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Structure Activity Relationship of Carboxylic Ester Antagonists of the Vitamin D_3 Receptor

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ABSTRACT

A 25-carboxylic ester analog of 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], ZK159222 (compound 1), was recently described as a novel type of antagonist of 1α,25$(OH)_2D_3$ signaling. In this study five derivatives of compound 1 (compounds 2–6) were selected because of their sensitivity in facilitating complex formation between the 1α,25$(OH)_2D_3$ receptor (VDR) and the retinoid X receptor on a 1α,25$(OH)_2D_3$ response element that was comparable to that of the natural hormone (0.2–0.9 nM). Most derivatives of compound 1 reacted as typical agonists, because they were able to promote ligand-dependent interaction of the VDR with the coactivator TIF2, stabilized the VDR preferentially in its agonistic conformation $c1_{LPD}$, and stimulated VDR-dependent gene activity with a potency similar to 1α,25$(OH)_2D_3$. In contrast, only compound 2 showed the antagonistic profile of compound 1, which includes the incompetence to induce a VDR-TIF2 contact, the stabilization of the antagonistic conformation $c2_{LPD}$, and only a very weak and insensitive functional activity. Accordingly, only compounds 1 and 2, but not compounds 3 to 6, showed prominent antagonistic effects in cellular systems. The comparison of the structures of the compounds indicates that the essential requirements for an antagonistic function are a cyclopropyl ring at carbon 25, a hydroxy group at carbon 24, and at least a butylester. Interestingly, compound 2 was an approximately 3 times more sensitive antagonist than compound 1 and even displayed a lower residual agonistic activity. In conclusion, only a very limited number of structural variations of compound 1 are possible to keep its antagonistic profile, but the tools presented here for their in vitro evaluation allow an accurate prediction of the effects and are suited to screening for even more potent 1α,25$(OH)_2D_3$ antagonists.

The nuclear hormone 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] is the natural agonist of the vitamin D_3 receptor (VDR) (Pike, 1991). VDR is a member of the nuclear receptor transcription factor superfamily (Mangelsdorf et al., 1995) and 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). The VDR contains a DNA-binding domain (DBD), which is formed by two zinc-finger motifs that are characteristic of the nuclear receptor superfamily (Glass, 1994), 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 (Moras and Gronemeyer, 1998). VDR-RXR-VDRE complexes are the molecular core of DNA-dependent 1α,25$(OH)_2D_3$-signaling pathways (Carlberg and Polly, 1998) and should be able to explain the physiological actions of 1α,25$(OH)_2D_3$, which are the regulation of calcium homeostasis and bone mineralization (DeLuca et al., 1990) and the control of cellular growth, differentiation, and apoptosis (Walters, 1992).

The most critical step in 1α,25$(OH)_2D_3$ signaling is the induction of a conformational change within the LBD of the VDR by interaction with 1α,25$(OH)_2D_3$ or its analogs. 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, 2000), 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 (Herdick et al., 2000). In contrast, if a given ligand stabilizes a VDR conformation that does not fulfill one of these criteria, so that the receptor will

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ABBREVIATIONS: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; AF-2, (trans)activation function 2; ANF, atrial natriuretic factor; DBD, DNA-binding domain; DR3, direct repeat spaced by three nucleotides; EC_{50}, half-maximal activation; GST, glutathione *S*-transferase; RXR, retinoid X receptor; LBD, ligand-binding domain; TIF2, transcriptional intermediary factor 2; VDR, 1α,25$(OH)_2D_3$ receptor; VDRE, 1α,25$(OH)_2D_3$ response element; DMSO, dimethyl sulfoxide.

be less or even not functional, and if in parallel this ligand shows an affinity for the VDR that is comparable to that of $1\alpha,25(OH)_2D_3$, it may have an antagonistic potential.

Approximately 2000 analogs of $1\alpha,25(OH)_2D_3$, 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 (Bouillon et al., 1995). Most of the modifications of $1\alpha,25(OH)_2D_3$ resulted in more or less active VDR agonists, whereas presently only two types of VDR antagonists, the 25-carboxylic ester ZK159222 (compound 1) from Schering (Berlin, Germany; Wiesinger et al., 1998) and the 26,23-lactone TEI-9647 from Teijin (Tokyo, Japan) (Miura et al., 1999), have been described. Interestingly, the two VDR antagonists appear to have different antagonistic mechanisms: TEI-9647 decreases the amount of VDR-RXR heterodimer complex formation (Ozono et al., 1999), and compound 1 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 (Herdick et al., 2000). The latter mechanism, which is based on an incorrect positioning and blocking of the AF-2 domain, has also been suggested for antagonists of other members of the nuclear hormone receptor superfamily, such as the estrogen receptor (Shiau et al., 1998) and the progesterone receptor (Vegeto et al., 1992).

