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Structural Evaluation of the Agonistic Action of a Vitamin D Analog with Two Side Chains Binding to the Nuclear Vitamin D Receptor

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ABSTRACT

The vitamin D receptor (VDR) is one of the endocrine members of the nuclear receptor superfamily and has a characteristic high affinity for its natural ligand 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$]. From a mechanistic point of view, the most interesting analog of 1α,25$(OH)_2D_3$ is the one that carries two side chains, referred to as Gemini. In this study, molecular dynamics (MD) simulations of the Gemini-VDR complex were performed that demonstrated that the binding of a ligand with a 25% increased volume does not disturb the overall structure of the ligand-binding domain (LBD). It was found that one of the two side chains takes exactly the same position as the single side chain of the natural ligand, which suggests that the molecular mechanism of the agonism of Gemini is identical to that of 1α,25$(OH)_2D_3$. VDR single and double point mutants at L227, A303, I313, and L397 and in vitro and ex vivo assessment of their agonistic action confirmed the predictions of the MD simulations. Moreover, it was found that the second side chain of Gemini can choose between two binding positions within the ligand-binding pocket of the VDR. These two newly identified "corners" were characterized most specifically by the amino acids pairs L227/A303 and I313/L397. Therefore, Gemini is an important model compound that allows further insight into the molecular actions of the VDR but is, in parallel, also a promising precursor for the design of even more potent 1α,25$(OH)_2D_3$ analogs.

Nuclear receptors (NRs) form a large family of eukaryotic transcription factors (48 genes in the human genome) that regulate development and metabolism through control of gene expression (Mangelsdorf et al., 1995). NRs are characterized by a highly conserved DNA-binding domain and a less conserved ligand-binding domain (LBD). The LBD is formed by 11 to 13 α-helical structures, and its overall architecture seems to be very similar for all NRs, because it is folded into a three-layered antiparallel α-helical sandwich that comprises a central core layer of the helices 5, 9, and 11 that is sandwiched between two additional layers of helices creating a ligand-binding pocket (Wurtz et al., 1996). Binding of an agonistic ligand to the LBD initiates the dissociation of corepressor proteins and an enhanced interaction with dimerization partners, which consequently increases the amount of complex formation with DNA. However, the major consequence of an agonist-induced conformational change of the LBD is a repositioning of the most carboxy-terminal α-helix (helix 12), which contains a short transactivation function 2 (AF-2) domain (Bourguet et al., 2000). A glutamate residue of the AF-2 domain forms, together with a lysine residue in helix 3, a charge clamp that positions the NR interaction domain of coactivator proteins to a hydrophobic cleft on the surface of the LBD (Feng et al., 1998). The correct positioning of helix 12 is critical for the distance of the glutamate to the lysine residue and influences in this way the efficacy of the NR-coactivator interaction. Coactivator proteins in turn contact components of the basal transcriptional machinery resulting in an enhanced transcription of NR target genes (Freedman, 1999).

Classic endocrine NRs, such as the receptors for steroid hormones, vitamin D, thyroid hormone, and retinoic acid, bind their ligands with a K_d value on the order of 1 nM or lower (Beato et al., 1995). In contrast, adopted orphan NRs that are sensors for lipids, such as certain polyunsaturated

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ABBREVIATIONS: NR, nuclear receptor; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; AF-2, (trans)activation function-2; ANF, atrial natriuretic factor; DR3, direct repeat spaced by 3 nucleotides; GST, glutathione *S*-transferase; MD, molecular dynamics; RXR, retinoid X receptor; LES, locally enhanced sampling; LBD, ligand-binding domain; RMSD, root-mean-square deviation; TIF2, transcriptional intermediary factor 2; VDR, 1α,25$(OH)_2D_3$ receptor; VDRE, 1α,25$(OH)_2D_3$ response element; RO272310, 1,25-dihydroxy-21(3-hydroxy-3-methylbutyl)-cholecalciferol; RO4383582, 1,25-dihydroxy-20*S*, 21(3-hydroxy-3-methylbutyl)-23-yne-26,27-hexafluoro-cholecalciferol; RO4383586, 1,25-dihydroxy-20*R*,21(3-hydroxy-3-methylbutyl)-23-yne-26,27-hexafluoro-cholecalciferol; DMEM, Dulbecco's modified Eagle's medium.

fatty acids, oxysterols, bile acids, and other cholesterol derivatives, interact with their ligands in a concentration range of 1 μM or higher (Chawla et al., 2001). The clear difference in the K_d values of endocrine and adopted orphan NRs is related to the much larger ligand-binding pocket of the latter. On the average, the ligand-binding pocket of an adopted orphan NR is filled to only 30 to 40% with ligand, whereas nuclear hormones occupy the ligand-binding pockets of their specific receptors to 60% or more. This tight adaptation of hormones to their receptors seems to restrict the possibilities to increase the ligand volume during the creation of effective synthetic analogs.

The vitamin D receptor (VDR) belongs to the endocrine NRs (Carlberg, 1995), because it binds the biological active form of the secosteroid vitamin D, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], with a K_d value of 0.1 nM. However, in contrast to other endocrine NRs, the ligand binding pocket of VDR is, with 697 Å^3, rather large and only 56% filled by the natural ligand (Rochel et al., 2000). 1α,25$(OH)_2D_3$ plays an important role in calcium resorption and bone formation (DeLuca et al., 1990) and was also shown to act as a regulator of cellular proliferation, differentiation, and apoptosis (Walters, 1992). VDR acts preferentially as a heterodimer with the retinoid X receptor (RXR) 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 and Polly, 1998). VDR-RXR-VDRE complexes are the molecular cores of DNA-dependent 1α,25$(OH)_2D_3$ signaling (Carlberg et al., 2001) and the stabilization of the agonistic conformation of the LBD of the VDR is the most critical step in this signaling process. This is achieved by a hydrogen bond between the C25-hydroxyl group of 1α,25$(OH)_2D_3$ and H397 of the receptor (Rochel et al., 2000) and is supported by an additional, minor important hydrogen bond with H305 (Väisänen et al., 2002b). In the presence of agonist, H397 is able to form van der Waals contacts with F422 of the AF-2 domain, which keeps helix 12 in an optimal position for the charge clamp between E420 (helix 12) and K246 (helix 3).

