MOLECULAR ENDOCRINOLOGY

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Mol. Endocrinol. 2006 20:3093-3104 originally published online Aug 10, 2006; , doi: 10.1210/me.2006-0215

0888-8809/06/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 20(12):3093–3104

doi: 10.1210/me.2006-0215

Characterization of Vitamin D Receptor Ligands with Cell-Specific and Dissociated Activity

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Although the main role of 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is to regulate calcium homeostasis, the valuable therapeutic applications of this compound have led to the search of new 1,25-$(OH)_2D_3$-vitamin D receptor (VDR) ligands with less side effects. In this work we have characterized seven 1,25-$(OH)_2D_3$ derivatives (ZK136607, ZK161422, ZK157202, ZK159222, ZK168492, ZK191732, and ZK168289). ZK157202 is an agonist that gives a pattern similar to that of 1,25-$(OH)_2D_3$ or ZK161422 in limited trypsin digestion assays, is able to recruit p160 and VDR-interacting protein 205 coactivators, is as potent as 1,25-$(OH)_2D_3$ to stimulate vitamin D response element-dependent transcription in HeLa cells, and acts as a superagonist in human embryonic kidney 293T cells. This compound is also more potent than the natural ligand to transrepress the activation of the retinoic acid receptor β2 promoter by retinoic acid and the response of the collagenase promoter to 4α-12-*O*-tetradecanoylphorbol 13-acetate. ZK136607, ZK168492, ZK191732, and ZK168289 have a profile similar to that of the partial antagonist ZK159222. They induce an antagonistic-type proteolytic pattern, do not recruit classical coactivators, and have little transactivation potency. However, they act in a cell context-dependent manner because they lack activity in HeLa cells while presenting some agonistic activity in human embryonic kidney 293T cells, or *vice versa*. Furthermore, some of these compounds have a dissociated activity: they cannot transactivate but they are as potent as 1,25-$(OH)_2D_3$ in transrepression assays. Together our results demonstrate the existence of novel VDR ligands with variable biological functions and dissociated activity. They should represent useful tools for studying VDR function and could have therapeutic utility. (*Molecular Endocrinology* 20: 3093–3104, 2006)

MOST OF THE biological actions of 1α, 25-dihydroxyvitamin D_3 [calcitriol, 1,25-$(OH)_2D_3$] are mediated by the receptor VDR (vitamin D receptor), a member of the nuclear receptor superfamily of ligand-dependent transcription factors (1). VDR acts preferentially as a heterodimer with RXR (retinoid X receptor) through binding to specific DNA sequences located at regulatory regions of target genes, referred to as vitamin D response elements (VDREs), normally composed of two copies of the consensus AGGTCA motif arranged as a direct repeat spaced by three nucleotides (DR3). The nuclear receptors exhibit a modular structure with several functional domains. The ligand binding domain (LBD) contains the COOH-terminal activation function (AF)-2 motif responsible for ligand-dependent transcriptional activation. In this process, a critical step in nuclear receptor signaling is the specific ligand-triggered induction of a conformational change within the LBD (2). This conformational change results in ordered recruitment of coactivators. The p160 coactivators [steroid receptor coactivator (SRC)-1/nuclear coactivator 1, transcriptional intermediary factor (TIF)2 or p300/cAMP response element binding protein-binding protein/activator for thyroid hormone and retinoid receptors (ACTR)] have intrinsic histone acetyltransferase activity and recruit additional histone acetyltransferase and histone methyltranferase enzymes that alter chromatin structure and modulate gene transcription (3, 4). The receptors also recruit ATP-dependent chromatin remodeling complexes, in the case of VDR through direct interaction with WSTF, a component of the WINAC complex (5). In addition, the subunit VDR-interacting protein (DRIP)205/thyroid hormone receptor-associated protein 220 (6) of the DRIP/ thyroid hormone receptor-associated protein/Mediator multiprotein complex is also recruited to the core AF-2 receptor region in response to ligand binding. It has been suggested that this complex

First Published Online August 10, 2006

Abbreviations: ACTR, Activator for thyroid hormone and retinoid receptors; AF-2, activation function 2; CAT, chloramphenicol acetyltransferase; c1LPD, conformation 1 of the limited limited protease digestion; DRIP, VDR-interacting protein; GHF-1, GH transcription factor-1; GST, glutathione-*S*-transferase; HEK, human embryonic kidney; LBD, ligand-binding domain; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; RA, retinoic acid; RAR, RA receptor; RARE, RA response element; RXR, retinoid X receptor; SRC, steroid receptor coactivator; TIF, transcriptional intermediary factor; TPA, 4α-12-*O*-tetradecanoylphorbol 13-acetate; VDR, vitamin D receptor; VDRE, vitamin D response element; VDRM, VDR modulator.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

could recruit the holoenzyme of the RNA polymerase II to the target promoter (7).

In contrast to positively regulated genes, the mechanism by which nuclear receptors control the transcription of negatively regulated genes is less well understood. Proposed mechanisms include: competition of nuclear receptors with other transcription factor binding sites (8–10); receptor binding to the so called negative regulatory elements in which the receptor interacts with other factors and recruits corepressors in a ligand-dependent manner (11); direct interactions of nuclear receptors with transcription factors such as the jun component of the AP-1 complex, precluding a productive interaction with coactivators or basal factors; and competition for limiting amounts of transcriptional cofactors (1).

It is widely accepted that the fundamental role of 1,25-$(OH)_2D_3$ is to regulate calcium homeostasis (12). However, many other biological functions of this vitamin have been reported. Indeed, 1,25-$(OH)_2D_3$ plays an important role in promoting cellular differentiation, and in inhibiting the growth of several primary and cultured cancer cell types, including T cell leukemias, breast, prostate, and colon. It has also been proposed that 1,25-$(OH)_2D_3$ and its synthetic analogs could be useful in renal failure, vitamin D-dependent rickets type I, osteoporosis, psoriasis, and certain autoimmune disorders such as multiple sclerosis or type 1 diabetes mellitus, although clinical data proving their efficacy are not yet available (13, 14).

Although these findings suggest new therapeutic possibilities for 1,25-$(OH)_2D_3$, deleterious side effects such as hypercalcemia and soft tissue calcification prevent the use of 1,25-$(OH)_2D_3$ as a therapeutic agent. Therefore, a great deal of effort is being made to develop new 1,25-$(OH)_2D_3$ analogs to dissociate immunosuppressive/growth inhibitory/differentiation properties and calcemic effects (15–18). Very recently, novel noncalcemic, tissue selective, nonsecosteroidal vitamin D receptor modulators (VDRMs) with improved therapeutic indices have been obtained and characterized (19).

