

Gene Regulatory Potential of $1\alpha,25$ -Dihydroxyvitamin D_3 Analogues With Two Side Chains

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Abstract The nuclear hormone $1\alpha,25$ -dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) acts through the transcription factor vitamin D receptor (VDR) via combined contact with the retinoid X receptor (RXR), coactivator proteins, and specific DNA binding sites (VDREs). Ligand-mediated conformational changes of the VDR are the core of the molecular switch of nuclear $1\alpha,25(OH)_2D_3$ signalling. Studying the interaction of $1\alpha,25(OH)_2D_3$ analogues with this molecular switch should allow the characterization of their potential selective biological profile. A $1\alpha,25(OH)_2D_3$ analogue with two side chains (Ro27-2310 or Gemini) was found to stabilize functional VDR conformations and VDR–RXR heterodimers on a VDRE with a slightly lower sensitivity than the natural hormone. A 19-nor derivative of Gemini (Ro27-5646) showed similar sensitivity whereas 5,6-*trans* (Ro27-6462) 3-*epi* (Ro27-5840) and 1 α -fluoro (Ro27-3752) derivatives were equal to each other, but approximately 30-times less sensitive than Gemini. A des-C,D derivative of Gemini (Ro28-1909) showed only residual activity at maximal concentrations. In contrast to $1\alpha,25(OH)_2D_3$, Gemini and its derivatives showed a differential preference in stabilizing VDR conformations which was found to be modulated by DNA coactivator and corepressor proteins. An analysis of the gene regulatory potential of the VDR agonists in cellular reporter gene systems demonstrated the same ranking as in the *in vitro* systems, but Gemini and its 19-nor derivative were found to be more sensitive than $1\alpha,25(OH)_2D_3$ which indicates that the natural hormone is selectively metabolized. This study used straightforward methods for the *in vitro* and *ex vivo* evaluation of the gene regulatory potential of $1\alpha,25(OH)_2D_3$ analogues. Gemini was highlighted as an interesting drug candidate which could not be optimized through obvious chemical modifications in its A-ring. *J. Cell. Biochem. Suppl.* 36:179–190, 2001. © 2001 Wiley-Liss, Inc.

Key words: vitamin D receptor; vitamin D analogues; receptor conformations; gene regulation; receptor agonists

The nuclear hormone $1\alpha,25$ -dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), which is the biologically active form of vitamin D_3 , is a secosteroid that regulates calcium homeostasis and bone mineralization [DeLuca et al., 1990], but also plays a

role in controlling cellular growth differentiation and apoptosis [Walters, 1992]. More than 2,000 analogues of $1\alpha,25(OH)_2D_3$, mainly containing modifications of the side chain, have been developed with the goal at improving the biological profile of the natural hormone for a potential therapeutic application [Bouillon et al., 1995] but only a few of them showed to be promising drug candidates.

The genomic effects of $1\alpha,25(OH)_2D_3$ and its analogues are mediated through the vitamin D receptor (VDR) [Carlberg and Polly, 1998] which is a member of the nuclear receptor transcription factor superfamily [Mangelsdorf et al., 1995]. VDR binds as a heterodimer with the retinoid X receptor (RXR) [Carlberg, 1996] to specific sequences in promoter regions of $1\alpha,25(OH)_2D_3$ target genes, referred to as $1\alpha,25(OH)_2D_3$ response elements (VDREs)

Abbreviations used: $1\alpha,25(OH)_2D_3$, $1\alpha,25$ -dihydroxyvitamin D_3 ; AF-2, (trans)activation function-2; ANF, atrial natriuretic factor; DR3, direct repeat spaced by 3 nucleotides; EC₅₀, half-maximal concentration; FBS, fetal bovine serum; GST, glutathione S-transferase; LPD, limited protease digestion; NCoR, nuclear receptor corepressor; RXR, retinoid X receptor; SRC-1, steroid receptor coactivator-1; VDR, $1\alpha,25(OH)_2D_3$ receptor; VDRE, $1\alpha,25(OH)_2D_3$ response element.

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[Carlberg, 1995]. 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) [Carlberg and Polly, 1998]. The VDR consists of several functional domains that include the DNA-binding domain (DBD) and the ligand-binding domain (LBD). The LBD of the VDR is formed by 12 α -helical structures of which the last one, helix 12, contains a short transactivation function 2 (AF-2) domain.

