stimulation onset 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.

Cell proliferation assays

MCF-7 cells were seeded at a density of 1.5×10^4 cells ml^{-1} in 24-well plates and grown in phenol red-free DMEM supplemented with 5% (w/v) charcoal-treated foetal calf serum (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 final concentration of ethanol did not exceed 0.0025% (v/v). After 5 days 1 $\mu Ci\,ml^{-1}$ [^{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 $\mu g\,ml^{-1}$ non-labelled thymidine, 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

Traditional competition assays using radiolabelled ligand do not allow for visualization of receptor conformational changes (Mørk Hansen *et al*., 1996). In contrast, limited protease digestion has been shown to be a powerful method for characterizing functional VDR conformations (Nayeri & Carlberg, 1997). The interaction of the receptor with ligand protects the LBD against protease digestion. Some biologically potent VD analogues have demonstrated a higher functional affinity to VDR than the natural hormone with this method (Nayeri *et al*., 1996b). In addition, limited protease digestion has been used recently to monitor the kinetics of VDR stabilization by ligand (van den Bemd *et al*., 1996).

Amino acids L417, V418, E420 and F422 within the AF-2 domain of the human VDR have recently been shown to be essential for high affinity ligand interaction [V418 and F422 (Nayeri *et al*., 1996a; Nayeri & Carlberg, 1997)] and for contacting co-activator proteins [L417 and E420 (Jurutka *et al*., 1997; Masuyama *et al*., 1997)]. Here, the respective point mutated VDR proteins were compared with wild type VDR for their ability to form functional conformations that were stabilized by VD (as a control), EB1089 or the EB1089 metabolites EB1435, EB1436, EB1445, EB1446 or EB1470 (Figure 2). [^{35}S]-methionine labelled, *in vitro* translated proteins, high ligand concentrations (10 μM) and trypsin were used to perform limited protease digestion assays. In contrast to other analogues that are known to stabilize three VDR conformations (named 1, 2 and 3) (Nayeri *et al*., 1996a; Nayeri & Carlberg, 1997), VD and EB1089 were only able to stabilize conformations 1 and 3, which were represented by protease-resistant VDR fragments of an approximate size of 33 and 29 kD, respectively. With these two compounds a higher amount of VDR molecules were stabilized in conformation 1 than in conformation 3. Interestingly, the ratio of ligand-dependent stabilization of conformations 1 and 3 appears to be characteristic for each of the EB1089 metabolites. At high pharmacological concentrations all EB1089 metabolites

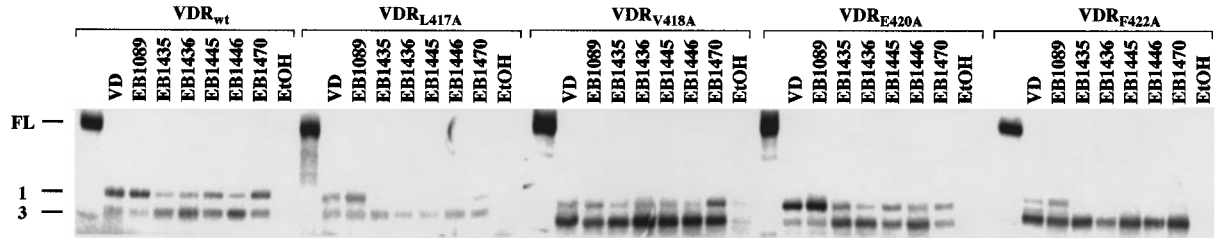

Figure 2 Ligand-dependent conformations of VDR with AF-2 point mutations. The [^{35}S]-methionine-labelled VDR proteins L417A, V418A, E420A and F422A were obtained by *in vitro* transcription/translation of respective DNA templates generated by site-directed mutagenesis of wild type (wt) receptor. They were preincubated with either of VD (10 μM), EB1089 (10 μM), its metabolites (10 μM) or solvent (ethanol). Limited protease digestion was performed by adding trypsin to a final concentration of 25 $\mu g\,ml^{-1}$ for 20 min at room temperature. Samples were electrophoresed through a 12% (w/v) SDS-polyacrylamide gel, dried and autoradiographed. FL indicates full-length receptor and 1 and 3 the protease-resistant VDR fragments. In the presence of solvent, protease-resistant VDR fragments were not observed. A representative gel is shown.

