Carboxylic ester antagonists of 1α,25-dihydroxyvitamin D_3 show cell-specific actions

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Background: The nuclear hormone 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) acts through the transcription factor vitamin D receptor (1α,25$(OH)_2D_3$ receptor, VDR) via combined contact with the retinoid X receptor (RXR), coactivator proteins and specific DNA binding sites (1α,25$(OH)_2D_3$ response elements, VDREs). Ligand-mediated conformational changes of the VDR are the basis of the molecular mechanisms of nuclear 1α,25$(OH)_2D_3$ signaling. Cell-specific VDR antagonists would allow to dissect and fine regulate the pleiotropic 1α,25$(OH)_2D_3$ endocrine system affecting the regulation of calcium homeostasis, bone mineralization and other cellular functions.

Results: Two carboxylic ester analogues of 1α,25$(OH)_2D_3$, ZK159222 and ZK168281, which have additional cyclopropyl rings and allylic alcohol substructures in their side chain, were characterized in different 1α,25$(OH)_2D_3$ target tissues as functional antagonists of 1α,25$(OH)_2D_3$ signaling. In all tested systems, ZK168281 showed lower residual agonistic activity and higher antagonistic effects than ZK159222, but the strength of these effects was cell-specific. Both antagonists were shown to act via the same mechanisms: they selectively stabilize an antagonistic conformation of the ligand-binding domain of the VDR within VDR–RXR–VDRE complexes, which then inhibits the interaction of the VDR with coactivator proteins and an induction of transactivation. Interestingly, cells that have been treated with antagonists were found to contain VDR–RXR heterodimers in a different conformation than cells that were stimulated with an agonist. Moreover, the strength of the functional antagonism of ZK159222 and ZK168281 appears to depend on the VDR/RXR expression ratio and high RXR levels were found to reduce the antagonistic effect of both compounds. In support of this observation, the overexpression of an transactivation function 2 (AF-2) deletion mutant of RXR resulted for both ZK159222 and ZK168281 in a reduced agonistic activity and an increased antagonistic effect.

Conclusions: A novel, more potent VDR antagonist, ZK168281, was identified, which stabilizes VDR–RXR heterodimers in living cells in a different conformation than agonists. In addition, the VDR/RXR ratio was found as the major discriminating factor for understanding cell-specific effects of VDR antagonists.

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Introduction

The nuclear hormone 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) mainly regulates calcium homeostasis and bone mineralization [1], but also plays a role in controlling cellular growth, differentiation and apoptosis [2]. Various analogues of 1α,25$(OH)_2D_3$, 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]. Nearly all of the approximately 2000 presently known 1α,25$(OH)_2D_3$ analogues have been characterized as more or less potent agonists of the 1α,25$(OH)_2D_3$ receptor (VDR) [4], which is a member of the nuclear receptor transcription factor superfamily [5]. For other members of the superfamily, such as the estrogen receptor (ER), the progesterone receptor (PR) and the retinoic acid receptor (RAR), synthetic antagonists are known for a while [6], whereas only two types of VDR antagonists, the 25-carboxylic ester ZK159222 from Schering [7,8] and the 26,23-lactone TEI-9647 from Teijin [9,10], have been described.

The VDR acts preferentially as a heterodimer with the retinoid X receptor (RXR) [4] 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) [11]. Simple VDREs consist of two hexameric nuclear receptor-binding sites, which are commonly arranged as a direct repeat with three spacing nucleotides (DR3-type

VDREs) [11]. The VDR contains a DNA-binding domain (DBD), which is formed by two zinc-finger motifs that are characteristic for the nuclear receptor superfamily [12], and a ligand-binding domain (LBD) that is formed by 12 α-helical structures, of which the last one, helix 12, contains a short transactivation function 2 (AF-2) domain [13]. VDR–RXR–VDRE complexes are the molecular core of DNA-dependent 1α,25(OH)$_2$D$_3$ signaling [14] and the induction of a conformational change within the LBD of the VDR by interaction with 1α,25(OH)$_2$D$_3$ or its analogues 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 [15], an enhancement of the interaction with RXR (and consequently an increased amount of complex formation with a VDRE [16]) and a stimulation of the interaction with coactivator proteins of the p160-family, such as SRC-1, transcriptional intermediary factor 2 (TIF2) and RAC3, via the AF-2 domain [17]. The AF-2 domain was described to be repositioned after ligand binding to the LBD [13] and provides together with amino acids of helices 3 and 5 an interface for the binding of coactivators [18].

The two presently known VDR antagonists appear to act via different mechanisms: TEI-9647 decreases the amount of VDR–RXR heterodimer complex formation [10], whereas ZK168281 is not able to promote an interaction of the VDR with coactivator proteins of the p160-family neither in solution nor in a complex with RXR on DNA [7]. A comparison of the crystal structure of agonist- and antagonist-bound ER suggests that antagonists block AF-2 function by disrupting the topography of the AF-2 surface [19]. This mechanism of incorrect positioning and blocking of the AF-2 domain has also been suggested for other nuclear receptors [20–22]. Several of these antagonists, such as the partial ER antagonist tamoxifen, show cell-specific effects as extreme as a shift from antagonism to agonism [23]. For a potential therapeutic application of antagonists a description and understanding of such cell-specific effects is highly important.

In this study, a structural relative of ZK159222, ZK168281, is described as a novel, more potent VDR antagonist. The two compounds showed the same mechanism of action and their functional antagonism was found to be cell-specific. The VDR/RXR ratio was found as the major discriminating factor for understanding this cell specificity.

