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A 20-Epi Side Chain Restores Growth-Regulatory and Transcriptional Activities of an A Ring–Modified Hybrid Analog of 1α,25-Dihydroxyvitamin D_3 Without Increasing Its Affinity to the Vitamin D Receptor

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Abstract 1α-hydroxymethyl-25-hydroxyvitamin D_3 and 1β-hydroxymethyl-3α,25-hydroxyvitamin D_3, two analogs with modifications restricted to the A ring, bind poorly to vitamin D receptor (VDR). The effective doses required for 50% of maximal binding activity (ED_{50}) are 7×10^{-7} M for the former and 8×10^{-8} M for the latter, and the ED_{50} for their growth-inhibitory activities is greater than 10^{-6} M. Unexpectedly, a hybrid analog with 20-epi configuration at its side chain and a 1β-hydroxymethyl group but not a 1α-hydroxymethyl group inhibits malignant cell growth with an ED_{50} of 7×10^{-9} M. To determine if the restored biological activity of the hybrid analog is associated with better binding to VDR, we performed competitive binding assays in vitro with calf thymus VDR and in vivo with recombinant human VDR. We found that the 20 epi side chain reduced the affinity of the 1β- and the 1α-hydroxymethyl hybrid analogs for VDR in vitro and in vivo fourfold to tenfold. To determine whether the 1β-hydroxymethyl analogs induced a VDR-mediated transcription, we tested the induction of reporter gene expression through the osteocalcin vitamin D response element (VDRE) in ROS 17/2.8 cells and the induction of binding activity of VDR to VDRE in COS-1 cells. We found that the ED_{50} for transcriptional activity of 1β-hydroxymethyl-3α,25-hydroxyvitamin D_3 was greater than 10^{-6} M, but its 1α diastereomer had barely detectable transcriptional activity. The 20-epi side chain preferentially increased the transcriptional activity of the 1β-hydroxymethyl hybrid analog to an ED_{50} of 10^{-8} M, but the 1α-hydroxymethyl hybrid analog remained inactive. To confirm that this transcriptional activity was dependent on the VDR, we repeated the assay in VDR-negative CV-1 cells and compared ligand-dependent expression of the VDRE/growth hormone reporter in the presence of either wild-type or transcriptionally inactive mutant VDR expression vectors. Transcription was induced by the 1β-hydroxymethyl compounds only in the presence of wild-type VDR. Thus, we conclude that it is possible, by adding a 20 epi side chain, to restore growth-inhibitory and VDR-mediated transcriptional activities without increasing binding to the VDR of A ring–modified analogs.

Key words: 1α,25-dihydroxyvitamin D_3, hybrid analogs, 20-epi analogs, vitamin D receptor, growth inhibition, transcription

The actions of 1α,25-dihydroxyvitamin D_3 ($1,25D_3$ or compound 1) are diverse and include regulation of phosphate metabolism, calcium mobilization, inhibition of malignant cell growth in culture and in vivo, and induction of cell differentiation [Reichel et al., 1989; Bouillon et al., 1995]. Because of the last two characteristics, $1,25D_3$ has been considered for treatment of malignancy and has been used with partial success in animal models of leukemia [Honma et al., 1983; Potter et al., 1985] and breast [Saez et al., 1993] and colon cancer [Eisman et al., 1987; Kawaura et al., 1990]. However, for clinical chemotherapy it is important to develop analogs of $1,25D_3$ in which the growth-regulatory activity is sufficiently separated from the calcium-regulating activity so that the toxic side effects

Abbreviations used: $1,25D_3$, 1α,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; VDRE, vitamin D response element; ED_{50}, effective dose required for 50% of maximal response.

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of the ligand are eliminated. To do so, it is necessary to identify the chemical and stereochemical features of the ligand that are responsible for growth inhibition and for calcium regulation and, if possible, to modify the latter without affecting the former so that growth regulation remains intact while calcium-regulatory activities are diminished. It has been shown that the calcium-regulating activity of $1,25D_3$ depends on the presence and stereochemistry of hydroxyl groups at the 1 and 3 positions [Norman et al., 1993; Bouillon et al., 1995]. Recently it was demonstrated that the 1β-hydroxylated analogs antagonize some aspects of the calcium-regulating activity of $1,25D_3$ [Norman et al., 1993]. The clinical advantage of analogs modified only in the A ring, however, is insignificant because they have little or no detectable growth-inhibitory activity [Posner et al., 1992, 1993; Norman et al., 1993]. The absence of this activity is believed to be due to the compounds' diminished affinity to the nuclear vitamin D receptor (VDR).

In our search for a way to improve the growth-regulatory activities of analogs with modifications restricted to the A ring, we considered further modifying these analogs by adding side chains with chemical and stereochemical changes that enhance antiproliferative and differentiating activities. To date the most active of these analogs are ligands with 20-epi side-chain stereochemistry [Binderup et al., 1991]. Therefore, we synthesized two compounds with 20-epi side chains plus either 1α-hydroxymethyl or 1β-hydroxymethyl-3α-hydroxyl groups in their A rings [Posner et al., 1994, 1995]. We report here that the 20-epi configuration of the side chain restored the antiproliferative activity preferentially of the 1β-hydroxymethyl-3α-hydroxyl analog. This modification also restored transcriptional activities mediated through the VDR. However, these enhanced activities are not associated with increased affinity to the VDR.