stabilized conformation 1 to a lower extent and conformation 3 to a higher extent than EB1089. The pattern that was observed with the mutant E420A qualitatively resembled that of wild type VDR, whereas with mutant VDR proteins L417A, V418A and F422A, the ability to stabilize conformation 1 was clearly lower with any of the seven ligands.

In order to quantify a difference in the ligand-dependent stabilization of functional VDR conformations, limited protease digestion was performed with wild type VDR and graded ligand concentrations (Figure 3). The relative amount of conformation 1 and 3 was quantified individually and plotted over ligand concentration. The natural hormone VD

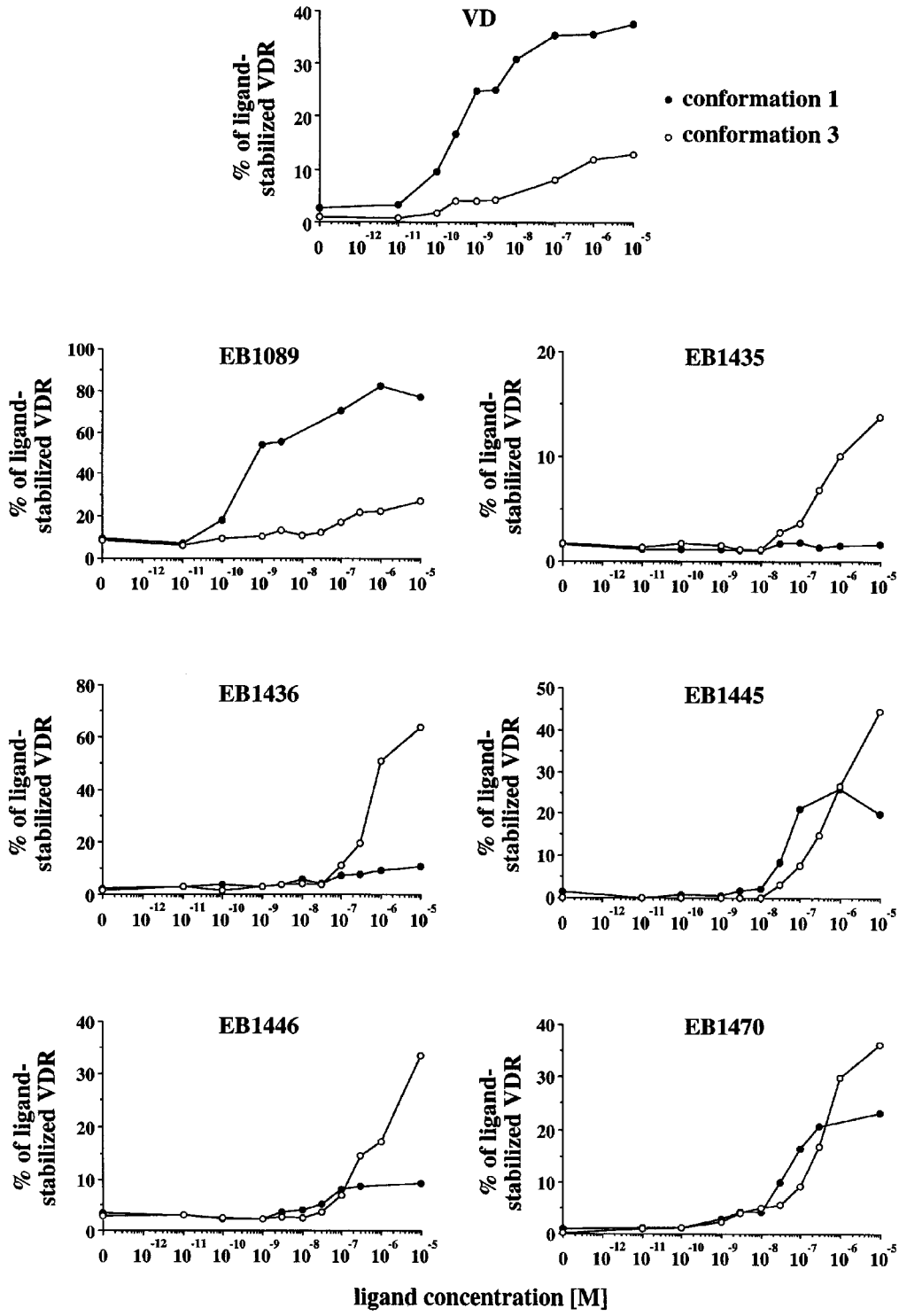

Figure 3 Concentration-dependent stabilization of functional VDR conformations is characteristic for EB1089 and its metabolites. Two μl of *in vitro* synthesized [^{35}S]-methionine-labelled wild type VDR was preincubated with the indicated concentrations of VD, EB1089 and its metabolites. Trypsin, to a final concentration of 25 μg ml^{-1}, was added and the mixtures were further incubated for 10 min at room temperature. Samples were electrophoresed through a 12% (w/v) 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.

