High-Affinity Nuclear Receptor Binding of 20-epi Analogues of 1,25-Dihydroxyvitamin D₃ Correlates Well With Gene Activation

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Abstract   The hormone 1,25-dihydroxyvitamin D₃ (VD) has the potential for clinical use in several diseases, such as cancer, osteoporosis, and psoriasis. The action of VD is mediated by primary responding genes that contain in their promoter region a binding site for the transcription factor VDR. Most of the known VD response elements are formed by a direct repeat of two hexameric core binding motifs spaced by three nucleotides (DR3) bound by a heterodimer of VDR and the retinoid X receptor (RXR). Various VD analogues have been developed in order to optimize the therapeutic profile of VD. This report presents a novel experimental system that may help in the understanding of the structural basis for the high potency of a VD analogue like KH1060, which is a 20-epi-22-oxa-derivative of VD. In human breast cancer cells, MCF-7, the half-maximal gene activation values for KH1060 and seven of its structural precursors were determined on a DR3-type VD response element. These eight analogues cover conservative structural changes from 20-epi-VD (MC1288) to KH1060. With a modified version of the limited protease digestion assay the functional affinity of the analogues to VDR was measured. The functional receptor affinity of the eight analogues was found to be directly proportional to their potency in VDR-RXR-mediated gene activity.   © 1996 Wiley-Liss, Inc.

The seco-steroid hormone 1,25-dihydroxyvitamin D₃ (VD) is well known to regulate calcium and phosphate transport in the intestine and the mobilization of mineral from the bone [Walters, 1992; Bikle and Pillai, 1993]. In addition to this classic function, VD modulates the proliferation and differentiation of several normal and malignant cell types [Mangelsdorf et al., 1984; Colston et al., 1992]. The nuclear receptor for VD, VDR [Pike, 1991; Walters, 1992], is a transcription factor that belongs to the nuclear receptor superfamily [Evans, 1988; Green and Chambon, 1988], which also contains the nuclear receptors for all-*trans*- and 9-*cis*-retinoic acid, RAR and RXR, respectively, for thyroid hormone and for steroids. All genes that are directly transcriptionally regulated by VD, i.e., primary VD responding genes, contain within their pro-

moter region a binding site for VDR, referred to as the VD response element. Since VDR binds as a dimer to DNA, VD response elements are formed by two hexameric core binding sites in either directly repeated or inverted palindromic orientation [Carlberg, 1995]. Accordingly to the 3-4-5 rule of Umesono et al. [1991] most of the known VD response elements are direct repeats spaced by three nucleotides (DR3), but DR4- and DR6-type structures are also known. Recently, the two first natural VD response element with an inverted palindromic orientation of their core binding motifs that are spaced by 9 nucleotides (IP9s) have been identified [Schräder et al., 1995]. It was shown for some, and is probably true for most of the about 20 known natural VD response elements, that VDR binds as heterodimer with RXR [Carlberg, 1995].

Well established primary VD responding genes are osteocalcin, osteopontin, and calbindins, but also peptide signaling molecules such as interleukin-2 (IL-2), tumor necrosis factor (TNF), transforming growth factor-β (TGF-β), calcitonin, and
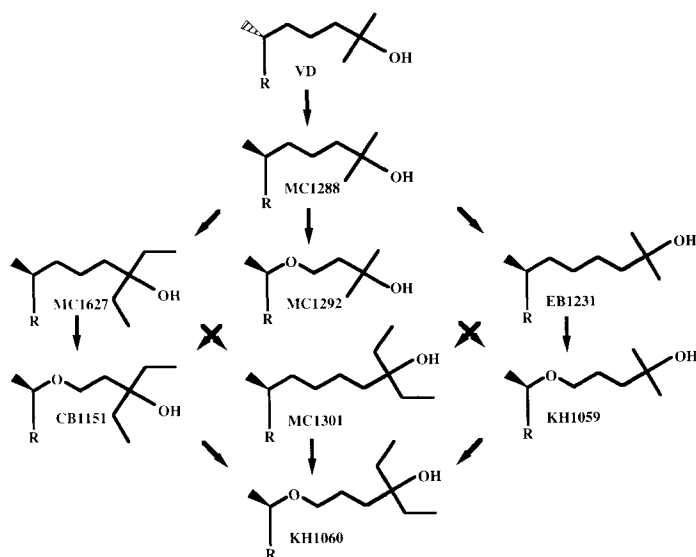
Fig. 1. The structure of VD in comparison with its 20-epi analogues. The structure of the side chain of the compounds are shown; R represents the rest of the VD molecule. MC1288 is the 20-epi isomer of VD. A second modification step led to the analogues MC1627, MC1292, and EB1231 and a third modification provided the compounds CB1151, MC1301, and KH1059, which converge by the fourth modification into KH1060. It is important to note that this scheme does only represent the structural relation between the VD analogues, but not a synthesis scheme.