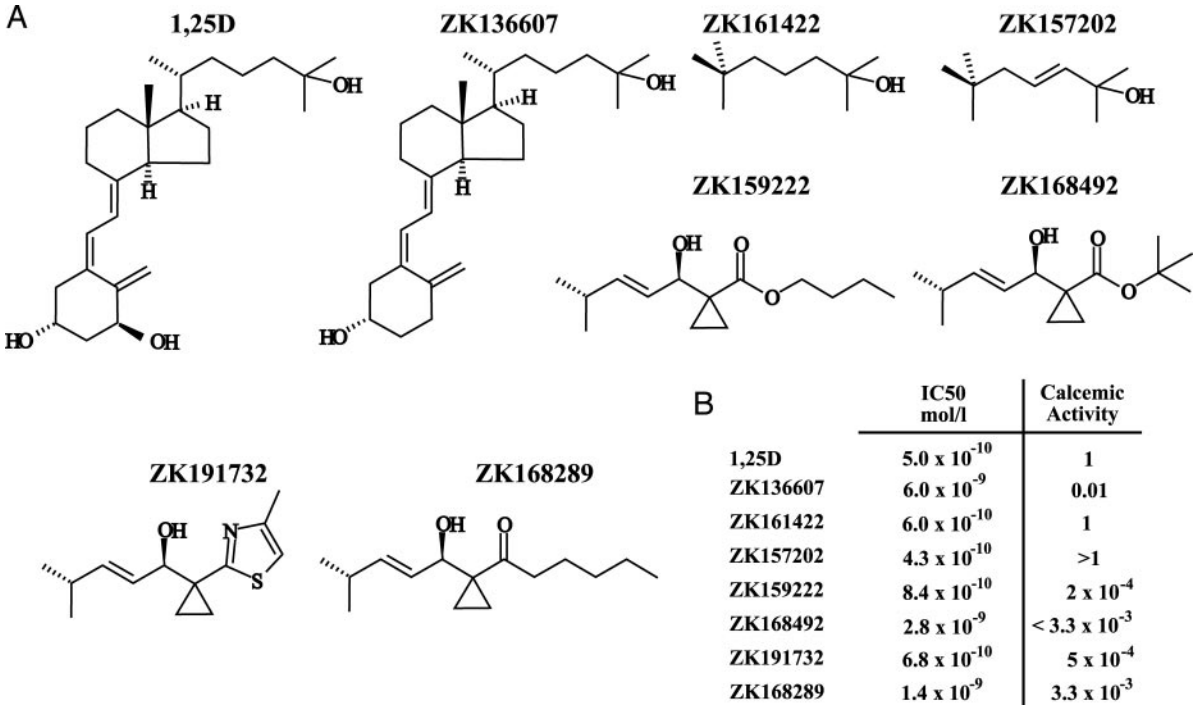
In this study, we have compared the biological actions of seven 1,25-$(OH)_2D_3$ derivatives synthesized by Schering AG (ZK136607, ZK161422, ZK157202, ZK159222, ZK168492, ZK191732, ZK168289). We have analyzed them for their agonistic and antagonistic profile *in vitro* by monitoring the consequences of ligand binding on receptor conformation and on the recruitment of coactivator complexes. We have also studied the effects of these compounds on transactivation and transrepression of target gene promoters in HeLa and human embryonic kidney (HEK) 293T cellular systems. In our study, ZK161422, described as an agonist (20) and ZK159222, described as an antagonist with residual agonistic activity (21), were chosen to compare with the effects promoted by the other compounds and by the natural ligand. Our results show that ZK157202 as well as ZK161422 have a clear agonist profile and that they are even more potent than 1,25-$(OH)_2D_3$ in both transactivation and transrepression. However, other compounds have a profile similar to that of the ZK159222 partial antagonist. They induce an antagonistic-type proteolytic pattern, they are unable to stimulate the recruitment of classical coactivators, and they have little transactivation potency. However, the agonistic effect appears to depend on the cell context, and some of these compounds have a dissociative activity: they cannot transactivate but they are as potent as 1,25-$(OH)_2D_3$ in transrepression assays. These dissociated 1,25-$(OH)_2D_3$ analogs, here identified, are potential pharmacological tools in the treatment and prevention of diseases in which VDRs play a role.

RESULTS

Effect of 1,25-$(OH)_2D_3$ Analogs on Receptor Conformation

The chemical structure, binding affinities and calcemic activity of the 1,25-$(OH)_2D_3$ analogs used are shown in Fig. 1. In an *in vitro* binding assay, unlabeled ZK161422, ZK157202, ZK159222, and ZK191732 bound to VDR with a potency similar to that of 1,25-$(OH)_2D_3$, whereas the other three ligands, ZK136607, ZK168492, and ZK168289, bound the receptor with 1 order of magnitude lower than 1,25-$(OH)_2D_3$. On the other hand, the calcemic activity of ZK161422 was similar to that of the natural ligand, and that of ZK157202 was ever higher, whereas the remaining VDR ligands presented a markedly reduced calcemic activity, measured both as urine calcium levels (Fig. 1) and serum calcium levels (data not shown).

The ability of a ligand to induce transactivation of the nuclear receptor can be described as a combination of affinity, kinetics, and effectiveness at producing an optimal protein conformation that facilitates the interaction with coactivator proteins, which consequently results in stimulation of transcriptional activity through various additional protein-protein interactions. We performed limited protease digestion assays, in which the interaction of a nuclear receptor with its ligand protects the LBD against protease digestion, as a method for characterizing functional VDR conformations. In this assay, VDR was subjected to limited proteolysis with trypsin in the presence of a saturating concentration of 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ analogs (10 μM). 1,25-$(OH)_2D_3$ generates a predominant 28-kDa fragment and a minor 23-kDa fragment represented in Fig. 2A as c1LPD or c3LPD, respectively (conformations 1 and 3 of the limited protease digestion). The agonist ZK161422, as well as the ZK157202 compound, induces the same proteolytic pattern as 1,25-$(OH)_2D_3$. However, the antagonist ZK159222, as well as ZK136607, ZK168492, ZK191732, and ZK168289, generates an additional fragment at 25 kDa (designated c2LPD in Fig. 2A). c1LPD, c2LPD, and c3LPD contain major parts of the LBD and its carboxyl-



B

	IC50 mol/l	Calcemic Activity
1,25D	5.0×10^{-10}	1
ZK136607	6.0×10^{-9}	0.01
ZK161422	6.0×10^{-10}	1
ZK157202	4.3×10^{-10}	>1
ZK159222	8.4×10^{-10}	2×10^{-4}
ZK168492	2.8×10^{-9}	$< 3.3 \times 10^{-3}$
ZK191732	6.8×10^{-10}	5×10^{-4}
ZK168289	1.4×10^{-9}	3.3×10^{-3}

Fig. 1. Structure and Characteristics of 1,25-$(OH)_2D_3$ Analogs

A, Chemical structures of 1,25-$(OH)_2D_3$ and the ZK compounds. Only the side chains that are different from that of the natural ligand are depicted for some of the analogs. B, VDR ligand binding and calcemic activities. VDR ligand binding is expressed as IC_{50} [concentration of ligand required to inhibit the binding of labeled 1,25-$(OH)_2D_3$ by 50%]. For the calcemic activities, the value 1, obtained with 0.03 μg/kg/d of 1,25-$(OH)_2D_3$, is used as a reference. The doses that were equipotent with this concentration of 1,25-$(OH)_2D_3$ are given for the ZK compounds. The maximal concentration used was 10 μg/kg/d. 1,25-$(OH)_2D_3$ is shown as 1,25D.

