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Different Molecular Mechanisms of Vitamin D_3 Receptor Antagonists

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ABSTRACT

Two structurally different antagonists of the nuclear hormone 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], the 25-carboxylic ester ZK159222 and the 26,23-lactone TEI-9647, have recently been described. In this study, the molecular mechanisms and the efficacy of both antagonists were compared. ZK159222 showed similar potency and sensitivity to 1α,25$(OH)_2D_3$ in ligand-dependent gel shift assays using the vitamin D receptor (VDR), the retinoid X receptor, and specific DNA binding sites, whereas TEI-9647 displayed reduced potency and >10-fold lower sensitivity in this assay system. Limited protease digestion and gel shift clipping assays showed that the two antagonists stabilized individual patterns of VDR conformations. Both antagonists prevented the interaction of the VDR with coactivator proteins, as demonstrated by GST-pull-down and supershift assays; like the natural hormone, however, they were able to induce a dissociation of corepressor proteins. Interestingly, ZK159222 demonstrated functional antagonism in reporter gene assays both in HeLa and MCF-7 cells, whereas TEI-9647 functioned as a less sensitive antagonist only in MCF-7 cells. In conclusion, the two 1α,25$(OH)_2D_3$ analogs act in part via different molecular mechanisms, which allows us to speculate that ZK159222 is a more complete antagonist and TEI-9647 a more selective antagonist.

The pleiotropic endocrine system of the secosteroid hormone 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] is affecting the regulation of calcium homeostasis, bone mineralization, and other cellular functions, such as proliferation, differentiation, and apoptosis (Walters, 1992). The genomic actions of 1α,25$(OH)_2D_3$ are mediated by its nuclear receptor vitamin D receptor (VDR) (Carlberg, 1996), which is a member of the nuclear receptor superfamily (Mangelsdorf et al., 1995). The VDR acts preferentially as a heterodimer with the retinoid X receptor (RXR) (Carlberg, 1996) on specific DNA sequences in promoter regions of 1α,25$(OH)_2D_3$ target genes, referred to as 1α,25$(OH)_2D_3$ response elements (VDREs) (Carlberg, 1995). Simple VDREs consist of two hexameric nuclear receptor-binding sites, which are commonly arranged as direct repeats with three spacing nucleotides (DR3-type VDREs) (Carlberg, 1995). The VDR contains a DNA-binding domain (DBD), which is formed by two characteristic zinc-finger motifs (Glass, 1994), and a ligand-binding domain (LBD), which is formed by 12 α-helical structures. The last of these structures, helix 12, contains a short *trans*-activation function 2 (AF-2) domain (Moras and Gronemeyer, 1998).

VDR-RXR-VDRE complexes are the molecular cores of DNA-dependent 1α,25$(OH)_2D_3$ signaling (Carlberg and Polly, 1998) and the induction of a conformational change within the LBD of the VDR by interaction with its ligand is the most critical step in this signaling process. The major consequences of an agonist-induced conformational change of the VDR are an induction of the dissociation of corepressor proteins, such as NCoR and Alien (Polly et al., 2000), an enhancement of the interaction with RXR (and consequently an increased amount of complex formation with a VDRE) (Quack and Carlberg, 2000b), and a stimulation of the interaction with coactivator proteins of the p160-family, such as SRC-1, TIF2, and RAC3, via the AF-2 domain (Herdick et al., 2000a). The AF-2 domain is repositioned after ligand binding to the LBD (Moras and Gronemeyer, 1998) and provides,

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ABBREVIATIONS: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; VDR, 1α,25-dihydroxyvitamin D_3 receptor; VDRE, 1α,25-dihydroxyvitamin D_3 response element; RXR, retinoid X receptor; DR3, direct repeat spaced by three nucleotides; DBD, DNA binding domain; LBD, ligand binding domain; AF-2, *trans*-activation function-2; TIF2, transcriptional intermediary factor 2; ANF, atrial natriuretic factor; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DOTAP, *N*-[1-(2,3-Dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate.

together with the amino acids of helices 3 and 5, an interface for the binding of coactivators (Feng et al., 1998).

Agonism and antagonism of natural and synthetic nuclear hormones are closely related processes. Molecules that selectively activate or inhibit a specific nuclear receptor are of considerable biological significance and may have important clinical applications. For some members of the superfamily, such as the estrogen, progesterone, and retinoic acid receptors, synthetic antagonists have been known for a while (Fuhrmann et al., 1998). Nearly all of the approximately 2000 known synthetic analogs of $1\alpha,25(OH)_2D_3$ have been characterized as more or less potent VDR agonists, whereas only two types of VDR antagonists are known. These are the 25-carboxylic ester ZK159222 from Schering (Wiesinger et al., 1998; Herdick et al., 2000b) and the 26,23-lactone TEI-9647 from Teijin (Miura et al., 1999; Ozono et al., 1999). ZK159222 seems to act in manner similar to that of estrogen receptor and retinoic acid receptor antagonists, for which crystal structure analysis demonstrated that the bulky ligand extensions push helix 12 of the respective LBDs into an antagonistic position (Shiau et al., 1998; Bourguet et al., 2000). In this antagonistic LBD conformation, the topography of the AF-2 surface is disrupted and the interaction with coactivators is blocked. In contrast, the main antagonistic mechanism of TEI-9647 seems to be a reduced interaction of VDR with its partner receptor RXR and the coactivator SRC-1 (Ozono et al., 1999). This suggests that TEI-9647 induces a different conformation in the VDR than ZK159222.

In this study, the two VDR antagonists, ZK159222 and TEI-9647, were compared directly for their potency and sensitivity in inducing VDR-RXR-VDRE complexes and their ability to stabilize VDR conformations and to induce association with coactivators and dissociation of corepressors. Moreover, the antagonistic potential of both compounds on VDR-mediated gene regulation was compared in two different cell lines. The results demonstrate that the two antagonists act in part via different molecular mechanisms.