A DNA-bound VDR–RXR heterodimer can be considered as the molecular switch of $1\alpha,25(\text{OH})_2\text{D}_3$ signalling. A critical step in this signalling process is the specific ligand-triggered induction of a conformational change within the LBD of the VDR. This conformational change induces the dissociation of corepressors such as NCoR or Alien [Polly et al., 2000] and facilitates the interaction with coactivator proteins with members of the p160-family such as SRC-1, TIF2, and RAC3 [Herdick et al., 2000]. This VDR–coactivator interaction then further facilitates recruitment of other factors to form a larger complex that modulates chromatin structure and initiates transcription [Spencer et al., 1997]. This also involves the recently described DRIP/ARC cofactor complexes [Näär et al., 1999; Rachez et al., 1999] which appear to contact the VDR and other nuclear receptors preceding their interaction with p160-family of coactivators [Freedman, 1999].

Several $1\alpha,25(\text{OH})_2\text{D}_3$ analogues represent interesting model agonists that are useful for studying the action of the VDR. These investigations focus on the interaction of $1\alpha,25(\text{OH})_2\text{D}_3$ and its analogues with the receptor via the induction of a conformational change in the LBD. Traditional competition assays using radio-labelled ligand provide an idea of the receptor–ligand interaction affinity, but do not allow for the visualization of receptor conformational changes [Mørk Hansen et al., 1996], whereas the limited protease digestion assay in which interaction of a nuclear receptor with ligand protects the LBD against protease digestion [Leng et al., 1993] has proven to be a powerful method for characterizing functional VDR conformations [Nayeri et al., 1995, 1996a; Peleg et al., 1995, 1996]. In addition, ligand-dependent gel shift assays are able to characterize the ligand-induced complex formation of VDR–RXR heterodimers on a VDRE [Quack

and Carlberg, 2000; Quack et al., 1998]. In turn, both methods are very well suited for the in vitro evaluation of VDR agonists and allow an extrapolation of their gene regulatory potential. The analysis of the effects of VDR agonists on the core of the molecular switch of $1\alpha,25(\text{OH})_2\text{D}_3$ signalling (the LBD of the VDR) in a cellular system is performed most straightforwardly by the mammalian one-hybrid assay in which a transiently transfected GAL4_{DBD} VDR_{LBD} fusion protein regulates reporter gene activity [Polly et al., 2000].

In this report the gene regulatory potential of a novel $1\alpha,25(\text{OH})_2\text{D}_3$ analogue called Gemini or Ro27-2310, which is the first analogue that contains two side chains at carbon 20, was studied in vitro and ex vivo by the above mentioned methods. Moreover, obvious chemical modifications of the A-ring of Gemini such as 5,6-*trans*, 3-*epi*, 19-*nor*, 1 α -*fluoro*, and *des-C,D* derivatives were analyzed for their potential to optimize the gene regulatory profile of Gemini.

MATERIALS AND METHODS

Compounds

Gemini (Ro27-2310) is a 21-(2-methyl-2-hydroxy-butyl) derivative of $1\alpha,25(\text{OH})_2\text{D}_3$, the remaining compounds are 5,6-*trans* (Ro27-6462), 3-*epi* (Ro27-5840), 19-*nor* (Ro27-5646), 1 α -*fluoro* (Ro27-3752), and *des-C,D* (Ro28-1909) derivatives of Gemini. The synthesis of Gemini and its derivatives has been described elsewhere [Uskokovic et al., 1997; Manchand and Uskokovic, 1999, 2000; Norman et al., 2000]. All compounds were dissolved in 2-propanol. For in vitro assays dilutions were made in DMSO whereas for cellular assays dilutions were made in ethanol. The structures of all seven compounds are shown in Figure 1.