Results

Antagonistic and agonistic effects of the carboxylic ester 1α,25(OH)$_2$D$_3$ analogues (ZK159222 and ZK168281, for structures see Figure 1) were compared in Cos-7 (SV40-transformed African Green monkey kidney), Saos-2 (human osteogenic sarcoma), HaCaT (human immortalized keratinocytes) or MCF-7 (human breast cancer) cells by applying a saturating concentration of 1α,25(OH)$_2$D$_3$ (10 nM) alone or in combination with ZK159222 or ZK168281 at a 100-fold higher concentration (1 μM). Reporter gene assays resulted for all four cell lines in a comparable induction pattern of luciferase activity: 10 nM of the natural hormone provided maximal induction, 1 μM of ZK159222 and ZK168281 reached only 3–40% of this value and the combination of 1α,25(OH)$_2$D$_3$ with ZK159222 or ZK168281 provided 20–60% of the maximal level, i.e. both compounds show a clear antagonistic effect (Figure 2). In all tested cell lines the residual agonistic effect of ZK168281 was found to be lower than that of ZK159222 and the corresponding antagonistic effect of ZK168281 was higher than that of ZK159222, thus ZK168281 appears to be a more effective antagonist than ZK159222. Interest-

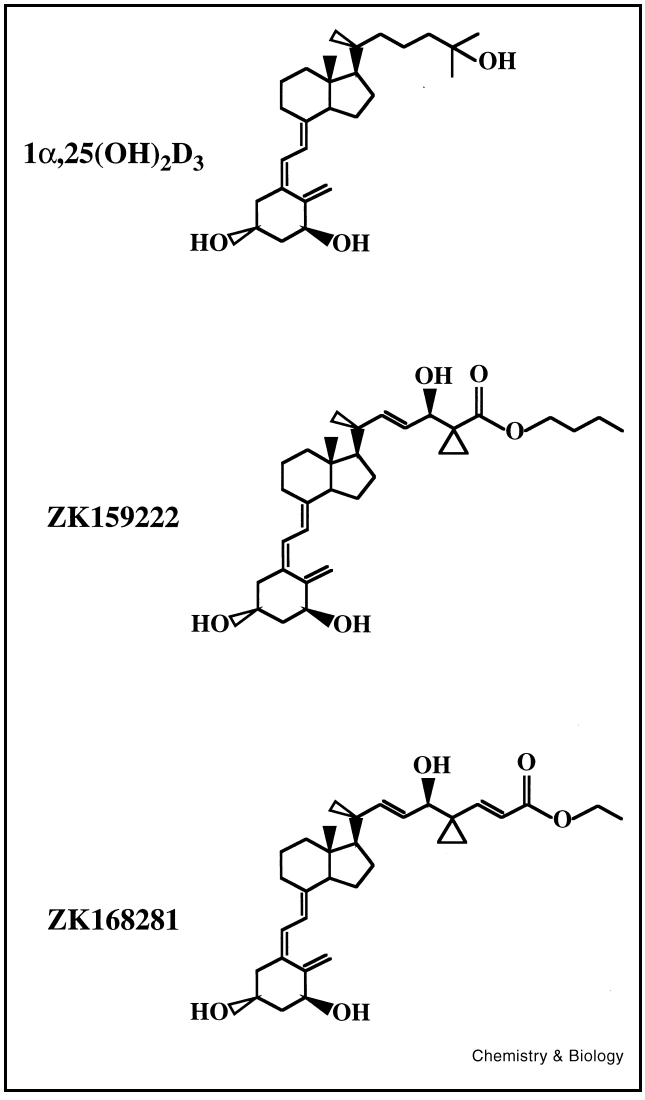


Figure 1. Structure of 1α,25(OH)$_2$D$_3$ and its antagonistic analogues. ZK159222 and ZK168281 are carboxylic esters of 1α,25(OH)$_2$D$_3$.

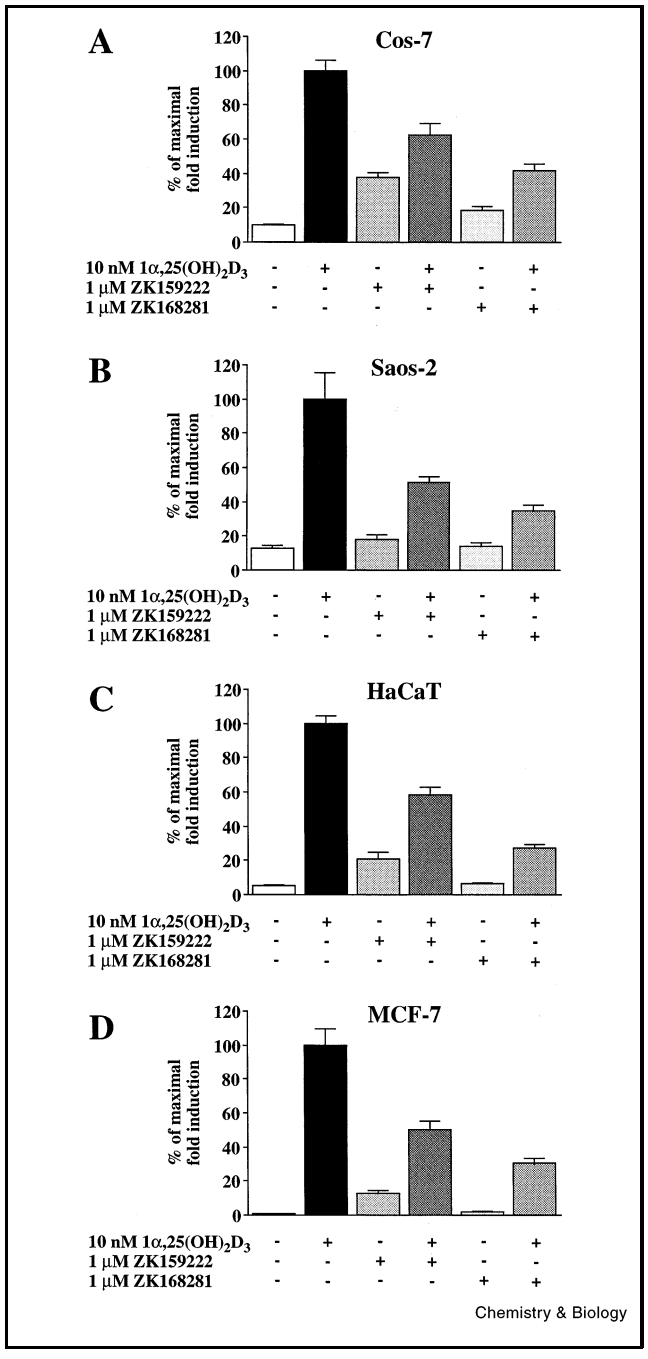


Figure 2. Cell-specific differences in functional antagonism. Luciferase reporter gene assays were performed with extracts from Cos-7 **(A)**, Saos-2 **(B)**, HaCaT **(C)** or MCF-7 **(D)** cells that were transiently transfected with a luciferase reporter gene construct driven by four copies of the rat ANF DR3-type VDRE together with the expression vectors for VDR and RXR (each 1 μg). Cells were treated for 16 h with indicated concentrations of $1\alpha,25(OH)_2D_3$, ZK159222 and ZK168281 alone or in combination. Stimulation of normalized luciferase activity was calculated in comparison to solvent-induced controls. The data are presented in reference to maximal induction of reporter gene activity. Columns represent the mean of triplicates and the bars indicate standard deviation.

ingly, the effects of ZK159222 and ZK168281 differ between the tested cell lines. The residual agonistic effect of ZK159222 and ZK168281 in MCF-7 cells (Figure 2D) is significantly lower than in Cos-7 cells (Figure 2A), whereas in HaCaT (Figure 2C) and Saos-2 (Figure 2B) cells agonistic activities between these extremes were observed. The corresponding antagonistic effect in the different cell lines showed the same ranking, i.e. the antagonism of both compounds is higher in MCF-7 than in Cos-7 cells. Taken together, both analogues act as functional antagonists of $1\alpha,25(OH)_2D_3$, but the strength of their antagonism is cell-specific.