Materials and Methods

Compounds. The natural hormone $1\alpha,25(OH)_2D_3$ and its 25-carboxylic ester analog ZK159222 (Wiesinger et al., 1998) were synthesized at the Medicinal Chemistry Department at Schering AG, whereas the 26,23-lactone analog TEI-9647 (Miura et al., 1999) was synthesized at the Teijin Institute for Bio-Medical Research. The side chain structures of all three VDR ligands are shown in Fig. 1. All compounds were dissolved in 2-propanol; further dilutions were made in dimethyl sulfoxide (for in vitro assays) or in ethanol (for cell culture assays).

DNA Constructs. The full-length cDNAs for human VDR (Carlberg et al., 1993) and human RXRα (Levin et al., 1992) were subcloned into the SV40 promoter-driven pSG5 expression vector (Stratagene, Heidelberg, Germany). These constructs are suitable for T_7 RNA polymerase-driven in vitro transcription/translation of the respective cDNAs. The DBD of the yeast transcription factor GAL4 (amino acids 1 to 147) was fused with the cDNA of the human VDR LBD (amino acids 109 to 427). For the mammalian one-hybrid assay, the luciferase reporter gene was driven by three copies of the GAL4 binding site fused to the *tk* promoter (Hörlein et al., 1995); for the reporter gene assays in MCF-7 cells, the luciferase gene was driven by four copies of the DR3-type VDRE of the rat atrial natriuretic factor (ANF) gene promoter fused to the *tk* promoter (Kahlen and Carlberg, 1996). The nuclear receptor interaction domain of human TIF2 (spanning amino acids 646–926) (Voegel et al., 1996) and mouse NCoR (spanning amino acids 1679–2453) (Hörlein et al., 1995) were subcloned into the GST-fusion vector pGEX (Amersham Pharmacia Biotech, Freiburg, Germany).

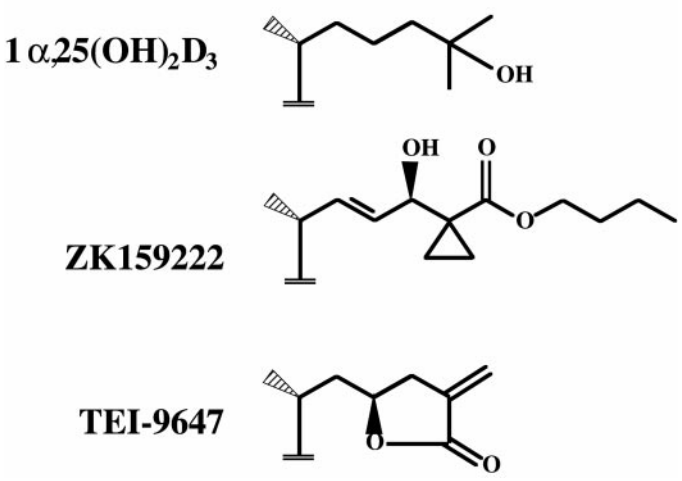


Fig. 1. Side chain structure of $1\alpha,25(OH)_2D_3$ and its antagonistic analogs. ZK159222 is a 25-carboxylic ester and TEI-9647 is a 26,23-lactone of $1\alpha,25(OH)_2D_3$.

In Vitro Protein Translation and Bacterial Protein Overexpression. In vitro translated VDR and RXR proteins were generated by transcribing their respective linearized pSG5-based cDNA expression vector with T_7 RNA polymerase and translating these RNAs in vitro using rabbit reticulocyte lysate as recommended by the supplier (Promega, Mannheim, Germany). Bacterial overexpression of GST-$TIF2_{646–926}$ was facilitated in the *Escherichia coli* BL21(DE3)pLysS strain (Stratagene) by induction with 0.25 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C, whereas expression of GST-$NCoR_{1679–2153}$ was performed with 1.25 mM IPTG for 5 h at 25°C.

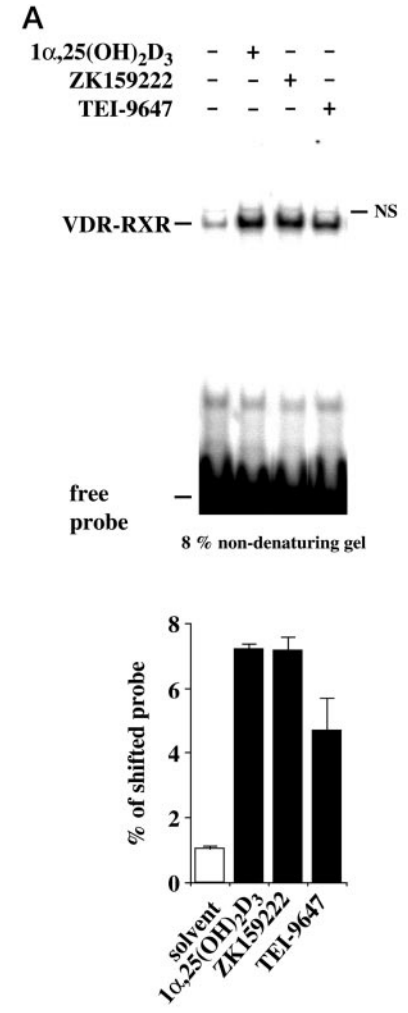
Limited Protease Digestion Assay. In vitro translated, ^{35}S-labeled VDR protein (2.5 μl), nonlabeled RXR (2.5 μl), and 1 ng of unlabeled rat ANF DR3-type VDRE were incubated with ligand for 15 min at room temperature in 20 μl of binding buffer [10 mM HEPES, pH 7.9, 1 mM dithiothreitol, 0.2 μg/μl poly(dI-C) and 5% glycerol]. The buffer was adjusted to 150 mM monovalent cations by addition of KCl. Trypsin (final concentration, 8.3 ng/μl; Promega) or chymotrypsin (final concentration, 16.7 ng/μl; Roche Diagnostics, Mannheim, Germany) was then added and the mixtures were further incubated for 15 or 10 min at room temperature, respectively. 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, and 0.025% bromphenol blue). The samples were denatured at 85°C for 3 min and electrophoresed through 15% SDS-polyacrylamide gels. 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, Supershift, and Gel Shift Clipping Assays. In vitro translated VDR-RXR heterodimers (approximately 5 ng of specific protein) were incubated with ligand for 15 min at room temperature in a total volume of 20 μl of binding buffer, which was adjusted to 150 mM by addition of KCl. Please note that the specific amount of VDR-RXR heterodimers was reduced compared with previous reports (Herdick et al., 2000b,c). For supershift assays, approximately 3 μg of bacterially expressed GST-$TIF2_{646–926}$ fusion protein was included in the incubation. Approximately 1 ng of the ^{32}P-labeled rat ANF DR3-type VDRE (50,000 cpm) was then added and incubation was continued for 20 min. For gel shift clipping assays, the endoprotease chymotrypsin (Roche Diagnostics) was added to a final concentration of 16.7 ng/μl and the incubation was continued for 10 min at room temperature. Pro-

