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The 1,25-Dihydroxyvitamin D_3 (VD) analogues MC903, EB1089 and KH1060 Activate the VD Receptor: Homodimers Show Higher Ligand Sensitivity than Heterodimers with Retinoid X Receptors

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The nuclear receptor for 1,25-dihydroxyvitamin D_3 (VD), VDR, belongs to the nuclear receptor superfamily. This ligand-inducible transcription factor mediates the genomic VD signalling pathways by binding to specific response elements in the promoter region of VD regulated genes. Two types of natural VD response elements are used as models for the VDR-mediated transcriptional activation: one is bound by VDR-homodimers and is found in the human osteocalcin gene promoter, and the other is bound by heterodimers of VDR with retinoid X receptors (RXRs) as in the mouse osteopontin promoter. Here, we demonstrate that the VD analogues MC903, EB1089 and KH1060, previously shown to be potent regulators of proliferation and differentiation, are able to act as ligands for VDR and replace VD as a ligand in both nuclear signalling pathways. We found that they have different potency and sensitivity in their ability to stimulate the hormone-dependent promoter element. MC903 and EB1089 provide about 20% higher induction of gene activity than VD in a gene reporter system, whereas KH1060 was more sensitive, inducing transcription at about 100-fold lower doses than VD. Interestingly, VD and its analogues induce VDR homodimer-mediated gene activity at a 3- to 4-fold lower concentration than that of VDR–RXR heterodimers. This suggests that the ligand concentration is an additional regulatory level in the discrimination between signalling pathways involving homo- and heterodimeric hormone receptors.

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INTRODUCTION

The physiologically active metabolite of the seco-steroid hormone vitamin D_3, 1,25-dihydroxyvitamin D_3 (VD), regulates calcium and phosphate metabolism, induces cellular differentiation and inhibits proliferation. Its cell-regulating properties provide VD with an interesting therapeutic potential in hyperproliferative diseases like psoriasis and cancer. VD also exerts immune suppressive effects in a number of animal models of graft rejection and autoimmune diseases [1].

The effects of VD are considered to be mediated by its nuclear receptor, VDR [2], which is a member of the large family of ligand-activated transcription factors termed the nuclear receptor superfamily [3]. VDR is found in many cells and tissues not traditionally involved in the regulation of calcium metabolism; it is present in various cancer cell lines, skin cells, muscle cells, hematopoietic cells and also in the pancreas, the brain and the pituitary [4]. Ligand-activated VDRs stimulate transcription by binding to DNA sequences, referred to as VD response elements (VDREs), in the promoter of target genes [5, 6]. The *in vitro* DNA binding affinity of VDRs is enhanced by the addition of nuclear extracts. This indicates the existence of nuclear co-factors for VDR [7, 8], one of which was demonstrated to be the retinoid X receptor (RXR) [9, 10]. Following the 3-4-5-rule [11], optimal VDREs for VDR–RXR heterodimers should be direct repeats

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(DRs) of two hexameric core binding sites spaced by three nucleotides. The high specificity of these DR3-type VDREs, represented by the mouse osteopontin VDRE [12] and artificial analogues, was confirmed by several groups [9, 10, 13]. To explain the functional activity of a DR6-type VDRE found in the human osteocalcin gene promoter [14, 15], we demonstrated that nuclear signalling by VD can also be transmitted through RXR-independent pathways mediated by VDR-homodimers [13, 16, 17]. VDR-homodimers also function on artificial VDREs formed by palindromes with no intervening nucleotide (P0) or inverted palindromes with 12 spacing nucleotides (IP12) [13, 16].

Recent interest has focused on the effects of VD in cell regulation and its therapeutical potential. The clinical use of VD is, however, limited due to its potent effects on intestinal calcium absorption and risk of associated side effects, such as hypercalcemia, hypercalciura and soft tissue calcification [18]. Therefore, a number of synthetic analogues have been developed which are less calcemic than the physiologically active VD. One such analogue is calcipotriol (MC903), which is currently in use as an antipsoriatic drug [19]. Newer analogues, EB1089 and KH1060, have been shown to have potent anticancer or immunosuppressive properties, respectively [20, 21]. Studies with a number of receptor preparations from various tissues have shown that these VD analogues are able to bind VDR [20, 21] and their function can be considered to be mediated by genomic pathways. However, a direct demonstration of ligand-mediated gene activation and indications for their different properties are still missing.