For a more detailed investigation, the two VDR antagonists were analyzed first in a series of assays with in vitro translated receptors (Figure 3). The stabilization of VDR conformations by $1\alpha,25(OH)_2D_3$, ZK159222 and ZK168281 was analyzed by limited protease digestion (LPD) assays using the endoprotease chymotrypsin. As shown previously with LPD assays using trypsin [16,24,25], also chymotrypsin provided up to three digestion products (Figure 3A). These three VDR fragments contain major parts of the LBD and are interpreted as the functional VDR conformations 1, 2 and 3 that mediate the agonistic ($c1_{LPD}$) [17], the antagonistic ($c2_{LPD}$) [7] and the non-agonistic ($c3_{LPD}$) [26] action of the receptor. The natural hormone stabilized 35% of all VDR molecules in $c1_{LPD}$ and 10% in $c3_{LPD}$, but no receptor molecules in $c2_{LPD}$. In contrast, ZK159222 and ZK168281 stabilized only 1–4% of all VDR molecules in $c1_{LPD}$, 10% in $c2_{LPD}$ and 8–16% in $c3_{LPD}$. In DNA-dependent LPD assays, i.e. when VDR–RXR heterodimers are formed on the rat atrial natriuretic factor (ANF) DR3-type VDRE, the absolute amount of stabilized VDR molecules slightly increased, but the ratio between the conformations stayed constant (data not shown).

Gel shift assays were performed with in vitro translated VDR–RXR heterodimers bound to the rat ANF DR3-type VDRE in the presence of increasing amounts of glutathione *S*-transferase (GST)-$TIF2_{646-926}$ fusion proteins and saturating concentrations (10 μM) of $1\alpha,25(OH)_2D_3$, ZK159222 and ZK168281 (Figure 3B). In the presence of $1\alpha,25(OH)_2D_3$, VDR–RXR–TIF2–VDRE complexes (supershifts) were observed, whereas in the presence of ZK159222 or ZK168281 no supershifts could be detected even at high coactivator concentrations. This indicates that both antagonists were not able to induce a change into the agonistic VDR conformation ($c1_{LPD}$), which would enable an interaction with coactivator proteins. However, in the absence of coactivators a comparable intensity (approximately 40% shifted probe) of dose-dependent VDR–RXR heterodimer complex formation on DNA was observed for $1\alpha,25(OH)_2D_3$, ZK159222 and ZK168281 (in relation to 16% shifted probe with solvent).