prolactin are known to be regulated by VD. A very interesting target gene for VD is the human atrial natriuretic factor (ANF), a hormone that is synthesized primarily in the cardiac atria (Li and Gardner, 1994). Very recently, in the promoter of the rat *ANF* gene, a potent DR3-type VD response element was identified that is bound with high affinity by VDR-RXR heterodimers [Kahlen and Carlberg, 1996].

Investigations of the clinical value of VD in such diseases as cancer and psoriasis have been hampered by its dose-limiting toxicity (hypercalcemia, hypercalciuria, and soft tissue calcification) [Vieth, 1990]. VD analogues with structural changes mainly in the side chain have been developed in order to obtain a more favorable therapeutic profile. During the past few years, a new class of analogues characterized by an altered stereochemistry at position C-20 in the side chain have been identified as potent inhibitors of cell proliferation and inducers of cell differentiation. Until now, the most potent analogue of this series has proved to be KH1060, a 20-epi-22-oxa-analogue of VD [Binderup et al., 1991]. Since the biological effects of VD and its analogues are believed to be based on gene regulatory events that include the binding of VDR, several attempts have been made to find the best correlation with VD response element-driven reporter gene activity. Recently, a functional analysis of KH1060 in comparison with VD, the antipsoriatic drug calcipotriol (MC903) [Binderup and Kragballe, 1992] and the highly antiproliferative analogue EB1089 [Mathiasen et

al., 1993] on the DR3-type VD response element of mouse osteopontin [Noda et al., 1990] and the DR6-type element of human osteocalcin [Morrison et al., 1989] has been reported [Carlberg et al., 1994]. This study showed that the ligand concentration that provides half-maximal gene activity (EC_{50} value) is about 100 times lower for KH1060 than for VD.

In the present study, gene regulatory activities of KH1060 and of seven other 20-epi analogues that cover conservative molecular modifications from VD to KH1060 (Fig. 1) were investigated. The modifications are the epimerization at position C-20, the exchange of the carbon atom at position C-22 by an oxygen, the one carbon elongation of the side chain and the exchange of the two methyl groups into ethyl groups. The functional dissociation constants (K_{df}) of the eight VD analogues to VDR were measured by a modified limited protease digestion assay. The potency of 20-epi VD analogues on a DR3-type VD response element was shown to correlate with their functional receptor affinity.

MATERIALS AND METHODS
Compounds

VD and its analogues were synthesized at the Department of Chemical Research (LEO Pharmaceutical Products, Denmark). The compounds were dissolved in 2-propanol at 4 mM. Dilutions were performed in ethanol (final concentration of ethanol in the medium: 0.1%).

DNA Constructs

For the ANF VD response element, the oligo-nucleotides ATTTCTAGAAGAGGTCATGAAG-GACATCTAGACCC (core sequence underlined) and GGGTCTAGATGTCCT were annealed. This semi-double-stranded DNA fragment was filled in using Klenow polymerase, digested with *Xba*I, and fused to the thymidine kinase (*tk*) promoter to drive the expression of the chloramphenicol acetyl transferase (CAT) reporter gene by subcloning into the *Xba*I site of pBLCAT2 [Luckow and Schütz, 1987]. The cDNA for human VDR has been subcloned into pSG5 (Stratagene) [Carlberg et al., 1993].

Transfection and CAT Assays

MCF-7 cells were seeded into 6-well plates (2×10^5 cells per well) and grown overnight in phenol red-free RPMI supplemented with 0.5% 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) 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 RPMI, the liposomes were added to the cells; 500 μl phenol red-free RPMI supplemented with 30% charcoal-treated FCS was added 4–8 h after transfection. At this time VD, VD analogues or their solvent ethanol were also added. The cells were harvested 40 h after onset of the stimulation, and CAT assays were performed as described [Pothier et al., 1992]. 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 controls.