More than 2000 analogs of 1α,25$(OH)_2D_3$ have been synthesized with the goal of improving the potency and specificity of the physiological effects of vitamin D (Bouillon et al., 1995). The large majority of these analogs have been modified at the side chain, which in most cases increases their metabolic stability and, in the case of potent agonists, increases the half-life of the VDR-ligand complex (van den Bemd et al., 1996; Bury et al., 2001b). However, most superagonists carry only minor modifications compared with the natural hormones and stabilize the same agonistic VDR conformation via the H397-F422 interaction (Tocchini-Valentini et al., 2001). A very interesting exception is Gemini, which is the first 1α,25$(OH)_2D_3$ analog that carries two side chains (Uskokovic et al., 1997; Herdick et al., 2000; Norman et al., 2000); i.e., this analog has a ~25% higher volume than the natural hormone. Gemini was shown to act as a potent agonist and seems to be able to bind the VDR in its agonistic conformation (Herdick et al., 2000; Herdick and Carlberg, 2000), but it is not obvious how a ligand with such a drastically increased volume can fit into the tight ligand-binding pocket of the VDR. Therefore, in this study, molecular dynamics (MD) simulations of the Gemini-VDR complex were performed on the basis of the X-ray structure of the 1α,25$(OH)_2D_3$-bound VDR-LBD (Rochel et al., 2000). One of the two side chains of Gemini was found to have the same location as in the natural hormone and contacts H397 and H305, whereas for the second side chain, two approximately equal positions were identified. The results of the MD simulations were confirmed by receptor mutagenesis, coactivator interaction studies in vitro, and functional assays in living cells. It was indicated that Gemini uses both possible positions and that the addition of fluor atoms improves the potential of Gemini even more. Taken together, this study shows that the ligand-binding pocket of the VDR is flexible enough to accommodate large ligands, such as Gemini, that act even more efficiently than the natural hormone.

Materials and Methods

Compounds. Gemini (RO272310) is a 21-(3-methyl-3-hydroxybutyl) derivative of 1α,25$(OH)_2D_3$. RO4383582 and RO4383586 are hexafluoro-derivatives of Gemini that differ in their configuration at carbon 20. The synthesis of Gemini and its derivatives was described elsewhere (Uskokovic et al., 1997; Manchand and Uskokovic, 1999; 2000; Norman et al., 2000), their structures are shown in Fig. 1. All compounds were dissolved in 2-propanol; further dilutions were made in dimethyl sulfoxide (for in vitro experiments) or in ethanol (for cell culture experiments).

MD Simulations. The initial coordinates for the MD simulations were obtained from the X-ray crystal structure of the 1α,25$(OH)_2D_3$-VDR-LBD complex (Brookhaven Protein Data Bank code 1DB1) determined to 1.8-Å resolution (Rochel et al., 2000). The missing amino acid residues of the X-ray structure (residues 118, 119, 375–377, and 424–427) were build using the Quanta98 molecular modeling package (Molecular Simulations Inc., San Diego, CA). The four residues missing from the C terminus (424–427) were built in an α-helical conformation ($\phi = -57°$, $\psi = -47°$). Crystallographic water molecules were included in simulation systems neutralized by placing four Na^+ ions in the positions of largest electrostatic potential as determined by the program CION of the AMBER6.0 simulation package (University of California, San Francisco, CA). Gemini was docked to the ligand-binding pocket of the LBD using the locally enhanced sampling (LES) method, which is a mean-field technique providing ability to focus on the interesting part of the system (Simmerling and Elber, 1995). In practice, multiple copies are employed on the part of the system in which conformational sampling may be critical, and the rest of the system is treated as a single structure. For the LES calculations, the protein-ligand complexes were solvated by adding a sphere of water molecules with a 25-Å radius from the mass center of the ligand. This resulted in 764 water molecules for the complexes. Only residues having atoms within 12 Å of the atoms of the ligand were allowed to move. The water molecules of the

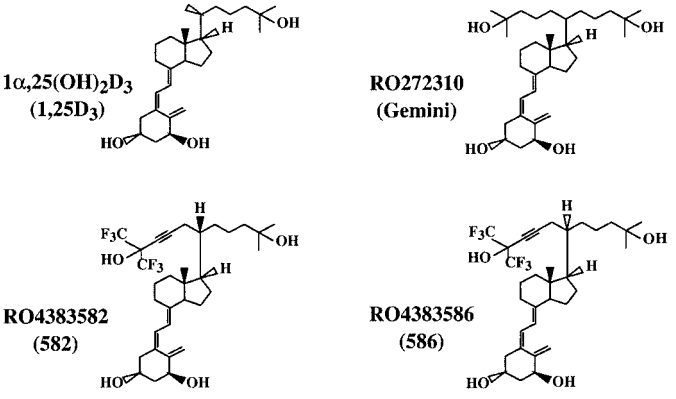

Fig. 1. Structure of 1α,25$(OH)_2D_3$, Gemini, and its derivatives. The short names of the ligands being used in Figs. 3 and 4 are indicated in parentheses.

complex were first energy-minimized for 2000 steps, heated to 300 K in 5 ps, and equilibrated by 30 ps at a constant temperature of 300 K. After that, the moving part of the protein-ligand complex was minimized for 2000 steps, the temperature of the system was increased to 300 K in 5 ps, and equilibrated for 75 ps using a time step of 2 fs. The system was then heated to 400 K in 5 ps and equilibrated for 40 ps using a time step of 1 fs and applying harmonic positional restraint of 2.0 kcal/mol. After that, the positional restraints were removed, velocities of the atoms were assigned to correspond to 340 K, and the system was cooled from 340 to 0 K in 160 ps.