DNA Constructs

The full-length cDNAs for human VDR [Carlberg et al., 1993] and human RXR α [Levin et al., 1992] were subcloned into the SV40 promoter-driven pSG5 expression vector (Stratagene, Heidelberg, Germany). These constructs are suitable for T₇ 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). For the mammalian one-hybrid-assay, the luciferase reporter

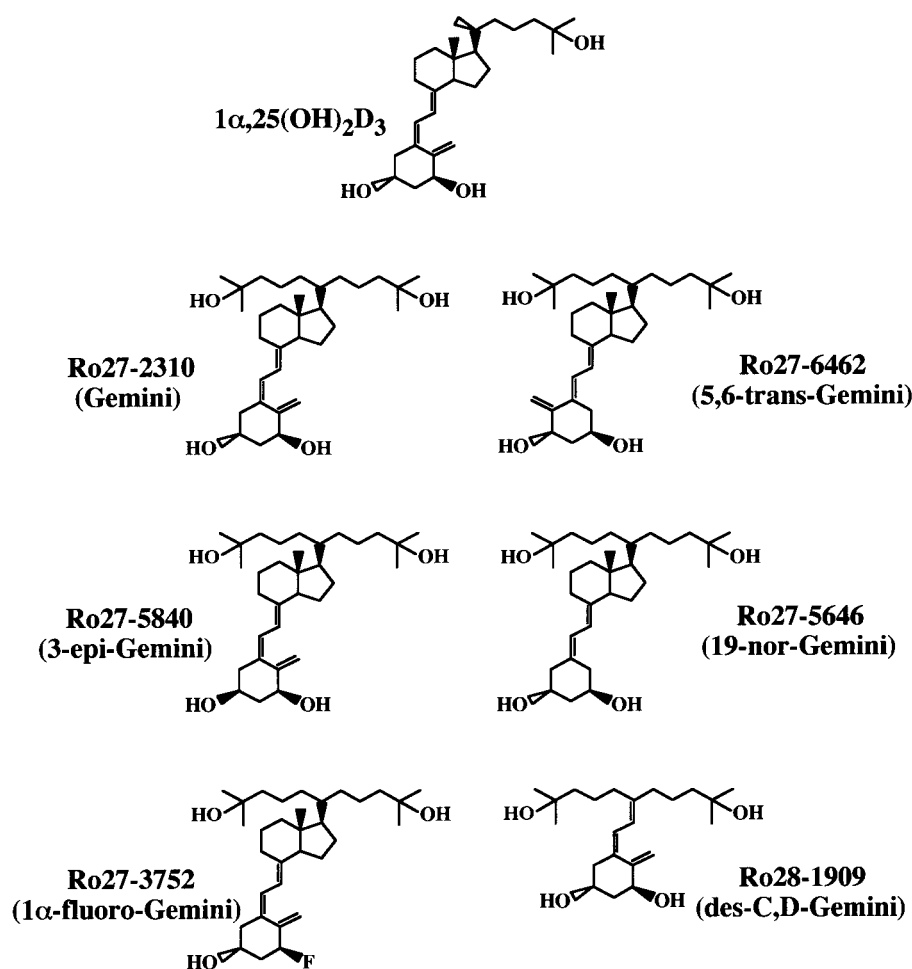


Fig. 1. Structure of 1 α ,25(OH) $_2$ D $_3$, Gemini and its derivatives.

gene was driven by three copies of the GAL4 binding site fused to the *tk* promoter [Hörlein et al., 1995] and for the reporter gene assays in Cos-7 cells the luciferase gene was driven by four copies of the DR3-type VDRE of the rat ANF promoter [Kahlen and Carlberg, 1996]. The nuclear receptor interaction domain of human SRC-1 (spanning from amino acids 596 to 790) [Onate et al., 1995] and mouse NCoR (spanning from amino acids 1,679 to 2,453) [Hörlein et al., 1995] were subcloned into the 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-SRC-1 $_{596-790}$ and GST-NCoR $_{1,679-2,453}$ was facilitated in the *E. coli* BL21(DE3)pLysS strain (Stratagene). GST-SRC-1 $_{596-790}$ expression was performed with isopropyl- β -D-thiogalactopyranoside (IPTG 0.25 mM) for 3 h at 37°C whereas, expression of GST-NCoR $_{1,679-2,453}$ was performed with 1.25 mM IPTG for 5 h at 25°C. The fusion proteins were tested for equal loading by Coomassie brilliant blue staining.