The ratio of known VDR agonists to VDR antagonists (approximately 2000:2) already suggests that structural requirements of a $1\alpha,25(OH)_2D_3$ analog to function as a VDR antagonist are stricter than that of a VDR agonists. However, because of a lack of straightforward in vitro screening systems, not every known $1\alpha,25(OH)_2D_3$ analog was tested for its potential antagonistic action. Therefore, in this study, five derivatives of compound 1 were analyzed for their agonistic and antagonistic profile in vitro, i.e., for an enhancement of VDR-RXR-VDRE complex formation, for promotion of VDR-coactivator interaction, and for stabilization of VDR conformations. Moreover, the compounds were tested for their agonistic and antagonistic effects in a cellular system. Only one derivative, compound 2, was identified as a novel VDR antagonist. However, compound 2 was approximately 3 times more sensitive than compound 1 and displayed a lower residual agonistic activity than the parent compound.

Materials and Methods

Compounds. Compound 1 (butyl-(5Z,7E,22E)-(1*S*,3*R*,24*R*)-1,3,24-trihydroxy-26,27-cyclo-9,10-secocholesta-5,7,10(19),22-tetraene-25-carboxylate) (Wiesinger et al., 1998) and compounds 2 to 6 are carboxylic esters of $1\alpha,25(OH)_2D_3$; the structures of their side chains are shown in Fig. 1. Compound 2 is formally derived from compound 1 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 compound 1. Compounds 3 to 5 also exhibit different substitution patterns at carbon 25. Compound 3 is the only compound without a ring system at carbon 25 but carries a geminal dimethyl situation at this position. Compounds 4 and 5 have been derived from compound 1 by enlargement of the ring system at carbon 25 by one or two methylene groups, respectively. Compound 6 is the corresponding ketone to the 24-alcohol compound 1. By this modification the geometry of the whole side chain is changed, providing a higher degree of rigidity. All compounds were dissolved in 2-propanol; further dilutions were made in dimethyl sulfoxide (DMSO; for in vitro experiments) or in ethanol (for cell culture experiments).

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 also 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 1–147) was fused with the cDNA of the human VDR LBD (amino acids 109–427). The luciferase reporter gene was driven by three copies of the GAL4-binding site fused to the *tk* promoter (Hörlein et al., 1995). The nuclear receptor interaction domain of human TIF2 (spanning from amino acids 646–926) (Voegel et al., 1996) was subcloned into the glutathione *S*-transferase (GST) fusion vector pGEX (Amersham-Pharmacia, Freiburg, Germany).

In Vitro Protein Translation and Bacterial Fusion 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 isopropyl-β-D-thio-galactopyranoside (0.25 mM) for 3 h at 37°C.

Gel Shift and Supershift Assay. In vitro translated VDR-RXR heterodimers were incubated with graded or saturating concentrations of $1\alpha,25(OH)_2D_3$ or compounds 1 to 6 for 15 min at room temperature in a total volume of 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 had been adjusted to 150 mM by addition of KCl. For supershift assays, 3 μg of bacterially expressed GST-TIF2$_{646–926}$ fusion protein were included in the incubation. Approximately 1 ng of the ^{32}P-labeled direct repeat spaced by three nucleotides (DR3-type) VDRE from the rat atrial natriuretic factor (ANF) promoter (50,000 cpm) was added to the protein-ligand mixture and incubation

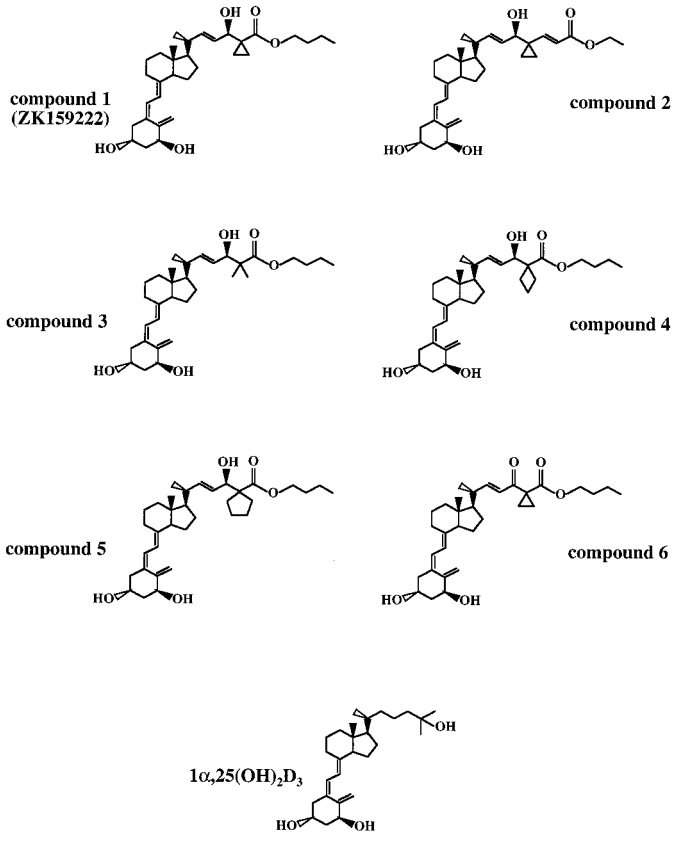

Fig. 1. Structures of compounds 1 to 6 and $1\alpha,25(OH)_2D_3$. Compounds 1 to 6 are carboxylic esters of $1\alpha,25(OH)_2D_3$.

was continued for 20 min. Protein-DNA complexes were resolved through 8% nondenaturing polyacrylamide gels in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) and were quantified on a FLA2000 reader (Fuji, Tokyo, Japan) using Image Gauge software (Raytest, Sprockhövel, Germany).