MATERIALS AND METHODS

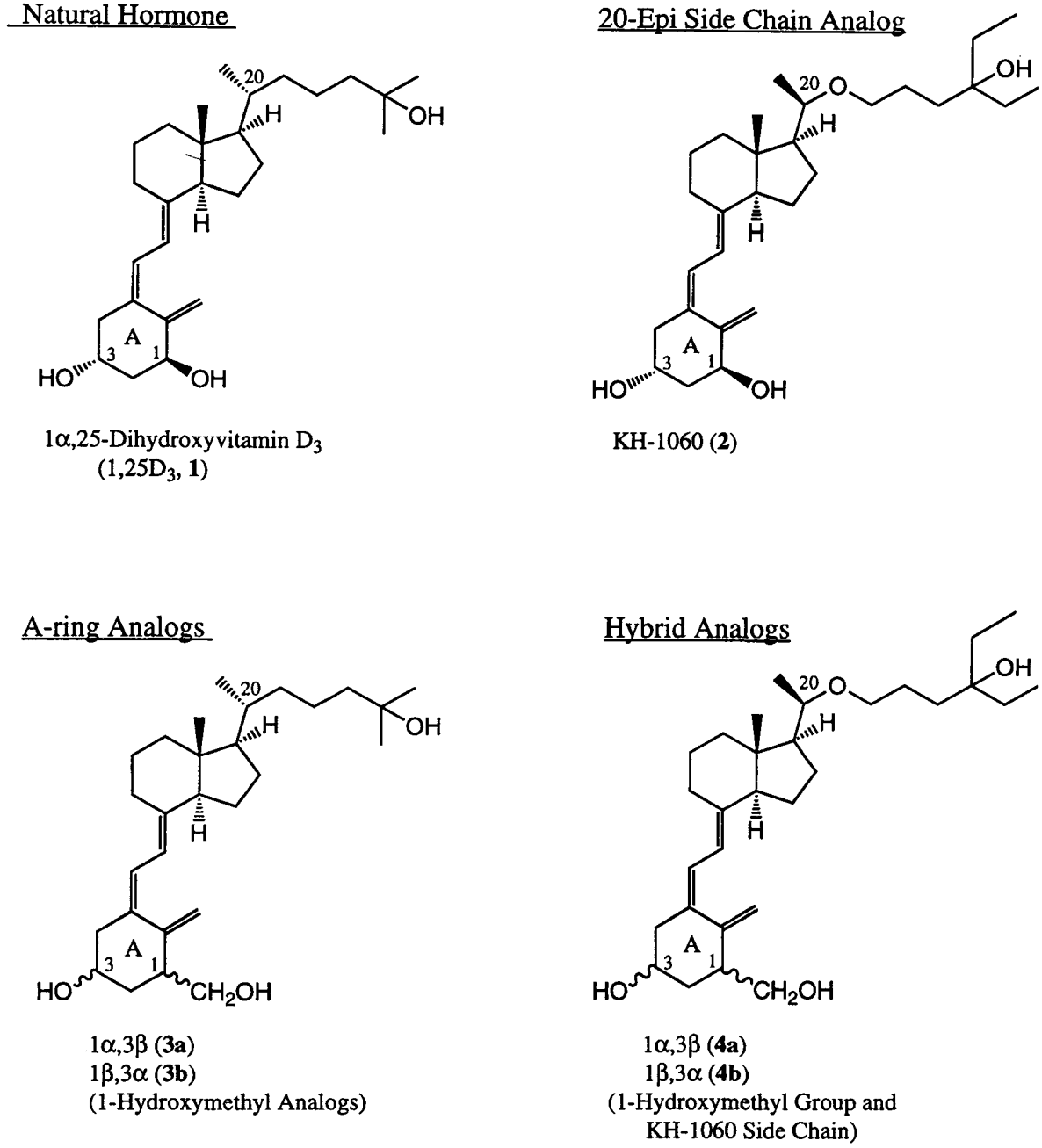
Reagents

[^{32}P] dCTP, [^{35}S]-methoinine, and 1,25(OH)$_2$[26,27-^{3}H]D_3 were purchased from Amersham Corp. (Arlington Heights, IL). The vitamin D analogs 20-epi-1α,25-dihydroxyvitamin D_3 (MC-1288) and 20-epi-22-oxa-24a,26a,27a-tri-homo-1α,25-dihydroxyvitamin D_3 (KH-1060) [Binderup et al., 1991] were a generous gift from Dr. L. Binderup (Leo Pharmaceuticals, Bellerup, Denmark). The A ring–modified analogs 1β-hydroxymethyl-3α,25-dihydroxyvitamin D_3 and 1α-hydroxymethyl-3β,25-dihydroxyvitamin D_3 were given the short names 3b and 3a, respectively. The 20-epi analog KH-1060 was called analog 2, and the resulting two hybrids with this side chain (Fig. 1) were given the short names 4a and 4b. The letter *a* indicates the presence of a hydroxymethyl group at the 1α position, whereas the letter *b* indicates the presence of a hydroxymethyl at the 1β position and a hydroxyl group at the 3α position. The synthesis strategy for the A ring–modified and hybrid analogs was described previously [Posner et al., 1992, 1993, 1994, 1995]. The structural formulas of these compounds are shown in Figure 1. Small samples of the hybrid analogs for further biological evaluation are available from G.H.P. upon request.

Cell Culture and Transfections

African green monkey kidney cell lines (COS-1 and CV-1) were maintained in Dulbecco's modified Eagle's medium (DMEM). Rat osteosarcoma ROS 17/2.8 cells were maintained in 50% DMEM and 50% F12. HL-60 cells were maintained in RPMI-1640. All the culture media were supplemented with 10% fetal bovine serum. For studies of growth regulation, HL-60 cells were grown in liquid cultures at a density of 10^5 cells/ml in 100 mm dishes without or with the ligands at the indicated concentration. More of the ligands was added after 48 and 96 h, and the cells were counted after 2, 4, and 6 days. For transfections, the cells were plated in 35 mm dishes at a density of 6×10^5 cells/dish (COS-1) in DMEM and 10% fetal bovine serum. Forty-eight hours later, ROS 17/2.8 cells were transfected with 2 μg of plasmid containing the vitamin D response element (VDRE, GGTGACTCACCGGGTGAACGGGGGCATT) from the human osteocalcin gene. This response element was attached to a thymidine kinase promoter/growth hormone fusion gene. CV-1 cells were transfected with the osteocalcin VDRE/reporter fusion gene (4 μg/dish) and the recombinant human VDR expression vectors or the recombinant human retinoid X receptor expression vector (2 μg/dish). COS-1 cells were transfected with 20 μg/dish of recombinant human VDR plasmid.

All transfections were performed by the DEAE dextran method [Peleg et al., 1989], and the cells were then treated for 1 min with 10% dimethyl sulfoxide. Ligands in serum-free medium were



1α,25-Dihydroxyvitamin D_3 ($1,25D_3$, **1**)

KH-1060 (**2**)

1α,3β (**3a**)
1β,3α (**3b**)
(1-Hydroxymethyl Analogs)

1α,3β (**4a**)
1β,3α (**4b**)
(1-Hydroxymethyl Group and KH-1060 Side Chain)

Fig. 1. Structural formulas of $1,25D_3$ and its analogs.

added for 1 h immediately after transfection (ROS 17/2.8 cells) or 24 h later (CV-1 cells) and then removed. The cells were then washed twice in phosphate-buffered saline (PBS) and added to DMEM with 10% serum. Medium samples for measurements of growth hormone were collected 24 h after ligand treatment. Growth hormone production from the reporter gene was measured by a radioimmunoassay as described by the manufacturer (Nichols Institute, San Juan Capistrano, CA).

Ligand Binding Assays

To compare the relative affinity of $1,25D_3$ and the analogs for VDR in vitro, whole cell homogenates from calf thymus were prepared as described previously [Reinhardt et al., 1984]. Radioreceptor assays were conducted with

increasing concentrations of unlabeled $1,25D_3$ (2.4×10^{-11} to 7.69×10^{-10} M) or analogs (4.65×10^{-9} to 2.17×10^{-5} M) in KTED (10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 0.3 M KCl, and 1 mM dithiothreitol). The homogenates were then aliquoted into tubes containing 1.2×10^{-10} M [^{3}H]$1,25D_3$, the mixtures were incubated for 1 h at 25°C, and then free ligand was separated from bound with dextran-coated charcoal and the radioactivity was measured by scintillation counting. Binding activity, expressed as a fraction of maximal binding (which was binding of [^{3}H]$1,25D_3$ in the absence of competitor), was plotted as a function of logarithmic value of competitor concentration.

