Antagonistic Action of a 25-Carboxylic Ester Analogue of 1α,25-Dihydroxyvitamin D₃ Is Mediated by a Lack of Ligand-induced Vitamin D Receptor Interaction with Coactivators

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A 25-carboxylic ester analogue of 1α,25-dihydroxyvitamin D₃ (1α,25-(OH)₂D₃), ZK159222, was described as a novel type of antagonist of 1α,25-(OH)₂D₃ signaling. The ligand sensitivity of ZK159222, in facilitating complex formation between 1α,25-(OH)₂D₃ receptor (VDR) and the retinoid X receptor (RXR) on 1α,25-(OH)₂D₃ response element (VDRE), was approximately 7-fold lower when compared with 1α,25-(OH)₂D₃. However, ZK159222 was not able to promote a ligand-dependent interaction of the VDR with the coactivator proteins SRC-1, TIF2, and RAC3, neither in solution nor in a complex with RXR on DNA. Functional analysis in HeLa and COS-7 cells demonstrated a 10-100-fold lower ligand sensitivity for ZK159222 than for 1α,25-(OH)₂D₃ and, most interestingly, a potency that was drastically reduced compared with 1α,25-(OH)₂D₃. A cotreatment of 1α,25-(OH)₂D₃ with a 100-fold higher concentration of ZK159222 resulted in a prominent antagonistic effect both in functional in vivo and in vitro assays. These data suggest that the antagonistic action of ZK159222 is due to a lack of ligand-induced interaction of the VDR with coactivators with a parallel ligand sensitivity, which is sufficient for competition with the natural hormone for VDR binding.

The main physiological role of 1α,25-dihydroxyvitamin D₃ (1α,25-(OH)₂D₃)-mediated gene transcription is the regulation of calcium homeostasis and bone mineralization (1), but the nuclear hormone also plays a role in controlling cellular growth, differentiation, and apoptosis (2). Various analogues of 1α,25-(OH)₂D₃, which mainly contain modifications of the side chain, have been developed with the goal to improve the biological profile of the natural hormone for a potential therapeutic application (3). The genomic effects of 1α,25-(OH)₂D₃ and its analogues are principally mediated through the 1α,25-(OH)₂D₃ receptor (VDR) (4), which is a member of the nuclear receptor transcription factor superfamily (5). VDR binds as a heterodimer with the retinoid X receptor (RXR) (6) to specific sequences in promoter regions of 1α,25-(OH)₂D₃ target genes, referred to as 1α,25-(OH)₂D₃ response elements (VDREs) (7). Simple VDREs consist of two hexameric nuclear receptor binding sites, which are commonly arranged as a direct repeat with 3 spacing nucleotides (DRE3-type VDREs) (4). The VDR consists of several functional domains, which includes the DNA-binding domain (DBD) and the ligand-binding domain (LBD). The LBD of the VDR is formed by 12 α-helical structures, of which the last one, helix 12, contains a short transactivation function 2 (AF-2) domain. A critical step in 1α,25-(OH)₂D₃ signaling is the specific ligand-triggered induction of a conformational change within the LBD of the VDR. This conformational change induces the dissociation of corepressors, such as NCoR (8) or Alien (9), and facilitates the interaction with coactivator proteins with members of the p160 family, such as SRC-1/ERAP160/NCoA1 (10, 11), TIF2/Grip-1/NCoA2 (12, 13), and RAC3/AIB1/ACTR/pCIP (14–16). This VDR-coactivator interaction then further facilitates recruitment of other factors to form a larger complex that modulates chromatin structure and initiates transcription (17). This also involves the recently described DRIP/ARC cofactor complexes (18, 19), which appear to contact the VDR and other nuclear receptors preceding their interaction with p160 family of coactivators (20).

For some members of the nuclear hormone receptor superfamily, such as the estrogen receptor (ER) and the progesterone receptor, antagonists in addition to agonists have been known for some time (21). Recently, 25-carboxylic ester and 26,23-lactone 1α,25-(OH)₂D₃ analogues have been described as the first types of VDR antagonists (22, 23). The molecular mechanisms of action of ER antagonists have been explained by incorrect positioning and blocking of the AF-2 domain (24, 25). In contrast, the molecular mechanisms of the antagonistic action of the 26,23-lactone analogue have been explained by reduced VDR-RXR heterodimer complex formation (26).