Limited Protease Digestion Assay. In vitro translated, ^{35}S-labeled VDR protein (1 μl), 5.5 μl of 50 mM Tris, pH 7.9, and 1 μl of ligand (or 1 μl of DMSO as a control) were preincubated for 15 min at room temperature. Then, 2.5 μl of trypsin (Promega, final concentration 12 ng/μl) were added, and the mixtures were further incubated for 30 min at room temperature. The digestion reactions were stopped by adding 10 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 by phosphorimaging.

Mammalian One-Hybrid Assay. HeLa human cervix carcinoma cells were seeded into six-well plates (10^5 cells/ml) and grown overnight in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% charcoal-treated fetal bovine serum. Liposomes were formed by incubating 1 μg of the GAL4-binding site-driven luciferase reporter gene construct and 1 μg of the expression vector for the $GAL4_{DBD}VDR_{LBD}$-fusion protein with 15 μg of DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate; 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 15% charcoal-treated fetal bovine serum (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, Mannheim, Germany), and the constant light signal luciferase reporter gene assay was performed as recommended by the supplier (Roche Diagnostics). Induction factors were calculated as the ratio of luciferase activity of ligand-stimulated cells to that of solvent controls.

Results

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 compounds 1 to 6 or 1α,25$(OH)_2D_3$ as a reference (for structures, see Fig. 1), to determine the ligand-dependent stabilization of VDR-RXR-VDRE complexes (Fig. 2). A comparable amount (approximately 30% shifted probe) of dose-dependent VDR-RXR heterodimer complex formation on the VDRE was observed with all seven compounds and provided half-maximal activation (EC_{50}) values of 0.18 nM for the natural hormone, 0.2 nM for compound 3, 0.3 nM for compound 4, 0.35 nM for compound 2, 0.4 nM for compound 1, 0.65 nM for compound 5, and 0.9 nM for compound 6. This indicates that all selected carboxylic ester analogs show a sensitivity for the stabilization of VDR-RXR-VDRE complexes that is comparable to that of 1α,25$(OH)_2D_3$, which implies that their affinity for the VDR is in a similar range. This confirms results from traditional ligand-binding assays (Wiesinger et al., 1998; data not shown).

Supershift assays were performed with in vitro translated VDR-RXR heterodimers bound to the rat ANF DR3-type VDRE in the presence of GST-$TIF2_{646–926}$ (as a representative member of the p160 family of coactivators) and saturating concentrations (10 μM) of 1α,25$(OH)_2D_3$ or compounds 1 to 6, to analyze a potential VDR-coactivator interaction (Fig. 3). All compounds showed an approximately 3-fold induction of VDR-RXR-VDRE complex formation (confirming the data presented in Fig. 2), but only in the presence of 1α,25$(OH)_2D_3$ and compounds 3 to 6 were VDR-RXR-VDRE-TIF2 complexes observed, whereas in the presence of compounds 1 and 2 no supershift could be detected.

The supershift experiments (Fig. 3) suggest that compounds 1 and 2 stabilize the VDR in a nonagonistic conformation. To challenge this idea, limited protease digestion assays were performed with in vitro translated VDR and graded concentrations of compounds 1 to 6 or 1α,25$(OH)_2D_3$ (Fig. 4). This assay displayed a dose-dependent stabilization of up to three VDR fragments, $c1_{LPD}$ (28 kDa), $c2_{LPD}$ (25 kDa), and $c3_{LPD}$ (23 kDa), that contained major parts of the LBD [from the trypsin-cutting site after arginine 173 to either the carboxy terminus at position 427 ($c1_{LPD}$), to arginine 402 ($c2_{LPD}$), or to arginine 391 ($c3_{LPD}$)] and represent the functional VDR conformations 1, 2, and 3, respectively (Peleg et al., 1995; Nayeri et al., 1996; Liu et al., 1997; Nayeri and Carlberg, 1997). The natural hormone and the compounds 3 to 5 stabilized at saturating concentrations up to 70% of all VDR molecules in $c1_{LPD}$ with EC_{50} values of 0.3, 1, 1.8, and 30 nM, respectively, whereas compound 6 mediated a stabilization of 35% of the VDR input in $c3_{LPD}$ with an EC_{50} value of 1 μM. However, none of these five compounds stabilized reasonable amounts of the VDR in $c2_{LPD}$. In contrast, compounds 1 and 2 stabilized at maximal concentrations 50 to 60% of all VDR molecules in $c2_{LPD}$ with EC_{50} values of 130 and 50 nM, respectively. Moreover, the two compounds were found to stabilize each less than 20% of the VDR input in $c1_{LPD}$ and $c3_{LPD}$.