To assess receptor occupancy by the ligand in vivo, monolayers of VDR-transfected COS-1 cells were washed three times with PBS and incubated for 1 h with ligand in serum-free medium, and then the medium was discarded. The cells were washed again three times in cold PBS, scraped into 10 ml of PBS, centrifuged, resuspended in KTED, and homogenized. Aliquots (0.2 ml) of the homogenates were incubated on ice for 3–4 h with 0.2 pmol of [^{3}H]$1,25D_3$ with or without a hundredfold excess of unlabeled ligand. To assess the number of unoccupied VDR sites, the free ligand was separated from the bound by treatment with a hydroxyapatite slurry as described previously [Peleg et al., 1995].

Ligand-Induced Sensitivity to Proteases

Synthetic human VDR labeled with [^{35}S]methionine (1,000 Ci/mmol) was prepared in reticulocyte lysates (Promega Corp., Madison, WI) by in vitro coupled transcription/translation of the human VDR cDNA inserted into the vector pGEM4. The receptor was incubated with $1,25D_3$, KH-1060, and MC-1288 (10^{-8} M) or with the A ring–modified analogs (1 μM) for 10 min at room temperature. Then 20 μg/ml trypsin was added, and the mixtures were incubated for another 10 min. The digestion products were analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the gels were dried and autoradiographed.

Preparation of Nuclear Extracts

To test the ligand-induced DNA binding activity of VDR, nuclear extracts were prepared from COS-1 cells 48 h after transfection with the human VDR plasmid. Ligands (1, 2, 3b, and 4b) dissolved in ethanol were added in serum-free medium for 1 h, and then the cells were harvested immediately after ligand treatment or 24 h later. The cells were scraped from individual dishes into PBS, washed twice in PBS, and resuspended in 1 ml of buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol) and incubated on ice for 30 min. After they had swollen, the cells were homogenized with five to ten strokes in Dounce homogenizer and centrifuged for 30 s at 14,000*g*. The supernatants were then discarded, and the nuclear pellets were resuspended in 50 μl of buffer C (20 mM Hepes, pH 7.9, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, and 0.5 mM dithiothreitol), set on ice for 30 min, homogenized again, and centrifuged for 30 s at 14,000*g*. The nuclear extracts were then collected, frozen in dry ice immediately, and stored at −80°C for further analysis.

Electrophoretic Mobility Shift Assays (EMSAs)

A *Hind*III fragment containing the mouse osteopontin VDRE (TGCTCGGGTAGGGTTCACGAGGTTCACTCGAC) was labeled with ^{32}P to a specific activity of $1–5 \times 10^8$ cpm/μg. Each binding reaction mixture contained 50 mM KCl, 12 mM Hepes-NaOH, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 12% (vol/vol) glycerol, 0.5 ng of the DNA probe, 10 μg of nuclear proteins, and 1 μg of poly (dI-dC) as nonspecific competitor DNA. The binding reactions were performed at room temperature for 30 min. The complexes were resolved by electrophoresis through 4% polyacrylamide gels at 4°C, and complexes were detected in the dried gels by autoradiography.

RESULTS

The 20-Epi Side Chain Restored Antiproliferative Activity of A Ring–Modified Analogs

The calcium-regulating activities of A ring–modified analogs with normal side chains are very low [Norman et al., 1993; Bouillon et al., 1995]. To test whether these modifications left any growth-regulatory activities, we compared the growth inhibitory effect of the 1α- and 1β-hydroxymethyl analogs 3a and 3b on HL-60 myeloid leukemia cells with that of $1,25D_3$. We found that in this culture system growth inhibition of 80% was reached after 6 days (Fig. 2A). Although the concentration required for 40% inhibition of cell growth (ED_{50}) was 5×10^{-7} M with analog 3b (Fig. 2B), an actual maximal growth inhibition of 80% was not reached with 3b even at 10^{-6} M, whereas $1,25D_3$ had an ED_{50}

of 5×10^{-10} M. Therefore, this A-ring modification reduced the growth-inhibitory activity by at least a thousandfold. The analog 3a was almost completely inactive even at 10^{-6} M. Greater ligand concentrations were not used because they are insoluble in aqueous media.

To increase the antiproliferative activity of these compounds, we synthesized two new analogs that contained the highly potent 20-epi side chain of KH-1060, as shown in Figure 1, and A rings containing either the 1α-hydroxymethyl or the 1β-hydroxymethyl-3α-hydroxyl groups [Posner et al., 1994]. The growth-inhibitory activities of these hybrid analogs were also tested and compared to those of KH-1060 and $1,25D_3$. We found that at 10^{-7} M significant growth inhibition associated with morphological changes and the induction of cell adherence occurred in cultures containing $1,25D_3$, KH-1060, and hybrid analog 4b (Fig. 2A). Hybrid analog 4a, although showing some growth inhibition by day 6, did not induce morphological changes or cell adherence (morphological data not shown). The ED_{50} for growth inhibition (Fig. 2B) of the hybrid 1β-hydroxymethyl analog with a 20-epi side chain (4b) was 7×10^{-9} M, significantly greater than that of the 1β-hydroxymethyl analog (3b) with an unmodified side chain. The hybrid analog with the 1α-hydroxymethyl group (4a) had very low growth-inhibitory activity even at 10^{-6} M. Therefore, the 20-epi configuration of the side chain preferentially enhanced the growth-regulatory activities of the analog with 1β-hydroxymethyl and 3α-hydroxyl groups. Although these restored activities were still fourteenfold lower than the growth inhibition induced by $1,25D_3$, it should be noted that the calcemic activity of the 1β-hydroxymethyl hybrid analog is 200-fold lower than that of $1,25D_3$ [Posner et al., 1995].

Binding of the 1-Hydroxymethyl/20-Epi Hybrid Analogs to VDR

Because the growth-inhibitory activity of $1,25D_3$ and its analogs is considered a VDR-mediated event, it was important to determine whether the restoring of the growth-regulatory activity of the 1β-hydroxymethyl analog by adding a 20 epi side chain also increased affinity to the VDR. First we performed binding assays in which [^{3}H]$1,25D_3$ binding to calf thymus VDR was competed by the unlabeled analogs (Fig. 3). We found that the concentration of the 1β-hydroxymethyl analog 3b required to compete