terminal truncations including from the trypsin-cutting site after arginine 173 to either the carboxy terminus at position 427 (c1LPD), to arginine 402 (c2LPD), or to arginine 391 (c3LPD) (21). The 28- and 23-kDa fragments reported here are thought to be the same previously referred to as the 34- and 30-kDa fragments (22). The 28-kDa (21) or 34-kDa (22) fragments contain a 19-residue portion of the hinge region and the entire LBD. The other shorter fragments have the same N terminus (after the arginine 173 trypsin-cutting site), but result from further trypsinization near the C terminus. An increase in the intensity of these fragments could be explained as a failure of the ligand to coordinate the active closed conformation of the helices 10–12 of the LBD, leaving them more susceptible to proteolytic cleavage. It has been suggested that these shorter fragments are indicative of a transcriptionally inactive state (22). In agreement with others (23), our results suggest that ZK157202 could be a potential agonist because the conformational change in VDR induced by this analog is very similar to that induced by 1,25-$(OH)_2D_3$ and the ZK161422 agonist (in which the 28- or 30-kDa fragments are predominant). In contrast, the proteolytic pattern of VDR observed in the presence of ZK136607, ZK168492, ZK191732, and ZK168289 is similar to that resulting from binding of the antagonist ZK159222 to the VDR. ZK191732 has already been demonstrated to behave as an antagonist of 1,25-$(OH)_2D_3$-induced differentiation of Caco-2 cells (24).

Analysis of VDR-Coactivator Interactions in the Presence of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ Analogs

As an additional approach, we have analyzed the agonistic or antagonistic potential of the 1,25-$(OH)_2D_3$ analogs based on their ability to induce an interaction with coactivator proteins. For this purpose, we have performed glutathione-*S*-transferase (GST) pull-down and supershift assays. GST pull-down assays were performed with bacterially produced GST-TIF2 (624–1287), GST-ACTR (621–821), GST-SRC1 (570–780), and GST DRIP205 (1770–2556) (fusion proteins containing the coactivators nuclear receptor interaction domain) and *in vitro* translated ^{35}S-labeled VDR in the presence of 100 nM 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs (Fig. 2B). Supershift assays were performed with the same coactivator proteins used in the pull-down assays, *in vitro* translated VDR-RXR heterodimers, and a consensus DR3-type VDRE in the presence of a saturating concentration of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs (1 μM) (Fig. 2C).

In both assays, 1,25-$(OH)_2D_3$, the agonist ZK161422, and the ZK157202 compound were able to

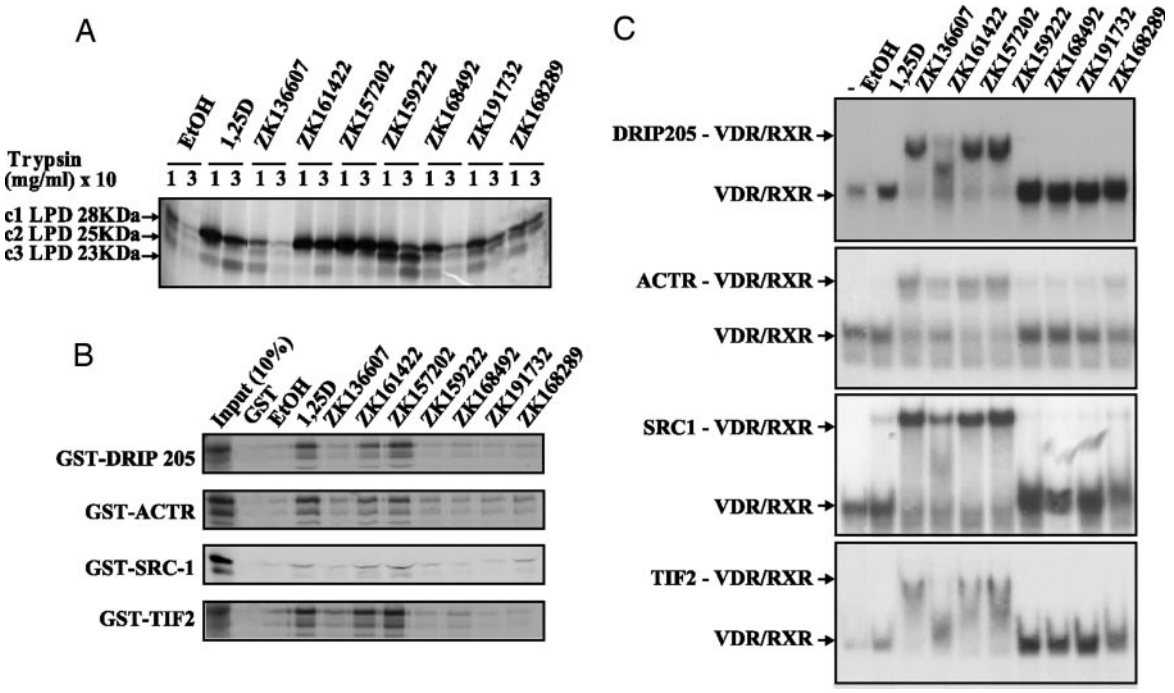


Fig. 2. Effect of 1,25-$(OH)_2D_3$ Analogs on VDR Conformation and Coactivator Recruitment

A, Limited protease digestion assay. *In vitro* translated [^{35}S]VDR preincubated with EtOH (as a negative control), 10 μM of 1,25-$(OH)_2D_3$ (as a positive control), and 10 μM 1,25-$(OH)_2D_3$ analogs (ZK compounds) were digested with two concentrations of trypsin (10 or 30 μg/ml). B, Pull-down assays performed with *in vitro* translated [^{35}S]VDR and the indicated GST coactivators. VDR was incubated with EtOH (as a negative control), 100 nM 1,25-$(OH)_2D_3$ (as a positive control), and 100 nM 1,25-$(OH)_2D_3$ analogs. C, Gel retardation assays were performed with *in vitro* translated VDR/RXR heterodimers that were preincubated in the presence of bacterially expressed GST coactivators with EtOH (as a negative control), 1 μM 1,25-$(OH)_2D_3$ (as a positive control), 1 μM ZK ligands, and the ^{32}P-labeled DR3-type VDRE. 1,25-$(OH)_2D_3$ is shown as 1,25D.

induce the interaction of VDR with the coactivators. In contrast, ZK159222, ZK168492, ZK191732, and ZK168289 were not able to promote significant coactivator recruitment, either in solution or in a complex with RXR on DNA. These results correlate with the conformational change observed in the limited protease digestion assays described above.

Interestingly, ZK136607 was not able to induce interaction of VDR with the coactivators in solution but showed a slight capacity to promote VDR-coactivator interaction in a complex with RXR on DNA. Although this analog stabilized the VDR conformation c2LPD, it is possible that in the presence of RXR and DNA, ZK136607 is able to generate a more agonistic conformation in VDR, which allows a partial recruitment of coactivators by this receptor. This ligand also shows a reduced affinity by VDR as compared with 1,25-$(OH)_2D_3$ in binding assays (see Fig. 1), but the concentration used in the supershift assays is high enough to saturate the receptor; therefore, its reduced capacity of coactivator recruitment cannot be secondary to its decreased binding affinity.