Limited Protease Digestion Assay

In vitro translated [35 S]-labelled VDR protein (2.5 μ l) together with unprogrammed lysate (2.5 μ l) or in vitro translated RXR (2.5 μ l) and 1 ng of unlabelled rat ANF DR3-type VDRE were incubated with graded or saturating concentrations of ligand for 15 min at room temperature in 20 μ l binding buffer (10 mM Hepes [pH 7.9],

1 mM DTT, 0.2 $\mu\text{g}/\mu\text{l}$ poly(dI-C), and 5% glycerol). The buffer was adjusted to 150 mM of monovalent cations by addition of KCl. In indicated cases, 5 μg of GST-SRC-1₅₉₆₋₇₉₀ or GST-NCOR₁₆₇₉₋₂₄₅₃ fusion protein were added and incubation was continued for 15 min. Trypsin (Promega final concentration 8.3 ng/ μl) was then added and the mixtures were further incubated for 15 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).

Ligand-Dependent Gel Shift Assay

In vitro translated VDR–RXR heterodimers were incubated with graded concentrations of ligand for 15 min at room temperature in a total volume of 20 μl binding buffer. The buffer had been adjusted to 150 mM by addition of KCl. Approximately 1 ng of [³²P]-labelled DR3-type VDRE from the rat ANF promoter (50,000 cpm) was added to the protein–ligand mixture and incubation was continued for 20 min. Protein–DNA complexes were resolved through 8% non-denaturing polyacrylamide gels in 0.5 \times TBE (45 mM Tris 45 mM boric acid 1 mM EDTA [pH 8.3]) and were quantified by phosphorimaging.

Transient Transfections and Reporter Gene Assay

HeLa human cervix carcinoma cells and Cos-7 SV40-transformed African Green monkey kidney cells were seeded into 6-well plates (10⁵ 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 a GAL4 binding site-driven luciferase reporter gene construct and 1 μg of an expression vector for a GAL4_{DBD}VDR_{LBD}-fusion protein (for mammalian one-hybrid assays in HeLa cells) or 1 μg of the DR3-type VDRE-driven reporter plasmid and 1 μg of each pSG5-based receptor expression vectors for VDR and RXR (for Cos-7 cells) and 1 μg of the reference plasmid pCH110 (Amersham–Pharmacia) with 15 μg N-[1-(2,

3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP, Roth Karlsruhe, Germany) 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 graded concentrations of 1 α ,25(OH)₂D₃, Gemini or its derivatives were also added. The cells were lysed 16 h after onset of stimulation using the reporter gene lysis buffer (Roche Diagnostics, Mannheim, Germany) for both types of assays and the constant light signal luciferase reporter gene assay was performed as recommended by the supplier (Canberra–Packard, Dreieich, Germany). The luciferase activities were normalized with respect to β -galactosidase activity and induction factors were calculated as the ratio of luciferase activity of ligand-stimulated cells to that of solvent controls.

RESULTS

Gemini and Its Derivatives

Different derivatives of Gemini (Ro27-2310) were synthesized that show modifications in the A-ring (for structures see Fig. 1). The interest in a 5,6-*trans* modification (Ro27-6462) of the A-ring was based on promising antiproliferative effects of 1 α ,25(OH)₂-16-ene-5,6-*trans* D₃ in prostate and leukemia cell lines (H.P. Koeffler, unpublished results). The 3-epi derivative (Ro27-5840) is a bone cell metabolite of Gemini (S. Reddy, unpublished results). The 19-nor (Ro27-5646) and 1 α -fluoro (Ro27-3752) derivatives of Gemini represent standard A-ring modifications. The des-C,D modification of Gemini (Ro28-1909) is a starting point for investigations on the length of the link between the A-ring and the side chain.

Stabilization of VDR Conformations

Conformations of the VDR bound by graded concentrations of Gemini its derivatives or 1 α ,25(OH)₂D₃ as a control were analyzed by limited protease digestion assays (Fig. 2). For all seven compounds the assay provided two digestion products c1_{LDPD} and c3_{LDPD} which have been previously characterized to represent VDR fragments from amino acids 174 to 427 and from 174 to 390, respectively, i.e., major parts of the LBD and its carboxy-terminal truncation