tein-DNA complexes were resolved through 8% nondenaturing polyacrylamide gels in 0.5× Tris/borate/EDTA buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) and were quantified on a Fuji FLA2000 reader.

GST-Pull-Down Assays. GST-pull-down assays were performed by coincubation of a 50% GST-TIF2$_{646–926}$- or GST-NcoR$_{1679–2153}$-Sepharose bead slurry with in vitro translated, ^{35}S-labeled VDR and ligand in incubation buffer (20 mM HEPES, pH 7.9, 200 mM KCl, 1 mM EDTA, 4 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Nonidet P40, and 10% glycerol) for 20 min at 30°C. GST-fusion protein-Sepharose slurries were routinely preblocked in incubation buffer containing bovine serum albumin (1 μg/μl). In vitro translated proteins that were not bound to GST-fusion proteins were washed away with incubation buffer. GST-fusion protein-bound proteins were detected by electrophoresis through 10% SDS-polyacrylamide gels and were quantified on a Fuji FLA2000 reader.

Transfection and Luciferase Reporter Gene Assays. MCF-7 human breast cancer cells were seeded into six-well plates (10^5 cells/ml) and grown overnight in phenol red-free DMEM supplemented with 10% charcoal-treated fetal bovine serum (FBS). Liposomes were formed by incubating 1 μg of the reporter plasmid and in indicated cases each 1 μg of pSG5-based receptor expression vectors for VDR and RXR with 15 μg DOTAP (Roth, Karlsruhe, Germany) for 15 min at room temperature in a total volume of 100 μl. After dilution with 900 μl of phenol red-free DMEM, the liposomes were added to the cells. Phenol red-free DMEM supplemented with 30% charcoal-treated FBS (500 μl) was added 4 h after transfection. VDR ligands were also added at that time. HeLa human cervix carcinoma cells were cultured, seeded, and transfected under the same conditions as MCF-7 cells, but for the mammalian one-hybrid assay, the expression vector for the GAL4$_{DBD}$VDR$_{LBD}$-fusion protein and a GAL4 binding site driven



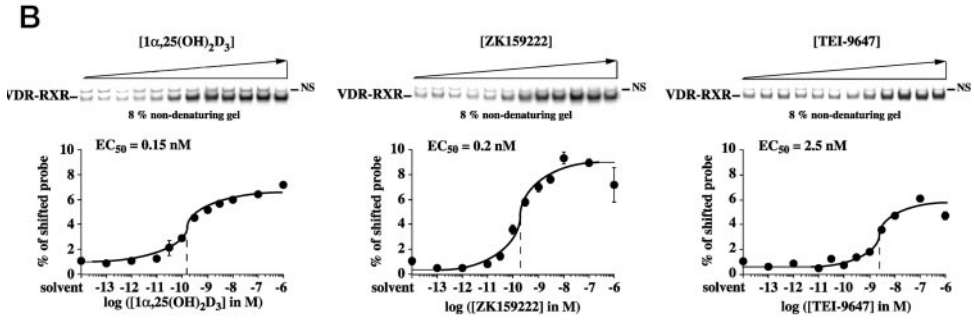


Fig. 2. Induction of VDR-RXR heterodimer complex formation on a VDRE by VDR antagonists. Gel shift experiments were performed with in vitro translated VDR-RXR heterodimers that were preincubated at room temperature with saturating (1 μM, A) or graded (B) concentrations of 1α,25(OH)$_2$D$_3$, ZK159222, or TEI-9647 and the ^{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. Complete gels (A) or VDR-RXR-VDRE complexes (B) are shown as representative experiments. The amount of VDR-RXR-VDRE complexes in relation to free probe was quantified with the use of a Fuji FLA2000 reader. Columns (A) or data points (B) represent the mean of triplicates and the bars indicate S.D. The EC$_{50}$ values for VDR-RXR-VDRE complex formation were determined from the respective dose-response curves (B).

luciferase reporter gene construct were used in transfections. The cells were lysed 16 h after onset of stimulation using the reporter gene lysis buffer (Roche Diagnostics) for both types of assays and the constant light signal luciferase reporter gene assay was performed as recommended by the supplier (Canberra-Packard, Dreieich, Germany). The luciferase activities were normalized with respect to protein concentration and induction factors were calculated as the ratio of luciferase activity of ligand-stimulated cells to that of solvent controls.

Results

Gel shift experiments were performed to compare the effect of the natural VDR agonist 1α,25(OH)$_2$D$_3$ and its antagonistic analogs, the 25-carboxylic ester ZK159222 and the 26,23-lactone TEI-9647 (for side chain structures, see Fig. 1), on VDR-RXR complex formation of the rat ANF DR3-type VDRE (Fig. 2). At saturating concentrations, 1α,25(OH)$_2$D$_3$ and ZK159222 both induced VDR-RXR-VDRE complex formation approximately 7-fold greater than that of the solvent control, whereas TEI-9647 showed a lower induction of 4.5-fold (Fig. 2A). This indicates that the potency of TEI-9647 to induce VDR-RXR-VDRE complex formation is lower than that of ZK159222 and the natural ligand. The dose-dependent stabilization of VDR-RXR-VDRE complexes provided for comparable EC$_{50}$ values of 0.15 and 0.2 nM for 1α,25(OH)$_2$D$_3$ and ZK159222, respectively, whereas TEI-9647 showed a clearly higher EC$_{50}$ value of 2.5 nM (Fig. 2B). This suggests also that the sensitivity of TEI-9647 for inducing VDR-RXR-VDRE complex formation is lower than that of ZK159222 and the natural ligand.