Effect of VDR Ligands on Transactivation Assays

We performed transient transfections with a VDRE-containing heterologous promoter (4×VDRE TK-Luc) in human HEK 293T (Fig. 3A) and HeLa cells (Fig. 3B), transfected with receptors in the presence of graded concentrations of 1,25-$(OH)_2D_3$ or ZK compounds. 1,25-$(OH)_2D_3$ induced reporter activity in a typical dose-dependent manner, achieving the maximal effect (20-fold induction) at 10 nM in HEK 293T cells or at 100 nM in HeLa cells. As expected, the agonist ZK161422 was as potent as 1,25-$(OH)_2D_3$ to transactivate the DR3-containing plasmid. In addition, at low doses, ZK157202 was even more potent than 1,25-$(OH)_2D_3$. This superagonistic effect was more marked in HEK 293T cells, achieving the maximal action at 1 nM. In contrast, the other compounds showed a null or partial agonist activity. As expected, ZK159222 considered as a partial antagonist, only weakly activated the promoter at the highest dose used. ZK136607 showed null (HEK 293T cells) or low activity (Hela cells), and ZK168492 also showed more activity in HeLa than in HEK 293 cells. In contrast, in HEK 293T, but not in HeLa cells, ZK191732 and ZK168289 activated the promoter although always with less potency than 1,25-$(OH)_2D_3$. Although a weaker increase of reporter activity was found in HeLa cells that were not transfected with receptors, the transactivation profile obtained with the different compounds was similar to that shown in Fig. 3B (data not shown), demonstrating

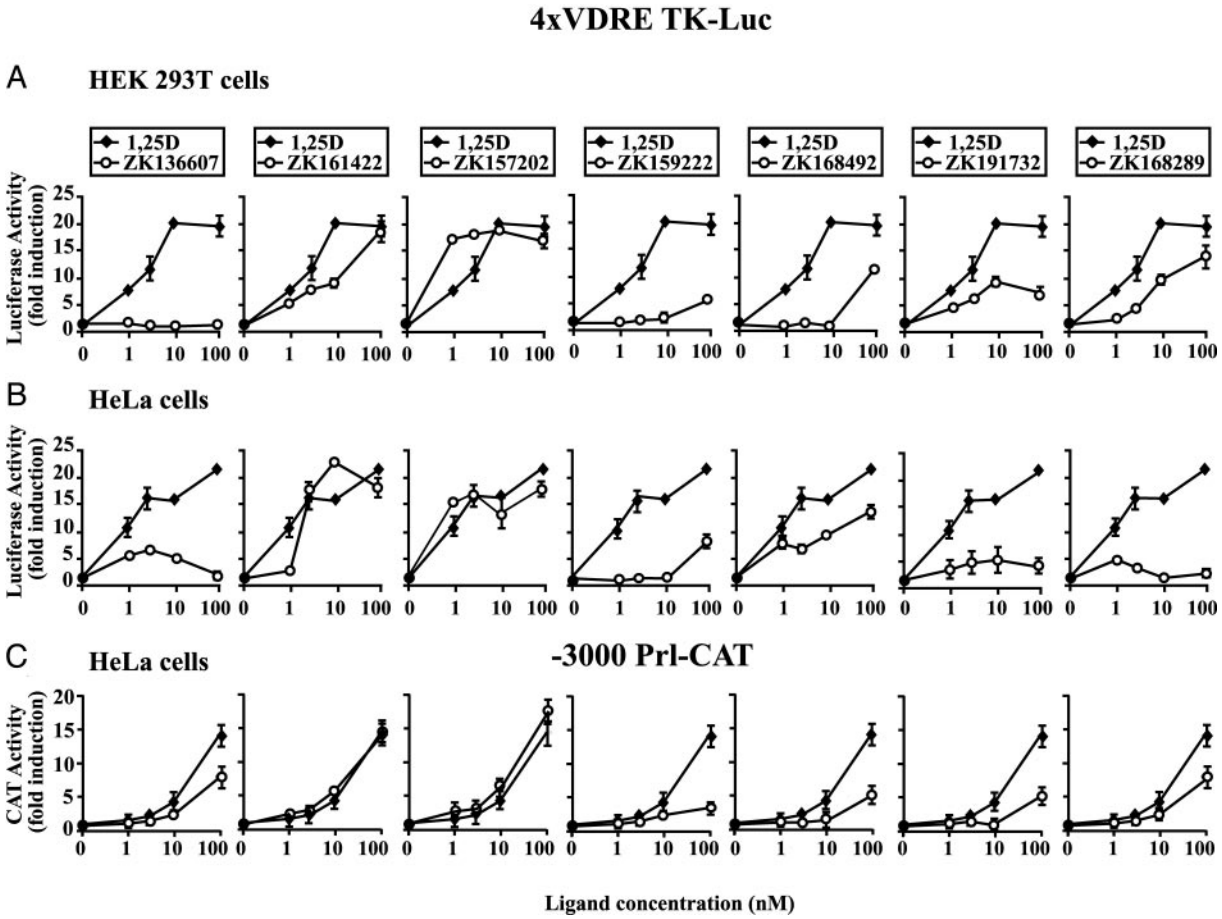


Fig. 3. Influence of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ Analogs on Transactivation

A, HEK 293T cells were cotransfected with 40 ng of 4×VDRE TK-Luc and 12.5 ng of VDR and RXR. B, HeLa cells were cotransfected with 500 ng of 4×VDRE TK-Luc and 80 ng of VDR and RXR. C, HeLa cells were cotransfected with 2.5 μg of −3000 Prl-CAT and expression vectors for the pituitary transcription factor GHF-1/Pit-1 (0.4 μg) and VDR (2.5 μg). Cells were treated for 48 h with graded concentrations of 1,25-$(OH)_2D_3$ (1,25D) and ZK compounds. CAT or Luc activity is expressed as fold induction over the values obtained in EtOH-treated control cells.

that the results obtained are applicable to a situation in which cells express low endogenous receptor levels.

We also performed transient transfections with a prolactin promoter construct (−3000 Prl-CAT), which contains a VDRE (25), as a model to analyze the role of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs on a natural complex promoter (Fig. 3C). Although HeLa cells, a cervical carcinoma cell line, do not express endogenous prolactin, expression of the pituitary-specific transcription factor GHF-1 (GH transcription factor-1)/Pit-1 and VDR causes a marked prolactin promoter stimulation by 1,25-$(OH)_2D_3$ that allows the analysis of transcriptional regulation in this heterologous cell system (25, 26). The dose of VDR ligands required to obtain the maximal activity was higher in the case of the prolactin promoter. Thus, the treatment of HeLa cells with 10 nM 1,25-$(OH)_2D_3$ strongly increased the heterologous promoter (Fig. 3B), whereas this concentration only promoted approximately one third of the maximal prolactin promoter activation (Fig. 3C). This discrepancy could be explained because the amount of transfected VDR used to activate the prolactin promoter in response to 1,25-$(OH)_2D_3$ was 3 times higher than that used to activate the heterologous promoter. ZK157202 was again more potent than 1,25-$(OH)_2D_3$, and the activity of ZK161422, ZK159222, and ZK168492 was similar to that found with the 4×VDRE TK-Luc construct in this cell type. Interestingly, ZK136607, ZK191732, and ZK168289 showed more agonistic potency in the context of the prolactin promoter.