Limited Protease Digestion Assay

Linearized cDNA for human VDR was used for in vitro transcription as recommended by the supplier (Promega). Ten μg of in vitro transcribed VDR RNA was mixed with 175 μl rabbit reticulocyte lysate (Promega), 100 units 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 2–5 h. For the determination of the functional dissociation constants (K_{df}), 2.5 μl of this in vitro-translated VDR were preincubated for 30 min with graded ligand concentrations 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 15 min at room temperature. To observe multiple trypsin-resistant VDR fragments, 1 μl ^{35}S-labeled VDR was preincubated with 10 μM ligand and then digested with 50 μg/ml trypsin for 30 min. 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% sodium dodecyl sulfate (SDS), 0.025% bromophenol blue), and the samples were denatured at 95°C for 5 min, electrophoresed through a 12% or 15% SDS–polyacrylamide gel (acrylamide/N,N'-methylene-bisacrylamide weight ratio 33:1), electrotransferred to a nitrocellulose filter, air-dried, and autoradiographed overnight. The protease-sensitive VDR fragment was localized and excised from the filter; radioactivity was measured in a scintillation counter.

RESULTS

A potent DR3-type VD response element, recently identified in the promoter of the rat *ANF* gene [Kahlen and Carlberg, 1996], was selected as central part of an assay system for structure–activity relationship studies of VD analogues. The response element was cloned in front of the *tk* promoter driving the CAT reporter gene; this heterologous promoter construct was transfected into human MCF-7 breast cancer cells. This cell line is an appropriate model system for the analysis of VD signaling pathways, since it endogenously expresses VDR, RXR, RAR, and T_3R [Carlberg et al., 1993; Schräder et al., 1994, 1995]. The transfected MCF-7 cells were stimulated with increasing concentrations of the eight VD analogues (the structures of their side chains are given in Fig. 1), and the plot of CAT reporter gene activity over ligand concentration provided typical sigmodial shaped dose-response curves. From these curves, the concentrations of half-maximal gene activity (EC_{50} values) were calculated. It is obvious that the analogues show clearly higher differences in their EC_{50} values than in their maximal induction at saturating ligand concentrations. Figure 2 presents the eight dose-response curves in the order of increasing EC_{50} values: they differ between 2.2 pM for KH1060 and 27 nM for MC1292, i.e., in a factor of about 10,000. By contrast, the maximal inductions differ only between 2.5-fold for
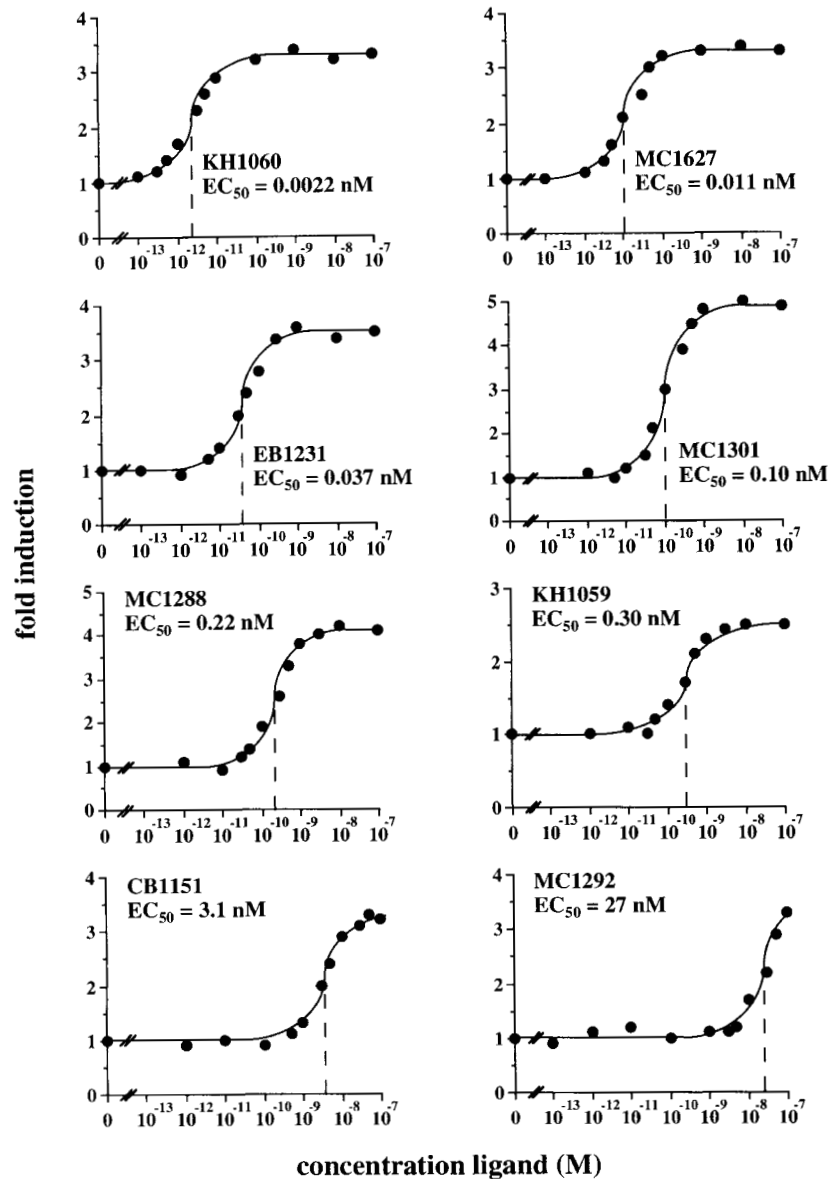
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Fig. 2. Activation of the rat ANF VD response element by 20-epi VD analogues. MCF-7 cells were transfected with the CAT reporter construct containing the rat ANF VD response element and treated with increasing concentrations of the indicated 20-epi VD analogues. CAT activities were determined 40 h later and stimulation was calculated in comparison to solvent-induced controls. Each data point represents the mean of triplicates; the standard deviation was always <10%. The dose-response curves are presented in the order of increasing EC_{50} values.