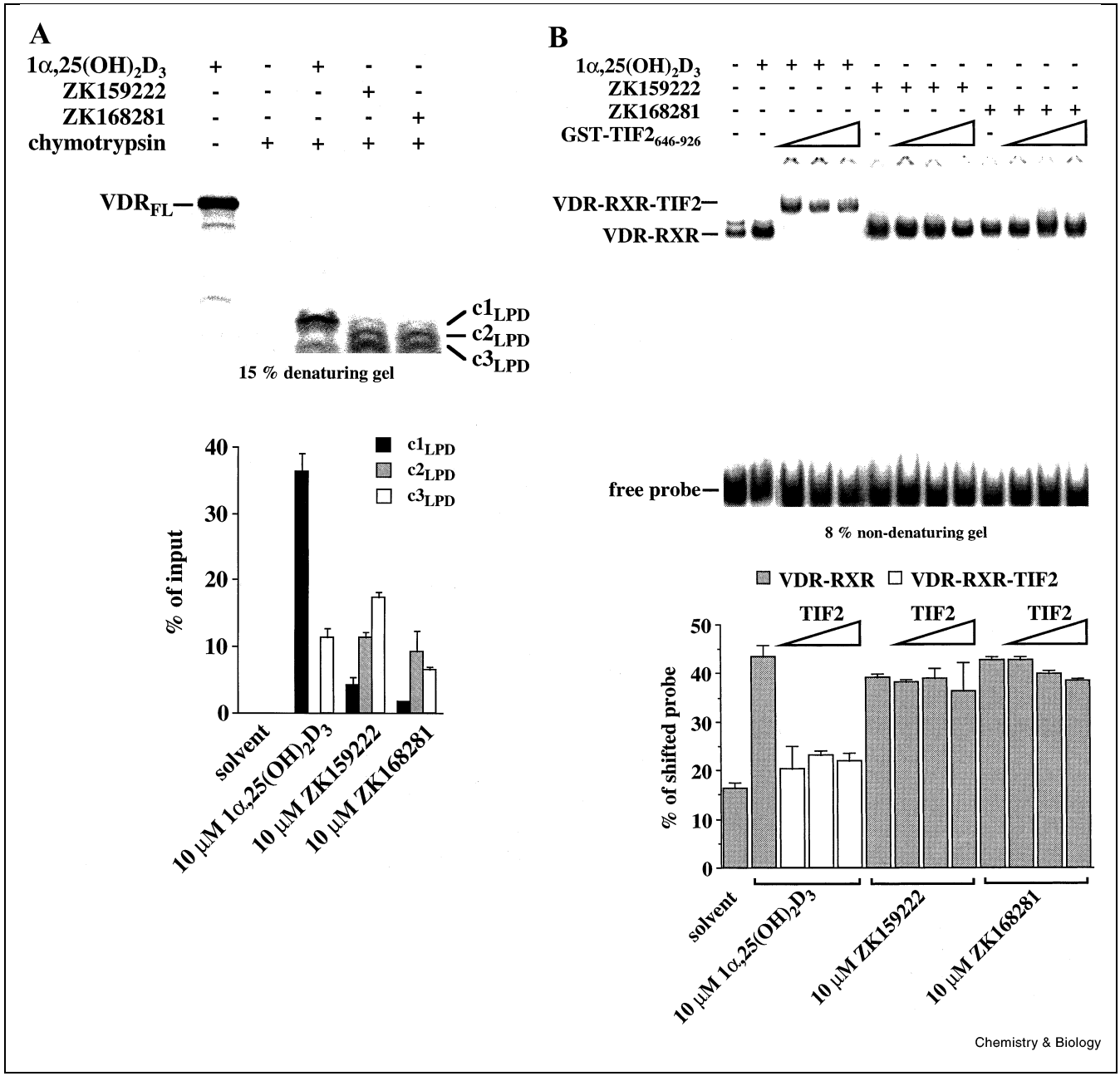


Figure 3. In vitro characterization of ZK159222 and ZK168281. LPD assays **(A)** were performed by preincubating in vitro translated ^{35}S-labelled VDR with saturating concentrations (10 μM) of 1α,25(OH)$_2$D$_3$, ZK159222 and ZK168281 (or solvent as a control). After digestion with chymotrypsin, samples were electrophoresed through 15% SDS–polyacrylamide gels. Supershift experiments **(B)** were performed with in vitro translated VDR–RXR heterodimers that were preincubated with increasing amounts of bacterially expressed GST–TIF2$_{646–926}$ (1, 3 and 6 μg), saturating concentrations (10 μM) of 1α,25(OH)$_2$D$_3$, ZK159222 and ZK168281 (or solvent as a control) and the ^{32}P-labelled rat ANF DR3-type VDRE. VDR–RXR heterodimers were separated from free probe through 8% non-denaturing polyacrylamide gels. GSC experiments **(C)** were performed with in vitro translated VDR–RXR heterodimers that were preincubated with saturating concentrations (10 μM) of 1α,25(OH)$_2$D$_3$, ZK159222 and ZK168281 (or solvent as a control). After digestion with chymotrypsin, VDR–RXR heterodimer conformations were separated from free probe through 8% non-denaturing 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 ZK168281) and 3 (c3$_{LPD}$) in relation to VDR input **(A)**, of VDR-RXR-VDRE or VDR-RXR-VDRE-TIF2 ('supershift') complexes in relation to free probe **(B)** and of DNA-complexed VDR–RXR heterodimer conformations 1 (c1$_{GSC}$) and 2 (c2$_{GSC}$) in relation to non-digested VDR–RXR–VDRE complexes **(C)** was quantified by phosphorimaging. In all cases columns represent the mean of triplicates and the bars indicate standard deviations.

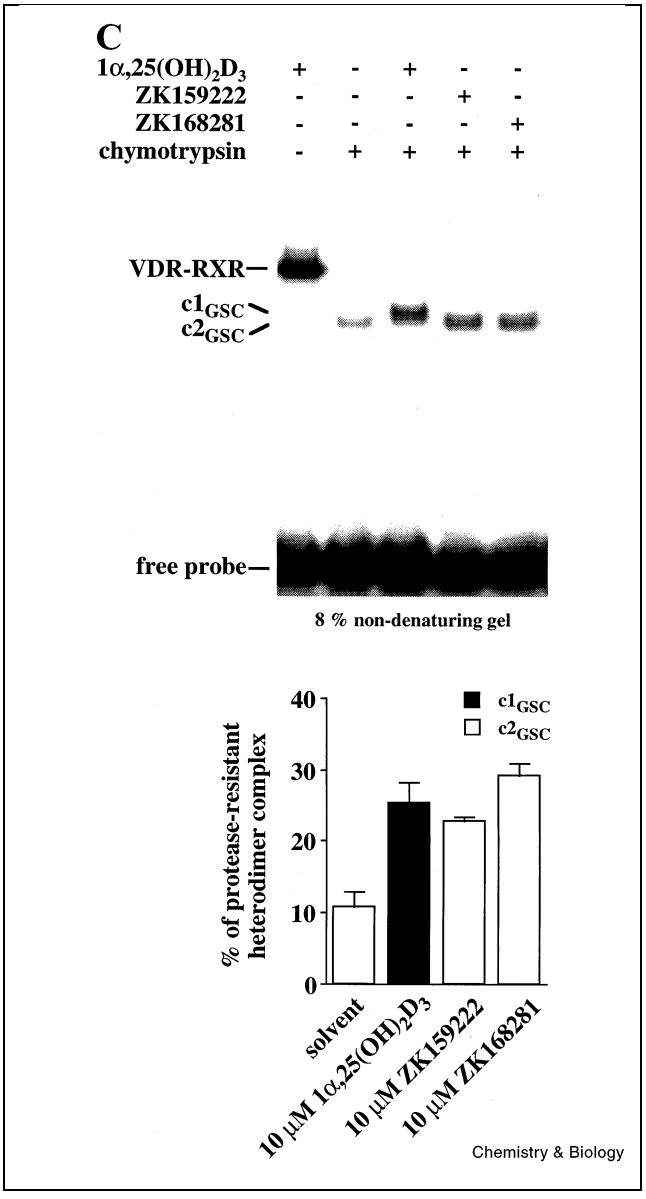


Figure 3 (*continued*)

Gel shift clipping (GSC) assays were performed with in vitro translated VDR–RXR heterodimers bound to the rat ANF DR3-type VDRE in the presence of saturating concentrations (10 μM) of $1\alpha,25(OH)_2D_3$, ZK159222 and ZK168281 (Figure 3C). After the digestion with chymotrypsin two VDR–RXR heterodimer conformations, referred to as $c1_{GSC}$ and $c2_{GSC}$ [26], could be discriminated. Interestingly, the natural hormone stabilized 25% of all DNA-binding VDR–RXR heterodimers in $c1_{GSC}$ (gel shift assay as reference), whereas in the absence of ligand only 10% of the heterodimers were stabilized in $c2_{GSC}$. Interestingly, both antagonists also stabilized approximately 25% of the pool of DNA-bound VDR–RXR heterodimers, but in $c2_{GSC}$, i.e. in a different conformation than observed with VDR agonists. Taken together, both antagonists show in these in vitro assays clear differences to the natural hormone (as a representative agonist) concerning the stabilization of specific conformations of the VDR-LBD and the whole VDR–RXR heterodimer complex, which then prevents the interaction with coactivator proteins.