The in vitro profile of compounds 1 and 2 (as shown in Figs. 2–4) suggests that their effects in cell culture systems should be clearly different from that of the five remaining compounds. This was tested by mammalian one-hybrid assays (Fig. 5), which 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. 5). The cells were stimulated with graded concentrations of compounds 1 to 6 or 1α,25$(OH)_2D_3$ for testing their agonistic potential (Fig. 5A). In this assay system, compounds 3 to 6 and 1α,25$(OH)_2D_3$ provided a 23- to 41-fold induction of reporter gene activity with EC_{50} values of 0.1, 0.2, 0.32, 7, and 1 nM, respectively. In contrast, stimulation with compounds 1 and 2 resulted in only a 6- to 12-fold increase of reporter gene activity and EC_{50} values of 110 and 60 nM, respectively. Antagonistic effects of compounds 1 to 6 were tested by a stimulation of the cells with a maximal concentration (1 μM) of the six compounds in the absence or presence of 10 nM 1α,25$(OH)_2D_3$ (Fig. 5B). Compounds 3 to 6 alone provided 47 to 96% of the maximal induction of reporter gene activity, and by costimulation with 1α,25$(OH)_2D_3$ (resulting in 80–97% of maximal activity) no significant antagonistic effect compared to 1α,25$(OH)_2D_3$-stimulated cells (41-fold induction) could be observed. In contrast, a combined stimulation of compounds 1 and 2 together with 1α,25$(OH)_2D_3$ resulted in only 31 and 14% of maximal induction of VDR-driven reporter gene activity, respectively, i.e., in a significant antagonistic effect. Finally, cells were stim-

ulated with 10 nM 1α,25(OH)$_2$D$_3$ in the presence of increasing concentrations of compounds 1 or 2 (Fig. 5C), which provided an obvious antagonistic effect for both compounds. Moreover, compound 2 was not only found to be a more potent antagonist than compound 1 at maximal concentrations but also showed these effects at an approximately 3 times lower concentration.

Discussion

Molecules that selectively activate or inhibit a specific nuclear receptor are of considerable biological significance and may have important clinical applications. Agonism and antagonism of natural and synthetic nuclear hormones are closely related processes. Both agonists and antagonists have to bind with reasonably high affinity to their respective nuclear receptor. In this study, a series of derivatives of the recently identified VDR antagonist ZK159222 (compound 1; Wiesinger et al., 1998; Herdick et al., 2000) was selected because their affinities for the VDR were similar. These compounds were analyzed by in vitro methods that are most appropriate for monitoring functional consequences of ligand binding, such as stabilization of VDR-RXR-VDRE complex