In this study, a 25-carboxylic ester analogue of 1α,25-(OH)₂D₃, ZK159222, was characterized as a novel type of 1α,25-(OH)₂D₃ antagonist. ZK159222 was not able to promote a ligand-dependent interaction of the VDR with coactivator proteins of the p160 family neither in solution nor in a complex with RXR on DNA. Moreover, functional in vivo assays in HeLa, COS-7, and MCF-7 cells demonstrated that a cotreatment of 1α,25-(OH)₂D₃ with a 100-fold higher concentration of ZK159222 showed a prominent antagonistic effect, which was confirmed with in vitro coactivator interaction assays.
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**EXPERIMENTAL PROCEDURES**

**Compounds**

1α,25-(OH)2D3 and ZK159222 (butyl-7,22E,15,3,24R-1,3,24-trihydroxy-26,27-cyclo-9,10-secocholesta-5,7,10(19),22-tetraene-25-carboxylate) (22) were dissolved in 2-propanol; further dilutions were made in Me2SO. ZK159222 is a 25-carboxylic ester of 1α,25-(OH)2D3; the side chain structure of both compounds is shown in Fig. 1.

**DNA Constructs**

Mammalian Expression Constructs—The full-length cDNAs for human VDR (27) and human RXRα (28) were subcloned into the SV40 promoter-driven pSG5 expression vector (Stratagene, Heidelberg, Germany). These constructs are also suitable for T7 RNA polymerase-driven in vitro transcription/translation of the respective cDNAs.

**VDR-driven Reporter Gene Construct**—Four copies of the DR3-type VDRE from the rat atrial natriuretic factor (ANF) gene promoter (29) were fused with the thymidine kinase (tk) minimal promoter driving the luciferase reporter gene.

**GAL4 Fusion Construct**—The DBD of the yeast transcription factor GAL4 (amino acids 1–147) was fused with the cDNA of the human VDR LBD (amino acids 109–427). For the mammalian one-hybrid assays the luciferase reporter gene was driven by three copies of the GAL4 binding site fused to the tk promoter (8).

**GST Fusion Protein Constructs**—The nuclear receptor interaction domain of the human SRC-1 (spanning from amino acids 673–1106) (14), human TIF2 (spanning from amino acids 646–920) (12), human RAC3 (spanning from amino acids 673–1106) (14) were subcloned into the GST fusion vector pGEX (Amersham Pharmacia Biotech, Freiburg, Germany).

**Limited Protease Digestion Assay**

In vitro translated, 35S-labeled VDR protein (2.5 µl) alone or together with 2.5 µl in vitro translated RXR and 1 ng of unlabeled rat ANF DR3-type VDRE were incubated with ligand for 15 min at room temperature in 20 µl of binding buffer (10 mM Hepes (pH 7.9), 1 mM dithiothreitol, 0.2 µg/ml poly(dI–C), and 5% glycerol). The buffer was adjusted to 150 mM of monovalent cations by addition of KCl. Trypsin (Promega, final concentration 8.3 ng/ml) was then added, and the mixtures were further incubated for 15 min at room temperature. The digestion reactions were stopped by adding 25 µl of protein gel loading buffer (0.25 M Tris (pH 6.8), 20% glycerol, 5% mercaptoethanol, 2% SDS, 0.025% bromphenol blue). The samples were denatured at 85 °C for 3 min and electrophoresed through a 15% SDS-polyacrylamide gel. The gels were dried and exposed to a Fuji MP2040s imager screen. The individual protease-sensitive VDR fragments were quantified on a Fuji FLA2000 reader (Tokyo, Japan) using Image Gauge software (Raytest, Sprockhövel, Germany).