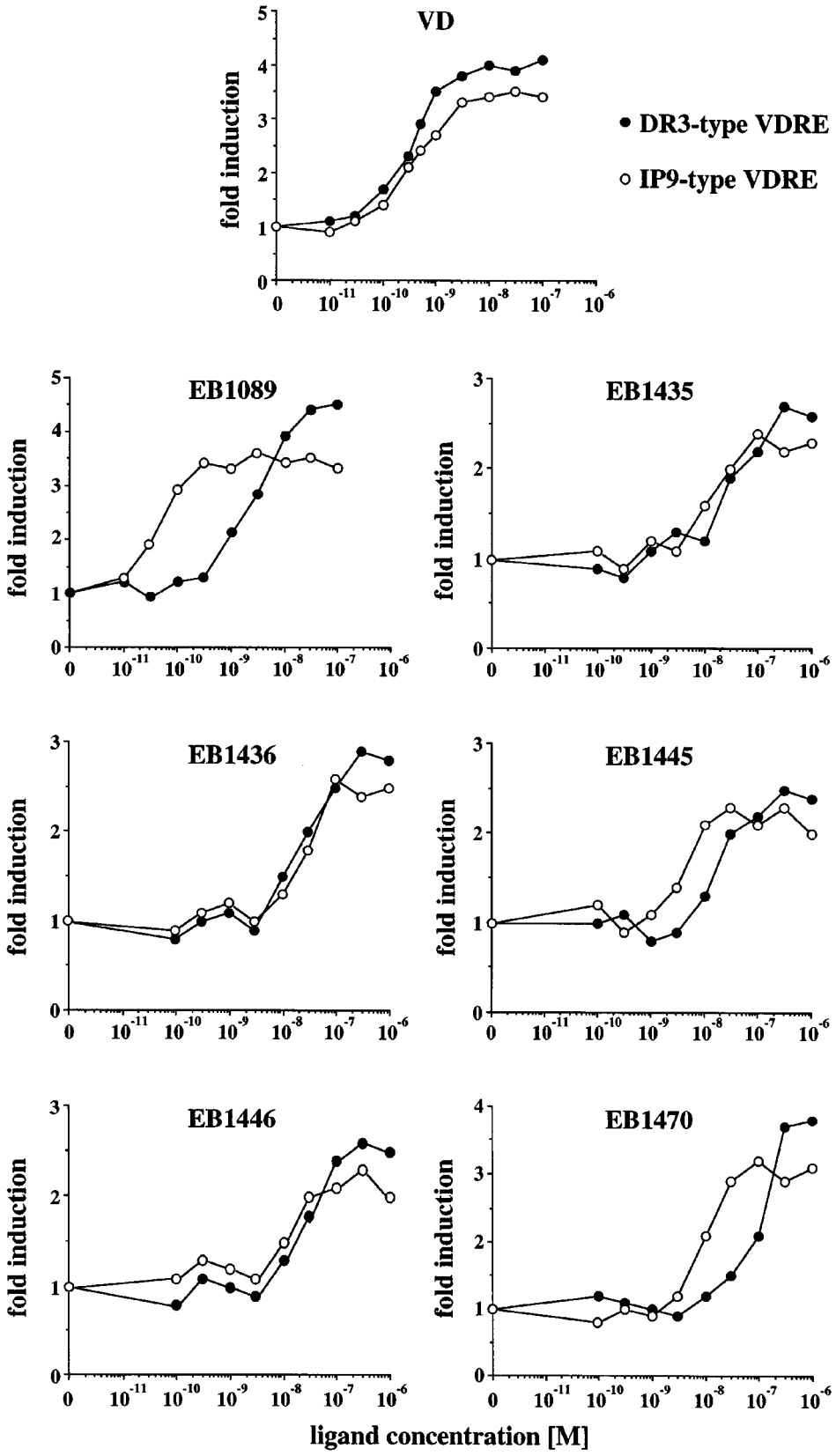

Figure 4 Selectivity of EB1089 and its metabolites for gene activation from DR3- and IP9-type VDREs. MCF-7 cells were transfected with *tk* promoter/CAT reporter gene constructs containing either the DR3-type VDRE of the rat ANF gene (solid circles) or the IP9-type VDRE of the mouse c-*fos* gene (open circles). Cells were treated for 40 h with graded concentrations of VD, EB1089 and its metabolites. Stimulation of CAT activity was calculated in comparison to solvent-induced controls. Each data point represents the mean of triplicates.

started to stabilize conformation 1 at a low concentration of 0.01 nM; at saturating concentrations nearly 40% of all receptor molecules were stabilized in this conformation. For the stabilization of conformation 3, higher VD concentrations were needed and only approximately 10% of all receptor molecules were stabilized in this conformation. EB1089 provided qualitatively a very similar result being able to stabilize at saturating concentrations nearly 80% of all receptor molecules in conformation 1. Moreover, 30 nM of EB1089 were necessary to stabilize conformation 3 with a plateau level of approximately 20% of all VDR molecules. The five EB1089 metabolites showed a dose-dependent stabilization pattern of VDR conformations that clearly differed from that of EB1089. Concentrations greater than 10 nM of EB1445 and EB1470 were needed to stabilize approximately 20% of the VDR molecules in conformation 1, whereas concentrations greater than 30 nM also stabilized conformation 3 in up to 45% of all receptor