The present study describes the activation of both the VDR–RXR heterodimer- and the VDR homodimer-pathway by these VD analogues. Compared to VD the analogues show different potency and sensitivity in their induction of gene activity. Interestingly, VD and its analogues are recognized with higher sensitivity by VDR homodimers than by VDR–RXR heterodimers.

EXPERIMENTAL

DNA constructs

Two pairs of oligonucleotides containing the VDREs of the mouse osteopontin gene promoter (−759 to −741) [12] and of the human osteocalcin gene promoter (−514 to −493) [15], respectively, were synthesized, purified, phosphorylated and annealed to yield the two double-stranded DNA-fragments:

DR3: 5′ ctagaAA**GGTTCA**CGA**GGTTCA**CGt 3′
3′ tTT**CCAAGT**GCT**CCAAGT**GCagatc 5′

DR6: 5′ ctagaTT**TGGTGA**CTCACC**GGGTGA**ACt 3′
3′ tA**AACCACT**GAGTGG**CCCACT**TGagatc 5′

They were fused to the *tk* promoter to drive the expression of the chloramphenicol acetyltransferase (CAT) reporter gene by subcloning into the *Xba*I site of pBLCAT2 [22]. The cDNAs for human VDR and human RXRα have been subcloned into pSG5 (Stratagene).

Compounds

VD and its analogues MC903, EB1089 and KH1060 were synthesized in the Department of Chemical Research (LEO Pharmaceutical Products, Denmark). The compounds were dissolved in 2-propanol at 4 mM. Dilutions were performed prior to use in 2-propanol (final concentration of 2-propanol in the medium: 0.025%).

Cell culture, transfection and CAT assays

For MCF-7 (human breast cancer) cells, 2×10^5 cells per well were seeded into 6-well plates and grown overnight in phenol red-free RPMI 1640 (Life Technologies) supplemented with 10% charcoal-treated foetal calf serum (FCS). For *Drosophila* SL-3 cells [23], 5×10^5 cells per well in a 6-well plate were grown overnight in Schneider's medium (Life Technologies) supplemented with 15% charcoal-treated FCS. Liposomes were formed by incubating 1 μg of the reporter plasmid and 1 μg of the reference plasmid pCH110 (Pharmacia) with 11 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. For the transfection of SL-3 cells, 0.25 μg of a pSG5-(Stratagene) based VDR- and RXRα-expression vector were also included. After dilution with 0.9 ml phenol red-free RPMI 1640 (or Schneider's medium for SL-3 cells), the liposomes were added to the cells. 500 μl phenol red-free RPMI 1640 supplemented with 10% charcoal-treated FCS (or 500 μl Schneider's medium supplemented with 15% charcoal-treated FCS for SL-3 cells) were added 8 h after transfection. At this time various concentrations of VD, VD-analogues, 9-*cis* retinoic acid (RA), combinations of them or solvent were also added. After further 16 h the cells were harvested and CAT-assays were performed as described previously [24]. The CAT activities were normalized to β-galactosidase activity and induction factors were calculated as the ratio of CAT activity of ligand-stimulated cells to that of mock-induced control. Each condition was analysed at least in triplicate and data are shown as mean with standard deviation.

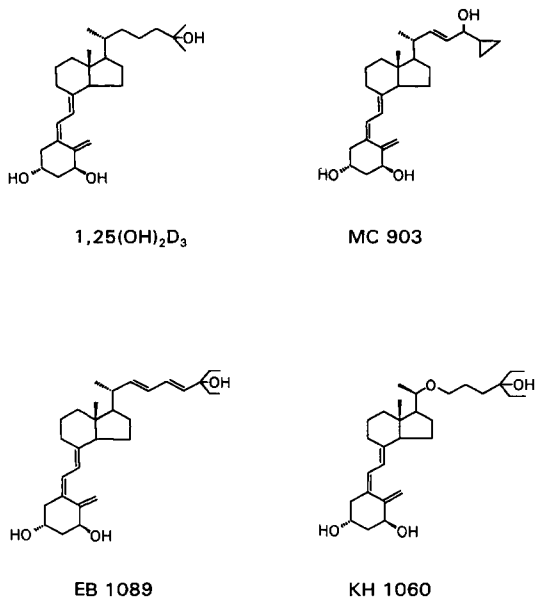


Fig. 1. The structure of the three VD analogues MC903 (calcipotriol), EB1089 and KH1060 in comparison with VD.