The ability of compounds ZK136607, ZK159222, ZK168492, and ZK191732 to antagonize 1,25-$(OH)_2D_3$-dependent transactivation was tested in HEK 293T cells (Fig. 4, *upper panel*) and that of compounds ZK136607, ZK159222, ZK191732, and ZK168289 in Hela cells (Fig. 4, *lower panel*). In these assays the cells, transfected with the 4×VDRE TK-Luc plasmid, were treated with a maximal concentration (1 μM) of the compounds in the absence and presence of 10 nM of 1,25-$(OH)_2D_3$. In HeLa cells, ZK191732 showed the most significant antagonistic effect, reducing 1,25-

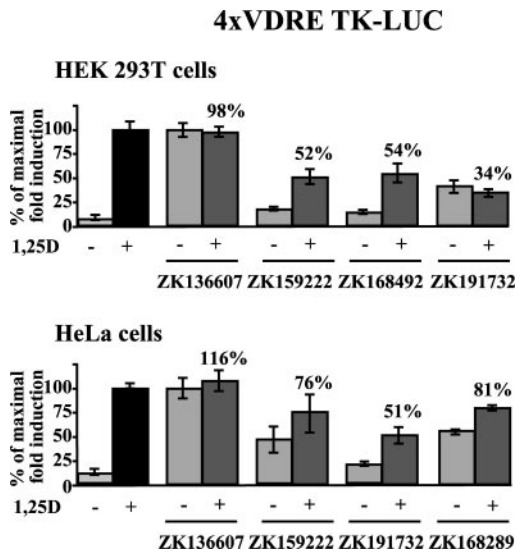


Fig. 4. Antagonism of 1,25-$(OH)_2D_3$-Dependent Transactivation

HEK 293T cells (*upper panel*) and HeLa cells (*lower panel*) were transfected with the 4×VDRE TK-Luc reporter as in Fig. 3. Cells were treated with 1 μM of the ZK analogs indicated in the absence and presence of 10 nM 1,25-$(OH)_2D_3$ (1,25D), and luciferase activity was determined after 48 h. Data are expressed relative to the maximal induction obtained in cells treated with 1,25-$(OH)_2D_3$ alone that were considered as 100%.

$(OH)_2D_3$-dependent promoter activation by 51%, whereas ZK159222 and ZK168289 alone provided approximately 50–60% of the maximal induction of reporter activity, and only a weak antagonistic effect was observed after 1,25-$(OH)_2D_3$ cotreatment. ZK136607, which had a null or very weak agonistic effect at lower doses (see Fig. 3), activated strongly reporter activity at 1 μM and consequently no antagonist effect was observed in the presence of 1,25-$(OH)_2D_3$ in either HEK 293T or HeLa cells. As occurred with the agonistic effects, antagonist potency was also cell context dependent. Thus, ZK159222 and ZK191732 showed more antagonistic activity in HEK 293T cells than in HeLa cells. ZK191732 was the most antagonistic compound in HEK 293T cells, and the combination of this compound with 1,25-$(OH)_2D_3$ resulted in only 34% of maximal induction.

Dissociated Activity of VDR Ligands

We have used the retinoic acid receptor (RAR)β2 (R140-Luc) and collagenase (−73Col-Luc) promoters as models to study transrepression by VDR ligands (Figs. 5 and 6). We have previously shown that 1,25-$(OH)_2D_3$ exerts a repressive effect on retinoic acid (RA)-dependent transactivation of the RARβ2 promoter. Competition for DNA binding site and titration of coactivator proteins are mechanisms suggested to explain this repression (9, 10). In HEK 293T (Fig. 5A) or Hela cells (Fig. 5B), as expected, the agonist ZK161422 and the potential superagonist ZK157202 were able to transrepress even more strongly than 1,25-$(OH)_2D_3$. It was expected that the VDR ligands promoting an incorrect positioning of the AF-2 surface, which does not allow the interaction of VDR with coactivators, should not transrepress RA-dependent transactivation. Surprisingly, the compounds ZK136607, ZK159222, ZK168492, ZK191732, and ZK168289, with null or weak agonistic activity, were also able to promote transrepression. Furthermore, inhibition was more evident at low doses, and some of these compounds with a low transactivation capacity were even more potent than 1,25-$(OH)_2D_3$ to transrepress the RARβ2 promoter. Cell-specific differences in the potency of the VDR ligands to inhibit the retinoic acid (RA) response were also observed. For instance, ZK136607 was more effective than the natural ligand in HeLa cells, whereas it was less potent in HEK 293T cells.

We also analyzed the transrepression exerted by the 1,25-$(OH)_2D_3$ analogs on 4α-12-*O*-tetradecanoylphorbol 13-acetate (TPA)-dependent transactivation of the collagenase promoter in HEK 293T (Fig. 6A) and HeLa cells (Fig. 6B). The results obtained with the −73Col-Luc construct were very similar to those obtained with the RARβ2 promoter in HEK 293T cells: ZK161422 and ZK157202 were more active than 1,25-$(OH)_2D_3$, and all the compounds with an antagonistic profile were able to transrepress the effect of TPA. However, in Hela cells, ZK136607, ZK191732, and ZK168289 transrepressed weakly in comparison with 1,25-$(OH)_2D_3$ and, paradoxically, only at low doses. Thus, the dissociated effect on transrepression *vs.* transactivation also appears to depend on the cellular context.

DISCUSSION

In this study, several 1,25-$(OH)_2D_3$ derivatives have been analyzed for their agonistic or antagonistic potential. Results obtained from the limited protease assays suggest that ZK136607, ZK168492, ZK191732, and ZK168289 could have low agonistic activity because they stabilize the VDR conformation c2LPD, which keeps helix 12 in a displaced position that does not allow an interaction of VDR with coactivators. The latter mechanism, which is based on an incorrect positioning and blocking of the AF-2 domain, has also been suggested for antagonists of other members of the nuclear hormone receptor superfamily, such as the estrogen receptor (27). In contrast with these compounds, ZK157202 appears to stabilize c1LPD even more than 1,25-$(OH)_2D_3$. In the presence of this compound, the shorter fragment c3LPD was only observed when a high dose of trypsin was used. This result suggests that this compound could be a potential superagonist. This agrees with the concept that superagonists are able to stabilize the agonistic conformation for a much longer time than the natural agonist (28).

Although ligand binding increases formation of c1-, c2-, and c3LPD, these bands are also detected in the