molecules. Concentrations of EB1435, EB1436 and EB1446 greater than 30 nM stabilized conformation 3 with maximal levels of 60% (EB1436), 35% (EB1446) and 15% (EB1435) of all VDR molecules. Interestingly, data generated with the latter three compounds showed that less than 10% of all receptor molecules were stabilized in conformation 1 even at maximal ligand concentration.

Further functional analyses of VD (as a control), EB1089 and its metabolites were performed in human MCF-7 breast cancer cells to stimulate concentration-dependent gene transcription from DR3- and IP9 type VDREs (Figure 4). On both response elements, VD produced rather similar dose response curves showing DR3-type VDREs to be only slightly more active. The values of half-maximal activity (EC_{50}) was on both types of VDREs in the order of 0.5 ± 0.2 nM. In contrast, EB1089 showed a clear promoter selectivity with EC_{50} values of approximately 0.1 ± 0.05 nM on the IP9-type VDRE and approximately 3 ± 0.5 nM on the DR3-type VDRE. Although the DR3- and IP9-type VDREs that have been used in this study differ slightly from those, that were used in previous reports (Nayeri *et al*., 1995; Schräder *et al*., 1997), the promoter selectivity of EB1089 was reproduced well. The maximal inducibility of both types of VDREs by EB1089 metabolites compared to EB1089 was found to be reduced by 10 to 50% and respective EC_{50}-values were 10–30 fold higher than for EB1089. More importantly, EB1445 and EB1470 showed only faint IP9-type VDRE selectivity and the three other metabolites did not display any promoter selectivity.