The stabilization of VDR-RXR conformations by 1α,25(OH)$_2$D$_3$, ZK159222 and TEI-9647 was analyzed by gel shift clipping assays that were performed with VDR-RXR heterodimers bound to the rat ANF DR3-type VDRE (Fig. 3). After the digestion with chymotrypsin, two protein-DNA complexes could be discriminated, which are interpreted as representatives of VDR-RXR heterodimer conformations, referred to as c1$_{GSC}$ and c2$_{GSC}$ (Quack and Carlberg, 2000a,b). Interestingly, the natural hormone stabilized 50% of all DNA-binding VDR-RXR heterodimers in c1$_{GSC}$ (gel shift assay as reference), whereas in the absence of ligand, only 15% of the heterodimers were stabilized in c2$_{GSC}$. ZK159222 stabilized 45% of the pool of DNA-bound VDR-RXR heterodimers in c2$_{GSC}$ (i.e., in a different conformation than observed with VDR agonists). Interestingly, TEI-9647 stabilized approximately 15% of all DNA-bound VDR-RXR heterodimers in conformations c1$_{GSC}$ and c2$_{GSC}$.

The effect of 1α,25(OH)$_2$D$_3$, ZK159222, and TEI-9647 on the stabilization of VDR conformations was tested by DNA-dependent limited protease digestion assays using VDR-RXR heterodimers bound to the rat ANF DR3-type VDRE (Fig. 4). In contrast to the gel shift clipping assay (Fig. 3), in limited protease digestion assay, the VDR and not the DNA is radioactively labeled. Digestion with chymotrypsin (Fig. 4A) as well as with trypsin (Fig. 4B) provided up to three digestion products. These three VDR fragments contain major parts of the LBD and are interpreted as the functional VDR conformations 1, 2, and 3, which mediate the agonistic (c1$_{LPD}$) (Herdick et al., 2000a), the antagonistic (c2$_{LPD}$) (Herdick et al., 2000b), and the nonagonistic (c3$_{LPD}$) (Herdick and Carlberg, 2000) action of the receptor, respectively. When using chymotrypsin, the natural hormone stabilized 29% of all VDR molecules in c1$_{LPD}$ and 18% in c3$_{LPD}$, but no receptor molecules in c2$_{LPD}$ (Fig. 4A). In contrast, ZK159222 and TEI-9647 stabilized only 6 to 11% of all VDR molecules in c1$_{LPD}$, but 8% in c2$_{LPD}$ and 12 to 21% in c3$_{LPD}$. By comparison, in the absence of ligand, only 7% of the VDR molecules were stabilized in c1$_{LPD}$ and c3$_{LPD}$ and none in c2$_{LPD}$. Interestingly, the stabilization of the VDR with TEI-9647 resulted in a protein fragment c2$_{LPD}$ with a significantly slower migration than in the case of ZK159222. The digestion with trypsin (Fig. 4B) resulted for 1α,25(OH)$_2$D$_3$ in a stabilization of 60% of all VDR molecules in c1$_{LPD}$ and 9% in c3$_{LPD}$, whereas ZK159222 and TEI-9647 stabilized 21 to 34% in