**Gel Shift and Supershift Assay**

In vitro translated VDR-RXR heterodimers were incubated with graded or saturating concentrations of 1α,25-(OH)2D3 and ZK159222 for 15 min at room temperature in a total volume of 20 µl of binding buffer. The buffer had been adjusted to 150 mM by addition of KCl. For supershift assays, approximately 3 µg of bacterially expressed GST-SRC-1, GST-TIF2, or GST-RAC3 fusion protein were included in the incubation. Approximately 1 ng of the 32P-labeled VDRE from the rat ANF promoter (50,000 cpm) was added to the protein–ligand mixture, and incubation was continued for 20 min. Protein-DNA complexes were resolved through 6 or 8% non-denaturing polyacrylamide gels in 0.5 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA (pH 8.3)) and were quantified by phosphorimaging.

**GST Pull-down Assays**

GST pull-down assays were performed by co-incipitation of a 50% GST-TIF2p246–926-Sepharose bead slurry with in vitro translated 35S-labeled VDR and indicated concentrations of 1α,25-(OH)2D3 and ZK159222 in PPI buffer (20 mM Hepes (pH 7.9), 200 mM KCl, 1 mM EDTA, 4 mM MgCl2, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 10% glycerol) for 20 min at 30 °C. GST fusion protein-Sepharose slurries were routinely preblocked in PPI buffer containing bovine serum albumin (1 µg/µl) prior to use in pull-down assays. In vitro translated proteins, that were not bound to GST fusion proteins, were washed away with PPI buffer. GST fusion protein bound VDR was detected by electrophoresis through 10% SDS-polyacrylamide gels and quantified by phosphorimaging.

**RESULTS**

Conformations of the VDR, bound by saturating concentrations (10 µM) of 1α,25-(OH)2D3 or the 25-carboxylic ester analogue ZK159222 (for structures see Fig. 1), were analyzed by limited protease digestion assays, which provided two digestion products, c1LPD and c3LPD, for 1α,25-(OH)2D3 and an additional digestion product, c2LPD, for ZK159222 (Fig. 2A). The VDR fragments c1LPD, c2LPD, and c3LPD have been previously characterized to contain major parts of the LBD and its carboxy-terminal truncations, which represent the functional VDR conformations 1, 2, and 3, respectively (30–33). With monomeric VDR, 1α,25-(OH)2D3 stabilized 50% and ZK159222 33% of all VDR molecules in c1LPD, which is known to be the most ligand-sensitive VDR conformation (30). In the presence of RXR and DNA, i.e., when VDR-RXR heterodimers are formed on a specific VDRE, the amount of c1LPD stabilization is increased to 75 and 48% of VDR input, respectively. The amount of VDR molecules that were in conformations c2LPD and c3LPD did not exceed 20%, both under DNA-independent and DNA-dependent conditions. However, due to the fact that ZK159222 stabilized three VDR conformations, the total amount of ligand-stabilized VDR molecules was equal for both compounds at saturating ligand concentrations. Ligand-dependent gel shift assays were performed with in vitro translated VDR-RXR heterodimers bound to the rat ANF DR3-type VDRE and graded concentrations of 1α,25-(OH)2D3 or ZK159222 (Fig. 2B). A comparable intensity (approximately 30% shifted probe) of dose-dependent VDR-RXR heterodimer complex formation on DNA was observed for both compounds providing EC50 (half-maximal activation) values of 0.14 and 1.0 nM for 1α,25-(OH)2D3 and

![Fig. 1. Structure of 1α,25(OH)2D3 and ZK159222.](http://www.jbc.org/figure)
ZK159222, respectively. For comparison, traditional ligand binding assays using \(^\text{\textsuperscript{3}H}\) labeled 1\(_{\alpha}\),25-(OH)\(_2\)D\(_3\) and purified VDR from pig intestine provided \(K_d\) values of 0.45 and 0.61 nM for 1\(_{\alpha}\),25-(OH)\(_2\)D\(_3\) and ZK159222, respectively (data not shown). Taken together, this series of \textit{in vitro} experiments characterize ZK159222 as an VDR agonist that is 2–10-times less sensitive than the natural hormone 1\(_{\alpha}\),25-(OH)\(_2\)D\(_3\).