For periodic box simulations, the LBD-ligand complexes were solvated by 10,778 water molecules in a periodic box of 69 × 61 × 87 Å. The minimization and equilibration of water molecules were done as in the water cap simulations. Then the whole system was again energy-minimized for 2000 steps, heated to 300 K in 5 ps, and equilibrated by 300 ps at 300 K and pressure of 101.325 Pa. After that, 2-ns production simulations were started. Bonds involving hydrogen atoms were constrained to their equilibrium lengths using the SHAKE algorithm. The time step of 1.5 fs was used in periodic box simulations. The electrostatics were treated using the particle-mesh Ewald method and continuum model correction was applied for energy and pressure. The center of mass velocity was removed each 75 ps. All the simulations were done using the AMBER6 program and the force field described by Cornell et al. (1995).

DNA Constructs and Point Mutagenesis. The full-length cDNAs for human VDR (Carlberg et al., 1993) and human RXRα (Levin et al., 1992) were subcloned into the simian virus 40 promoter-driven pSG5 expression vector (Stratagene, LaJolla, CA). These constructs are also suitable for T_7 RNA polymerase-driven in vitro transcription/translation of the respective cDNAs. The point mutants of VDR were generated using the Quik Change site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. The luciferase reporter gene was driven by four copies of the rat atrial natriuretic factor (*ANF*) gene promoter DR3-type VDRE (core sequence, AG**AGGTCA**TGA**AGGACA**) (Kahlen and Carlberg, 1996) fused to the *tk* promoter (bold characters indicate the hexameric core binding motifs). The NR interaction domain of the human coactivator transcription intermediary factor 2 (spanning amino acids 646–926) (Voegel et al., 1996) was subcloned into the glutathione *S*-transferase (GST) fusion vector pGEX (Amersham Biosciences, Uppsala, Sweden).

In Vitro Protein Translation and Bacterial Fusion Protein Overexpression. In vitro translated VDR and RXR proteins were generated by transcribing their respective 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, Madison, WI). ^{35}S-Labeled VDR was generated by translation in the presence of [^{35}S]methionine. 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.

GST Pull-Down Assay. The GST pull-down assay was performed with 40 μl of a 50% Sepharose bead slurry of GST-TIF2$_{646–926}$ (preblocked with 1 μg/μl bovine serum albumin) and 20 ng of in vitro translated, ^{35}S-labeled VDR (wild-type or mutant) in the presence of indicated ligands. VDR proteins were incubated in immunoprecipitation buffer (20 mM HEPES, pH 7.9, 200 mM KCl, 1 mM EDTA, 4 mM $MgCl_2$, 1 mM dithiothretiol, 0.1% Nonidet P-40 and 10% glycerol) for 20 min at 30°C. In vitro translated proteins that were not bound to GST-fusion proteins were washed away with immunoprecipitation buffer. GST-fusion protein bound ^{35}S-labeled VDRs were resolved by electrophoresis through 10% SDS-polyacrylamide gels and quantified on an FLA3000 reader (Fuji, Tokyo, Japan) using Image Gauge software (Fuji).

Supershift Assay. Heterodimers formed by in vitro translated VDR (wild type or mutant) and RXR were incubated with saturating concentrations of 1α,25(OH)$_2$D$_3$ or its analogs for 15 min at room temperature in a total volume of 20 μl of binding buffer [10 mM HEPES, pH 7.9, 1 mM dithiothretiol, 0.2 μg/μl poly(dI-C), and 5% glycerol]. The buffer had been adjusted to 150 mM by addition of KCl. Three micrograms of bacterially expressed GST-TIF2$_{646–926}$ fusion protein were included in the incubation. Approximately 1 ng of the ^{32}P-labeled DR3-type VDRE from the rat *ANF* gene was then added to the protein-ligand mixture and incubation was continued for 20 min. Protein-DNA complexes were resolved through 8% nondenaturing polyacrylamide gels in 0.5× Tris borate/EDTA (45 mM Tris, 45 mM boric acid, and 1 mM EDTA, pH 8.3) and were quantified on a Fuji FLA3000 reader.

Transfection and Luciferase Reporter Gene Assay. 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 5% charcoal-treated fetal bovine serum. Liposomes were formed by incubating 1 μg of the reporter plasmid and 1 μg each of pSG5-based receptor expression vectors for VDR (wild-type or mutant) and RXR with 10 μg of *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, VDR 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 (Canberra-Packard, Groningen, The Netherlands). 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