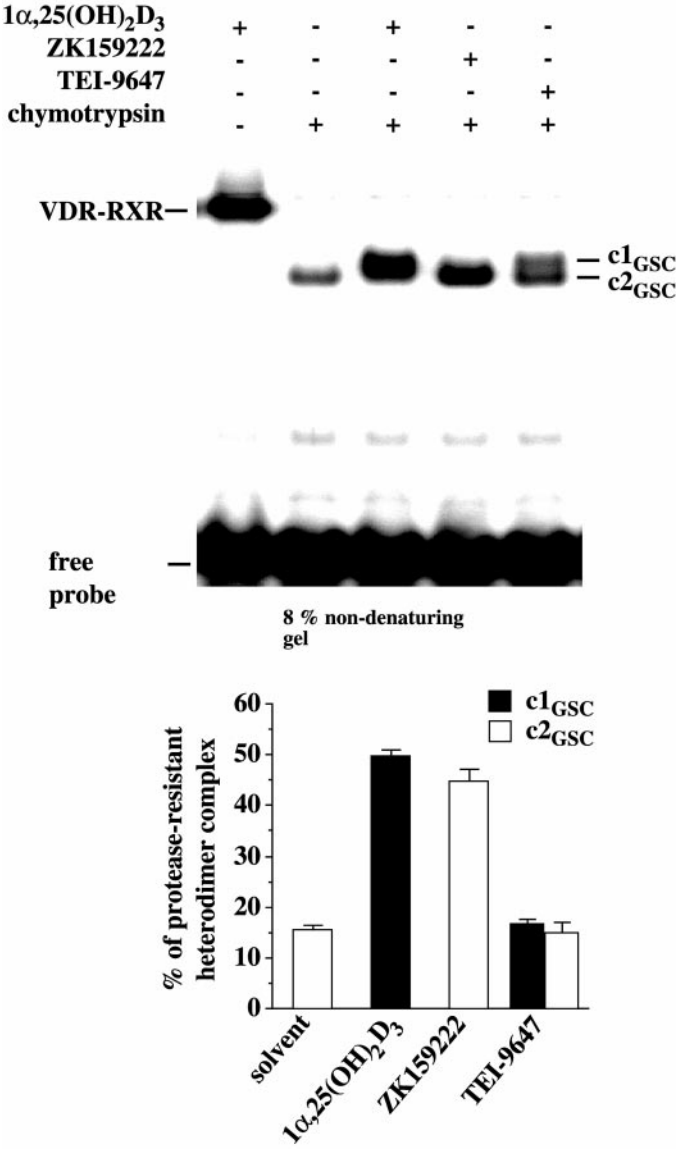


Fig. 3. Stabilization of VDR-RXR heterodimer conformations by VDR antagonists. Gel shift clipping experiments were performed with in vitro translated VDR-RXR heterodimers that were preincubated with saturating concentrations (1 μM) of 1α,25(OH)$_2$D$_3$, ZK159222, or TEI-9647 (or solvent as a control). After digestion with chymotrypsin, VDR-RXR heterodimer conformations were separated from free probe through 8% nondenaturing polyacrylamide gels. Representative experiments are shown. The amount of DNA-complexed VDR-RXR heterodimer conformations 1 (c1$_{GSC}$) and 2 (c2$_{GSC}$) in relation to nondigested VDR-RXR-VDRE complexes (C) was quantified with the use of a Fuji FLA2000 reader. Columns represent the mean of triplicates and the bars indicate S.D.

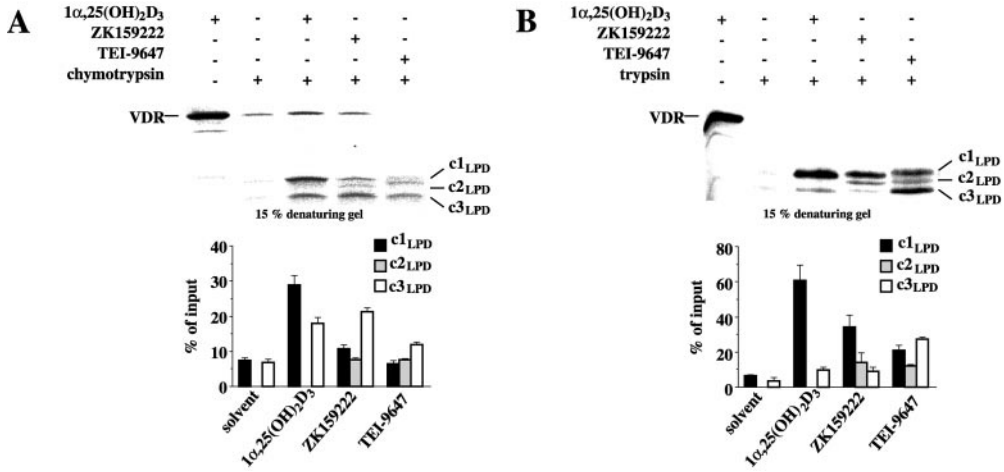


Fig. 4. Stabilization of VDR conformations by VDR antagonists. Limited protease digestion assays were performed by preincubating in vitro translated ^{35}S-labeled VDR with saturating concentrations (1 μM) of 1α,25(OH)$_2$D$_3$, ZK159222, or TEI-9647 (or solvent as a control). After digestion with chymotrypsin (A) or trypsin (B), samples were electrophoresed through 15% SDS-polyacrylamide gels. Representative experiments are shown. The amount of ligand-stabilized VDR conformations 1 (c1$_{LPD}$), 2 (c2$_{LPD}$, only in case of ZK159222 and TEI-9647), and 3 (c3$_{LPD}$) in relation to VDR input was quantified with the use of a Fuji FLA2000 reader. Columns represent the mean of triplicates and the bars indicate S.D.

c1$_{LPD}$, 12 to 14% in c2$_{LPD}$, and 9 to 27% in c3$_{LPD}$. Interestingly, the migration of the VDR fragment that represents c1$_{LPD}$ was clearly slower with TEI-9647 than with ZK159222 or the natural hormone. This is in accordance with a recent publication by Bula et al. (2000). In the absence of ligand, 8% of the VDR molecules were stabilized in c1$_{LPD}$, none in c2$_{LPD}$, and 5% in c3$_{LPD}$.

The modulation of the interaction of the VDR with coactivators and corepressors by 1α,25(OH)$_2$D$_3$, ZK159222, and TEI-9647 was analyzed by GST-pull-down assays (Fig. 5). The assays were performed with bacterial produced GST-TIF$_{646-926}$ and GST-NCoR$_{1679-2453}$ fusion proteins (each containing the nuclear receptor interaction domains). 1α,25(OH)$_2$D$_3$ mediated a precipitation of up to 20% of the VDR input, whereas in the presence of ZK159222 and TEI-9647, the precipitation of VDR protein was not significantly higher than that of solvent control (3%) (i.e., they did not induce any interaction with coactivators) (Fig. 5A). In contrast, like the natural hormone, both ZK159222 and TEI-9647 were able to induce VDR-corepressor dissociation (Fig. 5B). However, even in the presence of saturating concentrations of the natural agonists as well as of the two antagonists, reasonable proportions of VDR-corepressor complexes remained intact.

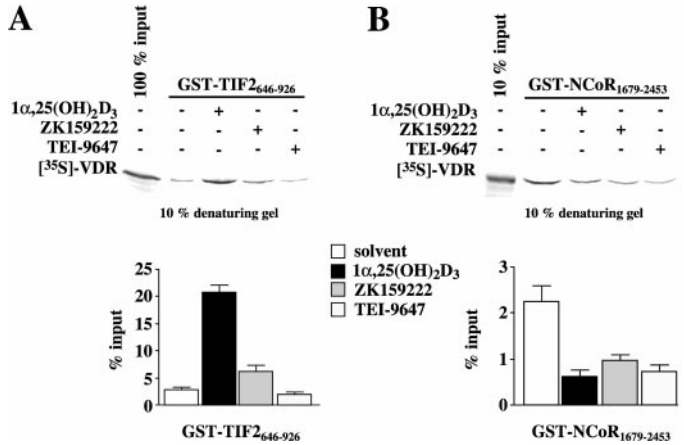


Fig. 5. VDR antagonist triggered interaction of VDR with coactivators and corepressors. GST-pull-down assays were performed with in vitro translated, ^{35}S-labeled VDR and bacterially expressed GST-TIF2$_{646-926}$ (A) or GST-NCoR$_{1679-2453}$ (B). The VDR was preincubated with saturating (1 μM) concentrations of 1α,25(OH)$_2$D$_3$, ZK159222, or TEI-9647 (or solvent as a control). After precipitation and washing, samples were electrophoresed through 10% SDS-polyacrylamide gels. Representative experiments are shown, but please note that the gel in A was exposed for much shorter time than that in B. The percentage of precipitated VDR with respect to input was quantified with the use of a Fuji FLA2000 reader. Columns represent the mean of triplicates and the bars indicate S.D.