[Quack and Carlberg, 2000]. Therefore, $c1_{LPD}$ and $c3_{LPD}$ are considered to represent the functional VDR conformations 1 and 3, respectively [Peleg et al., 1995; Nayeri et al., 1996b; Liu et al., 1997; Nayeri and Carlberg, 1997]. Gemini and its derivatives were found not to be able to stabilize monomeric VDR in $c1_{LPD}$, whereas the natural hormone stabilized 50% of all VDR molecules this conformation with a half-maximal concentration (EC_{50}) of 3 nM. In contrast Gemini and its derivatives were found to stabilize up to 60% of all VDR molecules in $c3_{LPD}$ whereas $1\alpha,25(OH)_2D_3$ stabilized less than 20% of the VDR pool in this conformation. However, the natural hormone (EC_{50} -value of 0.7 nM) was found to be more sensitive than Gemini (10 nM) and its 19-nor derivative (30 nM) in stabilizing $c3_{LPD}$. The 1α -fluoro 3-epi and 5,6-*trans* derivatives of Gemini even showed a lower sensitivity (EC_{50} -values of 180 nM, 220 nM, and 300 nM, respectively) and the des-C,D derivative can be considered as non-functional (EC_{50} -value of 10 μ M or higher).

Limited protease digestion assays were performed with in vitro translated VDR in the presence of RXR, the DR3-type VDRE of the rat atrial natriuretic factor (ANF) promoter the coactivator SRC-1 and the corepressor NCoR, in order to assess the effect of DNA and cofactors on the potency of Gemini and its derivatives. The percentage of stabilized VDR conformations $c1_{LPD}$ and $c3_{LPD}$ was quantified at saturating concentrations of $1\alpha,25(OH)_2D_3$, Gemini and its derivatives (10 μ M) (Fig. 3A–D). When analyzing monomeric VDR in solution (Fig. 3A), Gemini and its derivatives exclusively stabilized $c3_{LPD}$ (30–40% of the VDR input, see also Fig. 2), whereas the natural hormone preferentially stabilized $c1_{LPD}$. The complex formation of VDR with RXR on the VDRE (Fig. 3B) resulted for all VDR agonists in a selectively increased stabilization of $c1_{LPD}$, whereas the amount of $c3_{LPD}$ appeared to be not significantly affected: $1\alpha,25(OH)_2D_3$ stabilized up to 70% of all VDR molecules in $c1_{LPD}$, whereas in the presence of Gemini or its derivatives approximately each 40% of the VDR pool were stabilized in $c1_{LPD}$ and $c3_{LPD}$. Interestingly, the addition of SRC-1 to the protein–DNA complex (Fig. 3C) conferred Gemini and its derivatives with a similar profile than the natural hormone, i.e., a preferential stabilization of $c1_{LPD}$ (approximately 50% of all VDR molecules) in relation to $c3_{LPD}$ (approximately 20%). In contrast, the addition of NCoR

to the VDR–RXR–VDRE complex (Fig. 3D) resulted for all VDR agonists in the inverse effect, i.e., a low amount of VDR molecules were stabilized in $c1_{LPD}$ (10–20%), which is for Gemini and its derivatives even lower than the amount of VDR molecules that were stabilized in $c3_{LPD}$ (approximately 30%). However, the des-C,D derivative of Gemini (Ro28-1909) showed at none of these conditions a stabilization of $c1_{LPD}$ or $c3_{LPD}$ that was remarkably different to that of the solvent control.

Ligand-Triggered VDR–RXR Complex Formation on DNA

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 $1\alpha,25(OH)_2D_3$, Gemini and its derivatives (Fig. 4). A comparable intensity (approximately 10–15% shifted probe) of dose-dependent VDR–RXR heterodimer complex formation on DNA was observed for all compounds with the exception of the des-C,D derivative. The concentration-dependent evaluation of the VDR–RXR complex formation on DNA resulted in a similar ranking of EC_{50} -values as the analysis of VDR conformations (see Fig. 2) and provided values of 0.2 nM for $1\alpha,25(OH)_2D_3$, 0.3 nM for Gemini, 0.6 nM for Ro27-5646, 4 nM for Ro27-6462, 6 nM for Ro27-3752, and 10 nM for Ro27-5840. Interestingly, in gel shift assays a more sensitive reaction of the VDR agonists was observed than in the DNA-independent limited protease digestion assay.