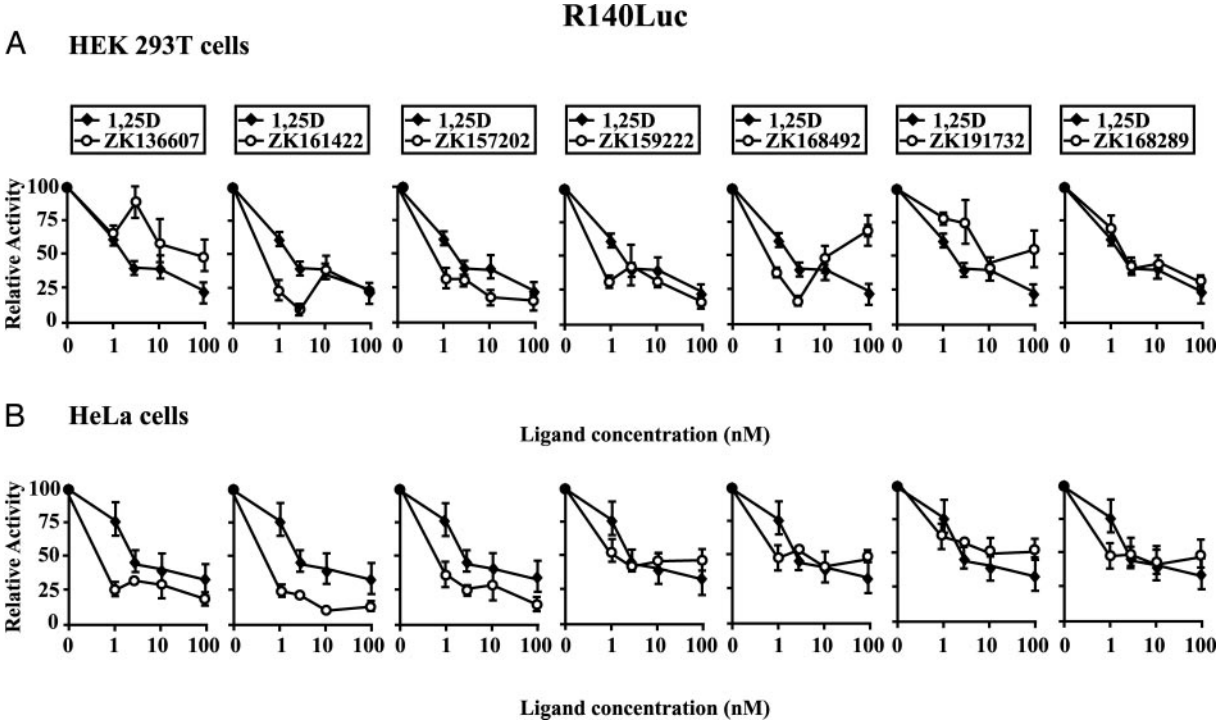


Fig. 5. Influence of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ Analogs on RA-Dependent Transactivation of the RARβ2 Promoter

A, HEK 293T cells were cotransfected with 200 ng of the RARβ2 promoter (R140-Luc), and expression vectors for VDR (12.5 ng), RXR (12.5 ng), and RAR (2.5 ng). B, HeLa cells were cotransfected with 500 ng of the RARβ2 promoter and VDR (80 ng), RXR (80 ng), and RAR (16 ng). After transfection the cells were treated for 48 h with 1 μM RA alone or in combination with increasing concentrations of 1,25-$(OH)_2D_3$ (1,25D) or ZK compounds, as indicated. Results are shown as a percentage of the luciferase activity obtained in the cells treated with RA alone.

absence of ligand, in agreement with the idea that different VDR conformations exist (29). The production of c3LPD has been recently linked to a putative VDR alternative binding pocket that is proposed to be kinetically favored by vitamin D sterols (30). Interestingly, it has been proposed that occupation by an appropriately shaped ligand can lead to the onset of either rapid or genomic VDR-mediated responses (29).

In all crystal structures of VDR bound to agonist ligands, a single conformation of the complex is observed: the position and conformation of the activation helix 12 is strictly maintained (31–33). In this agonistic conformation, precise positioning of helix 12 via the H397-F422 interaction creates a distance of 19Å between the negatively charged E420 on the surface of helix 12 and the positively charged K246 on the surface of helix 3. This charge clamp structure is essential for contacting the LXXLL motif of the NR interacting box of coactivator proteins. In fact, only ZK161422 and ZK157202, but not the other compounds, were able to induce, as 1,25-$(OH)_2D_3$ does, coactivator recruitment in solution and in the presence of RXR and DNA.

On the other hand, it has been suggested that the partial antagonist ZK159222 or the full antagonist ZK168281 adopt an structure in which the last four carbon atoms extend toward helices 3 and 12 and steric contacts are observed with A231 (helix 3) and V418 (helix 12), suggesting that most likely the activation helix will not be optimally positioned (31, 32). Molecular dynamics (MD) simulations of VDR's LBD have also demonstrated that the extended side chain of both antagonists prevents the H397-F422 interaction and places helix 12 in a position in which the distance between residues K246 and E420 deviates from the optimized value of 19Å. This decreases the affinity for coactivators or even makes the interaction impossible. It has been proposed that antagonism by the 26,23-lactone 1,25-$(OH)_2D_3$ analog (TEI-9647) or by compounds ZK159222 and ZK168281, which contain an extended side chain, results from disturbing the helix 12 position (31). This is consistent with our results: ZK168492, ZK191732, and ZK168289 have an extended side chain as does the ZK159222 1,25-$(OH)_2D_3$ antagonist, and none of these compounds were able to induce recruitment of p160 coactivators or DRIP205 either in solution or bound to RXR on DNA. Thus, these VDR ligands could act as antagonists, even though some agonistic activity could be observed at high concentrations. Although ZK136607 has a chemical structure very similar to that of the natural ligand, it has little potency to induce coactivator recruitment and to transactivate. This compound binds VDR with a 10-fold lower affinity than the natural hormone, and a higher concentration appears to be necessary to achieve an agonistic behavior. In fact,

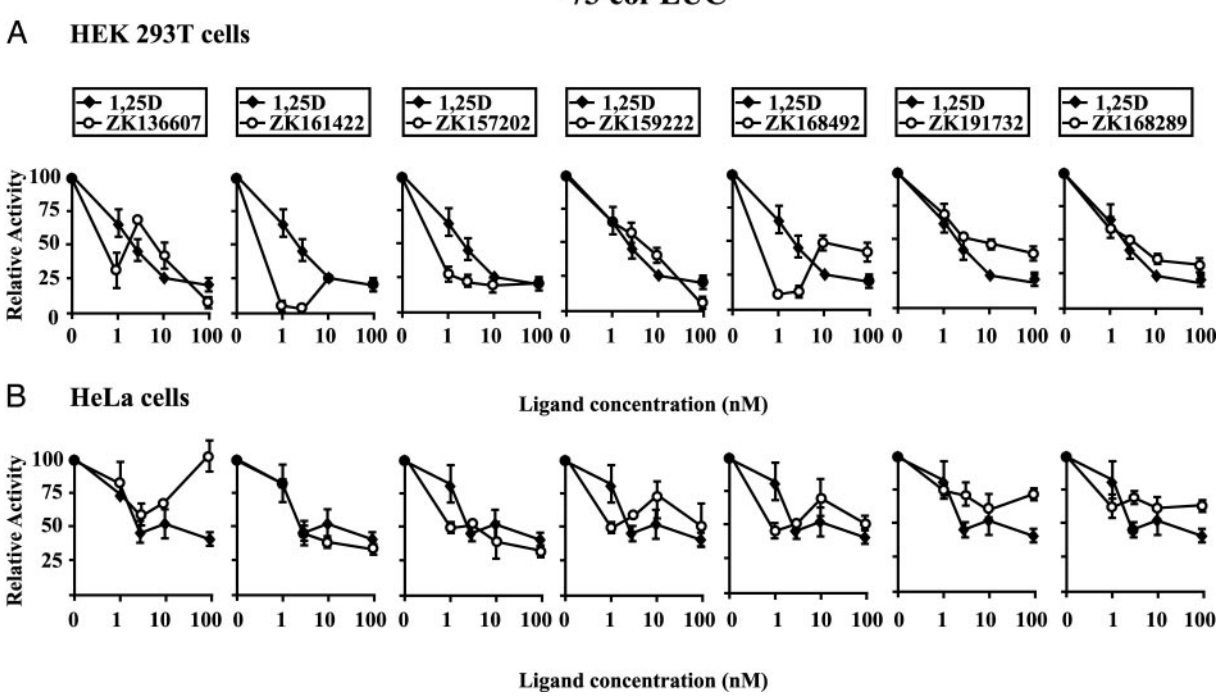


Fig. 6. Influence of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ Analogs on TPA-Dependent Transactivation of the Collagenase Promoter