As a preparation for MD simulations Gemini was docked into the crystal structure of the VDR-LBD (Rochel et al., 2000), which has been completed by molecular modeling with the amino acid residues 118, 119, 375–377, and 424–427. Twelve different LBD-Gemini conformations were simulated with the LES method by systematically rotating both side chains of Gemini in steps of 20° around the C17–C20 bond, whereas the first side chain stays at the same position as within the 1α,25(OH)$_2$D$_3$-VDR-LBD structure. This conformational analysis resulted in two possible positions of the extra side chain of Gemini, which were studied further with long periodic box MD simulations. The whole VDR-LBD view (Fig. 2A) shows the positions of the second side chain of Gemini in relation to its first one. Interestingly, the first side chain of Gemini keeps the same position than the single side chain of 1α,25(OH)$_2$D$_3$. The detailed views on Gemini in position 1 (Fig. 2, B and C) and position 2 (Fig. 2, D and E) were restricted to those 12 amino acid residues that are near the second side chain. Interestingly, in position 1, the extra side chain of Gemini points toward the same direction as the methyl-group (C21) at C20 of the 1α,25(OH)$_2$D$_3$-VDR-LBD crystal structure (Rochel et al., 2000), whereas in position 2, the second side chain is rotated by 120° in relation to its location in position 1. In detail, the dihedral angle of C16-C17-C20-C21 of 1α,25(OH)$_2$D$_3$ in the X-ray structure was −32.6°, the respective angles of Gemini in positions 1 and 2 were −29.5° and −69.8°, respectively, for the first side chain and 102.7° and 155.6°, respectively for the extra side chain. The C25-hydroxy group of the extra side chain of Gemini was found to form a hydrogen bond with Q400 in position 1 and with V300 in position 2.

The distance between the 12 amino acids of the binding site and the second side chain (Tab. 1) suggests that the amino acid residues M272, L309, I313, L393 and H397 are significantly closer to the extra side chain when Gemini is in position 1. In contrast, amino acid residues L227, L230, and A303 are closer to that side chain when the ligand is in position 2. The crystal structure of the 1α,25(OH)$_2$D$_3$-VDR-LBD complex and the simulated Gemini-VDR-LBD complex were compared via the root-mean-square deviation (RMSD) value, which was calculated from the last nanosecond of the 2-ns simulation. The RMSD value of the 12 C_α-atoms of the binding site of the extra side chain was found to be 0.62 Å (1.26 Å for all atoms) for position 1 and 0.67 Å (1.26 Å for all atoms) for position 2. Interestingly, a corresponding MD simulation of the 1α,25(OH)$_2$D$_3$-VDR-LBD complex provided already a RMSD-value of 0.45 Å for these 12 C_α-atoms. Moreover, the RMSD-value of all C_α-atoms of the VDR-LBD was 2.1 Å for position 1 and 1.9 Å for position 2; i.e., for both positions, only rather minor changes in the VDR-LBD structure were necessary to accommodate the second side chain of Gemini.

For an experimental confirmation of the model derived by MD simulations (Fig. 2) and for a possible discrimination of the two predicted binding positions of the extra side chain of Gemini, amino acid residues L227 and A303 were selected to be most characteristic for position 2, whereas the residues I313 and L393 should represent most specifically position 1 (see Table 1). In the full-length VDR expression construct, these four amino acids were mutated individually to phenylalanine to disturb most effectively with this bulky, aromatic side chain the binding of Gemini to the respective position. In three different assay systems, the four VDR mutants were compared with wild-type VDR for the agonistic potential of Gemini in relation to the natural hormone (Fig. 3). Moreover, hexafluoro-derivatives of Gemini, RO4383582, and RO4383586, in which one of the two side chains was constrained by a triple bond and made more electronegative by replacing six hydrogen atoms with fluor atoms (for structures see Fig. 1), were included in the comparison.

The GST pull-down assay (Fig. 3A) monitored the ligand-induced interaction of VDR with the coactivator TIF2 in solution. Compared with solvent control, all four ligands were able to increase the VDR_{wt}-TIF2 interaction from 5 to ~40%. The mutant VDR_{L227F} interacted more efficiently with TIF2 when it was induced by 1α,25(OH)$_2$D$_3$ than by Gemini, whereas both hexafluoro derivatives were comparable with each other and more effective than Gemini. On the mutant VDR_{A303F}, the agonistic action of both 1α,25(OH)$_2$D$_3$ and Gemini was nearly completely blocked, whereas both Gemini derivatives still showed approximately half of their maximal potential. The action of 1α,25(OH)$_2$D$_3$ on VDR_{A303F} was affected, because amino acid 303 is also near the binding site of the first side chain. Concerning the mutants VDR_{I313F} and VDR_{L393F}, the GST pull-down assay proved to be insufficiently stringent to allow discrimination among the four ligands. However, it could be shown that VDR_{I313F} has only