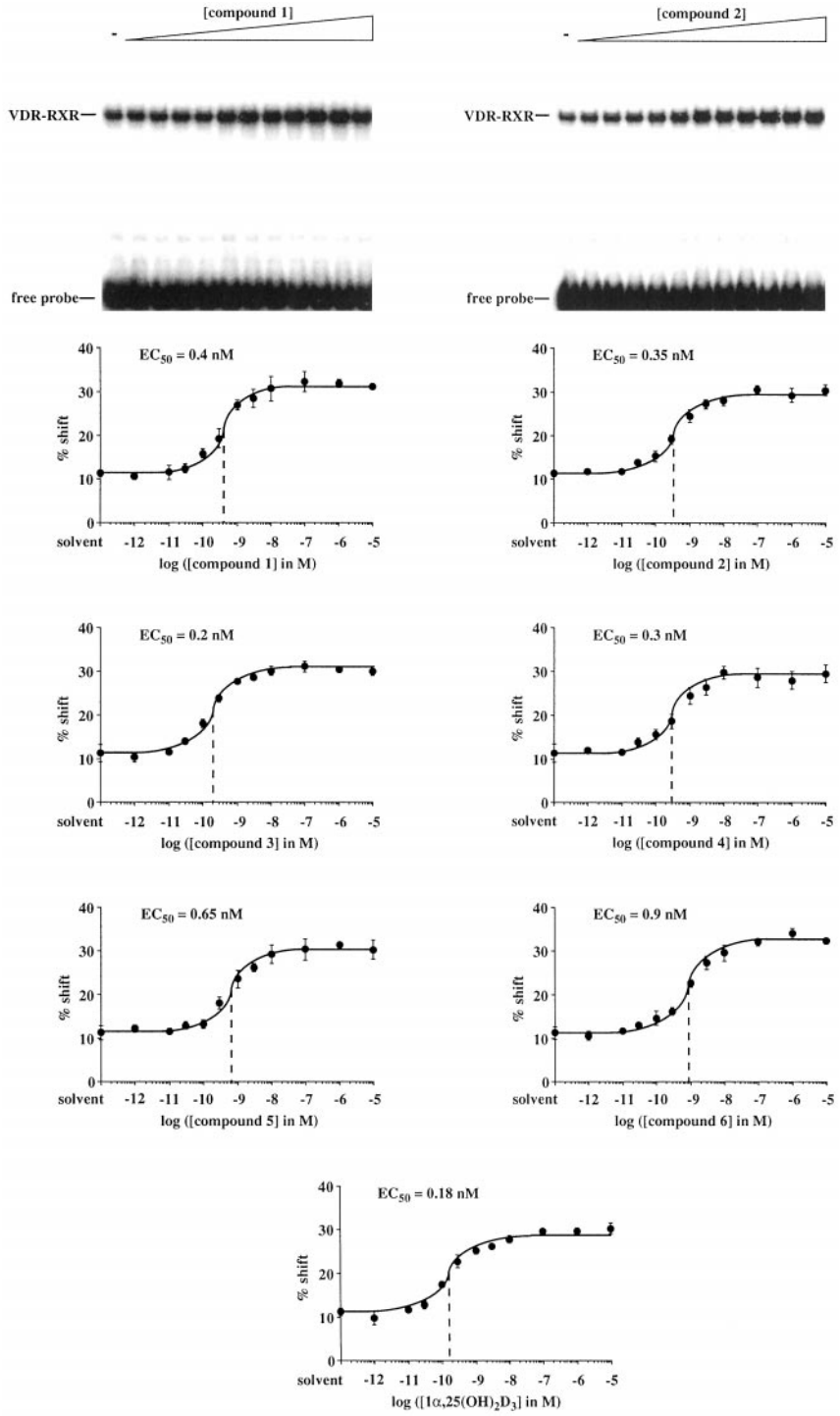

Fig. 2. Compounds 1 to 6 stabilize VDR-RXR heterodimer complex formation on a VDRE. Gel shift experiments were performed with in vitro translated VDR-RXR heterodimers, which were preincubated at room temperature with graded concentrations of compounds 1 to 6 or 1α,25(OH)$_2$D$_3$ (as a control) and the ^{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. The amount of VDR-RXR-VDRE complexes in relation to free probe was quantified by phosphorimaging. Representative experiments are shown for compounds 1 and 2. Data points represent the means 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.

formation and interaction with coactivator proteins. Compounds 1 to 6 induced the same increase of VDR-RXR-VDRE complex formation as the natural hormone (3-fold) with a sensitivity, i.e., with an EC_{50} value, that was identical or maximally 5 times lower than that of $1\alpha,25(OH)_2D_3$. This indicates that a potential antagonistic action of compounds 1 to 6 is not based on a different ability in VDR-RXR-VDRE complex formation, such as suggested for the 26,23-lactone antagonist (Ozono et al., 1999).

As already shown for the parent compound 1 (Herdick et al., 2000), the agonistic or antagonistic potential of its derivatives can be differentiated based on their ability to induce an interaction with coactivator proteins. In addition to the positive control compound 1, this assay highlighted compound 2 as the only potential antagonist, whereas the four other derivatives (compounds 3–6) and $1\alpha,25(OH)_2D_3$ were able to induce a supershift. This hint was strengthened by analyzing the stabilization of VDR conformations by compounds 1 to 6, which showed that only compounds 1 and 2, but not compounds 3 to 6 or the natural hormone, were able to stabilize the majority of the VDR molecules in the antagonistic conformation $c2_{LPD}$. In this conformation, helix 12 of the LBD appears to be positioned incorrectly such that the AF-2 domain on this helix is not able to interact with the LXXLL core interaction motifs of coactivator proteins (Shiau et al., 1998; Brzozowski et al., 1997). The prediction of the in vitro assays on the antagonistic potential of compounds 1 to 6 was confirmed in a cellular assay, in which compounds 1 and 2 showed only a weak agonistic action and were functional antagonists of $1\alpha,25(OH)_2D_3$-induced gene activity. If a VDR agonist is not metabolized, its EC_{50} value in a DNA-dependent in vitro assay, such as the gel shift assay, should be identical with that in a cellular assay, such as the mammalian one-hybrid assay. This appears to be the case for compounds 3 to 5. In contrast, the EC_{50} values of the natural hormone and compound 6 in the gel shift assay were 5- and 8-fold lower, respectively, than those in the related reporter gene assay in HeLa cells, which indicates that both VDR agonists are metabolized. The observation that compound 6, but not the structurally similar compounds 3 to 5, appear to be metabolized correlates with the finding that only compound 6 was able to stabilize the VDR in conformation $c3_{LPD}$. Compounds 1 and 2 also appear to be metabolized, because the effective concentrations for obtaining a half-maximal antagonistic effect (approximately 300 and 100 nM) are 15- and 5-fold higher than the values that would be expected from the ratio of their respective EC_{50} values in the gel shift assay compared with that of $1\alpha,25(OH)_2D_3$. Taken together, both in vitro assays, supershift and limited protease digestion, consistently indicated that compounds 1 and 2 are antagonists, because they are not able to stabilize the VDR in a conformation that enables interaction with coactivators, which in turn reduces the ability of transactivation. In contrast, compounds 3 to 6 appear to be functional VDR agonists, i.e., the relative minor modifications of their side chains were sufficient to restore agonism.