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Limited protease digestion assays \((A)\) were performed by preincubating \textit{in vitro} translated \textsuperscript{35}S-labeled VDR alone or in combination with the unlabeled RXR and the unlabeled DR3-type VDRE from the rat ANF gene promoter in the presence of saturating concentrations (10 \(\mu\)M) of 1\(_{\alpha}\),25-(OH)\(_2\)D\(_3\) or ZK159222. After digestion with trypsin, samples were electrophoresed through 15\% SDS-polyacrylamide gels. The amount of ligand-stabilized VDR conformations 1 (c1LPD), 2 (c2LPD), only in case of ZK159222), and 3 (c3LPD) in relation to VDR input was quantified by phosphorimaging. Gel shift experiments \((B)\) were performed with \textit{in vitro} translated VDR-RXR heterodimers that were preincubated at room temperature with graded concentrations of 1\(_{\alpha}\),25-(OH)\(_2\)D\(_3\) or ZK159222 and the \textsuperscript{32}P-labeled DR3-type VDRE from the rat ANF gene promoter. Protein-DNA complexes were separated from free probe through 8\% nondenaturing polyacrylamide gels. The amount of VDR-RXR-VDRE complexes in relation to free probe was quantified by phosphorimaging. Representative experiments are shown. Columns \((A)\) or data points \((B)\) represent the mean of triplicates, and the bars indicate standard deviation. The \(EC_{50}\) values for VDR-RXR-VDRE complex formation were determined from the respective dose-response curves \((B)\).
Fig. 3. ZK159222 does not stimulate VDR-coactivator interactions. GST pull-down assays (A) were performed with in vitro translated 35S-labeled VDR and bacterially expressed GST-TIF2646–926. The VDR was preincubated with saturating (10 μM) concentrations of 1α,25-(OH)2D3 or ZK159222. After precipitation and washing, samples were electrophoresed through 10% SDS-polyacrylamide gels. The percentage of precipitated VDR with respect to input was quantified by phosphor-imaging. Gel shift experiments (B) were performed with in vitro translated VDR-RXR heterodimers that were preincubated with approximately 3 μg of bacterially expressed GST-SRC-1596–790, GST-TIF2646–926 or GST-RAC3673–1106 at saturating (10 μM) concentrations of 1α,25-(OH)2D3 or ZK159222 and the 32P-labeled DR3-type VDRE from the rat ANF gene promoter together with the expression vectors for VDR and RXR (B) as schematically depicted above the respective figures. Cells were treated for 16 h with graded concentrations of 1α,25-(OH)2D3 and ZK159222. Stimulation of β-galactosidase-normalized luciferase activity was calculated in comparison to solvent-induced controls. Each data point represents the mean of triplicates, and the bars indicate standard deviations. The EC50 values for ligand-triggered reporter gene activities were determined from the respective dose-response curves.

Fig. 4. ZK159222 shows reduced potency in vivo. Luciferase reporter gene assays were performed with extracts from HeLa (A) or COS-7 (B) cells that were transiently transfected with a reporter gene construct-driven by three copies of the GAL4 binding site and an expression vector for a GAL4DBDVDRLBD fusion protein (A) or with a luciferase reporter gene construct-driven by four copies of the DR3-type VDRE from the rat ANF gene promoter together with the expression vectors for VDR and RXR (B) as schematically depicted above the respective figures. Luciferase activities were determined from the respective control wells. Each data point represents the mean of triplicates, and the bars indicate standard deviations. The EC50 values for ligand-induced reporter gene activities were determined from the respective dose-response curves.