KH1059 and 4.8-fold for MC1301, i.e., less than a factor of 2. For comparison, on the same response element and in the same cell line the EC_{50} value of VD and the maximal induction at saturating ligand concentration were determined as 0.48 nM and 3.7-fold, respectively [Kahlen and Carlberg, 1996].

In order to understand the basis for the large differences in EC_{50} values, the affinity of the eight analogues to the VDR was measured. In most cases, this is performed by displacing ^{3}H-labeled VD from partially purified VDR by increasing concentrations of nonlabeled test compounds. Unfortunately, the relative affinities obtained in this way in most cases did not show a good correlation with other biological data of the analogues [Peleg et al., 1995; Wiberg et al., 1995; Mørk Hansen et al., 1996]. Therefore, a different technique was applied, known as limited protease digestion assay, in which the digestion of the VDR-ligand complex with a limited amount of a protease as trypsin results in a
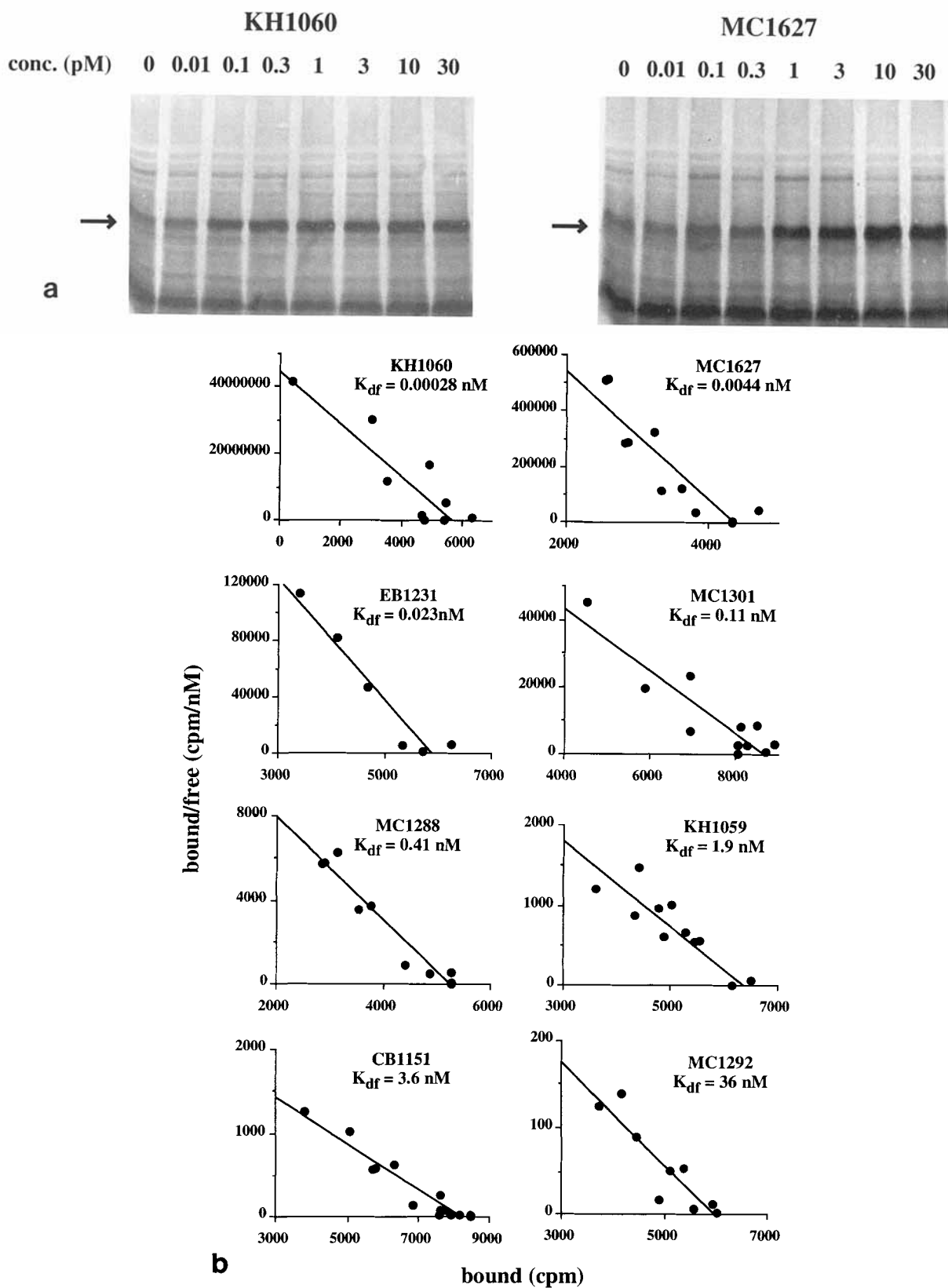
KH1060

conc. (pM)   0   0.01   0.1   0.3   1   3   10   30