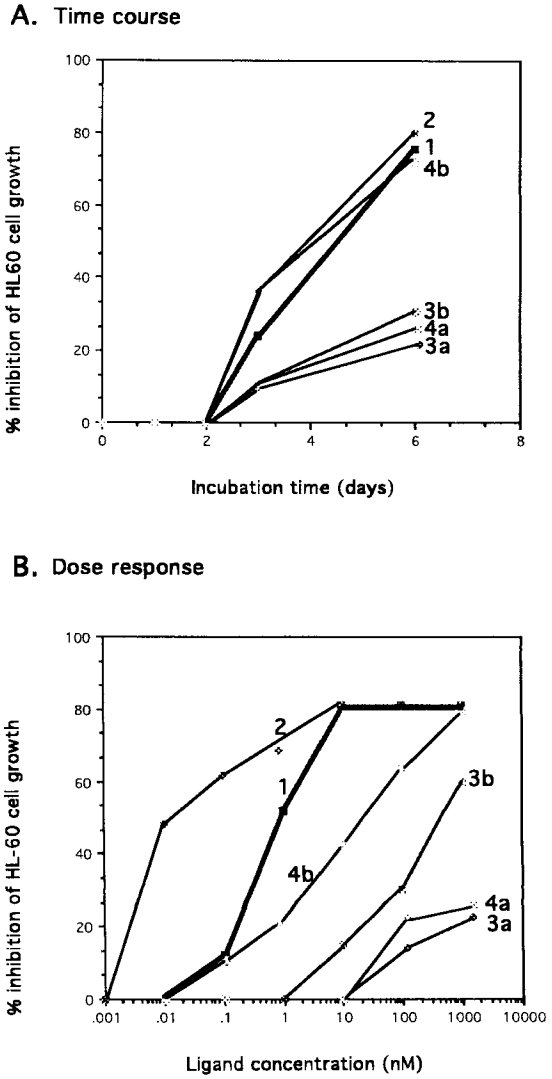


Fig. 2. Effect of 20-epi side chains on growth inhibition of HL-60 cells by A ring–modified analogs. HL-60 myeloid leukemia cells were incubated with $1,25D_3$ or analog for 6 days. The ligands were added immediately and again after 48 h and 96 h. The cells were counted after 48 h, 72 h, and 6 days of incubation. Shown are time-course plots for growth inhibition in the presence of 10^{-7} M of each ligand (**A**) and representative dose-response plots for growth inhibition after 6 days of incubation (**B**). Each point on the plots represents the average the results from duplicate cultures. The dose-response experiments were performed six times for compound 1, three times for compound 2, three times each for compounds 3b and 4b, and once each for compounds 3a and 4a.

50% of [^{3}H]$1,25D_3$ binding to VDR was 8×10^{-8} M, whereas the concentration of the hybrid analog with a 1β-hydroxymethyl group (4b) required was 2×10^{-7} M. Similar tests were also performed with the 1α-hydroxymethyl compounds 3a and 4a (Fig. 3). The concentrations required for 50% competition were 7×10^{-7} M

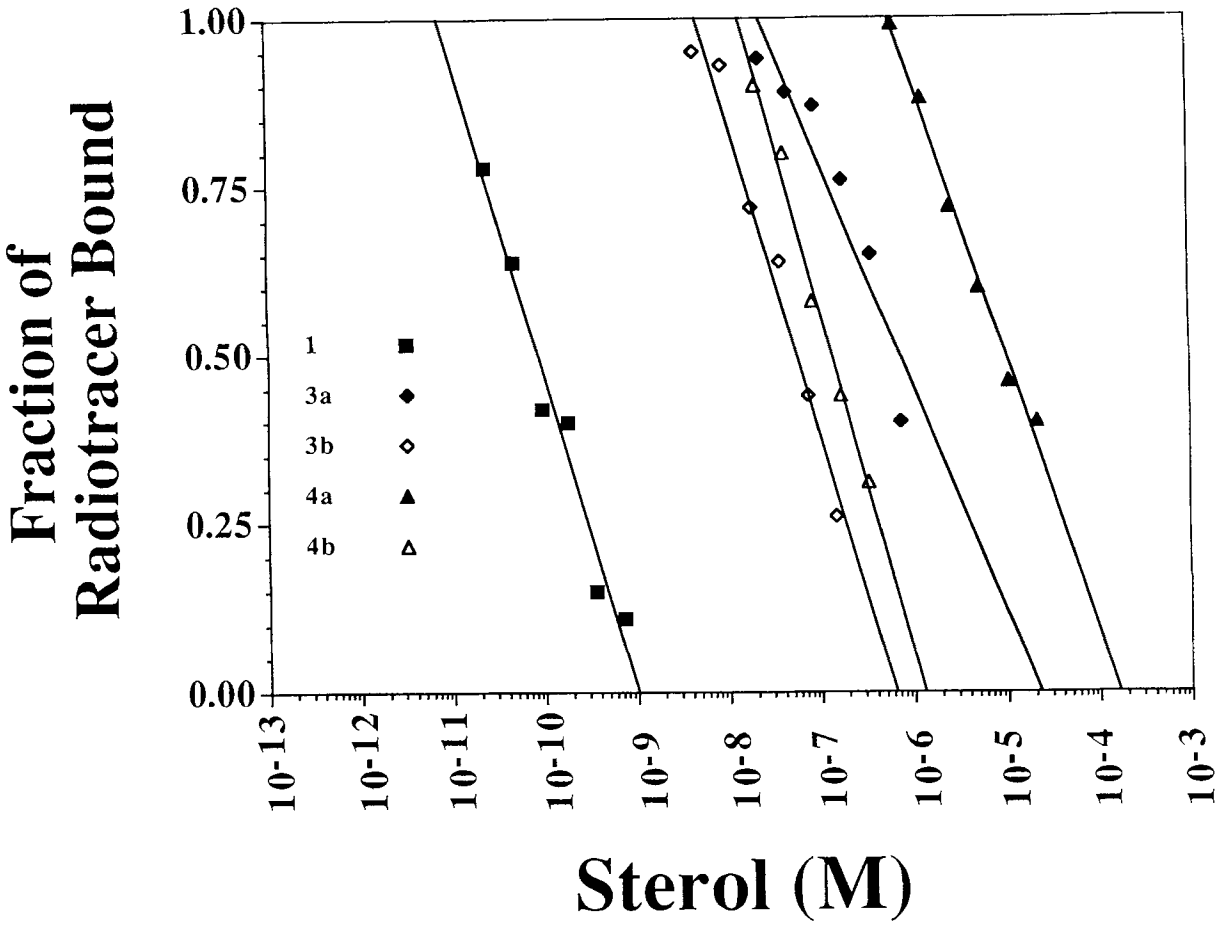


Fig. 3. Relative affinities of $1,25D_3$ and the 1-hydroxymethyl analogs to calf thymus VDR in vitro. Homogenates of calf thymus were incubated with a fixed amount of [^{3}H]$1,25D_3$ and increasing amounts of unlabeled competitors as indicated in Materials and Methods. At the end of the incubation period, bound ligand was separated from free with dextran-coated charcoal, and binding activity was measured by scintillation counting. The results are expressed as the fraction of [^{3}H]$1.25D_3$ binding in the absence of competitor (maximal binding) and are plotted against the amount of competitor used. The analogs' codes and their structural formulas are shown in Fig. 1.

for analog 3a and 10^{-5} M for 4a, the 1α-hydroxymethyl analog with a 20-epi side chain. The binding of the biologically inactive 1α-hydroxymethyl compounds to the VDR was therefore less efficient than binding of the 1β-hydroxymethyl compounds. The 20-epi side chain reduced the affinity of KH-1060 for VDR threefold to fourfold [Peleg et al., 1995]. Similarly, adding the 20-epi side chain decreased the affinity of the hybrid analogs to VDR. Therefore, we conclude that the restoration of growth-inhibitory activity by the 20-epi side chains was not associated with increased affinity to the VDR in vitro.