Finally, the effect of VD (as a control), EB1089 and its five metabolites on MCF-7 cell proliferation was assessed (Figure 5). As expected, EB1089 appeared to be the most potent analogue, being 116-times (IC_{50}-value 0.25 nM) more potent than its parent compound VD. The metabolites EB1445 and EB1470 were shown to be 26 times (IC_{50}-value 1.1 nM) and 10-times (IC_{50}-value 2.8 nM) more potent than VD, whereas the potencies of EB1435, EB1436 and EB1446 were found to be similar to that of VD.

Discussion

The assessment of the metabolic stability of a VD analogue, the identification of the respective metabolites and their functional characterization is very important for the evaluation of its therapeutic potential and putative side effects. EB1089 is a metabolically stable analogue showing a half-life in rats that is comparable (Kissmeyer *et al*., 1995) and in cell culture eve higher (Shankar *et al*., 1997) than that of VD. This stability is likely to be due to the two double bonds at positions C22 and C24 that block hydroxylation at these positions. The major metabolites of EB1089 were recently identified and investigated with respect to their binding affinity for the VDR and their effects on the proliferation of keratinocytes and monocytic leukemia cells *in vitro* (Kissmeyer *et al*., 1997; Shankar *et al*, 1997). These results clearly showed that all the EB1089 metabolites bind with lower affinity to the VDR and exert weaker antiproliferative effects than EB1089.

The basis of such a differential biological profile is likely to be the interaction of the respective compounds with the VDR. Therefore, the interaction with functional VDR conformations was primarily studied in this report. Functional VDR conformations can differ in their sensitivity to ligand, as conformation 1 already interacts with the ligand at low concentrations (Nayeri *et al*., 1996a; Nayeri & Carlberg, 1997). If conformation 1 is disturbed, e.g. by the point mutations L417A, V418A and F422A, in the case of EB1089, its metabolites, VD and most other VD analogues this results in a prominent loss of ligand binding affinity of the respective mutant VDR protein. Similarly, amino acid E420 has also been shown to be crucial for VDR-mediated transactivation,

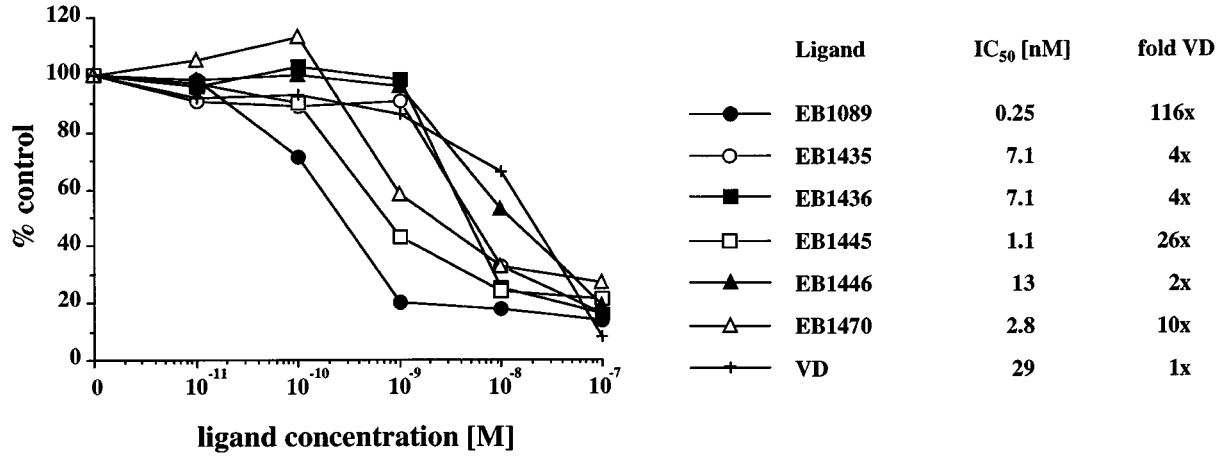

Figure 5 Anti-proliferative effects of EB1089 and its analogues on MCF-7 cells. Proliferation was assessed by [^{3}H]-thymidine incorporation after 5 days in culture with graded concentrations of the VD, EB1089 and its metabolites. Results are expressed as the % mean of control and are based on eight determinations. The IC_{50}-values are expressed in comparison to that of VD.