MC1627

0   0.01   0.1   0.3   1   3   10   30

a

KH1060
$K_{df} = 0.00028$ nM

MC1627
$K_{df} = 0.0044$ nM

EB1231
$K_{df} = 0.023$nM

MC1301
$K_{df} = 0.11$ nM

MC1288
$K_{df} = 0.41$ nM

KH1059
$K_{df} = 1.9$ nM

CB1151
$K_{df} = 3.6$ nM

MC1292
$K_{df} = 36$ nM

bound/free (cpm/nM)

b

bound (cpm)

Fig. 3. Binding of 20-epi VD analogues to in vitro translated VDR. In vitro synthesized [^{35}S]methionine-labeled VDR was preincubated with the indicated concentrations of KH1060 or MC1627 (**a**). Trypsin to a final concentration of 27 μg/ml was added and the mixtures were incubated for 30 min at room temperature. Samples were electrophoresed through a 12% SDS-polyacrylamide gel, electrotransferred to a nitrocellulose filter, air-dried, and autoradiographed. (*Arrow*) Resistant protein fragment. Respective bands were cut out from the filter and their radioactivity measured in a scintillation counter. These limited protease digestion assays were also performed with the other six 20-epi VD analogues. Scatchard plots of all eight data sets are presented in the order of increasing K_{df} values (**b**).