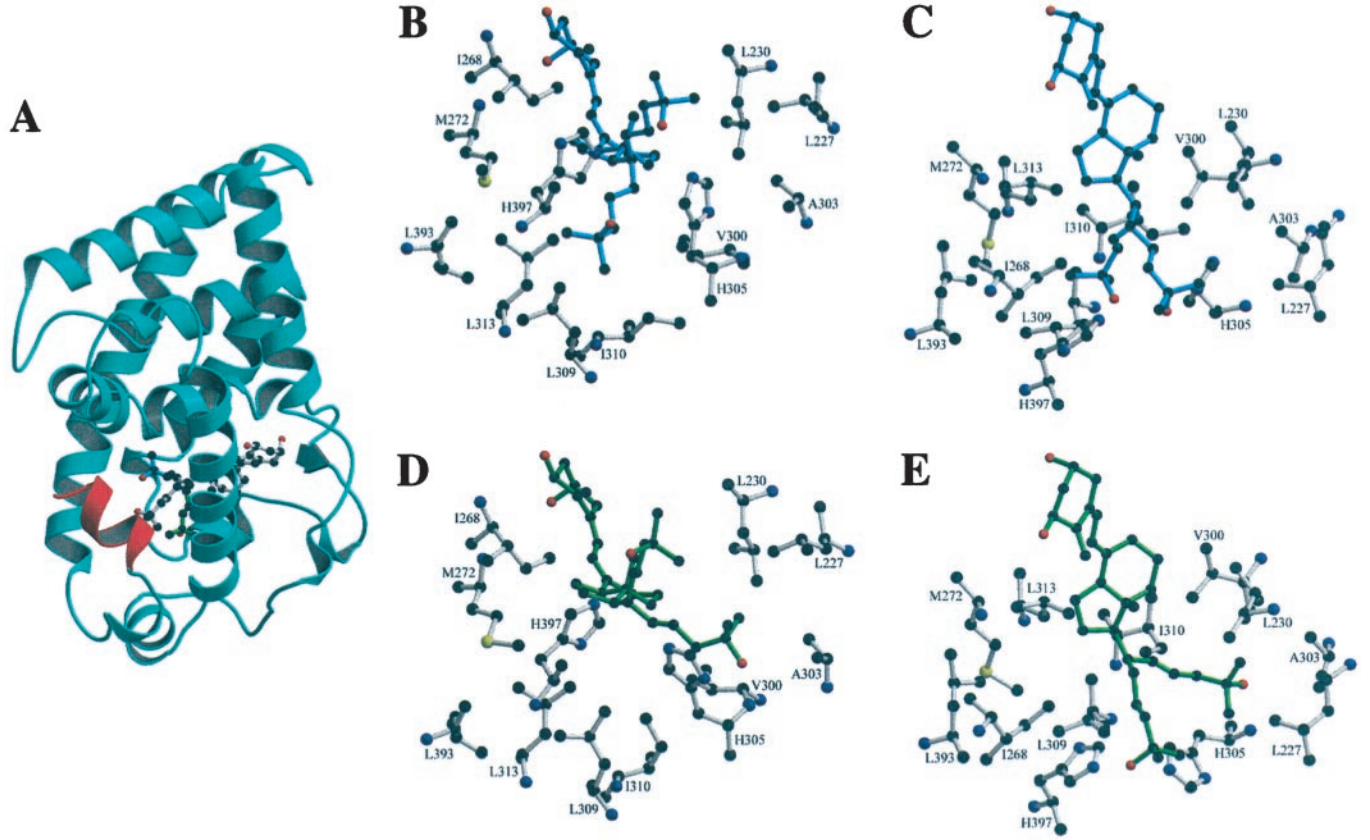

Fig. 2. MD simulations of Gemini-VDR-LBD complexes. A, view of the whole VDR-LBD complexed with 1α,25(OH)$_2$D$_3$ as reported by Rochel et al. (2000). The location of the extra side chain of Gemini in position 1 (blue) and position 2 (green) are after MD simulations. B, detailed view on Gemini (blue) in position 1. Only those 12 amino acids that are nearest the extra side chain of Gemini are shown. C, same as B, but rotated by 90° around the horizontal axis. D, Detailed view on Gemini (green) in position 2. E, same as D, but rotated by 90° around the horizontal axis. All Gemini structures are averages from the last 200 ps of 2-ns MD simulations.

half of the coactivator interaction potential of wild-type VDR, whereas VDR_{L393F} displayed nearly full activity.

In the supershift assay (Fig. 3B), the ligand-induced interaction of DNA-bound VDR-RXR complexes with TIF2 was assessed. The results were quite comparable with those from the GST-pull down assay (Fig. 3A); i.e., on wild-type VDR, all four ligands showed comparable effects, whereas on each of the four mutants, the hexafluoro derivatives were more effective than $1\alpha,25(OH)_2D_3$ and Gemini. Moreover, in agreement with the GST pull-down assay, the supershift assay demonstrated that on the mutant VDR_{L227F} $1\alpha,25(OH)_2D_3$ was clearly more effective than Gemini, whereas both ligands were blocked with VDR_{A303F}.

Finally, the reporter gene assay (Fig. 3C) analyzed the agonistic potential of the four ligands in the transiently transfected model cell line MCF-7 (human breast cancer). Also, this assay showed results similar to those of the two previous ones. The only remarkable difference is that Gemini and its derivatives showed activities in living cells slightly higher than those of the natural hormone. In agreement with the two previous assays, no significant difference between the two hexafluoro derivatives could be detected, and on all four VDR mutants, they proved to be more active than $1\alpha,25(OH)_2D_3$ and Gemini. On the mutants VDR_{L227F}, VDR_{I313F}, and VDR_{L393F}, $1\alpha,25(OH)_2D_3$ was found to be more active than Gemini, whereas with VDR_{A303F}, both showed low activity.

For a more detailed analysis, double point mutants of VDR were created in which each two amino acid residues were mutated, so that both are affecting Gemini only in position 1 ($VDR_{I313F/L393F}$) or only in position 2 ($VDR_{L227F/A303F}$) or each is influencing a different binding site ($VDR_{L227F/I313F}$ and $VDR_{L227F/L393F}$). Please note that the two other possible double point mutants ($VDR_{A303F/I313F}$ and $VDR_{A303F/L393F}$) were not considered useful, because VDR_{A303F} on its own already inhibited the action of $1\alpha,25(OH)_2D_3$ and Gemini (see Fig. 3). With the four double point mutants (Fig. 4), the same set of assays were performed as with the single point mutants (Fig. 3). All three assays provided comparable results exception of Gemini on mutant $VDR_{I313F/L393F}$, which was found to be quite active in the GST pull-down assay (Fig. 4A) but not in the supershift (Fig. 4B) or reporter gene assays (Fig. 4C). This deviation was most probably attributable to the lower stringency of the GST pull-down assay, which was done in solution without RXR, whereas the supershift and reporter gene assays were performed on DNA in the presence of RXR. However, a consistent observation was that on the mutants that affect only one position at the time ($VDR_{L227F/A303F}$ and $VDR_{I313F/L393F}$), the activity of Gemini was still significantly higher than on the crossover double mutants ($VDR_{L227F/I313F}$ and $VDR_{L227F/L393F}$). Similarly, both hexafluoro derivatives of Gemini showed still reasonable activity on $VDR_{L227F/A303F}$ and $VDR_{I313F/L393F}$, which decreased on $VDR_{L227F/I313F}$ and $VDR_{L227F/L393F}$. In contrast, $1\alpha,25(OH)_2D_3$ did not differentiate between the different type of double mutants.