The binding properties of the VDR in vivo and in vitro may be different; the greater antiproliferative activities of the 1β-hydroxymethyl hybrid analogs may be due to better uptake by target cells or better binding to VDR in vivo. To test these possibilities we incubated COS-1 cells transfected with the human VDR expression vector with increasing concentrations of the ligands and then measured the remaining unoccupied binding sites in homogenates of these cells. Figure 4 shows that the dose required to saturate 50% of the VDR binding sites with $1,25D_3$ or KH-1060 was 10^{-9} M, although KH-1060 is a hundredfold more potent growth-inhibitory agent. Likewise, the concentration required to saturate 50% of VDR binding sites was 3×10^{-8} M for the less active analog 3b but 3×10^{-7} M for the more potent hybrid analog 4b. Therefore, it seems that, both in vivo and in vitro, the binding of compounds with 20-epi side chains was not proportional to their biological activity and that

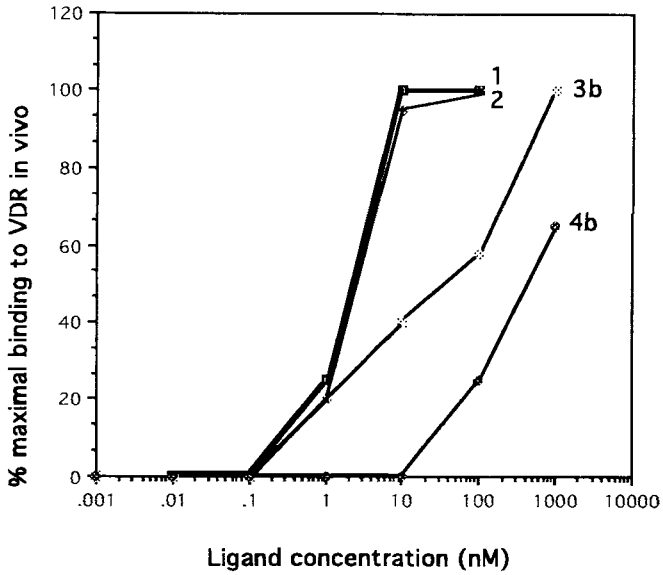


Fig. 4. Saturation analysis of VDR binding sites in intact cells. Human VDR-transfected COS-1 cells were incubated in serum-free medium for 1 h with increasing concentrations of either $1,25D_3$ or analogs. The cells were then washed twice with cold PBS and homogenized, and the number of remaining unoccupied binding sites was determined. The results were expressed as the percentage of binding sites occupied in cells exposed to 10^{-7} M $1,25D_3$.

the hybrid analogs with 20-epi side chains bound to the VDR less efficiently than the hydroxymethyl ligands with natural side chains.

Effect of Hybrid Analogs on VDR Conformation

The assays described above determine affinity to VDR by competition with [^{3}H]$1,25D_3$ and therefore actually assess binding of the analogs to the $1,25D_3$ binding site. However, because of the significant structural changes in the A ring–modified ligands (and the even more pronounced modifications in the hybrid ligands), we must consider the possibility that the actual contact points of these ligands with VDR have shifted. In that case, they may bind strongly to a somewhat different binding site on VDR while competing poorly with $1,25D_3$. This possibility can be tested in two ways. The first involves synthesizing radiolabeled analogs for direct binding analysis, a costly and a time-consuming approach. Another approach is to take advantage of the conformational changes induced in steroid hormone receptors (including VDR) by their natural ligands and analogs. These conformational changes alter the sensitivity of the receptor to protease digestion and can be used to detect direct interaction of synthetic ligands with the receptor as well as to determine if the new ligands modify receptor conformation differently from the natural hormone. In a recent study, we showed that 20-epi analogs and $1,25D_3$ induced different conformational changes in VDR [Peleg et al., 1995]. We linked those conformational changes to enhanced dimerization of VDR with retinoid X receptor and to enhanced DNA binding and transcription [Peleg et al., 1995]. We therefore tested the effect of the 1β-hydroxymethyl compounds on VDR conformation and compared it to that of their 1α-hydroxymethyl counterparts, to $1,25D_3$, and to the 20-epi analogs KH-1060 and MC-1288. In this assay, [^{35}S]methionine-labeled synthetic VDR was incubated with 10^{-6} M $1,25D_3$, KH-1060, and MC-1288 or with 10^{-6} M A ring–modified analogs and then digested with trypsin for 10 min. The proteolytic products were then separated by gel electrophoresis and detected by autoradiography. Figure 5A shows that the proteolytic products of $1,25D_3$-treated VDR were 34 and 28 kDa. The digestion of VDR/MC-1288 complexes led to proteolytic products of same sizes and with a similar intensity, but an additional polypeptide of 32 kDa was also detected. The digestion of VDR/KH-1060 complexes led to the same proteolytic products as VDR/$1,25D_3$ complexes, but the 28 kDa fragment was barely detectable. Prominent 34 kDa and weaker 28 kDa fragments were seen with the two 1β-hydroxymethyl analogs (Fig. 5B), but the intensity of the 34 kDa band was significantly less than that

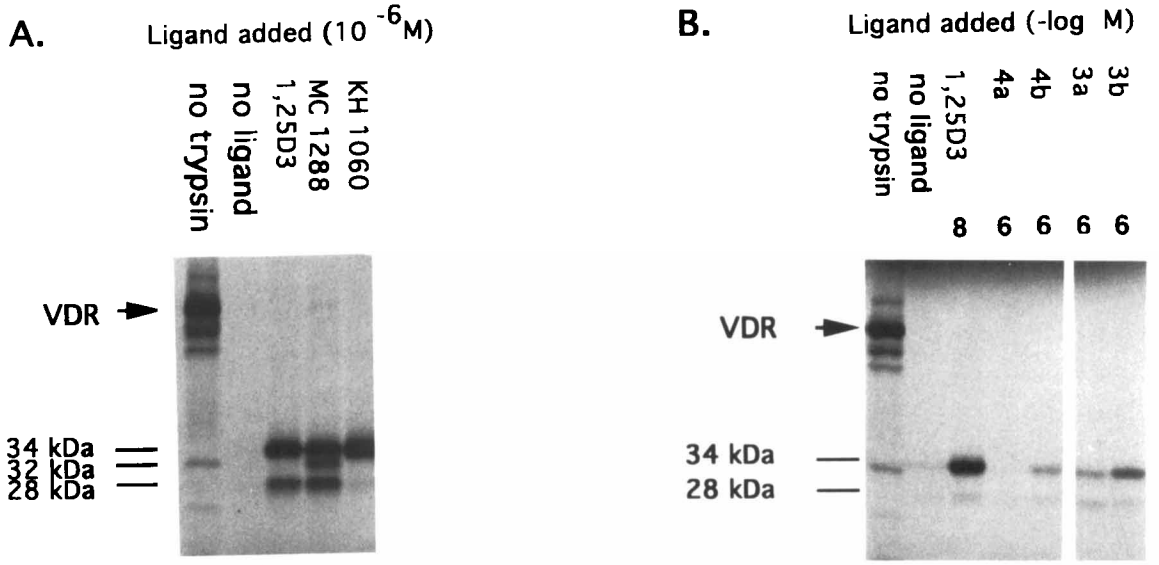


Fig. 5. Ligand-induced stabilization of VDR against trypsin digestion. In vitro–translated VDR labeled with [^{35}S]methionine was incubated without or with 1,25D_3 and the 20 epi analogs MC-1288 and KH-1060 (10^{-6} M) (**A**) or the A ring–modified analogs (10^{-6} M) (**B**) before digestion with 20 μg/ml trypsin. The digestion products were analyzed by sodium dodecyl sulfate gel electrophoresis. The undigested VDR (*arrow*) and the ligand-dependent proteolytic products 34 kDa, 32 kDa, and 28 kDa are indicated.