The next series of experiments, gel shift and GSC assays with the rat ANF DR3-type VDRE, were performed with nuclear extracts from Cos-7 and MCF-7 cells that overexpressed VDR and RXR (Figure 4). Before scraping, the cells were preincubated with saturating concentrations of $1\alpha,25(OH)_2D_3$, ZK159222 and ZK168281 for 1 h, but in the following assays no extra ligand was added. Gel shift assays with nuclear extracts from Cos-7 cells (Figure 4A) and MCF-7 cells (Figure 4B) provided the same protein–DNA complexes than gel shift assays with in vitro translated VDR–RXR heterodimers. With Cos-7 nuclear extracts a clear ligand-dependent increase of VDR–RXR–VDRE complex formation by $1\alpha,25(OH)_2D_3$ (sixfold induction in comparison to solvent-treated cells) and by ZK159222 and ZK168281 (threefold induction) was observed, whereas with MCF-7 nuclear extracts no ligand effect was found. Interestingly, GSC assays with nuclear extracts (Figure 4C,D) also provided the same VDR–RXR heterodimers conformations as in vitro translated material (Figure 3C). Nuclear extracts from cells that have been treated with the agonist $1\alpha,25(OH)_2D_3$ contain VDR–RXR heterodimers in conformation $c1_{GSC}$, whereas in cells that have been treated with antagonists VDR–RXR heterodimers are in conformation $c2_{GSC}$.

In contrast to MCF-7 cells and other $1\alpha,25(OH)_2D_3$ target cells, Cos-7 cells do not express endogenous VDR and only relatively low levels of endogenous RXR (data not shown). This cell line is therefore well suited for analyzing the effect of transiently overexpressed VDR, RXR and TIF2 proteins on the antagonistic effect of ZK159222 and ZK168281 (Figure 5). Cos-7 cells were transfected with varied amounts of expression vectors for VDR, RXR and the coactivator TIF2 and then stimulated with saturating concentrations of $1\alpha,25(OH)_2D_3$ (10 nM) alone or in combination with ZK159222 or ZK168281 at a 100-fold higher concentration (1 μM). Reporter gene assays provided patterns of luciferase activity (Figure 5, for a better comparison all patterns were normalized to respective maximal induction by $1\alpha,25(OH)_2D_3$) that are very comparable to that presented in Figure 2A, i.e. ZK168281 showed a lower agonistic activity and higher antagonistic effect than ZK159222. The overexpression of increasing amounts of both VDR and RXR (Figure 5A) as well as the overexpression of increasing amounts of TIF2 at a constant expression level of VDR and RXR (Figure 5B) showed no significant effect on the pattern. In contrast, overexpression of increasing amounts of RXR at a constant, low expression

level of VDR (Figure 5C) resulted in a significant increase of the agonistic activity of ZK159222 (and to a lower extent also of ZK168281) and a respective decrease of the antagonistic effect (at higher RXR concentrations ZK159222 showed a significant antagonistic effect). Taken together, the strength of the antagonistic effect of ZK159222 and ZK168281 depends on the VDR/RXR expression ratio and high RXR levels appear to reduce the antagonism of both compounds.

In order to test the involvement of RXR in functional antagonism of ZK159222 and ZK168281, the effect of the overexpression of an AF-2 deletion mutant of RXR (RXR_{444*}) in comparison to wild type RXR (RXR_{wt}) was tested in Cos-7 cells (Figure 6). The cells were transfected with expression vectors for VDR, RXR_{wt} or RXR_{444*} and then stimulated with saturating concentrations of $1\alpha,25(OH)_2D_3$ (10 nM) alone or in combination with ZK159222 or ZK168281 at a 100-fold higher concentration (1 μM). Reporter gene assays provided for VDR–RXR_{wt} heterodimers the usual luciferase activity patterns (compare Figs. 2A and 5). Interestingly, the overexpression of RXR_{444*} and the following formation of VDR–RXR_{444*} heterodimers resulted for antagonists in a reduced agonistic activity and in an increased antagonistic effect. This observation suggests that RXR_{wt} allows less of an antagonistic effect for VDR–RXR heterodimers, when the VDR is occupied by ZK159222 or ZK168281. Alternatively, when VDR is complexed with RXR_{444*}, the absence of the AF-2 domain results in a heterodimer responding more to the antagonistic actions of ZK159222 and ZK168281.

Discussion

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. In this study, a dihomo derivative of the recently identified VDR antagonist ZK159222 [7,8] was characterized in different $1\alpha,25(OH)_2D_3$ target tissues as a novel functional antagonist. Both antagonists are able to stabilize the same amount of VDR–RXR heterodimers on DNA as the natural hormone, which indicates that in contrast to the 26,23-lactone antagonist the inhibition of VDR–RXR–VDRE complex formation is not a mechanism of action of carboxylic ester antagonists. A direct compar-