TABLE 1

Distances between the amino acid residues of the ligand-binding pocket and the second side chain of Gemini.

The LBD-Gemini structures are averages collected from the last 200 ps of 2-ns MD simulations.

Residue	Distance	
	Position 1	Position 2
	Å	
L227	9.0	4.1
L230	7.2	3.5
I268	5.8	6.8
M272	4.8	7.4
V300	3.6	4.0
A303	7.9	3.6
H305	4.1	3.9
L309	3.8	5.8
I310	3.8	4.2
I313	4.0	6.3
L393	5.1	11.2
H397	2.9	4.7

Discussion

After the first reports on Gemini (Uskokovic et al., 1997; Herdick et al., 2000; Norman et al., 2000) the analog has been accepted as a potent VDR agonist, but its mechanism of action and method of binding to the VDR-LBD was not understood. Therefore, the first important finding of the MD simulations was that one of the two side chains of Gemini takes exactly the same position as the single side chain of the natural ligand. Because the side chains of Gemini themselves are not modified, this observation suggests that the molecular mechanism of the agonism of Gemini is identical to that of $1\alpha,25(OH)_2D_3$. This means that the hydroxyl group at C25 of one of the two side chains contacts H397 and H305 just as it does in $1\alpha,25(OH)_2D_3$. Amino acid H397 interacts with F422, which in turn stabilizes helix 12, in particular E420, in a position that is optimal for interaction with coactivator proteins. However, it could be imagined that the extra side chain of Gemini either directly interferes with this optimal positioning of helix 12 or that it disturbs the overall structure of the LBD, so that it harms indirectly the stabilization of agonistic conformation of the VDR. Therefore, it is important that the MD simulation could indicate that the ligand-binding pocket of VDR has sufficient extra space to accommodate a second side chain. Compared with the $1\alpha,25(OH)_2D_3$-VDR-LBD crystal structure, only minor shifts of the C_α-atoms were necessary to fit Gemini in. This means that the binding of Gemini does not disturb the overall structure of the VDR-LBD.

Another interesting finding was that there are two different binding sites for Gemini within the VDR-LBD. In position 1, the extra side chain of Gemini points in the same direction as the C21-methyl group of $1\alpha,25(OH)_2D_3$, whereas in position 2, it is rotated by 120° in relation to the first position (see Fig. 2A). Mutated into bulky, nonpolar phenylalanines, the amino acids at positions 227, 303, 313, and 393 (being by pairs specific for one of the two binding positions of the extra side chain) were shown to disturb the action of Gemini significantly more than that of $1\alpha,25(OH)_2D_3$. This suggests that Gemini is using both binding sites. This conclusion is further supported by the analysis of double point mutants. It could be demonstrated that filling both binding sites of the extra side chain with one bulky phenylalanine is more severe than placing two phenylalanines together into one or the other binding site. Precise interactions of the second side chain of Gemini with the amino acids of the two new corners of the ligand-binding pocket await further cocrystallographic analysis.