protein fragment that is resistant to digestion [Leng et al., 1993; Keidel et al., 1994]. In the presence of a specific ligand, VDR is in a conformation that prevents the exposure of a cutting side to the protease; therefore, the relative amount of resistant protein fragment is directly proportional to the saturation of the receptor with ligand. These assays were performed with in vitro translated, [^{35}S]methionine-labeled VDR and graded concentrations of the eight analogues; representative results are shown for KH1060 and MC1627 in Figure 3a. Genomic effects of VD are generally considered to be based on conformational changes that the ligand induces to the receptor; however, the possibility cannot be exclude that the ligand binds to the receptor without causing functional effects. Therefore, the ligand concentration that provides 50% of protease-resistant VDR fragment was defined as functional dissociation constant (K_{df}), which value might be different from that of the traditional dissociation constant (K_d). The relative amounts of the protease-resistant VDR fragments were obtained by scintillation counting of the respective excised bands and normalization for the background of the solvent control. The Scatchard plots of the data are shown in Figure 3B and are arranged by increasing K_{df} values. For comparison, in the same experimental system the K_{df} value for VD was determined as 0.9 nM [Nayeri et al., 1995]. Interestingly, the order of the analogues arranged by increasing K_{df} values (Fig. 3b) is identical to that arranged by increasing EC$_{50}$ values (Fig. 2). It is

remarkable that for each 20-epi-analogue the absolute EC$_{50}$ and K_{df} values do not differ more than by a factor of 8, if one realizes that the first value is determined by VDR action in cultured MCF-7 cells and the second value with in vitro translated VDR in vitro.

Finally, whether the 20-epi conformation of the VD analogues induces a specific conformation in the VDR was analyzed. Therefore, the conditions of the limited protease digestion were modified by increasing the protease/VDR ratio, and the use of saturating ligand concentrations; moreover, the digestion products were resolved on 15% SDS-polyacrylamide gels (Fig. 4). In addition to the 34-kDa VDR fragment (No. 1) that was observed under standard conditions (Fig. 3A), two smaller fragments of about 32 and 30 kDa (Nos. 2 and 3) were obtained (molecular mass according to Peleg et al. [1995]). Under these modified assay conditions, fragments No. 1 and 3 were observed with VD and all eight analogues. In contrast, fragment No. 2 can be observed in reasonable quantities with the analogues MC1288, CB1151, MC1627, and MC1292, in low quantities with KH1060 and KH1059, but not at all with MC1301 and EB1231.

DISCUSSION

In this report, a novel experimental system for the study of VD analogue structure–activity relationships was introduced. The limited protease digestion assay enables the direct visualization and quantification of VDR–VD analogue
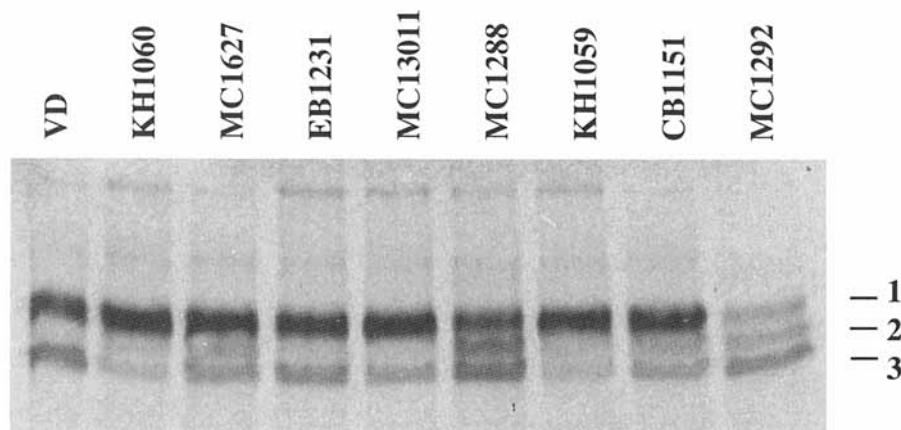

Fig. 4. Fine analysis of VDR–VD analogue interactions. Limited protease digestion assays were performed with in vitro translated VDR in the presence of 10 μM of the indicated ligands. Compared to results presented in Figure 3 the trypsin/VDR ratio was increased. Up to three different protease-resistant VDR fragments (1, 2, and 3) have been resolved on a 15% SDS-polyacrylamide gel.

interactions and has therefore a great advantage compared to the traditional indirect measurement by competition studies. In particular, for analogues that have higher affinity to the VDR than VD, the latter assay may provide misleading results. It was reported recently [Peleg et al., 1995] that MC1288 and even KH1060 bind with lower affinity to the VDR than VD, in contrast to the results presented in this report and the biological profile of the analogues.