induced by the ligands with natural A rings. At a concentration of 10^{-6} M, analog 3b induced more of the 34 kDa fragment than did the 1β-hydroxymethyl hybrid (4b). The 1α-hydroxymethyl diastereomer (3a) induced less proteolytic protection of VDR than 3b did, but its hybrid with a 20-epi side chain (4a) had completely lost this activity. Therefore, we conclude that these results correlate with the data from the binding/competition assays, which showed that the order of these ligands' affinity to VDR was 1β-hydroxymethyl analog, 3b > 1α-hydroxymethyl analog, 3a > 1β-hydroxymethyl hybrid analog, 4b > 1α-hydroxymethyl hybrid analog, 4a. We also conclude that hybrid analogs with modified A rings and with 20-epi side chains do not induce different conformational changes in VDR than 1,25D_3 or analog 3b do, although the 20-epi analogs with natural A rings do.

VDR Mediates Transcriptional Activity of Hybrid Analogs

Because the restored growth-inhibitory activity of the hybrid analog was not associated with better uptake by the cells or with better binding to the VDR, we considered the possibility that its enhanced growth inhibition was not mediated through the nuclear VDR. To test whether the VDR was activated by the A ring–modified analogs, we transfected ROS 17/2.8 cells with a thymidine kinase/growth hormone reporter gene containing the human osteocalcin VDRE [Ozono et al., 1990] and examined the ligand-dependent expression of this gene (Fig. 6A). We found that the 1α-hydroxymethyl compounds (3a and 4a) had little transcriptional activity at a concentration of 10^{-6} M. The analog 3b had detectable transcriptional activity at a concentration of 10^{-7} M and more activity at 10^{-6} M, but it did not reach the maximal activity of 1,25D_3 and KH-1060. On the other hand, the hybrid analog with 1β-hydroxymethyl and 3α-hydroxyl groups (4b) was significantly more active transcriptionally: it had an ED_{50} of 1.5×10^{-8} M. This transcriptional activity was a thousandfold lower than that of KH-1060 and tenfold lower than that of 1,25D_3. We concluded that transcriptional activity through the osteocalcin VDRE correlated better with the dose response for growth inhibition than with receptor binding. Furthermore, although the growth-regulatory responses of analog 3b and hybrid analog 4b could be attributed to the greater stability of these A ring–modified ligands during the 6 day incubation with HL-60 cells, this is not a likely explanation for their transcriptional activity in ROS 17/2.8 cells, because all unbound ligands were removed from the cultured cells after 1 h.

The results of the transfection experiments strongly suggested that all the compounds that

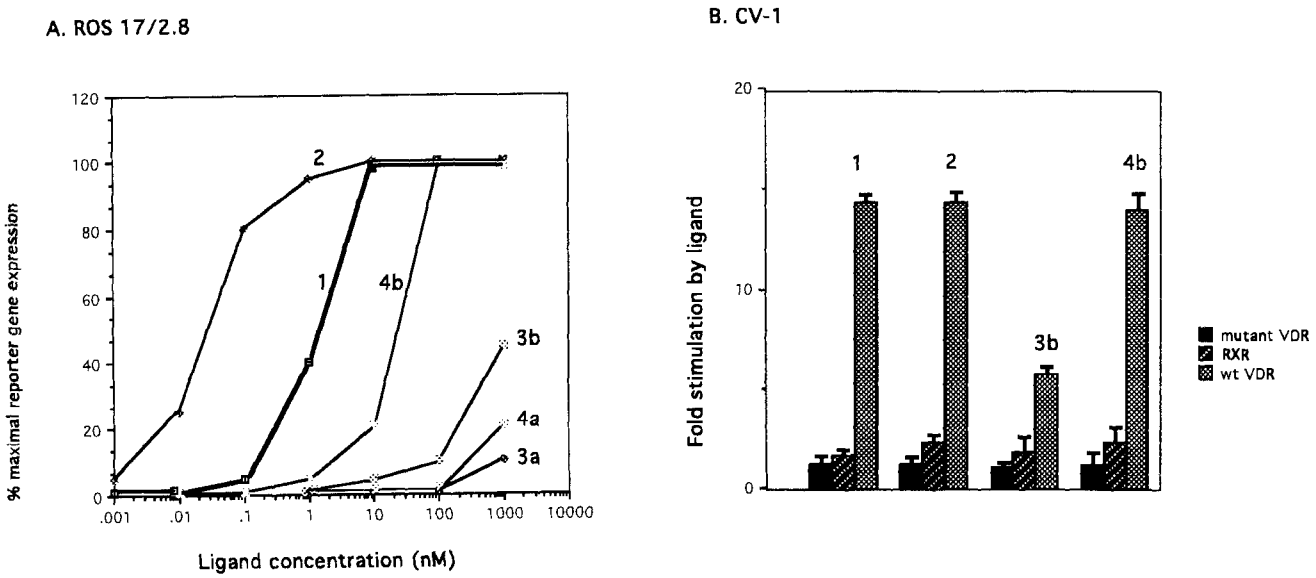


Fig. 6. Transcriptional activities of $1,25D_3$ and its analogs. **A:** ROS 17/2.8 cells were transfected by the DEAE-dextran method with a thymidine kinase–growth hormone fusion gene containing the osteocalcin VDRE. **B:** CV-1 cells were cotransfected with the osteocalcin VDRE and wild-type human VDR expression vector (wt VDR), with a transcriptionally inactive mutant VDR, or with an expression vector for human RXRα. Immediately after transfection (for ROS 17/2.8 cells) or 24 h later (for CV-1 cells), ligand was added to the cells in serum-free culture medium for 1 h and then removed, and the cells were washed twice with PBS, and DMEM with 10% fetal bovine serum was added. Forty-eight hours after transfection, the culture medium was collected and growth hormone levels were determined by a radioimmunoassay. Each point in the dose-response curve (A) and each bar (B) are the average of triplicate transfections. The ligand concentration added to CV-1 cells was 10^{-7} M. The results shown are representative of two to four individual experiments.

had growth-regulatory activity, including the 1β-hydroxymethyl analogs, also induced transcription through VDR. However, because the response element used in these experiments contains two motifs, DR3 and DR6, with blended binding properties for VDR, retinoid X receptor (RXR), thyroid hormone receptor, and retinoic acid receptor [Schrader et al., 1993, 1994], we could not exclude the possibility that nuclear receptor other than VDR is activated through this element by the hybrid compound. To rule out this possibility, we repeated the transfection experiment with CV-1 cells which do not have any detectable VDR and therefore are an ideal model for reconstitution of VDR activities. The CV-1 cells were cotransfected with the osteocalcin VDRE/reporter fusion gene and with the wild-type human VDR expression vector, with a transcriptionally inactive VDR mutant, or the RXR, and the effect of 1β-hydroxymethyl hybrid analogs on reporter-gene expression was examined. Figure 6B shows that transcription through the osteocalcin VDRE was induced by $1,25D_3$, KH-1060, 3b, and the hybrid analog 4b only in the presence of transfected wild-type VDR. We therefore conclude that the transcriptional activity of the two 1β-hydroxymethyl analogs 3b and 4b is definitely mediated through the VDR, but we cannot exclude the possibility that these ligands interact with another cellular component to promote more efficient VDR-mediated transcription.