To analyze the effect of 1α,25(OH)$_2$D$_3$, ZK159222 and TEI-9647 on the interaction of coactivators with DNA-bound VDR-RXR heterodimers, supershift assays were performed with bacterial produced GST-TIF2$_{646-926}$ fusion proteins (Fig. 6). In the presence of 1α,25(OH)$_2$D$_3$, VDR-RXR-TIF2-VDRE complexes (supershifts) were observed, whereas in the presence of ZK159222 or TEI-9647, no supershifts could be detected. Moreover, this assay confirmed that TEI-9647 shows a lower potential in inducing VDR-RXR-VDRE complex formation than 1α,25(OH)$_2$D$_3$ and ZK159222 (compare Fig. 2).

Finally, functional antagonism of ZK159222 and TEI-9647 was tested in different reporter gene assays using HeLa human cervix carcinoma and MCF-7 human breast cancer cells (Fig. 7). Mammalian one-hybrid assays were performed in HeLa cells that were transiently transfected with an expression vector for a fusion protein containing the DBD of the yeast transcription factor GAL4 and the LBD of the VDR together with a reporter gene construct containing a GAL4 binding site-driven luciferase gene (Fig. 7A). In this assay system, saturating concentrations of ZK159222 showed reasonable agonistic activity (30-fold induction of luciferase reporter gene activity over solvent control), which was significantly lower than that of the natural hormone (71-fold) or TEI-9647 (70-fold). Therefore, a combined treatment of cells with 1α,25(OH)$_2$D$_3$ and a 100-fold higher concentration of ZK159222 resulted in antagonistic effect (only 50-fold stimulation) compared with the treatment with 1α,25(OH)$_2$D$_3$ alone (71-fold stimulation). In contrast, TEI-9647 showed no antagonistic effect in this cellular system (80-fold stimulation). In MCF-7 cells that were transfected with only a reporter gene construct containing four copies of the rat ANF DR3-type VDRE (Fig. 7B), both ZK159222 and TEI-9647 showed clearly lower agonistic effects (2.0- and 1.5-fold induction over solvent control) than the natural hormone (18.4-fold). Moreover, the combination of the natural hormone with 100-fold higher concentrations of ZK159222 and TEI-9647 resulted in a significant antagonistic effect (2.6- and 5.8-

fold stimulation, respectively) for both compounds. When MCF-7 cells additionally overexpressed VDR and RXR (Fig. 7C), drastically higher stimulation factors were observed, but the agonistic action of ZK159222 (26-fold induction over solvent control) and TEI-9647 (34-fold) were still clearly lower than that of the natural hormone (360-fold). In this very responsive cellular system, stimulation was performed with constant saturating concentrations of $1\alpha,25(OH)_2D_3$ (100 nM) and graded concentrations of ZK159222 or TEI-9647 up to a 10-fold molar excess. Both compounds demonstrated dose-dependent antagonism. At 10-fold molar excess, ZK159222 showed a clear antagonistic effect (80-fold stimulation) and half-maximal antagonism at equimolar concentrations. In comparison, the antagonistic effect of TEI-9647 was less prominent; even at 10-fold molar excess (still 216-fold induction), less than half-maximal antagonism was obtained.