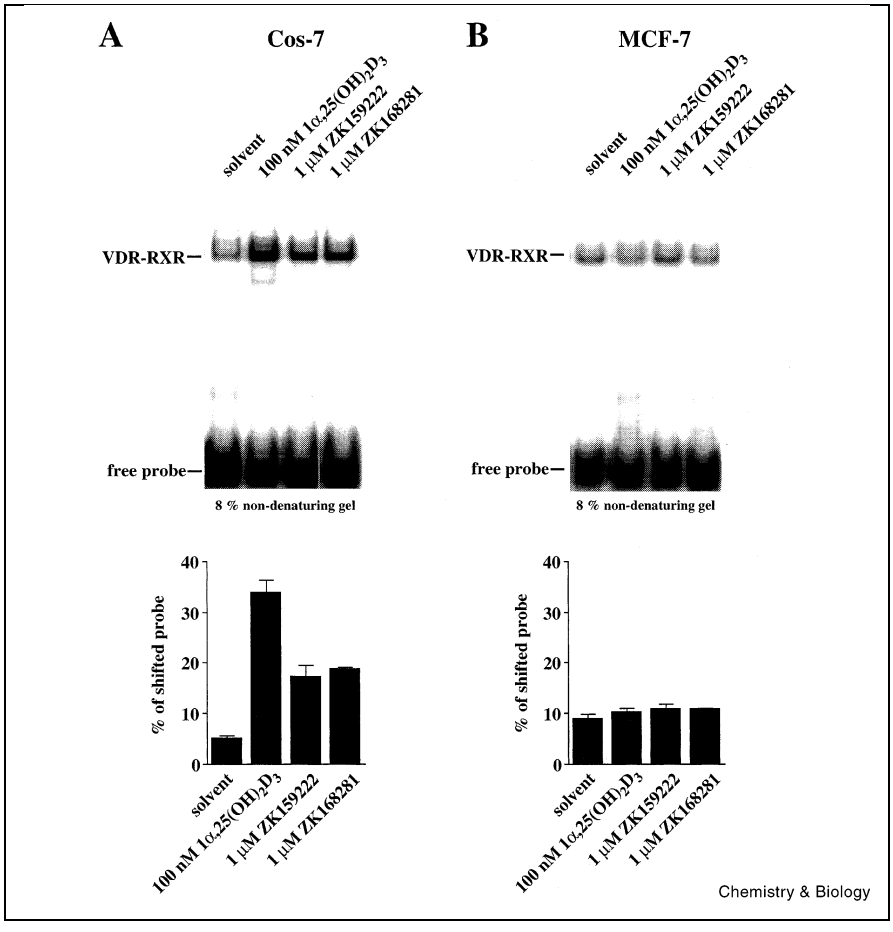


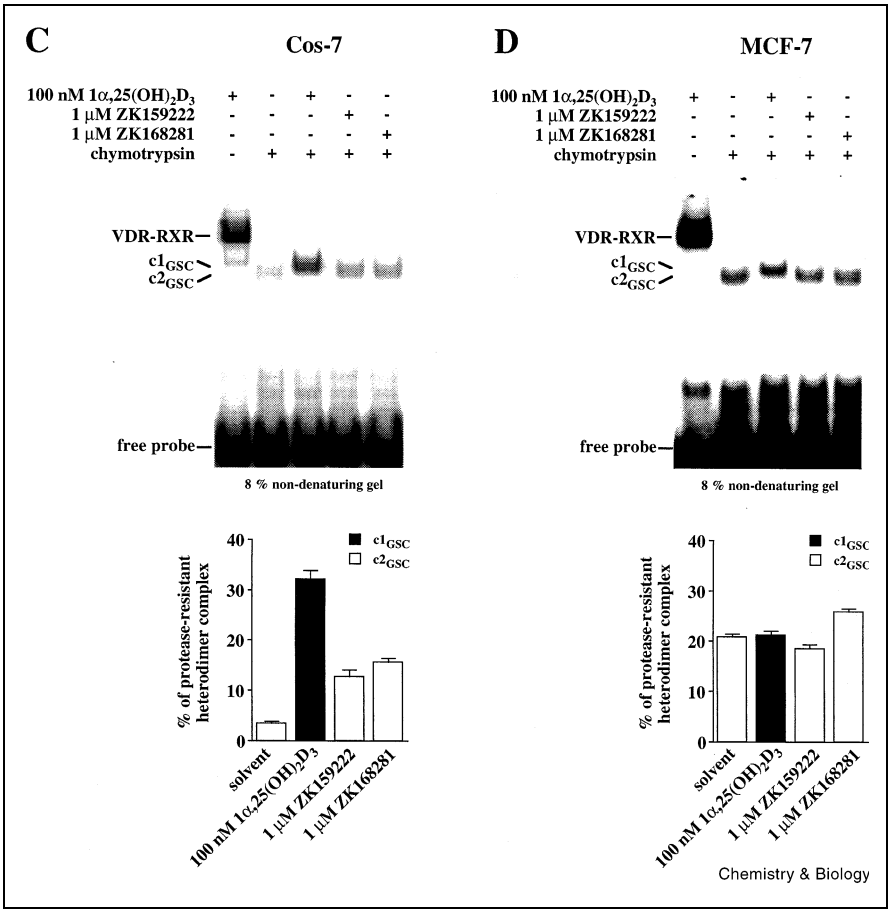
Figure 4. Ligand-responsiveness of VDR–RXR heterodimers in living cells. Gel shift **(A, B)** and GSC experiments **(C, D)** were performed with nuclear extracts from VDR- and RXR-overexpressing Cos-7 **(A, C)** and MCF-7 **(B, D)** cells were preincubated with saturating concentrations (100 nM) of $1\alpha,25(OH)_2D_3$, ZK159222 and ZK168281 (or solvent as a control). For GSC assays samples were digested with trypsin. In both cases protein–DNA complexes were separated from free probe through 8% non-denaturing polyacrylamide gels. Representative experiments are shown. The amount of DNA-complexed VDR–RXR heterodimers **(A, B)** and of VDR–RXR heterodimer conformations 1 ($c1_{GSC}$) and 2 ($c2_{GSC}$) in relation to non-digested VDR–RXR–VDRE complexes **(C, D)** was quantified by phosphorimaging. Columns represent the mean of triplicates and the bars indicate standard deviation.

ison of the functional antagonism of ZK159222 and ZK168281 in different cell lines indicated that ZK168281 is more potent, since it shows lower residual agonistic activity and a higher antagonistic effect. Moreover, ZK168281 provides functional antagonism already at approximately three times lower concentrations than ZK159222, which is also indicated by respective lower half maximal activation (EC_{50}) values in in vitro assays [27].

Both carboxylic ester antagonists function via the same mechanism as they selectively stabilize an antagonistic conformation ($c2_{LPD}$) of the VDR–LBD, which is not observed with VDR agonists. The observation that ZK159222 and ZK168281 cannot induce supershifts with TIF2 suggests that in this antagonistic conformation helix 12 of the LBD appears to be not positioned correctly, so that the AF-2 domain on this helix is not able to interact with the LXXLL (L = leucine, X = any amino acid) core nuclear receptor interaction motifs of coactivator proteins [19,21]. This lack of interaction with coactivators may be the main reason why ZK159222 and ZK168281 stimulate only residual agonistic activity.

GSC assays with the endoprotease chymotrypsin allowed for the first time a visualization of different conformations representing antagonist-complexed VDR–RXR heterodimers ($c2_{GSC}$) and agonist-complexed VDR–RXR heterodimers ($c1_{GSC}$). Interestingly, these different heterodimer conformations can not only be observed with receptors that were translated in vitro, but also with receptor complexes within nuclear extracts from different agonist- and antagonist-treated cells. This observation confirms that antagonist-specific conformations also exist in living cells, i.e. that an in vitro evaluation of antagonistic receptor conformations represents the in vivo situation. Therefore, GSC assays with nuclear extracts appear to be very appropriate for screening a larger group of $1\alpha,25(OH)_2D_3$ analogues and different cell lines or tissues for potential antagonists.