Gene Regulatory Potential in Cellular Systems

Mammalian one-hybrid assays were performed in HeLa cells that were transiently transfected with an expression vector for a fusion protein containing the DBD of the yeast transcription factor GAL4 and the LBD of the VDR together with a reporter gene construct containing a GAL4 binding site-driven luciferase gene and stimulated with graded concentrations of $1\alpha,25(OH)_2D_3$, Gemini and its derivatives (Fig. 5A). In this ex vivo assay system the sensitivity of Gemini and its derivatives had a similar ranking than that of the two in vitro assay systems (Figs. 2 and 4): EC_{50} -values of 0.1 nM for Gemini, 0.25 nM for Ro27-5646, 6 nM for Ro27-5840, 7 nM for Ro27-6462, 10 nM for Ro27-3752, and 300 nM for Ro28-1909 were determined. However, the induction of

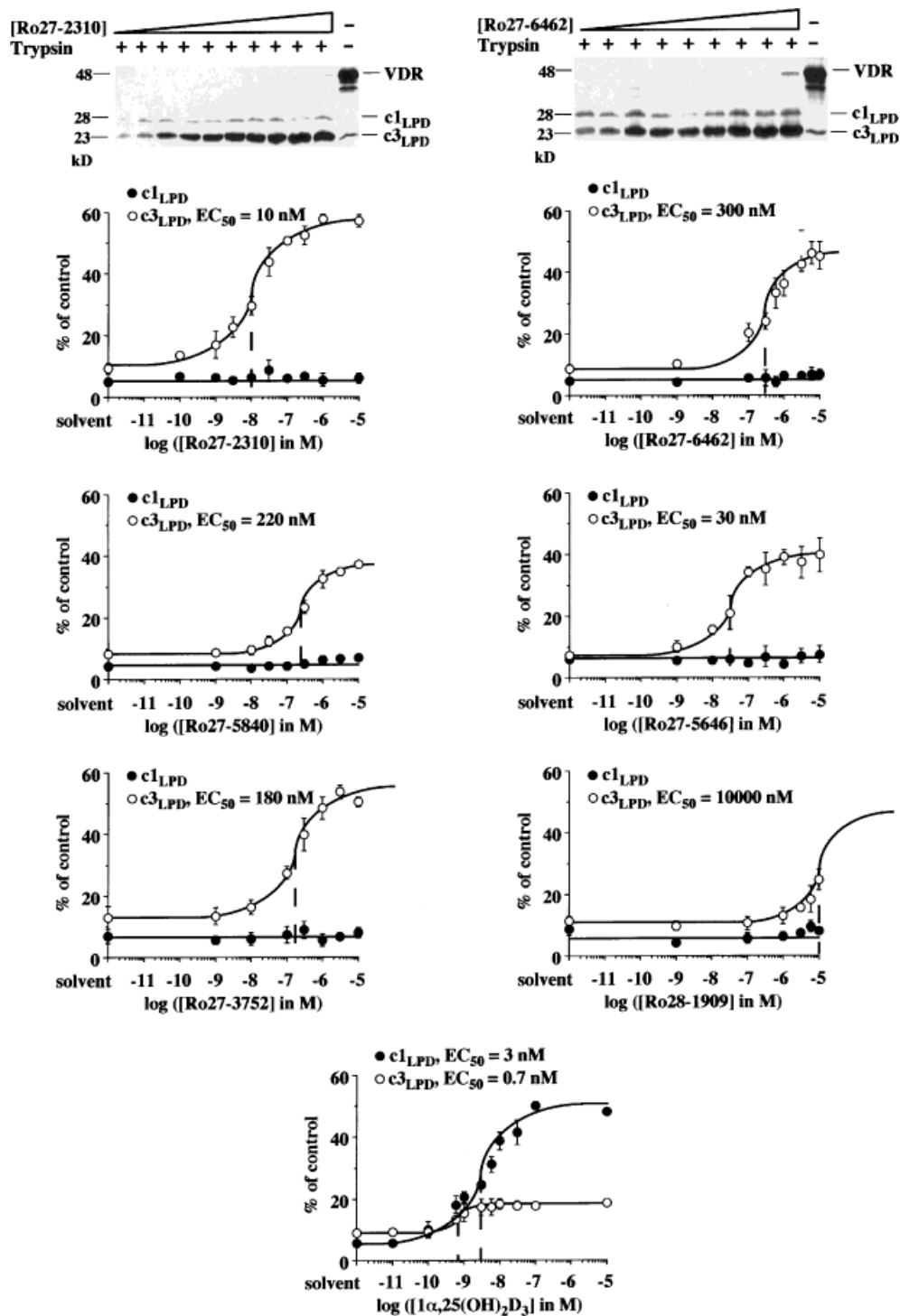


Fig. 2. Dose-dependent stabilization of VDR conformations. Limited protease digestion assays were performed by preincubating *in vitro* translated [³⁵S]-labelled VDR with graded concentrations of the indicated ligands. The amount of ligand-stabilized VDR conformations 1 (c1_{LPD}) and 3 (c3_{LPD}) in relation

to VDR input was quantified by phosphorimaging. Representative experiments are shown for Ro27-2310 and Ro27-6462. Data points represent the mean of triplicates and the bars indicate standard deviation.