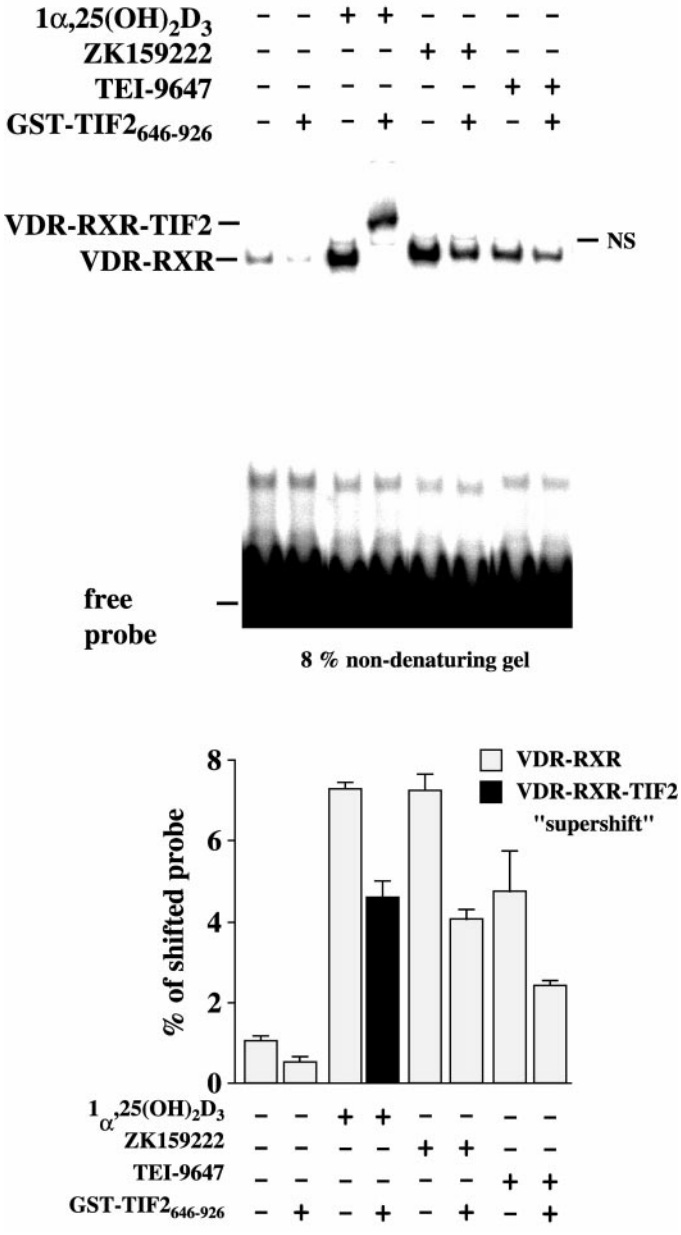


Fig. 6. VDR antagonists do not induce interaction of coactivators with VDR-RXR heterodimers. Supershift experiments were performed with in vitro translated VDR-RXR heterodimers that were preincubated with bacterially expressed GST-TIF2$_{646-926}$, saturating concentrations (1 μM) of $1\alpha,25(OH)_2D_3$, ZK159222, or TEI-9647 (or solvent as a control) and the ^{32}P-labeled rat ANF DR3-type VDRE. Protein-DNA complexes were separated from free probe through 8% nondenaturing polyacrylamide gels. Representative experiments are shown. The amount of VDR-RXR-VDRE or VDR-RXR-VDRE-TIF2 ("supershift") complexes in relation to free probe was quantified with the use of a Fuji FLA2000 reader. Columns represent the mean of triplicates and the bars indicate S.D.

Discussion

This study presents the first direct comparison of the two recently described VDR antagonists ZK159222 and TEI-9647, which are both $1\alpha,25(OH)_2D_3$ analogs with modifications in the side chain (Miura et al., 1999; Herdick et al., 2000b). Compared with the natural hormone, both compounds have relatively bulky ring structures in their side chains, which are assumed to be the main structural bases of their antagonistic action. However, ZK159222 carries a much longer side chain than TEI-9647, which suggests that there are differences in the molecular mechanisms of their antagonistic action. One major difference in the action of the two antagonists is that ZK159222 stabilizes the complex formation of VDR-RXR heterodimers on a VDRE with the same potency and nearly the same sensitivity as $1\alpha,25(OH)_2D_3$, whereas TEI-9647 shows both reduced potency and >10-fold reduced sensitivity (Fig. 2). DNA-bound VDR-RXR heterodimers are the core of $1\alpha,25(OH)_2D_3$ signaling (Carlberg and Polly, 1998), so that the reduced potency of TEI-9647 in VDR-RXR-VDRE complex formation results in a reduced agonistic potential of the compound. Moreover, this also suggests that TEI-9647 is stabilizing at least a part of the VDR molecules in a conformation that prevents binding to RXR, whereas the binding of ZK159222 seems not to affect the affinity of the VDR for its partner receptor. The different sensitivity of the antagonists means that equimolar amounts of ZK159222 would be able to replace nearly half of the VDR-bound $1\alpha,25(OH)_2D_3$ molecules, whereas a more than 10-fold molar excess of TEI-9647 would be required for obtaining the same effect. This explains the different antagonistic efficacy of both compounds, as demonstrated in VDR- and RXR-overexpressing MCF-7 cells (Fig. 7C).

The natural hormone and its agonistic analogs are known to stabilize a reasonable proportion of all DNA-bound VDR-RXR heterodimers in the agonistic conformation $c1_{GSC}$, whereas in the absence of ligand, only a clearly lower amount of heterodimers are stabilized in the nonagonistic conformation $c2_{GSC}$ (Quack and Carlberg, 2000a,b). Interestingly, ZK159222 stabilizes a similar proportion of the pool of VDR-RXR-VDRE complexes than $1\alpha,25(OH)_2D_3$, but in the nonagonistic conformation $c2_{GSC}$ (Fig. 3). TEI-9647 stabilizes a lower amount of VDR-RXR heterodimers than $1\alpha,25(OH)_2D_3$ and ZK159222, each half of them in conformations $c1_{GSC}$ and $c2_{GSC}$. This means that although TEI-9647 has a reduced potential in VDR-RXR-VDRE complex formation, it still stabilizes 15% of the DNA-VDR-RXR heterodimer pool in an agonistic conformation, whereas ZK159222 is not able to stabilize any VDR-RXR-VDRE complexes in this conformation. When looking more detailed on the VDR within these VDR-RXR-VDRE complexes (Fig. 4), both ZK159222 and TEI-9647 seem to stabilize a similar pattern of VDR conformations. Both antagonists stabilize a clearly lower amount of the VDR molecule pool in the agonistic conformation $c1_{LPD}$

than the natural hormone, whereas only ZK159222 and TEI-9647 specifically stabilize the antagonistic conformation $c2_{LPD}$. Interestingly, the stabilization of the VDR with TEI-9647 compared with the VDR with ZK159222 results in a slight migration difference between the VDR fragments that represent conformation $c2_{LPD}$ (Fig. 4), which suggests that the two antagonists stabilize different antagonistic conformations. A more detailed investigation of these different antagonistic conformations is in progress.

The comparison of ZK159222 and TEI-9647 for their ability to modulate the interaction of the VDR with coactivators and corepressors resulted in no significant difference between the two antagonists (Fig. 5). In contrast to the natural hormone and its agonistic analogs (Herdick et al., 2000a), both antagonists were unable to mediate a significant interaction of the VDR with coactivators. However, like $1\alpha,25(OH)_2D_3$ (Polly et al., 2000), the binding of the two antagonists to the VDR induces a dissociation of the majority of the VDR-corepressor complexes. This suggests that both antagonists stabilize the VDR in a conformation that blocks an interaction with coactivators, but this antagonistic conformation seems not to prevent the normal amount of VDR-corepressor dissociation. Even when the VDR is complexed with RXR on a VDRE, both antagonists were able to block effectively the interaction of the VDR with coactivators (Fig. 6). In the antagonistic conformation, helix 12 of the VDR-LBD seems to be positioned incorrectly, so that the AF-2 domain on this helix is unable to interact with the LXXLL (L, leucine; X, any amino acid) core nuclear receptor interaction motifs of coactivator proteins (Brzozowski et al., 1997; Shiau et al., 1998). This suggests that ZK159222 and TEI-9647 function as antagonists, because they both prevent an effective *trans*-activation through the VDR by blocking contacts with coactivator proteins of the SRC/p160 family (Herdick et al., 2000b).