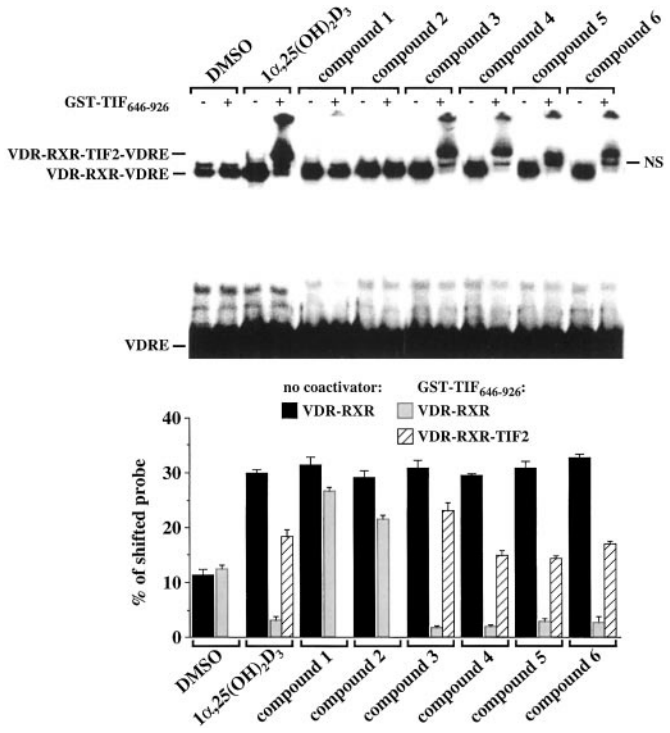

Fig. 3. Compounds 1 and 2 do not stimulate VDR-coactivator interactions. Gel shift experiments were performed with in vitro translated VDR-RXR heterodimers that were preincubated in the absence (−) or presence (+) of bacterially expressed GST-$TIF2_{646-926}$ with saturating concentrations (10 μM) of DMSO (as a negative control), $1\alpha,25(OH)_2D_3$ (as a positive control), compounds 1 to 6, 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. 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. Columns represent the means of triplicates and the bars indicate S.D. NS indicates a nonspecific complex.

The direct comparison of the two antagonists, compounds 1 and 2, in the cellular assay indicated that compound 2 is more potent, because it has a lower residual agonistic action and shows its antagonistic effects already at approximately 3 times lower concentrations than compound 1. The tendency that compound 2 is more sensitive than compound 1 was also indicated by 1.2- to 2.6-fold lower EC_{50} values in gel shift assays, limited protease digestion assays, and agonistic tests in the cellular system. By a comparison of the structures of compounds 1 and 2 with those of the four VDR agonists, compounds 3 to 6, clear structural requirements for the antagonistic profile can be deduced. It is obvious that a cyclopropyl ring in the center of the side chain at carbon 25 is essential for antagonistic behavior. Ring enlargement by one or two methylene units (compounds 4 and 5), as well as opening of the ring creating the geminal dimethyl situation (compound 3), changes the biological profile to an agonistic behavior. Thus, the high degree of strain generated by the cyclopropyl ring seems to be crucial for antagonism. Moreover, the length of the side chain dictates the biological profile. Shortening the side chain below the size of compound 1 results in agonistic activity, whereas elongation of the side chain preserves the antagonism (data not shown). The central portion of the side chain is rather sensitive to structural modifications. For exerting antagonistic activity, a 24-hydroxyl group, which must have the 24*R* configuration, is essential. Oxidation of this alcohol to the 24-ketone completely switches the biological properties to the agonistic side (compound 6). However, the distant parts of the side chain show a higher degree of structural flexibility, because the positioning of the carboxylic ester unit is rather variable (see