The functionality of the rat ANF DR3-type VD response element in a heterologous promoter system is at about the same than that of the mouse osteopontin VD response element [Carlberg et al., 1994]. The latter is very often used as typical VD response element [Cheskis and Freedman, 1994; Peleg et al., 1995]; the rat ANF element now may provide an equivalent alternative. It should be noted that results, which have been obtained in a heterologous promoter context with either the osteopontin or the ANF element, probably do not represent gene-specific responses to VD and its analogues. However, the emphasis of this report was to analyze gene regulatory properties of 20-epi-analogues on a common VD response element structure.

The fact that a close correlation of the eight 20-epi analogues was observed in the gene activation and the receptor affinity assay indicates that both assay systems provide useful data for the understanding of the effects of side chain modifications in VD analogues. At earlier times, it was assumed that the activity of a VD analogue is proportional to its receptor affinity [Stern, 1981], but later more complex relations were observed [Jones and Calverley, 1993]. However, at least in a defined series of 20-epi-analogues with closely related structures, as studied in this report, VDR binding correlates with gene expression. The biological effects of VD and its analogues are multistep processes, in which the transcriptional regulation of a primary VD responding gene is one of the early steps; however, later steps may disturb the linearity between receptor affinity and biological readout. The functional dissociation constants were measured on VDR monomers, whereas on the DR3-type VD response element VDR binds as heterodimer with RXR. However, although it is likely that RXR influences the ligand binding affinity of VDR, this effect appears not to have major selectivity for the eight tested VD analogues. Furthermore, the cellular uptake of the ligands, i.e., in first priority the affinity to the

vitamin D-binding protein (DBP), and their catabolism, i.e., mainly their 24-hydroxylation, have to be taken into account. However, the DBP affinity of most 20-epi analogues is low [Kissmeyer et al., 1995] and clearly lower than their affinity to the VDR, so that the presence of DBP in the FCS of the culture medium for MCF-7 cells certainly had only very minor effects on the results.

The 20-epimerization of VD, which forms MC1288, increases both the K_{df} value and the EC_{50} value by a factor of about 2 and appears to stabilize a different VDR conformation, represented by the three-band pattern in the modified limited protease digestion assay. This finding is in accordance with the higher activity generally found for 20-epi-analogues as compared to the corresponding normal compounds. However, on the basis of EC_{50} and K_{df} values, there is no clear-cut structure–activity pattern for the selected structural analogues between MC1288 and KH1060. The structural modifications in the side chain of VD addressed the importance of chain length, accessibility of the tertiary OH-group in the end of the side chain, and the influence of substitution of a carbon for an oxygen at position C-22. Side-chain elongation without heteroatom substitution (EB1231 and MC1301, see Fig. 1) led to more active compounds. Formal oxygen substitution at position C-22 in EB1231 decreased the activity (KH1059). By contrast, the same formal substitution in MC1301 leading to KH1060 immensely increased the activity. However, for the homologue EB1231 the same formal modification to MC1301 led to a decreased activity. Furthermore, the introduction of an oxygen at position C-22 in MC1627 led to a decreased activity, in contrast to the above mentioned activity increase for the MC1301 to KH1060 modification. Although these findings seem inconclusive with respect to structure–activity relationships, they reflect the known importance of small structural changes in activity. The very large conformational flexibility of these molecules is perhaps an explanation how seemingly small changes give drastic differences in activity. Analysis of analogues with restricted flexibility may lead to a better understanding of this question.

The modification of the limited protease digestion assay in extension of the protease digestion reaction towards an equilibrium provides as ma-

jor result a change from a two-band pattern to a three-band pattern, or vice versa. This may be interpreted as a conformational change in the ligand-bound VDR molecule, which gives a first sight on multiple conformations of the VDR. A comparable observation has been recently reported for the interaction of the estrogen receptor and antiestrogens [McDonnell et al., 1995]. However, the conformational in the VDR is not uniquely related to the 20-epi-conformation of the analogues as indicated by Peleg et al. [1995], since the analogues MC1301 and EB1231 show no and KH1059 and KH1060 show only a very weak protease resistant VDR fragment No. 2. Interestingly, MC1301 and EB1231 have an elongated side chain without an oxygen atom and KH1059 and KH1060 an elongated chain with oxygen atom, whereas the analogues that show a clear fragment No. 2 (MC1288, MC1627, MC1292, and CB1151) all have a shorter side chain. Studies that correlate the VDR conformations, represented by these two-band and three-band-patterns, with a specific activities of VDR as dimerization or transactivation, are in progress.

ACKNOWLEDGMENTS

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