RESULTS

The compounds MC903, EB1089 and KH1060 are analogues of VD with modifications in the side chain; their structures are given in Fig. 1. To investigate their ability to induce VDR-mediated transcriptional activity, we used reporter constructs consisting of the mouse osteopontin (DR3-type) VDRE [12] or the human osteocalcin (DR6-type) VDRE [14, 15], respectively, fused to the *tk* promoter driving the CAT gene. Here we used only the core sequence of the osteocalcin VDRE (position −514 to −493, see Experimental) to avoid interference with an adjacent imperfect direct repeat spaced by three nucleotides (compare with Refs [13, 26]). The clearest discrimination between VDR–RXR heterodimer- and VDR homodimer-mediated gene activation can be observed in *Drosophila* cells that are devoid of mammalian nuclear receptors by definition. As in previous reports [13, 16, 25–27], we carried out our transactivation studies in the *Drosophila* cell line SL-3 [23] (Fig. 2). Consistently with previous reports [13, 26], the DR3-type VDRE showed a "heterodimer-pattern" characterized by the observation that the expression of VDR and RXR, respectively, alone provides only low VD- or 9-*cis* RA-induced gene activity (both about 2.5-fold), whereas high induction is achieved in the presence of both receptors and both ligands (8.1-fold). In contrast, the DR6-type VDRE showed a "homodimer-pattern", where the VD-induction of VDRs (5.2-fold) could neither be enhanced by

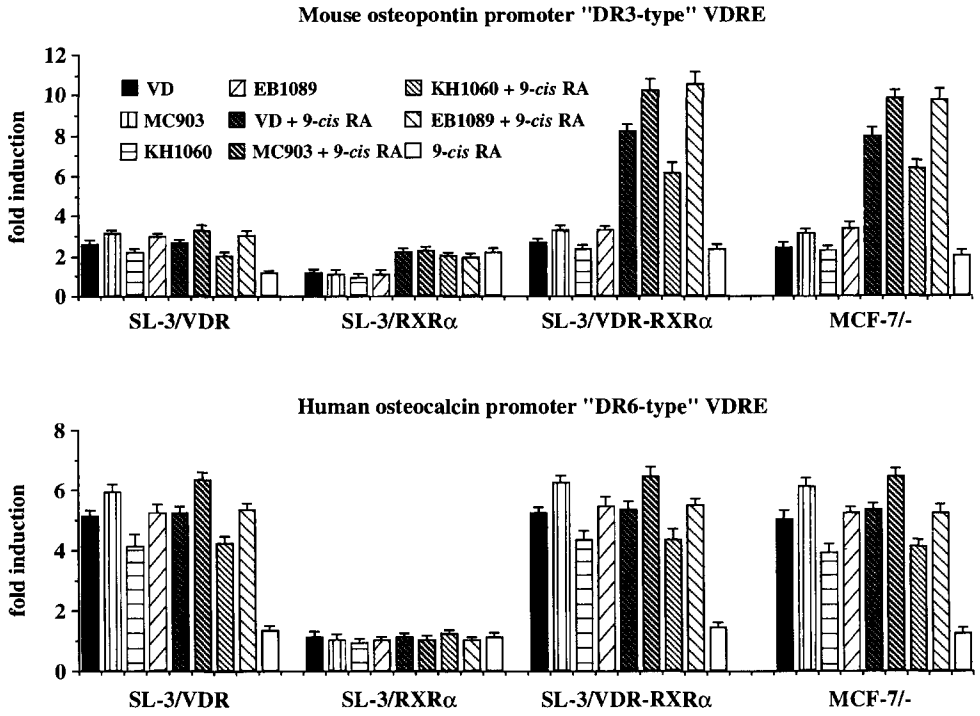


Fig. 2. The transcriptional activity of VDR homodimers and VDR–RXR heterodimers after induction with VD and its analogues. *Drosophila* SL-3 cells were co-transfected with the *tk* promoter/CAT gene constructs containing the mouse osteopontin or the human osteocalcin VDRE and the indicated receptor expression vectors for VDR and RXRα alone or in combination. Human MCF-7 cells endogenously express VDR and RXR and were transfected only with the indicated reporter constructs. The cells were treated for 16 h with VD, the VD analogues MC903, EB1089 and KH1060, respectively, alone or in combination with 9-*cis* RA or with 9-*cis* RA alone (final concentration 100 nM each) as indicated. Stimulation of CAT activity was calculated in comparison to solvent-induced controls. Columns represent mean values of at least three independent experiments; the bars indicate standard deviations.