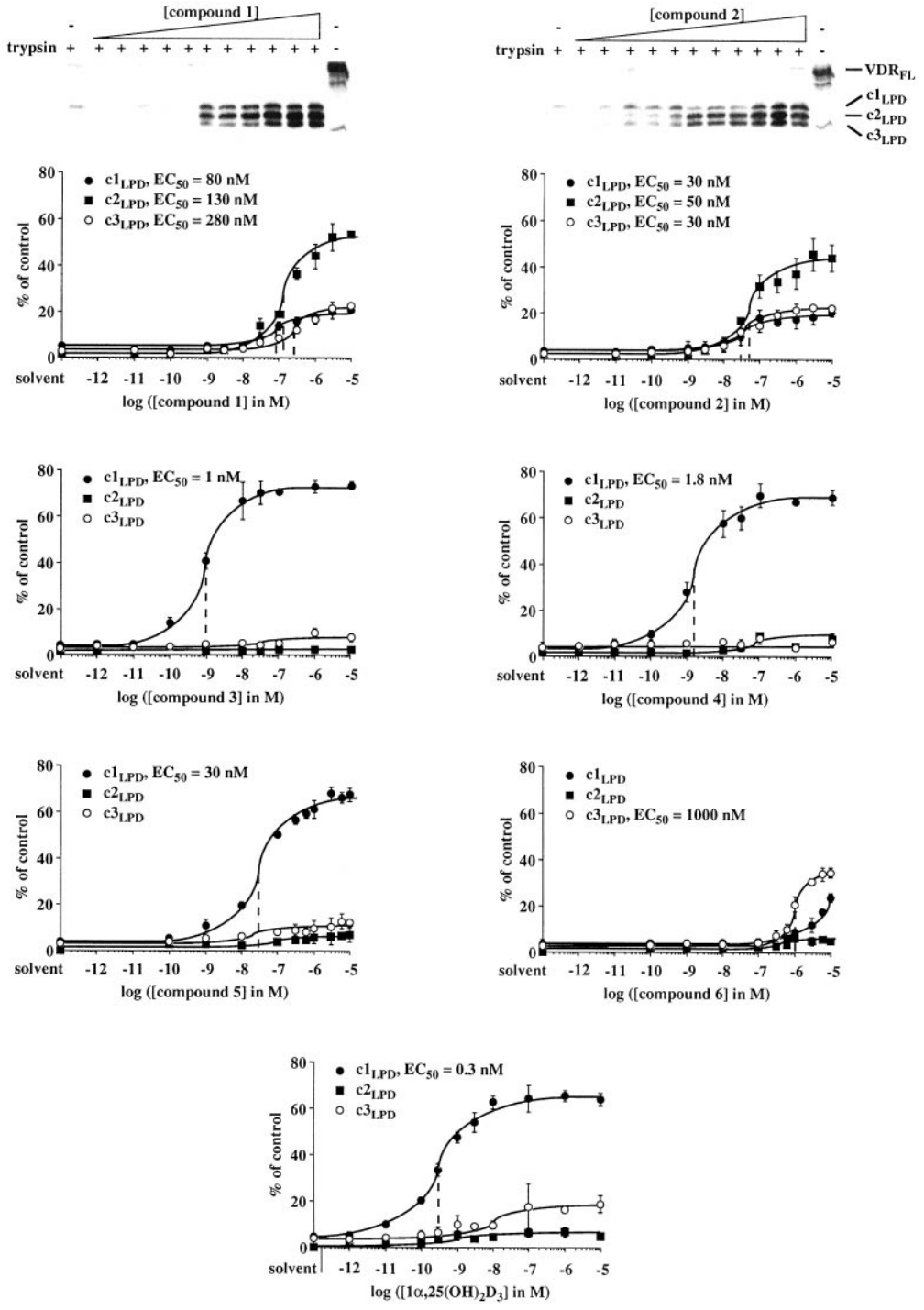

Fig. 4. Compounds 1 and 2 stabilize the VDR in the antagonistic conformation c2$_{LPD}$. Limited protease digestion assays were performed by preincubating in vitro translated ^{35}S-labeled VDR with graded concentrations of compounds 1 to 6 or 1α,25(OH)$_2$D$_3$ (as a control). After digestion with trypsin, samples were electrophoresed through 15% SDS-polyacrylamide gels. The amount of ligand-stabilized VDR conformations 1 (c1$_{LPD}$, filled circles), 2 (c2$_{LPD}$, filled squares), and 3 (c3$_{LPD}$, open circles) in relation to VDR input was quantified by phosphorimaging. Representative experiments are shown for compounds 1 and 2. Data points represent the means of triplicates and the bars indicate S.D. The EC$_{50}$ values for the stabilization of VDR conformations were determined from the respective dose-response curves.

compounds 1 and 2). Furthermore, planarization of the side chain by introducing double bonds is possible without loosing the antagonistic activity. Taken together, essential structural features for antagonists are the 24*R*-hydroxyl group and the 25-cyclopropyl ring together with a certain total length of the side chain. Introduction of more structural rigidity is allowed for the distant portion only.

In conclusion, compound 2, a dihomo derivative of the 25-carboxylic ester ZK159222 (compound 1), has been identified as a novel, more potent antagonist of the VDR. Compound 2 acts as an antagonist by stabilizing the antagonist-specific VDR conformation c2$_{LPD}$, which keeps helix 12 in a displaced position and thus does not allow an interaction of the VDR with coactivator proteins. The methods used in this