A, HEK 293T cells were cotransfected with 200 ng of the −73Col-Luc reporter plasmid and 12.5 ng of VDR and RXR. B, HeLa cells transfected with 500 ng of the reporter plasmid and 80 ng of VDR and RXR. After transfection the cells were treated for 48 h with 100 nM TPA alone or in combination with the indicated concentrations of 1,25-$(OH)_2D_3$ (1,25D) or ZK analogs. Data are shown as a percentage of the luciferase activity obtained in cells incubated with TPA alone.

this compound used at 100 nM was unable to promote binding of coactivators to VDR, but at 1 μM induced partial coactivators recruitment by VDR-RXR in the supershift assays (Fig. 2C).

Prevention of VDR-RXR complex formation on DNA could be a mechanism of antagonism in 1,25-$(OH)_2D_3$ signaling. However, strong binding to the VDRE in the presence of the different compounds was found (Fig. 2C), and we have results demonstrating that both 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs equally increase binding of the VDR/RXR heterodimer to DNA (data not shown). Therefore, prevention of complex formation is not the cause for the low transcriptional activity of some ligands. In contrast, the defective interaction with coactivators appears to be the main reason why ZK168492, ZK191732, and ZK168289 have a low potency in comparison with 1,25-$(OH)_2D_3$ to transactivate VDRE reporter genes. In contrast, ZK157202, which induced coactivator recruitment, was even more potent than 1,25-$(OH)_2D_3$ in activating either a heterologous reporter or a natural promoter and could be described as a superagonist. This compound is not metabolized by the C-24 oxidation pathway, which allows it to be retained longer inside target cells, showing a higher biological activity as compared with 1,25-$(OH)_2D_3$ or ZK161422 (23).

Interestingly, some of the VDR ligands used in this study act in a cell specific-dependent manner because they lack activity in HeLa cells while presenting some agonistic activity in HEK 293T cells, or *vice versa*. Furthermore, their ability to antagonize the response to 1,25-$(OH)_2D_3$ is also quantitatively different depending on the cell context. This would describe these compounds as VDRMs. It has been suggested that ligand-selective cofactor recruitment may underlie the novel pharmacological properties of ligands that show preference for osteoblasts over intestinal cells (18). Very recently, tissue-selective nonsecosteroidal VDRMs that function as potent agonists in keratinocytes, osteoblasts, and peripheral blood mononuclear cells, but show poor activity in intestinal cells, have been described (19). Different ligands could induce different conformational changes in the receptor leading to selective coactivator recruitment. For instance, the nonhypercalcemic nonsecosteroidal analogs show differential recruitment of the coactivator, peroxisomal proliferator-activated receptor coactivator 1, to E420A mutant VDR (19). Furthermore, the secosteroidal analog 22-*oxa*-1α,25-dihydroxyvitamin D_3 has been described to induce interaction of VDR with TIF2 but not with SRC-1 or amplified in breast cancer 1 (34). This is not the case with the compounds tested here, which showed a similar profile for the recruitment of different p160 coactivators and DRIP205 (Fig. 2), although the possibility that they could recruit other coactivators selectively cannot be dismissed.

A most important finding in this work was that the ZK136607, ZK159222, ZK168492, and ZK191732

ligands have a dissociated effect, *i.e*. they have low capacity to transactivate, but they have potency to transrepress. Interestingly, these compounds that were able to inhibit activation of the RARβ2 promoter by RA have very little calcemic activity. The RARβ2 promoter contains two RA response elements (RAREs), but only the proximal βRARE appears to be sufficient to confer 1,25-$(OH)_2D_3$-mediated repression (9). We have previously shown that VDR/RXR can bind to the βRARE with high affinity, but without a defined polarity (10). Binding of the heterodimer to this element is transcriptionally unproductive for activating the promoter in response to 1,25-$(OH)_2D_3$, and the competition between active RAR/RXR and inactive VDR/RXR for DNA binding could contribute to transrepression. Accordingly, the VDR ligands tested here could generate a more stable structure of VDR/RXR on the βRARE and reduce RA-dependent transactivation.

The VDR ligands analyzed also behave as VDRMs in transrepression because their potency appears to depend on the cell context. Whereas in HeLa and HEK 293T cells all compounds were able to repress the RA response (Fig. 5), cell-specific differences have been also observed. Thus, ZK136607 was more active in HeLa than in HEK 293T cells, being even more potent than 1,25-$(OH)_2D_3$ in blocking the RA response. Thus, in addition to DNA binding competition, other mechanism/s must contribute to the inhibition, among which cell-specific differences in the metabolism of the compounds could play a role. The finding, that the VDR AF-2 domain seems to be required for the dominant negative activity of VDR (9), suggests that titration of coactivators may also be involved in the inhibition of the RA response by VDR ligands. However, all the ZK compounds, even those with an antagonistic profile that do not induce coactivators recruitment, were able to transrepress the RA response. This finding suggests that other still unidentified cofactors that bind to both RAR and VDR could be involved in the transrepression by 1,25-$(OH)_2D_3$ in HeLa and HEK 293T cells. Furthermore, these unidentified cofactors, as opposed to the classical coactivators, do not appear to require an intact AF-2 surface to mediate transrepression. In agreement with this idea, it has been shown recently that β-catenin interacts with and activates VDR in a ligand-dependent manner, under conditions in which other coactivators do not. 1,25-$(OH)_2D_3$ induces interaction between β-catenin and the AF-2 VDR point mutant E420Q (35), which has very diminished capacity to bind classical coactivators (34). Moreover, the partial antagonist ZK 159222 was also able to induce β-catenin recruitment by VDR (35).

The noncalcemic VDR ligands examined were also able to repress the response of the AP-1-containing collagenase promoter to TPA. Again, there is not a clear correlation between their agonistic activity on a VDRE and their capacity for AP-1 transrepression, demonstrating that molecular determinants governing the transrepressive activity of VDR are likely to be distinct from those ruling its transactivation potential. Furthermore, also in this case the inhibitory effect of some of the ZK compounds was more marked in HEK 293T than in HeLa cells, showing that they can function as cell-specific VDR modulators. Because the AP-1 complex regulates the expression of several genes involved in oncogenic transformation and cellular proliferation, there is considerable interest in the identification of compounds able to down-regulate AP-1 activity and thereby oppose unregulated cell growth. A number of ligands for nuclear receptors display such AP-1-repressive activity, which seems to be the basis for their beneficial therapeutic effects. That the VDR ligands analyzed here are unable to stimulate transcription efficiently but have anti-AP-1 activity is a novel finding for 1,25-$(OH)_2D_3$ analogs, but has been already described for dissociated glucocorticocoids (37) and retinoids (38–42), which can inhibit AP-1-dependent transcription, while only weakly activating GRE- or RARE-based reporter genes. These compounds could have an added therapeutic interest because they could be devoid of the deleterious side effects secondary to activation of genes containing hormone response elements.