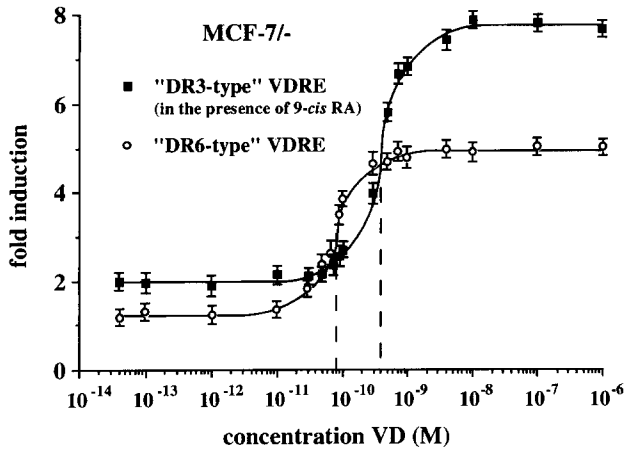
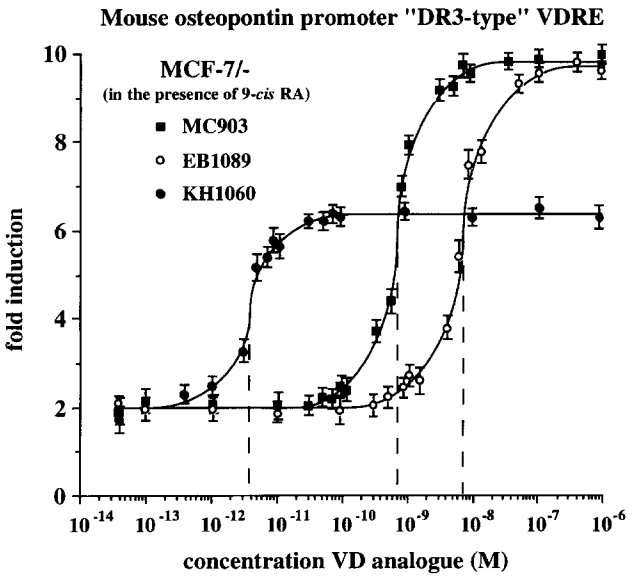


Fig. 3. Dose–response to VD of VDR homodimers and VDR–RXR heterodimers in MCK-7 cells. Human MCF-7 cells were transfected with the *tk* promoter/CAT gene constructs containing the mouse osteopontin ("DR3-type") or the human osteocalcin ("DR6-type") VDRE and treated with increasing concentrations of VD. Dose–responses on the DR3-type VDRE were in the presence of 100 nM 9-*cis* RA. CAT activities were determined 16 h later and stimulation was calculated in comparison to solvent-induced controls. Each data point represents the mean of triplicate determinations; the bars indicate standard deviations.

co-expression of RXR nor by addition of 9-*cis* RA (see also Ref. [13]). These two types of response patterns could also be observed with the endogenously expressed VDR and RXR of human MCF-7 cells (Fig. 2) and the respective stimulation factors were nearly identical to those observed in the *Drosophila* system (with equivalent relative expression levels of VDR and RXR in SL-3 cells). We also performed stimulations with MC903, KH1060 and EB1089, respectively, replacing VD. On both nuclear signalling pathways and in the *Drosophila* as well as in the mammalian system, we observed the same type of response with each VD-analogue and with VD. This indicates that all three compounds are able to replace VD as a specific ligand for VDR. However, the VD analogues differed from VD in the maximal induction of gene activity. In SL-3 cells, in the presence of 9-*cis* RA, VDR and RXR MC903 mediated a 10.2-fold induction of CAT activity on the osteopontin VDRE and a 6.2-fold induction on the osteocalcin VDRE; these activities are both higher than those observed with VD. On the DR3-type VDRE, EB1089 was also more potent (10.5-fold induction) than VD, whereas it showed nearly identical induction (5.4-fold) as VD on the DR6-type VDRE. In contrast, on both signalling pathways, KH1060 provided only 6.1-fold (on the osteopontin VDRE) and 4.3-fold induction (on the osteocalcin VDRE), i.e. lower stimulation of transcriptional activity than VD. In the MCF-7 system, we observed with all three VD-analogues the same effects than in the *Drosophila* system and obtained very similar induction values. This suggests that these VD-analogues also function in the human system as specific ligands of VDR homo- and heterodimers.