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43-fold, whereas 1 μM ZK159222 provided less than 20% of this activity (2–8-fold induction). Interestingly, a combined treatment of 10 nM 1α,25-(OH)2D3 and 1 μM ZK159222 resulted in only 27–40% of the activity of 1α,25-(OH)2D3 alone, i.e. in an obvious antagonistic effect of ZK159222 in vivo. Moreover, the remaining reporter gene activity that was obtained by a combined treatment of 10 nM 1α,25-(OH)2D3 with 1 μM ZK159222 is close to the partial agonistic activity of 1 μM ZK159222. The titration of ZK159222 at constant concentrations of 1α,25-(OH)2D3 (10 nM) indicated that a half-maximal antagonistic effect was obtained at 20–30 nM ZK159222, i.e. at concentrations two to three times higher than that of 1α,25-(OH)2D3. This fits well with the difference in EC50 values of 1α,25-(OH)2D3 and ZK159222 (compare Fig. 2B). In GST pull-down assays (Fig. 5D) and supershift assays (Fig. 5E) with GST-TIF2646–926, 100 nM 1α,25-(OH)2D3 mediated precipitation of up to 18% of in vitro translated 35S-labeled VDR protein and 10 nM 1α,25-(OH)2D3 provided a shift of nearly all VDRE-complexed VDR-RXR heterodimers into complexes with TIF22, respectively. In contrast, 10 μM ZK159222 did not provide a significant precipitation of VDR or a supershift of DNA-complexed VDR-RXR heterodimers (as already shown in Fig. 3, A and B). However, the combination of 100 nM 1α,25-(OH)2D3 with 10 μM ZK159222 provided a reduction of VDR precipitation by 40% (Fig. 5D). Moreover, a combination of 10 nM 1α,25-(OH)2D3 with 10 μM ZK159222 resulted in an equal amount of VDR-RXR-VDRE and VDR-RXR-VDRE-TIF22 complexes (Fig. 5E). The supershift experiments (Fig. 5E) have been performed at different doses of ZK159222 and indicated the dose dependence of the antagonistic action of ZK159222.
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FIG. 5. Antagonistic effects of ZK159222 in vivo and in vitro. Luciferase reporter gene assays were performed with extracts from COS-7 (A), HeLa (B), or MCF-7 (C) cells that were transiently transfected with a luciferase reporter gene construct-driven by four copies of the DR3-type.
DISCUSSION

1α,25-(OH)2D3-mediated transactivation is a multistep event, in which the binding of VDR-RXR heterodimers to a VDRE is one of the first critical steps. Interestingly, on this level the 1α,25-(OH)2D3 analogue that was characterized in this study, ZK159222, displayed the profile of a weak VDR agonist that requires an approximate 7-fold higher concentration than that of the natural hormone 1α,25-(OH)2D3 to stabilize VDR-RXR heterodimer complex formation on a DR3-type VDRE. ZK159222 was found to belong to the category of 1α,25-(OH)2D3 analogues that stabilize an additional third functional VDR conformation, which has also been described for some agonistic 20-epi analogues (32, 34). However, under the conditions used in this study agonistic analogues were not found to stabilize conformation c2LPD (data not shown). Like 1α,25-(OH)2D3 and most other potent VDR agonists, and in particular in DNA-bound heterodimeric complexes with RXR, ZK159222 also stabilizes the high affinity VDR conformation c1LPD most prominently (30, 33).

This study confirmed previous reports that VDR interacts in a ligand-dependent fashion with the three members of the p160 family, SRC-1, TIF2, or RAC3 (35, 36). 1α,25-(OH)2D3 stimulated this VDR-coactivator interaction, whereas ZK159222 was found to be unable to induce an interaction between VDR and SRC-1, TIF2, and RAC3, neither with VDR monomers in solution nor with heterodimeric complexes with RXR on DNA. This lack of interaction with coactivators appears to be the reason why the inducibility of ZK159222-stimulated reporter gene activity demonstrated to be very low in comparison to that of the natural hormone 1α,25-(OH)2D3. This effect could be observed in mammalian one-hybrid assays in HeLa cells, in VDRE-driven reporter gene assays performed in COS-7 cells that overexpressed VDR- and RXR-overexpressing COS-7 model system, as well as in the natural 1α,25-(OH)2D3 target cell line MCF-7. A combination of a saturating 1α,25-(OH)2D3 concentration with a 100-fold higher concentration of ZK159222 resulted in a significant antagonistic effect in all these in vitro and in vivo assay systems: the amount of 1α,25-(OH)2D3-induced VDR-coactivator interaction in solution and on DNA was found to be reduced as well as that of 1α,25-(OH)2D3-induced reporter gene activity. A 100-fold higher concentration of ZK159222 appears to be sufficient for effectively competing with the natural hormone in occupying the majority of the VDR molecules in these different experimental systems. This would then result in the observed reduced interaction with coactivators and the subsequent decrease of ligand-induced reporter gene activity.