Effect of the Hybrid Analog 4b on the Stability of VDR/DNA Complexes

Our earlier experiments with 20-epi analogs, including KH-1060, showed that these ligands promoted more efficient dimerization with RXR and this was associated with enhanced binding of VDR complexes to a VDRE. In that system, the analog-dependent binding of VDR complexes to DNA was not proportional to their affinities to VDR. To test whether hybrid analog 4b, which has a 20-epi side chain, maintained this characteristic of the parental compound KH-1060, we performed VDR/DNA interaction assays with cell extracts from VDR-transfected COS-1 cells. The cells were treated for 1 h with 10^{-8} M $1,25D_3$ or KH-1060 and 10^{-6} M 3b and 4b in serum-free medium. The ligands were then removed, and nuclear extracts were prepared immediately or 24 h later. DNA binding was examined by mobility shift assays using the osteopontin VDRE as a probe. Figure 7 shows that VDR binding to DNA was almost completely ligand-dependent. In extracts prepared immediately after ligand treatment, the intensity of the complexes was proportional to the ligand-binding activity of the tested compounds: $1,25D_3$ and KH-1060 induced more DNA-binding activity than the A ring–modified ligands 3b and 4b, and 3b induced more DNA-binding activity than the hybrid analog 4b. Interestingly, however, 24 h after ligand treatment the DNA-binding activity of 3b/VDR complexes was completely gone, whereas the DNA-binding activity of the other ligand/VDR complexes remained unchanged. We conclude that the binding of the hybrid analog with a 20-epi side chain (4b) stabilized the VDR-DNA complexes more effectively than binding of 3b did, although 3b binds to the VDR more avidly. A difference in catabolism of 3b and 4b is not the likely explanation for detection of DNA-binding activity 24 h after ligand removal. The mechanisms for this stabilization could be either a lower dissociation rate of the 4b hybrid analog from the VDR or a slower dissociation rate of the VDR-4b complexes from RXR.

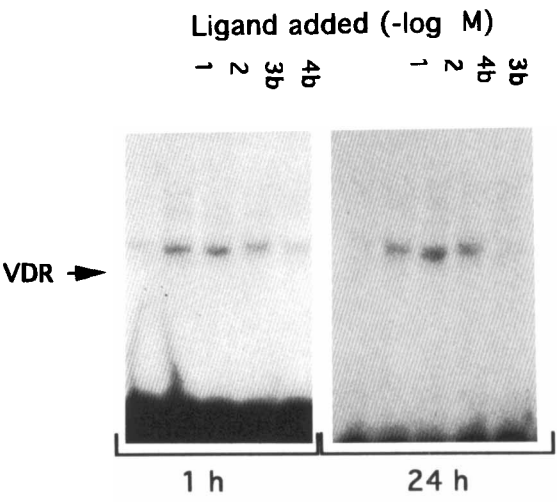


Fig. 7. Effect of the hybrid analog on stability of VDR-DNA complexes. Nuclear extracts were prepared from COS-1 cells transfected with human VDR expression vector and treated with the indicated ligand concentrations for 1 h. The extracts were prepared immediately after ligand removal (**left panel,** 1 h), or 24 h after its removal (**right panel,** 24 h). The extracts were incubated with the radiolabeled osteopontin VDRE, and the VDR-DNA complexes were separated from the free probe by polyacrylamide gel electrophoresis and visualized by autoradiography. un, no ligand was added.

DISCUSSION

This study is an extension of our recent efforts to establish a new approach to designing vitamin D analogs with diminished calcemic activities but with significant growth-inhibitory effects on cancer cells [Posner et al., 1992, 1993, 1994, 1995]. In the study described here we focused on analogs that have both chemical modifications in the A ring that diminish the calcium-regulating activity and side chains with 20-epi configuration that augment growth inhibition and differentiation. Because the A-ring modifications used in this study also diminish binding to the nuclear VDR and growth inhibition, it was not obvious that adding a modified side chain would restore these important biological functions. Unexpectedly, we found that the 20-epi side chain used in these experiments preferentially enhanced the growth-inhibitory and transcriptional activities of 1β-hydroxymethyl 3α-hydroxyl analogs 3b and 4b but not of 1α-hydroxymethyl analogs 3a and 4a. These results raise two questions: why the 1α compounds remain inactive and what the mechanism is by which the 20-epi side chain enhanced the VDR-mediated transcriptional activities of the weak agonist 3b.

The affinity of 3b to the VDR is 30 times greater than that of its 1α-hydroxymethyl counterpart (3a) even though the former has modifications in two hydroxyl groups, at carbons one and three. These findings suggest that the 1β stereochemistry of the hydroxymethyl group compensates for the loss of binding activity (and the biological activities) due to the 3α stereochemistry. The addition of the 20-epi side chain to the 1α-hydroxymethyl analog further reduced its affinity to the VDR by twentyfold, but this same side chain reduced the affinity of the 1β-hydroxymethyl hybrid analog 4b to VDR only threefold to fivefold. This lack of detectable binding of the 1α-hydroxymethyl hybrid analog with the 20 epi side chain (4a) to the VDR may partly explain its lack of biological activity.

It is more difficult to explain why the transcriptional activity of the 1β-hydroxymethyl hybrid with a 20-epi side chain (4b) was greater than that of the parental compound with a natural side chain (3b) although 4b's affinity to the VDR remained very low. The relationship between binding to the VDR and transcriptional activity was clearest when the dose-response curve for the transcriptional activity was aligned with the binding curve (Fig. 8). The two curves for $1,25D_3$ aligned perfectly, but, for the singly modified 1β-hydroxymethyl analog 3b, the dose response for transcription was shifted to the right of the binding curve. Our finding that the DNA binding activity of VDR/3b complexes was very unstable explained the poor transcriptional activity of this analog and strongly suggest that 1) the parental 1β-hydroxymethyl 3b analog is extremely unstable and is rapidly catabolized to even less active byproducts, 2) that the dissociation rate of 3b from VDR is very rapid, or 3) that 3b binds VDR at a site that does not allow efficient transcription activation. In any case, the predicted result is a short half-life for the transcriptionally active VDR/ligand complexes and poor transcriptional activity.