Having observed different potencies in VDR-mediated gene induction by VD as compared to its synthetic analogues, we next studied the influence of ligand concentration on the two VD signalling pathways. We stimulated MCF-7 cells transfected with either the



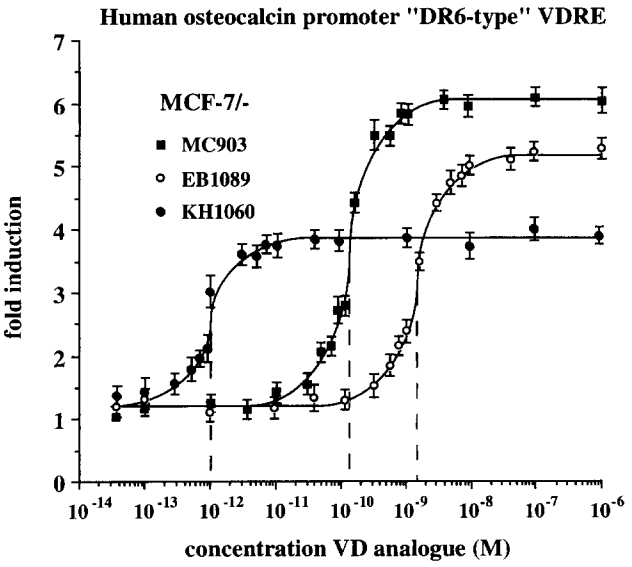


Fig. 4. Dose–response to VD analogues MC903, EB1089 and KH1060 of VDR homodimers and VDR–RXR heterodimers in MCF-7 cells. Human MCF-7 cells were transfected with the *tk* promoter/CAT gene constructs containing the mouse osteopontin ("DR3-type") or the human osteocalcin ("DR6-type") VDRE and treated with increasing concentrations of VD analogues as indicated. Dose–responses on the DR3-type VDRE were in the presence of 100 nM 9-cis RA. CAT activities were determined 16 h later and stimulation was calculated in comparison to solvent-induced controls. Each data point represents the mean of triplicate determinations; the bars indicate standard deviations.

osteopontin or the osteocalcin VDRE/*tk*/CAT reporter constructs with VD (Fig. 3) or its three analogues (Fig. 4), respectively, over a concentration range from 0.05 pM to 1 μM. For both signalling pathways, we observed typical sigmodial shaped dose–response curves that differed in their starting and end points and the concentration of half-maximal induction (EC_{50}). Due to co-stimulation by 9-*cis* RA on the osteopontin VDRE, the dose–response to VD started at 2-fold induction and reached a plateau of about 8-fold induction at concentrations higher than 10 nM (Fig. 3). We determined the EC_{50} value of VDR–RXR heterodimers for VD as about 300 pM. On the osteocalcin VDRE VDR homodimers reached a plateau at about 5-fold induction, but, interestingly, the EC_{50} value was found to be only 80 pM. Similar EC_{50} values were determined in the *Drosophila* system (data not shown). This suggests that VDR homodimers react about 4-fold more sensitively to VD stimulation than VDR–RXR heterodimers. The dose–response curves for the VD analogues MC903, EB1089 and KH1060 on the two VDRE reporter constructs reached their plateau at the induction levels described in Fig. 2. We determined the EC_{50} values for MC903 as 600 pM for VDR–RXR heterodimers and 140 pM for VDR homodimers. The respective EC_{50} values were 6000 and 1500 pM for EB1089 and 2.5 and 1 pM for KH1060. Therefore, the sensitivity of VDR homodimers for each of the three VD analogues was also higher than that of VDR–RXR heterodimers. The comparison of the EC_{50} values of VD (Fig. 3) with those of its three analogues (Fig. 4) indicates that VD induces gene activity already at 2- and 20-fold lower concentrations than MC903 and EB1089, respectively, but, interestingly, KH1060 was found to be 80- to 120-fold more sensitive than VD.