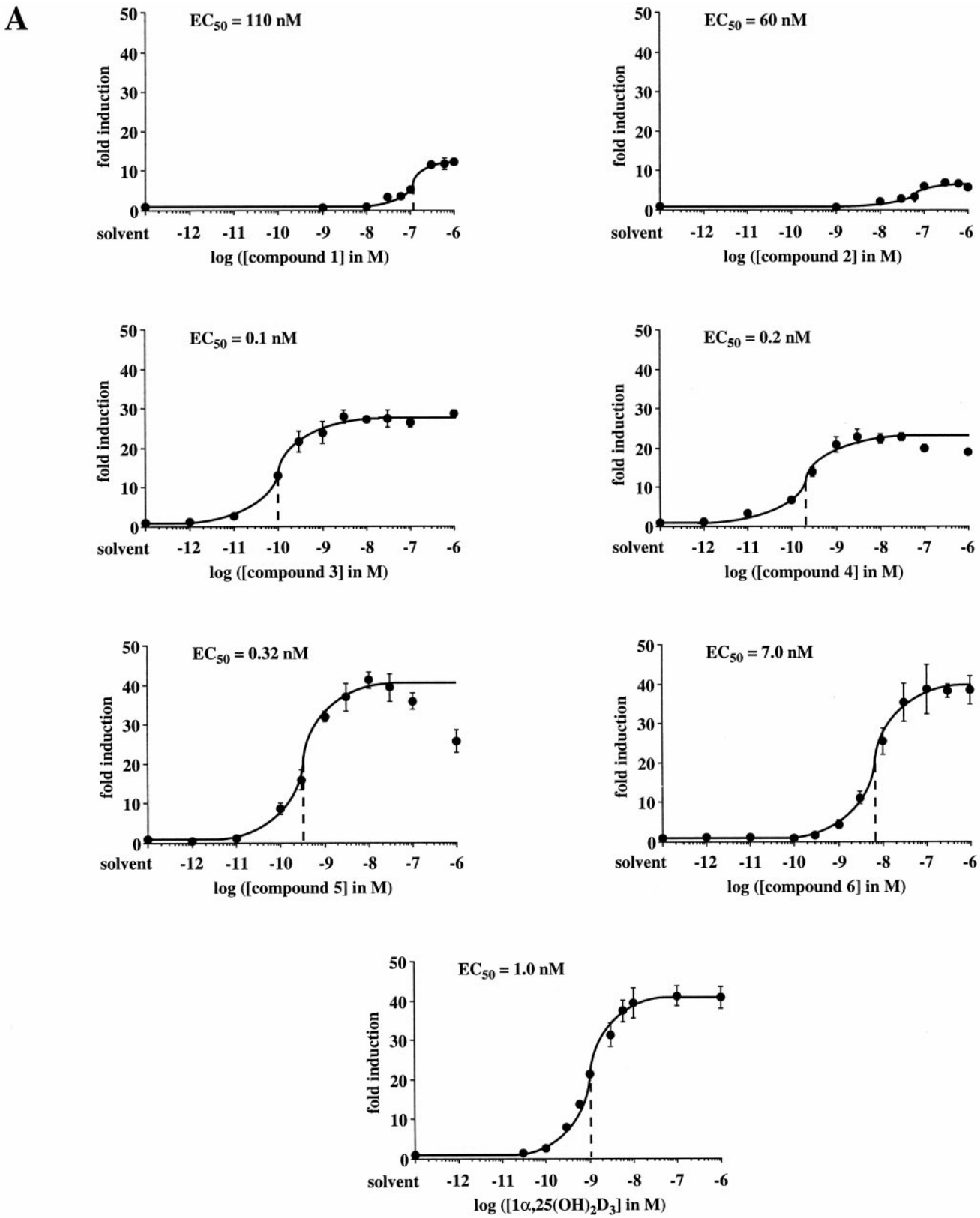

Fig. 5. Antagonistic effects of compounds 1 and 2 in vivo. Luciferase reporter gene assays were performed with extracts from HeLa cells that were transiently transfected with a reporter gene construct-driven by three copies of the GAL4-binding site and an expression vector for a $GAL4_{DBD}VDR_{LBD}$ fusion protein. The cells were treated for 16 h with graded (A and C) or saturating (1 μM, B) concentrations of compounds 1 to 6 or 1α,25$(OH)_2D_3$ (as a control) in the absence (−) or presence (+) of 10 nM 1α,25$(OH)_2D_3$ (as indicated). Stimulation of luciferase activity was calculated in comparison to solvent-induced controls and normalized to maximal induction with 1α,25$(OH)_2D_3$ (41-fold; B and C). Data points (A) or columns (B and C) represent the means of triplicates and the bars indicate S.D. The EC_{50} values for stimulation of VDR-driven gene activity (A) were determined from the respective dose-response curves.

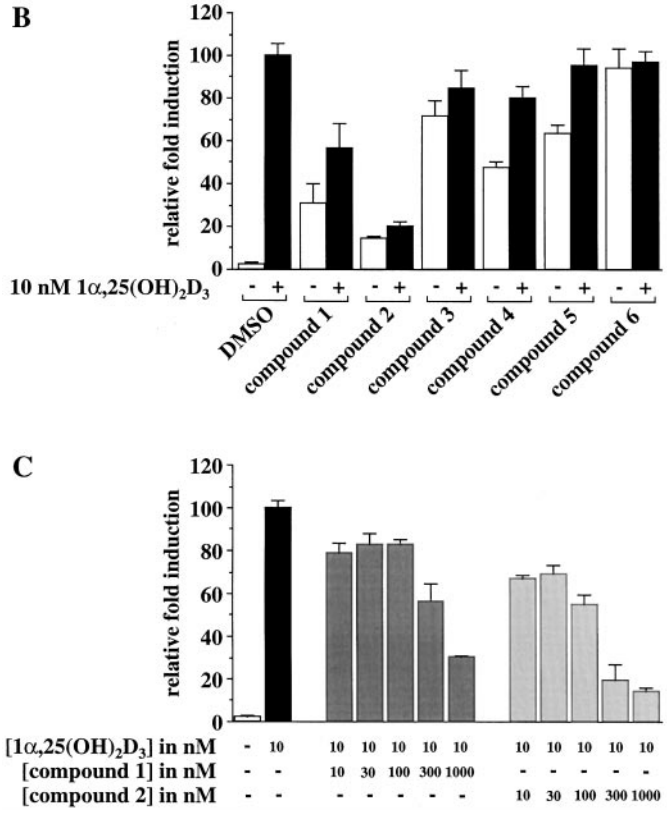

Fig. 5b.

study appear to be very appropriate for screening a larger group of $1\alpha,25(OH)_2D_3$ analogs for potential antagonists.

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