According to the analysis in in vitro systems, ZK159222 and TEI-9647 should be functional antagonists in all cellular systems. This seems to hold true for ZK159222, which shows antagonistic function in a mammalian one-hybrid system in HeLa cells, in a VDRE-driven reporter gene system in MCF-7 cells, as well as VDR- and RXR-overexpressing MCF-7 cells (Fig. 7). In all three cellular systems, the agonistic action of ZK159222 was relatively low, so that a 10- to 100-fold molar excess of ZK159222 over $1\alpha,25(OH)_2D_3$ reduced the strong agonistic action of the natural hormone. However, this functional antagonism was more effective in MCF-7 cells than in the VDRE- and RXR-independent mammalian one-hybrid system. In contrast, in the latter system, TEI-9647 even shows high agonistic effects, so that no functional antagonism can be observed (Fig. 7A). In MCF-7 cells, the agonistic potential of TEI-9647 was significantly lower than that of $1\alpha,25(OH)_2D_3$, so that at 100-fold molar excess, it works as an effective antagonist. However, at lower molar ratios between TEI-9647 and the natural hormone, TEI-9647 is a less effective antagonist than ZK159222, most probably because TEI-9647 has an affinity for the VDR at least 10-fold lower than that of ZK159222 and the natural hormone (Fig. 2B). The reduced potential of TEI-9647 in VDR-RXR complex formation seems to contribute significantly to its functional antagonism, because the latter was not detectable in the RXR-independent mammalian one-hybrid system in HeLa

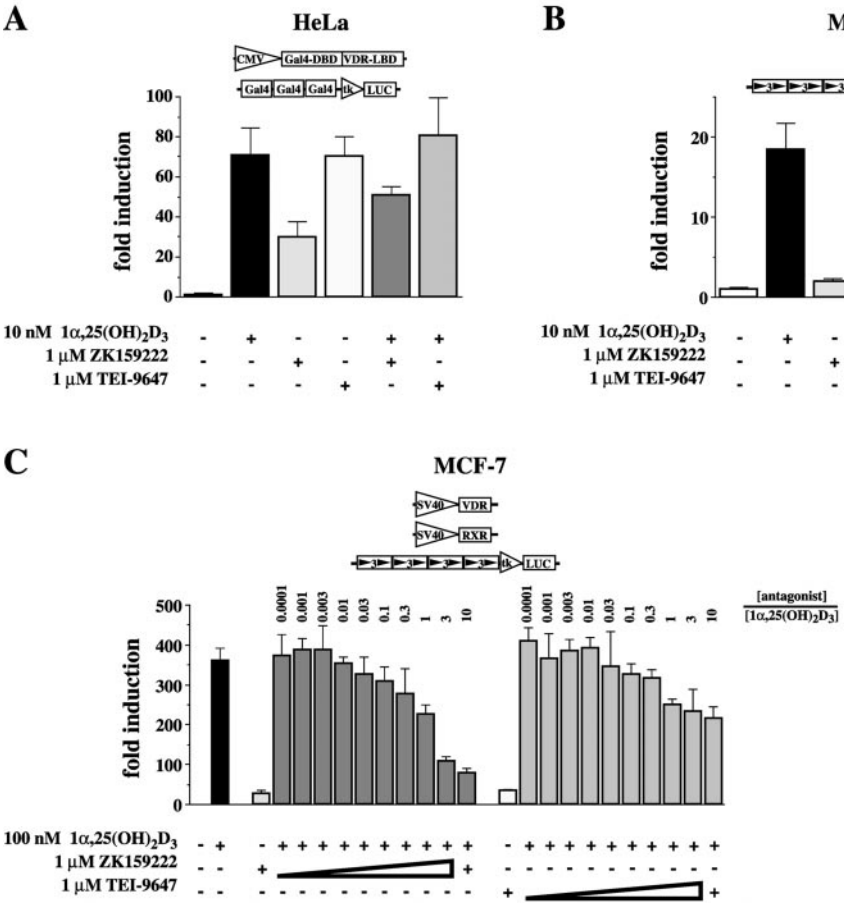


Fig. 7. Functional antagonism in HeLa and MCF-7 cells. Luciferase reporter gene assays were performed with extracts from HeLa (A) or MCF-7 (B and C) cells. HeLa cells were transiently transfected with a luciferase reporter gene construct driven by three copies of the GAL4 binding site and an expression vector for a $GAL4_{DBD}VDR_{LBD}$ fusion protein (A), whereas MCF-7 cells were transfected by a reporter gene construct driven by four copies of the rat ANF DR3-type VDRE alone (B) or together with expression vectors for VDR and RXR (C). Cells were treated for 16 h with indicated concentrations of $1\alpha,25(OH)_2D_3$, ZK159222, or TEI-9647 alone and in combination. The molar ratio of ZK159222 or TEI-9647 in relation to $1\alpha,25(OH)_2D_3$ is indicated above the columns (C). Stimulation of normalized luciferase activity was calculated compared with solvent-induced control cells. Columns represent the mean of triplicates and the bars indicate S.D.

cells. Moreover, ZK159222 shows a relatively high agonistic potential in the mammalian-one-hybrid system, which indicates that the VDR-LBD is not blocked for the interaction with all classes of coactivator proteins. The identity of these coactivator proteins is not yet known, but their expression level seems to be higher in HeLa than in MCF-7 cells.

In conclusion, ZK159222 and TEI-9647 are two structurally different VDR antagonists that in part show different mechanisms of action. ZK159222 seems to be a more complete antagonist, in that it demonstrates functional antagonism in all cellular systems that have been tested so far, whereas TEI-9647 seems to be a more selective antagonist that shows antagonism on VDR signaling in certain cellular systems. The latter may be of advantage for a potential clinical application of VDR antagonists, where a complete block of the vitamin D_3 endocrine system is mostly undesirable.

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