DISCUSSION

The therapeutically interesting regulatory functions of VD are mediated by its intracellular receptor, VDR, which binds to VDREs either as a homodimer or as a heterodimer with RXR. We have demonstrated that the three VD analogues MC903, EB1089 and KH1060 can stimulate via both nuclear VD signalling pathways. These VD-analogues are able to replace VD as a ligand, but with different potencies and sensitivities in their ability to activate gene transcription: MC903 and EB1089 being more potent, but less sensitive than VD, whereas KH1060 does not induce such high gene activity, but shows about 100-fold higher sensitivity than VD.

MC903 appears to be the closest functional analogue of VD in the present study. Its slightly lower sensitivity in gene activation is counterbalanced by its higher potency for induction. These findings correlate well with previous studies on effects of MC903 on U937 cell regulation and receptor binding, which showed that this analogue was equipotent with VD itself [19].

EB1089 has been shown to be 50–100 times more potent than VD as an inhibitor of cell proliferation and an inducer of cell differentiation in U937 and MCF-7 cells [21]. In the present study, however, 20-fold higher concentrations of EB1089 than of VD were needed to activate each of the two VD signalling pathways. These findings correlate with the decreased receptor binding affinity for EB1089 described previously [21]. The effects of EB1089 on cellular proliferation may be mediated through different VD responding genes. On the VDR–RXR heterodimer pathway, we obtained an about 25% higher gene induction by EB1089 than by VD. It is possible that this 25% difference observed with the heterologous DR3-type VDRE/*tk* promoter is further enhanced on the mRNA or protein level of natural responding genes. EB1089 has been shown to be about 50 times more potent than VD in transiently increasing c-*fos* and decreasing c-*myc* mRNA [21].

KH1060 is one of the most potent VD analogues described to date; it belongs to the group of 20-epi-VD analogues, which are very potent regulators of immune responses [28]. A special feature of KH1060 is its ability to inhibit activation of T lymphocytes at very low concentrations. The different regulatory levels of the processes are not known. Our finding of a 100-fold increased sensitivity for KH1060 in gene activation, suggests that its main role lies in transcriptional regulation, although it is not known which responsive genes are involved. However, the binding of KH1060 to VDR does not correlate with the 100-fold increased sensitivity of VDR-induced CAT-activity [28]. This may be explained by the different stereochemistry in the side chain compared to VD. Comparisons with other 20-epi-VD analogues will hopefully elucidate the role of these side chain modifications in transcriptional regulation.

All experiments were performed in the presence of FCS, containing the vitamin D binding protein (DBP). Depending on the affinity of the analogues for the DBP, different concentrations of free ligand may be available in cell cultures. However, the differences in sensitivity between the analogues in the CAT-assay cannot solely be related to differences in DBP-binding, as both KH1060 and EB1089 display similar low binding affinities for DBP (A.-M. Kissmeyer, I.S.M., S. Latini and L.B., unpublished results).

An additional finding of this study is the difference in sensitivity of VDR homodimers and VDR–RXR heterodimers to VD and its analogues. The interactions with their respective specific response elements may cause different conformational changes of the VDR in homo- and heterodimeric complexes. However, it is more likely that the difference in ligand sensitivity is caused by different protein–protein interactions. Due to ligand binding the two VDR molecules in VDR homodimers may react allosterically, whereas this is not possible in VDR–RXR heterodimers. The result of this allosteric reaction might be a more effective orientation

of the transactivation domains towards the basal transcriptional machinery. Although VDR homodimers provide lower maximal induction of gene activity than VDR–RXR heterodimers, their higher ligand sensitivity may provide them with physiological relevance in, e.g. early and late VD responding genes. Further studies are needed to investigate which natural VD responding genes are regulated, like the human osteocalcin gene, by VDR homodimers.

Recently we observed functionally active heterodimerization of VDR not only with RXR, but also with RA and thyroid hormone receptors ([26, 29, 30]). Studies on the concentration-dependent interference of VD with 9-*cis* RA, all-*trans* RA and thyroid hormone are in progress. Taken together, our studies indicate that the concentration of VD in the absence or presence of additional ligands is an important regulatory level in the modulation of VD responsive genes.

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