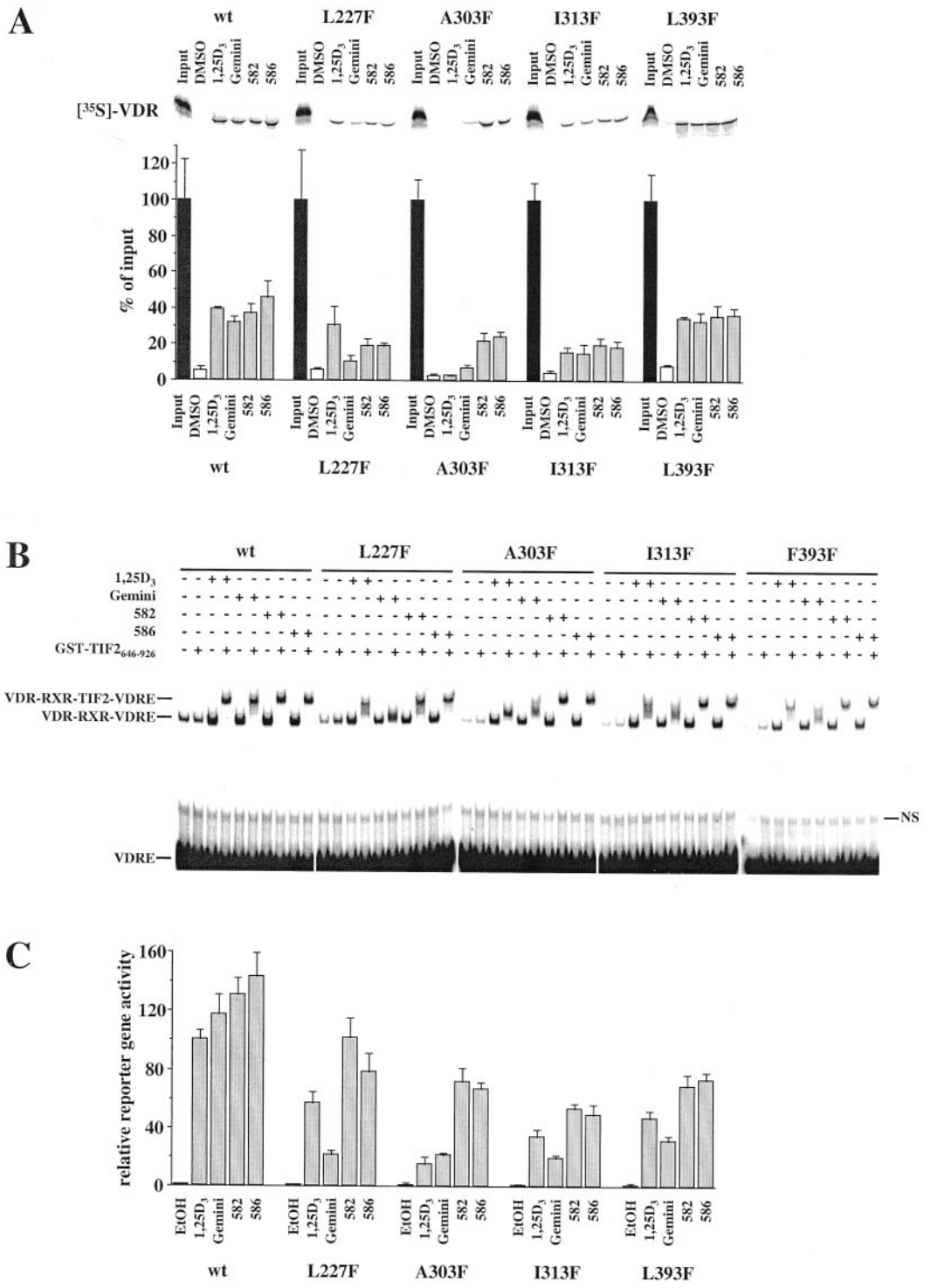

Fig. 3. Single point mutagenesis analysis of critical amino acid residues within the Gemini binding site. The amino acids L227, A303, I313, and L393 in VDR were mutated individually to phenylalanine. A, GST-pull down assays were performed with bacterially expressed GST-TIF2$_{646-926}$ and full-length, in vitro translated, ^{35}S-labeled wild-type or mutant VDR in the presence of solvent, 1α,25(OH)$_2$D$_3$, Gemini, RO4383582, or RO4383586 (each 1 μM). After precipitation and washing, the samples were electrophoresed through 10% SDS-polyacrylamide gels, and the percentage of precipitated VDRs with respect to input was quantified using a Fuji FLA3000 reader. Representative gels are shown. B, supershift experiments were performed with heterodimers of in vitro translated RXR and wild-type or mutant VDR that were preincubated in presence of bacterially expressed GST-TIF2$_{646-926}$ with solvent, 1α,25(OH)$_2$D$_3$, Gemini, RO4383582, or RO4383586 (each 1 μM) and the ^{32}P-labeled DR3-type rat ANF VDRE. Protein-DNA complexes were separated from free probe through 8% nondenaturing polyacrylamide gels. Representative experiments are shown. C, luciferase reporter gene assays were performed with extracts from MCF-7 cells that were transiently transfected with a reporter gene construct driven by four copies of the DR3-type rat ANF VDRE and expression vectors for RXR and wild-type or mutant VDR proteins. The cells were treated for 16 h with solvent, 1α,25(OH)$_2$D$_3$, Gemini, RO4383582, or RO4383586 (each 100 nM). Stimulation of luciferase activity was calculated compared with to solvent-induced controls and normalized to the activity of 1α,25(OH)$_2$D$_3$ on VDR$_{wt}$-RXR heterodimers. Columns represent the mean of three experiments, and the bars indicate S.D..