Cell-specific differences in the residual agonism of ZK159222 and ZK168281 appear to be largely related to the ratio of the expression levels of RXR and VDR. The agonistic activity of the VDR antagonists appears to increase, when the amount of RXR protein is in excess to VDR protein, whereas it decreases, when an AF-2 deletion mutant of RXR (RXR_{444*}) is overexpressed. This suggests



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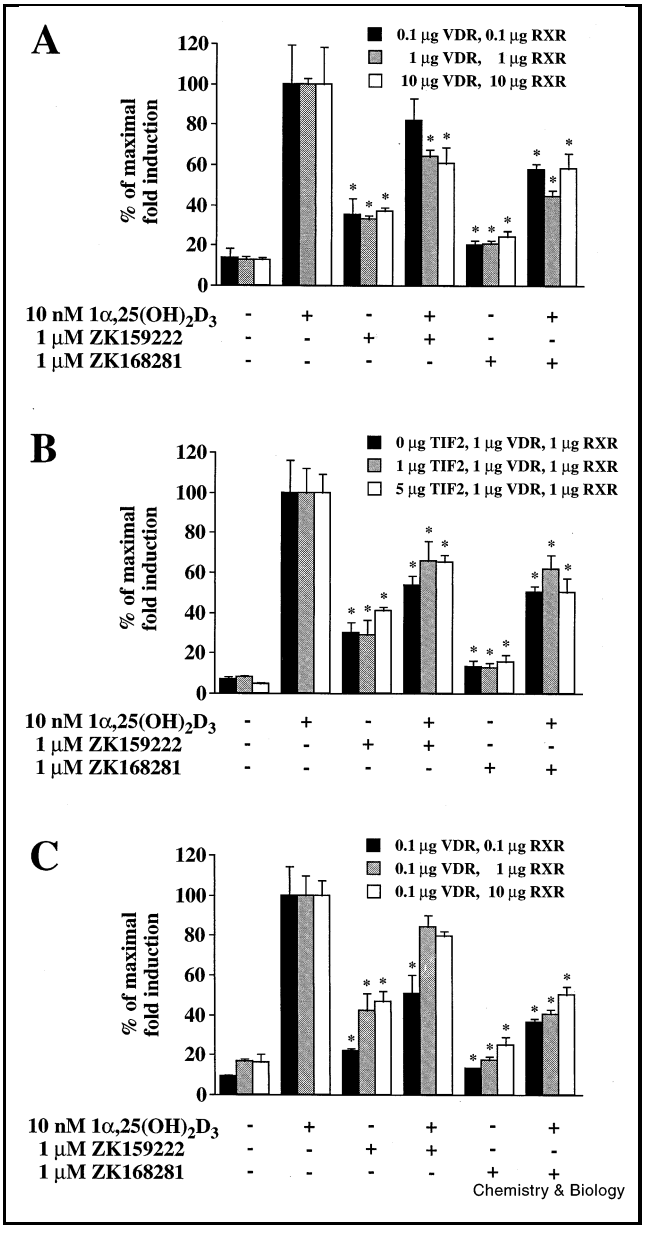

Figure 5. Functional antagonism at different VDR, RXR and TIF2 expression levels. Luciferase reporter gene assays were performed with extracts from Cos-7 cells that were transiently transfected with a luciferase reporter gene construct driven by four copies of the rat ANF DR3-type VDRE together with indicated amounts of expression vectors for VDR, RXR and TIF2. In different experimental series VDR and RXR were increased in parallel **(A)**, TIF2 was increased at constant amounts of VDR and RXR **(B)** and RXR was increased at constant amounts of VDR **(C)**. Cells were treated for 16 h with indicated concentrations of $1\alpha,25(OH)_2D_3$, ZK159222 and ZK168281 alone or in combination. Stimulation of normalized luciferase activity was calculated in comparison to solvent-induced controls. The data are presented in reference to maximal induction of reporter gene activity. Columns represent the mean of triplicates and the bars indicate standard deviation. A statistically significant ($P < 0.02$ according to Student's *t*-test) reduction of reporter gene activity in reference to the activity induced by 100 nM $1\alpha,25(OH)_2D_3$ is indicated by asterisks.
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that the partner receptor RXR, in particular its AF-2 domain, has a reasonable contribution to the agonistic activity of VDR–RXR heterodimers. It has been suggested for RAR–RXR heterodimers that the AF-2 domain of RXR mimics the LXXLL nuclear receptor interaction motif of coactivators and interacts in the absence of ligand with the AF-2 domain of RAR [28]. It may therefore be possible that the RXR AF-2 domain modulates the orientation of the VDR AF-2 domain, so that despite an antagonist binding a reasonable proportion of the VDR molecules shifts into the agonistic conformation. In addition, even in the absence of RXR ligands the RXR AF-2 domain may be able to contact coactivators. Both possibilities would increase the agonistic activity of antagonist-bound VDR–RXR heterodimers and may explain the modulatory effect of RXR expression levels. Each tissue or cell line can be assumed to have its individual expression level for VDR and RXR. MCF-7 cells have a relatively high VDR/RXR ratio (data not shown) and therefore provide a low agonistic activity of antagonists, whereas Cos-7 cells are known for their low VDR/RXR ratio, which results in a high agonistic

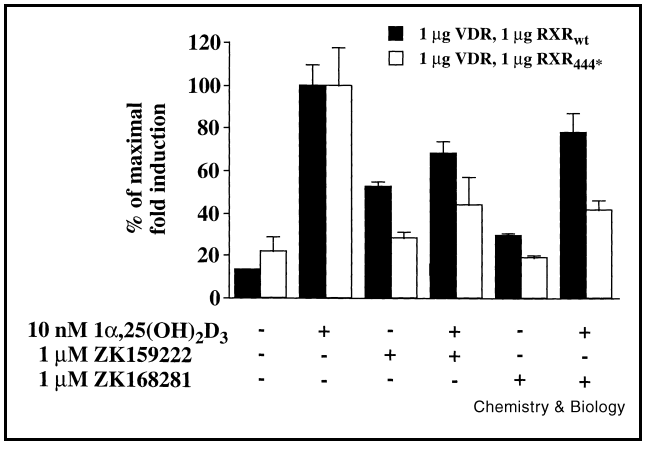

Figure 6. The impact of the AF-2 domain of RXR on functional antagonism. Luciferase reporter gene assays were performed with extracts from Cos-7 cells that were transiently transfected with a luciferase reporter gene construct driven by four copies of the rat ANF DR3-type VDRE together with indicated amounts of expression vectors for VDR, RXR_{wt} or RXR_{444*}. Cells were treated for 16 h with indicated concentrations of $1\alpha,25(OH)_2D_3$, ZK159222 and ZK168281 alone or in combination. Stimulation of normalized luciferase activity was calculated in comparison to solvent-induced controls. The data are presented in reference to maximal induction of reporter gene activity. Columns represent the mean of triplicates and the bars indicate standard deviations.