The antagonistic mechanism described here for ZK159222 is also likely to apply to other VDR ligands. If a compound, which does not necessarily have to be a classical 1α,25-(OH)2D3 analogue, binds with an affinity to the ligand binding cleft of the VDR that is in the order of the EC50 value of the VDR-1α,25-(OH)2D3 interaction, i.e., 0.1 nM, but in parallel does not enable the receptor to interact with coactivators, it may act as an antagonist of 1α,25-(OH)2D3 signaling. This suggests that the transactivation potency of a VDR-binding ligand, i.e., its fold induction, should be taken in relation to its interaction sensitivity with the VDR, i.e., its EC50 value. Structural relatives of ZK159222 also show a low potency in reporter gene assays, but their affinity for the VDR is even lower than that of ZK159222, such that a 1000-fold or higher excess of these compounds is needed to obtain an antagonistic effect (data not shown). Moreover, tissue-specific differences in coactivator expression as well as in analogue metabolism may cause tissue-specific differences in the extent of the antagonistic effects of VDR ligands. However, there may also be other mechanisms of antagonism in 1α,25-(OH)2D3 signaling, e.g., a prevention of VDR-RXR complex formation on DNA, as suggested for the 26,23-lactone 1α,25-(OH)2D3 analogue TEL-9647 (26).

The AF-2 domain in helix 12 was described to be repositioned after ligand binding to the LBD (37) and provides an interface for nuclear receptors together with amino acids of helices 3 and 5 for the binding of coactivators (38). A comparison of the crystal structure of agonist- and antagonist-bound ER suggests that antagonists block AF-2 function by disrupting the topology of the AF-2 surface (24). In analogy to this, one would expect that ZK159222 also stabilizes a VDR conformation that differs from the agonistic conformation c1LPD in which the AF-2 is functionally blocked (25). This could be c2LPD, which is stabilized only by ZK159222 and its antagonistic relatives and not by agonists, if physiological ionic strength conditions are used (data not shown). One could speculate that the rather long side chain of ZK159222 results in an alternative packing arrangement of ligand-binding pocket residues. This may then result in a conformation of the LBD, where helix 12 reaches the static region of the AF-2 surface, which was shown in the case of ER to mimic bound coactivator (25).

In conclusion, the 25-carboxylic ester 1α,25-(OH)2D3 analogue ZK159222 has been characterized in this report as a novel type of VDR antagonist with a partial agonistic character, where the mechanism of antagonistic action is based mainly on a lack of induction of VDR-coactivator interaction.

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VDRE from the rat ANF gene promoter together with the expression vectors for VDR and RXR (A, C) or with a reporter gene construct-driven by three copies of the GAL4 binding site and an expression vector for a GAL4DNAVP fusion protein (B). Cells were treated for 16 h with indicated concentrations of 1α,25-(OH)2D3 or ZK159222 alone and in combination. Stimulation of β-galactosidase-normalized luciferase activity was calculated in comparison with solvent-induced controls. GST pull-down assays (D) were performed with in vitro translated 35S-labeled VDR and bacterially expressed GST-TIF2646–928. The VDR was preincubated with indicated concentrations of 1α,25-(OH)2D3 or ZK159222 alone or in combination. After precipitation and washing samples were electrophoresed through 10% SDS-polyacrylamide gels. The percentage of precipitated VDR with respect to input was quantified by phosphorimaging. Gel shift experiments (E) were performed with in vitro translated VDR-RXR heterodimers that were preincubated with approximately 3 μg of bacterially expressed GST-TIF2646–928 the indicated concentrations of 1α,25-(OH)2D3 and ZK159222, and the 32P-labeled DR3-type VDRE from the rat ANF gene promoter. VDR-RXR heterodimers were separated from free probe through 6% nondenaturing polyacrylamide gels. The amount of VDR-RXR-VDRE or VDR-RXR-VDRE-TIF2 (supershift) complexes in relation to free probe was quantified by phosphorimaging. Representative experiments are shown (D, E). Columns represent the mean of triplicates, and the bars indicate S.D. *** and ** represent p < 0.001 and p < 0.01, respectively, compared with the activity of 1α,25-(OH)2D3 alone.
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