Because the vitamin D analog with a 1β-hydroxyl group acts as an antagonist of certain aspects of $1,25D_3$ action including growth inhibition and differentiation of certain types of leukemic cells [Norman et al., 1993; Bahtia et al., 1995], and because the transcriptional activity of the 1β-hydroxymethyl analog 3b is lower than expected from its binding to VDR, it occurred to us that this analog may also act as an antagonist, either through the VDR or independently of it. However, the 1β-hydroxymethyl compound (3b) did not act as an antagonist of the VDR-mediated transcriptional activity of $1,25D_3$ or of the growth inhibitory and differentiating activities in HL-60 cells (data not shown). Furthermore, it has already been shown that 3b's regulation of VDR-independent calcium channel activities is as effective as that of $1,25D_3$ [Yukihiro et al., 1994]. These findings suggest that there is an advantage to analogs with 1β-hydroxymethyl, 3α-hydroxyl groups: although they have diminished calcium regulating activity in vivo, they probably do not block any of the signal transduction pathways utilized by $1,25D_3$.

The transcriptional dose responses of the 20-epi analogs with natural A rings and of the 1β-hydroxymethyl hybrid 4b were shifted to the left of the binding curve, suggesting that a synergism occurs downstream from ligand binding to the receptor, possibly during self-dimerization or heterodimerization of the VDR with the RXR or during interaction of the VDR with transcriptional coactivators. Our studies of 20-epi analogs with natural A rings clearly indicated that all of them increased VDR-mediated transcriptional activity in a manner not proportional to their equilib-

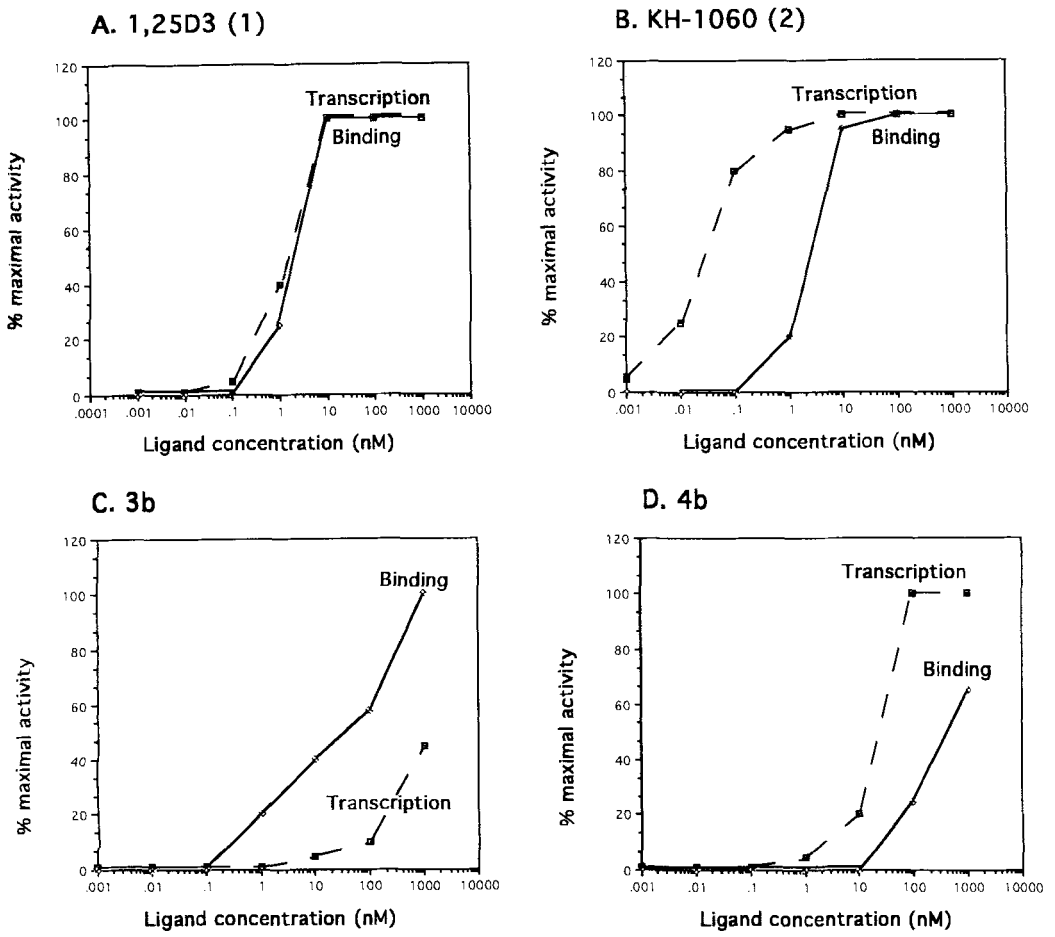


Fig. 8. A comparison of analog-occupied VDR binding sites in vivo and transcriptional activities. The binding data from Fig. 4 are graphed with the transcriptional activities from Fig. 6A.

rium dissociation constants from the VDR [Peleg et al., 1995]. This response pattern to 20-epi analogs was associated with induction of unique conformational changes in the VDR, enhanced dimerization with the RXR, and enhanced binding to VDRE [Peleg et al., 1995]. We predict that some of the unique properties of the 20-epi analogs are maintained in the hybrid. For example, the 20-epi side chain clearly stabilized the DNA-binding activity of VDR/4b complexes, but the hybrid analog 4b did not induce unique conformational changes in VDR monomers in solution as did the parental 20 epi analogs KH-1060 and MC-1288. We are now examining the possibility that the hybrid analog 4b modifies VDR conformation only after self-dimerization or heterodimerization with RXR have occurred.

We must consider the possibility that stability or properties of 4b's metabolites also play a role in the augmented biological activities of this hybrid analog. First, the changed stereochemistry of the side chain must influence its accessibility to the enzyme activities involved in the carbon-24 and carbon-23 oxidation pathways and downstream events that lead to the degradation of these compounds. For example, it has been shown that 24-hydroxylated products of 20-epi 1,25D_3 (MC-1288) accumulate in certain target cells and are not degraded to calcitriol acid [Dilworth et al., 1994]. If these products have biological activities similar to or greater than those of the parental compounds, that would contribute to the enhanced biological activities of the parental compounds. However, while this may have affected the growth-inhibitory assays, which require the continuous presence of ligands in the culture medium, it cannot explain the enhanced transcriptional activity and stabilization of DNA binding of VDR by the 20-epi analogs and by hybrid analog 4b, because in the assays of these processes accumulation of active metabolites was prevented by removal of excess, unbound li-

gands after a short incubation. We hypothesize that the results of these assays reflect more the stability of ligand-receptor complexes than the stability of the ligand itself.

Finally, it is possible that there is a very rapid metabolic process that converts the unbound or even the receptor-bound hybrid analog into a by-product with a high affinity for VDR and great transcriptional activity. However, our preliminary results showed that all the detectable catabolic products of the 1β-hydroxymethyl hybrid analog were inactive (data not shown).

In conclusion, it is possible to partially restore the VDR-mediated activities of A ring–modified ligands by adding a highly potent side chain. This approach is currently being used to develop more potent growth-inhibitory analogs of $1,25D_3$ with diminished calcemic activity by combining other types of powerful side chains with the A-ring modifications used in this study.

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