activity. Taken together, the fact that VDR preferentially forms heterodimers with RXR [4], whereas ER and PR each form homodimers [29], appears to result in different mechanisms for the cell-specific action of VDR antagonists versus ER or PR antagonists.

Significance

The nuclear hormone $1\alpha,25(OH)_2D_3$ exerts pleiotropic activities such as the regulation of calcium homeostasis, bone mineralization, cellular growth, differentiation and apoptosis. Approximately 2000 $1\alpha,25(OH)_2D_3$ analogues are presently known as agonists of the transcription factor VDR, but only the 25-carboxylic ester analogue (ZK159222) and a 26,23-lactone analogue (TEI-9647) were identified as VDR antagonists. In this study, a second carboxylic ester analogue (ZK168281) was characterized in different $1\alpha,25(OH)_2D_3$ target cells as a novel, more potent VDR antagonist. The strength of the functional antagonism of both carboxylic ester analogues was found to be cell-specific. The ratio of VDR/RXR expression was found to be the major discriminating factor for understanding this cell specificity. Both carboxylic ester analogues selectively stabilize an antagonistic conformation of the LBD of VDR within VDR–RXR heterodimers, which inhibits the interaction of the VDR with coactivator proteins and an induction of transactivation. Notably, the antagonistic conformation of VDR–RXR heterodimers could also be observed in nuclear extracts from ligand-treated cells and will allow a screening for further VDR antagonists.

Materials and methods

Compounds

ZK159222 [8] and ZK168281 are carboxylic esters of $1\alpha,25(OH)_2D_3$ and were both synthesized at the Medicinal Chemistry Department at Schering AG. The structures of both compounds are shown in Figure 1. ZK168281 is formally derived from ZK159222 by introduction of an ethylene unit between carbon 25 and the ester moiety. The ester side chain itself is shortened by two carbon atoms, thus providing a total side chain length that equals that of ZK159222. All compounds were dissolved in 2-propanol; further dilutions were made in DMSO (for in vitro assays) or in ethanol (for cell culture assays).

DNA constructs

Mammalian expression constructs. The full-length cDNAs for human VDR [30], human RXRα [31] and human TIF2 [32] were subcloned into the SV40 promoter-driven pSG5 expression vector (Stratagene, Heidelberg, Germany). The AF-2 deletion mutant of RXR (RXR_{444*}) was generated by introducing a stop codon at amino acid position 444 [33].

VDRE-driven reporter gene construct. Four copies of the DR3-type VDRE from the rat ANF gene promoter [34] were fused with the thymidine kinase (*tk*) minimal promoter driving the luciferase reporter gene.

GST fusion protein construct. The nuclear receptor interaction domain human TIF2 (spanning from amino acid 646 to 926) [32] was subcloned into the GST fusion vector pGEX (Amersham-Pharmacia, Freiburg, Germany).

In vitro protein translation, bacterial protein overexpression and nuclear extracts

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 isopropyl-β-D-thio-galactopyranoside (0.25 mM) for 3 h at 37°C. For the generation of nuclear extracts, Cos-7 cells were transfected with pSG5-based cDNA expression vectors for VDR and RXR and stimulated for 1 h with ligand. Cells were then collected and nuclear extracts were prepared as described [35].

LPD assay

In vitro translated, ^{35}S-labelled VDR protein (2.5 µl) was incubated with ligand for 15 min at room temperature in 20 µl binding buffer (10 mM HEPES [pH 7.9], 1 mM DTT, 0.2 µg/µl poly(dI-C) and 5% glycerol). The buffer was adjusted to 150 mM of monovalent cations by addition of KCl. Chymotrypsin (Roche Diagnostics, Mannheim, Germany, final concentration 16.7 ng/µl) was then added and the mixtures were further incubated for 10 min at room temperature. The digestion reactions were stopped by adding 25 µl protein gel loading buffer (0.25 M Tris, pH 6.8, 20% glycerol, 5% mercaptoethanol, 2% SDS, 0.025% bromophenol 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, supershift and GSC assay

In vitro translated VDR–RXR heterodimers (approximately 20 ng specific protein) were incubated with ligand for 15 min at room temperature in a total volume of 20 µl binding buffer, whereas nuclear extracts (5 µg total protein) were not pretreated with ligand. In both cases the buffer was adjusted to 150 mM by addition of KCl. For supershift assays, approximately 3 µg of bacterially expressed GST-$TIF2_{646-926}$ fusion protein were included in the incubation. Approximately 1 ng of the ^{32}P-labelled rat ANF DR3-type VDRE (50 000 cpm) was then added and incubation was continued for 20 min. For GSC assays, the endoprotease chymotrypsin (Roche Diagnostics) was added to a final concentration of 16.7 ng/µl and the incubation was continued for 30 min at room temperature. Protein–DNA complexes were resolved through 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 on a Fuji FLA2000 reader.

Transfection and luciferase assays

Cos-7 SV40-transformed African Green monkey kidney cells, Saos-2 human osteogenic sarcoma cells, HaCaT human immortalized keratinocytes or MCF-7 human breast cancer cells were seeded into 6-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 indicated amounts of pSG5-based receptor expression vectors for VDR, RXR and TIF2 with 15 µg *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP, Roth, Karlsruhe, Germany, 20 µg for HaCaT cells) for 15 min at room temperature in a total volume of 100 µl. After dilution with 900 µl 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. At this time, ligands were also added. The cells were lysed 16 h after onset of stimulation using the reporter gene lysis buffer (Roche Diagnostics) and the constant light signal luciferase reporter gene assay was performed as recommended by the supplier (Canberra-Packard, Dreieich, Ger-

many). 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.

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