but the data presented here show that it is apparently not involved in contacting the ligand. This may be explained by location of E420 on the external surface of the AF-2 domain-containing α-helix 12 of the LBD. The functional role of VDR conformation 3 is not yet resolved, but according to previously reported data (Nayeri *et al*., 1996a; Nayeri & Carlberg, 1997) and consistent with results presented here, conformation 3 seems to represent a low affinity conformation of the VDR. VD analogues that stabilize the VDR predominantly in conformation 3 can be considered as low affinity VDR ligands. The extent to which the functional VDR conformations are complexed with ligand is characteristic for each ligand, so that the analysis of all five EB1089 analogues at high concentration already predicts them as weak VDR agonists. This classification is confirmed by the dose-dependent analysis of VDR conformations 1 and 3. In contrast, prominent stabilization of conformation 1 by very low concentrations of EB1089 qualifies it as a very potent VDR ligand. EB1445 and EB1470 are ranked second despite 100 fold lower potency primarily due to their ability to stabilize up to 25% of all receptor molecules in conformation 1. The residual activity of the three remaining EB1089 metabolites is most likely due to their ability to the stabilizing of conformation 3, rather than the very minor stabilization of conformation 1.

Promoter selectivity is considered to be an important aspect of the selective functional profile of a VD analogue. The recently identified IP9-type VDRE selectivity of EB1089 (Schräder *et al*., 1995; 1997) could be an explanation of the strong antiproliferative effects demonstrated by the analogue. Therefore, it is interesting that side chain hydroxylation at positions C26 and C26a not only drastically reduce the ability of the EB1089 metabolites to stimulate transactivation mediated by VDR-RXR heterodimers, but apparently also destroy the selective recognition of different VDR-VDRE complexes. This may explain, why the EB1089 metabolites have a clearly reduced antiproliferative potential, but a calcemic effect that is similar or lower than that of the parent compound. The lack of promoter selectivity of the EB1089 metabolites EB1435, EB1436 and EB1446 and the weak selectivity of EB1445 and EB1470 correlates with the reduced ability to stabilize the VDR in the high affinity ligand binding conformation 1. This is also represented by ranking antiproliferative effects in breast cancer cells. EB1445 and EB1470 are both 25R-isomers of 26-hydroxy EB1089, i.e. the direction of the hydroxy groups at positions C1 and C25 are the same relative to the rest of the molecule, which may explain the residual activity of both compounds in comparison to the three other metabolites.

The prominent antiproliferative effect of EB1089 compared to that of the natural hormone VD (more than 100 fold more potent) is not reflected that drastically by the limited protease digestion and the reporter gene assay. On the IP9-type VDRE EB1089 is only five times more potent than VD and at high saturating concentrations EB1089 stabilizes only double as much VDR molecules in conformation 1 than VD does. According to these results VD should have an antiproliferative effect that is not different from that of EB1089. The difference is probably due to the fact that proliferation is measured after 5 days of incubation, where metabolic stability of VD and its higher affinity for the vitamin D binding protein (Kissmeyer *et al*., 1995) have a more reasonable influence than in rather short-term or *in vitro* assays.

Taken together, the functional characterization of EB1089 metabolites reported here demonstrated that hydroxylation of EB1089 at positions C26 and C26a resulted in molecules with clearly reduced potency to stabilize the high affinity conformation of the VDR and to stimulate transactivation *via* IP9- and DR3-type VDRE bound VDR-RXR heterodimers. This suggests that the potent antiproliferative effect of EB1089 is not related to the generation of metabolites, but rather to the compound itself.

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