In summary, we report here the characterization of VDR modulators that have not only cell-selective effects, but also have dissociated activity that distinguishes between transactivation and transrepression. Such compounds may be a valuable tool for studying molecular mechanisms of VDR signaling and, due to their low calcemic activity, they could be promising therapeutic agents.

MATERIALS AND METHODS

VDR Analogs

The chemical names for the 1,25-$(OH)_2D_3$ derivatives used were the following: ZK136607, (5Z,7E)-(3S)-9,10-seco-5,7,10(19)-cholestatriene-3,25-diol; ZK 157202, (5Z,7E,23E)-(1S,3R)-20-methyl-9,10-secocholesta-5,7,10(19),23-tetraene-1,3,25-triol; ZK159222, (5Z,7E,22E)-(1S,3R,24R)-1,3,24-trihydroxy-26,27-cyclo-9,10-secocholesta 5,7,10(19),22-tetraene-25-carboxylic acid butyl ester; ZK161422, (5Z,7E)-(1S,3R)-20-methyl-9,10-secocholesta-5,7,10(19)-triene-1,3,25-triol; ZK168289, (5Z,7E,22E)-(1S,3R,24R)-25-(1-oxohexyl)-26,27-cyclo-9,10-secocholesta-5,7,10(19),22-tetraene-1,3,24-triol; ZK168492, (5Z,7E,22E)-(1S,3R,24R)-1,3,24-trihydroxy-26,27-cyclo-9,10-secocholesta-5,7,10(19),22-tetraene-25-carboxylic acid 1,1-dimethyl ester; and ZK191732, (5Z,7E,22E)-(1S,3R,24R)-25-(1-methylthiazole-2-yl)-26,27-cyclo-9,10-secocholesta- 5,7,10(19),22-tetraene-1,3,24-triol.

VDR Binding Assay

The affinity of the 1,25-$(OH)_2D_3$ analogs was determined in a competition ligand-binding assay as previously described (43). Briefly, a VDR preparation was incubated with [methyl-^{3}H]1α25-vitamin D_3 (1,25-$(OH)_2D_3$) in the presence of increasing concentrations of unlabeled 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ analogs. Bound and unbound material was

separated by absorption of the free ligand to dextran-coated charcoal. The radioactivity remaining was counted, and binding data are expressed as the IC_{50} [*i.e.* the concentration of cold ligand required to inhibit 50% of labeled 1,25-$(OH)_2D_3$ binding].

Calcemic Activity

For measurement of total calcium levels, female mice 8–12 wk old were treated with compounds by sc injection for 5 d, and urine and serum were collected 24 h after compound administration. The vehicle-treated group served as the control. For measurement of total calcium, samples were diluted 1:2 with H_2O and analyzed for calcium (millimoles/liter) by flame photometer AFM 5051 (Eppendorf, Hamburg, Germany) against a standard curve prepared from a standard solution containing 5 mM calcium (Eppendorf) as previously described (43). Calcemic activity of the different VDR ligands was expressed relative to that of 1,25-$(OH)_2D_3$. Urine calcium levels in control animals were significantly elevated (from 2 to 6 mmol/liter) after treatment with 0.03 μg/kg/d of 1,25-$(OH)_2D_3$. Significant increases of serum calcium after 1,25-$(OH)_2D_3$ administration (from 2.7 to 3.0 mmol/liter) were found with 0.1 μg/kg/d (43). The maximal concentration of 1,25-$(OH)_2D_3$ and ZK analogs used was 10 μg/kg/d.

Expression Vectors and Transfections

Expression vectors for wild-type and mutant human RXRα, VDR, and RARα are cloned in pSG5 (9). The constructs GST-ACTR, GST-TIF-2, GST-SRC-1, and GST-DRIP205 code for the nuclear receptor-interacting domains of these proteins. These plasmids have already been described (36). The −3000 PRLCAT plasmid containing the 5′-flanking region of the rat prolactin promoter fused to choramphenicol acetyl transferase (CAT) was also previously described (25, 26). This reporter (5 μg) was transfected in HeLa cells by calcium phosphate in p60 dishes. The cells were cotransfected with 2.5 μg of VDR and 0.4 μg of the GHF-1/Pit-1 transcription factor. In 4×VDRE TKLuc construct the luciferase reporter gene is driven by four copies of DR3-type VDRE from the rat ANF gene promoter (20). The R140-Luc construct contains the fragment −124 to +14 of the human RARβ2 promoter, and the construct −73Col-Luc contains the collagenase promoter fused to luciferase. These plasmids were also cotransfected with VDR and RXR as is described for 4×VDRE TK-Luc. HEK 293T cells, grown in 24-well plates, were transfected with 40 ng of 4×VDRE TK-Luc, 200 ng of R140-Luc, or 200 ng of −73Col-Luc, and the expression vectors for VDR (12.5 ng), RXRα (12.5 ng), or RARα (2.5 ng) as indicated in the figure legends. HeLa cells, also grown in 24-well plates, were transfected with 500 ng of 4×VDRE TK-Luc, R140-Luc or −73Col-Luc and VDR (80 ng), RXRα (80 ng), and RARα (16 ng), as indicated in the figures. In all cases, after transfection cells were plated in medium containing hormone-stripped serum and, after an overnight incubation, treatments were started, and luciferase or CAT activity was determined after 48 h. Experiments were performed with triplicate cultures, and each experiment was repeated at least three times. Data are represented as means ± SDS.

Limited Proteolytic Digestion

Limited proteolytic assays were performed as described (36). *In vitro*-translated [^{35}S]VDR was incubated in the presence of ethanol or 10 μM 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ analogs for 20 min at room temperature. The receptors were then incubated for 2 min with 10 or 30 μg/ml of trypsin. Proteolysis was stopped by adding SDS sample buffer, and the proteolytic fragments were separated by SDS-PAGE in a 12% polyacrylamide gel and identified by autoradiography.

Gel Retardation Assays

Mobility shift assays were performed with 2.5 μl of *in vitro*-translated VDR and RXR in the presence and absence of 400–600 ng of recombinant GST-fused SRC1, ACTR, TIF 2, or DRIP205 and the consensus DR3 oligonucleotide 5′-AGCTCAGGTCAAGGAGGTCAG-3′ as previously described (25). 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs were used at 1 μM.

GST Pull-Down Assays

Protein-protein interactions were performed with 5 μl of *in vitro* translated [^{35}S]VDR and the fusion proteins of GST-ACTR, GST-TIF-2, GST-SRC-1, or GST-DRIP205, or GST as a control (25, 26). Fifteen minutes before and during the binding reaction, [^{35}S]VDR is incubated in the presence of 100 nM 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ analogs. The bound proteins were analyzed by SDS-PAGE in a 10% polyacrylamide gel and identified by autoradiography.

Acknowledgments

Received May 22, 2006. Accepted July 31, 2006.

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This work was supported by the Spanish Grants BFU2004 03165 from the Ministerio de Educación y Ciencia and FIS C03/10, and from the European Grant CRESCENDO (FP-018652). A.I.C was supported by a postdoctoral fellowship of the Ernst Schering Research Foundation.

Disclosure Summary: A.I.C., R.S.-M., A.M.J.-L., A.S, U.Z., and A.A. have nothing to declare.

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