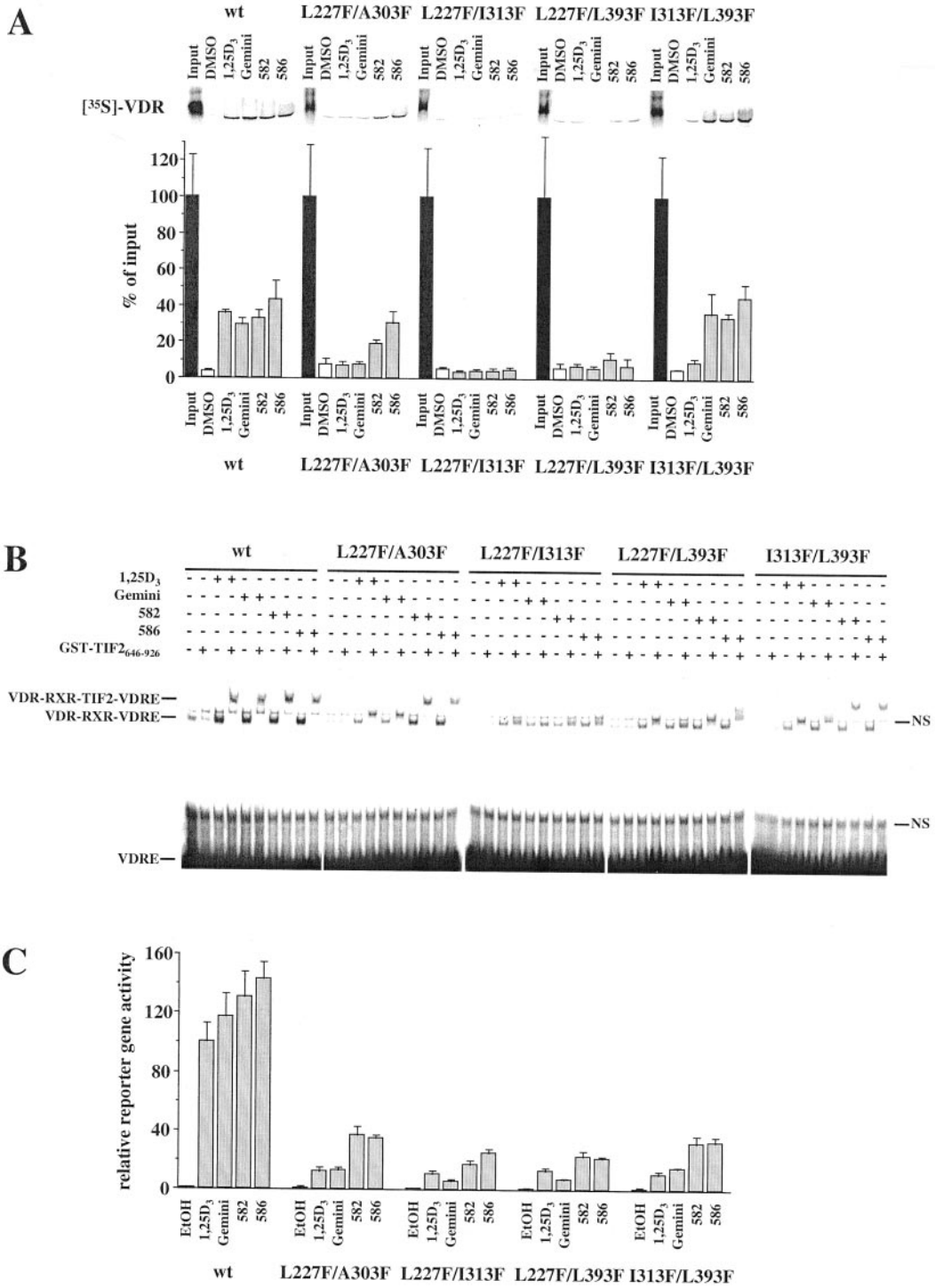

Fig. 4. Double point mutagenesis analysis of critical amino acid residues within the Gemini binding site. The amino acid combinations L227/A303, L227/I313, L227/L393, and I313/L393 in full-length VDR were mutated to phenylalanines. A, GST-pull down assays were performed with bacterially expressed GST-TIF2$_{646-926}$ and full-length in vitro translated, ^{35}S-labeled wild-type or mutant VDR in the presence of solvent, 1α,25(OH)$_2$D$_3$, Gemini, RO4383582, or RO4383586 (each 1 μM). After precipitation and washing, the samples were electrophoresed through 10% SDS-polyacrylamide gels and the percentage of precipitated VDRs with respect to input was quantified using a Fuji FLA3000 reader. Representative gels are shown. B, supershift experiments were performed with heterodimers of in vitro translated RXR and wild-type or mutant VDRs that were preincubated in the presence of bacterially expressed GST-TIF2$_{646-926}$ with solvent, 1α,25(OH)$_2$D$_3$, Gemini, RO4383582, or RO4383586 (each 1 μM) and the ^{32}P-labeled DR3-type rat ANF VDRE. Protein-DNA complexes were separated from free probe through 8% nondenaturing polyacrylamide gels. Representative experiments are shown. C, luciferase reporter gene assays were performed with extracts from MCF-7 cells that were transiently transfected with a reporter gene construct driven by four copies of the DR3-type rat ANF VDRE and expression vectors for RXR and wild-type or mutant VDR proteins. The cells were treated for 16 h with solvent, 1α,25(OH)$_2$D$_3$, Gemini, RO4383582, or RO4383586 (each 100 nM). Stimulation of luciferase activity was calculated compared with solvent-induced controls and normalized to the activity of 1α,25(OH)$_2$D$_3$ on VDR$_{wt}$-RXR heterodimers. Columns represent the mean of three experiments and the bars indicate S.D.

Gemini derivatives with asymmetric side chains should be able to discriminate between unequal binding sites. However, neither on wild-type VDR nor on any of the VDR mutants could a significant difference in the activity of the two stereoisomers of hexafluoro-Gemini be detected. Interestingly, the hexafluoro derivatives bound so efficiently to the VDR-LBD that, in contrast to Gemini, even two bulky phenylalanines could not completely diminish their activity. The superactivity of RO4383582 and RO4383586 relates to the high electronegativity of the fluor atoms, which provide the Gemini derivatives with higher affinity to the LBD than Gemini or the natural hormone. Therefore, the mutations of the LBD are less severe for RO4383582 and RO4383586 than for Gemini. Taken together, in contrast to all other known VDR ligands, Gemini and its derivatives have the choice between two positions to bind the LBD.

Gemini has been shown previously to stabilize the VDR also in a nonagonistic conformation (Herdick et al., 2000; Herdick and Carlberg, 2000; Bury et al., 2001a). The nonagonistic conformation of the VDR resembles its apo-form, which is characterized primarily by a different orientation of helix 12. In this conformation VDR is not able to interact with coactivator proteins but forms complexes with corepressors. A ligand that stabilizes the VDR-LBD in this conformation has no agonistic effect. However, in contrast to the antagonistic conformation of the VDR (Väisänen et al., 2002a), in which the receptor is permanently blocked to induce gene activity, the nonagonistic conformation can be converted into the agonistic conformation, when the binding partners of the VDR are exchanged. This study focused on the agonistic conformation of the Gemini-bound VDR-LBD, because as a starting point for the MD simulations, coordinates of the VDR were available only in its agonistic form, not its apo-form. However, it can be speculated that with a displaced helix 12, H397 will no longer have a dominant attraction for the C25-hydroxyl group of the side chains of Gemini. Then it may be possible that both side chains bind to the two newly identified corners of the ligand-binding pocket. In this way, Gemini may be able to bind to the LBD without inducing any agonistic action of the receptor.

In conclusion, this study could demonstrate that endocrine NRs, such as the VDR, could also interact efficiently with derivatives of their natural ligands that have a 25% increased volume. Gemini is an outstanding $1\alpha,25(OH)_2D_3$ analog, because it has the unique possibility to choose between two binding positions within the ligand-binding pocket of the VDR.

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