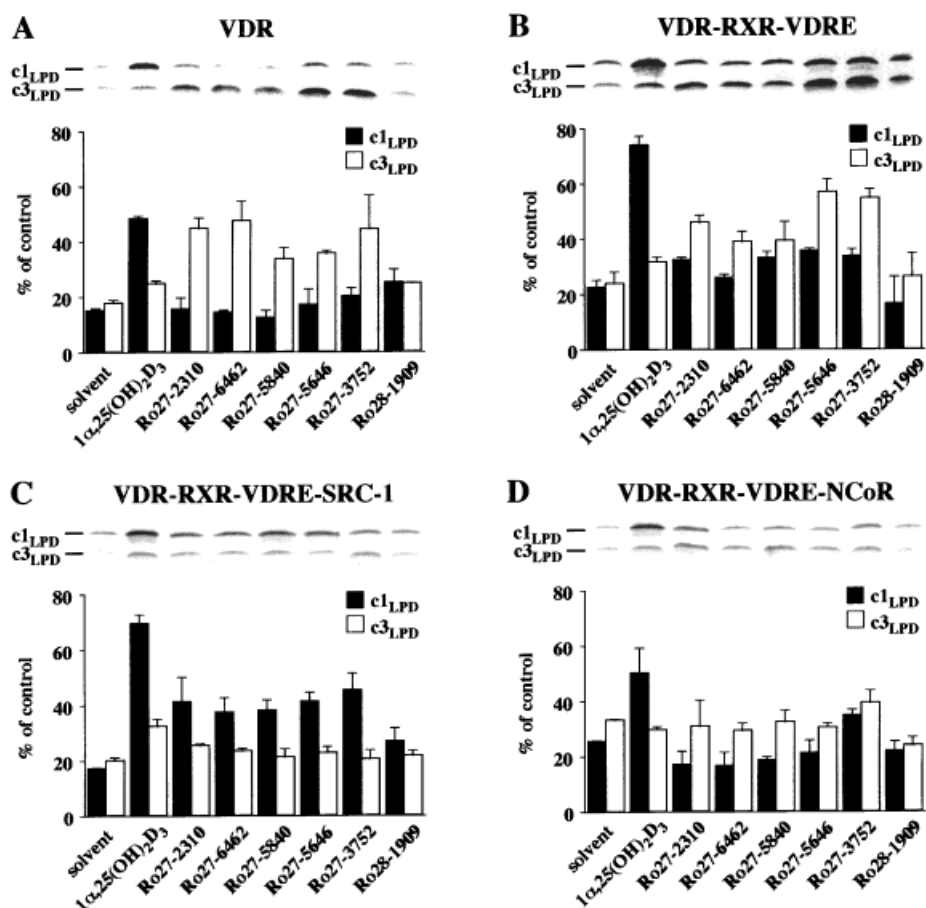


Fig. 3. DNA- and cofactor-dependent stabilization of VDR conformations. Limited protease digestion assays were performed by pre-incubating *in vitro* translated [³⁵S]-labelled VDR alone (A) or in combination with the unlabelled RXR and the unlabelled DR3-type VDRE from the rat ANF gene promoter (B) and bacterially expressed GST-SRC-1_{596–790} (C) or GST-

NCOR_{1679–2453} (D) in the presence of saturating concentrations (10 μM) of indicated ligands. Representative experiments are shown. The amount of ligand-stabilized VDR conformations 1 (c1_{LPD}) and 3 (c3_{LPD}) in relation to VDR input was quantified by phosphorimaging. Columns represent the mean of triplicates and the bars indicate standard deviation.

reporter gene activity at saturating ligand concentrations were found to be 35–50-fold for Gemini, Ro27-5646, and Ro27-3752, but only 15–25-fold for the three remaining Gemini derivatives. Interestingly, with an EC₅₀-value of 0.9 nM, 1α,25(OH)₂D₃ showed to be less sensitive in this assay system than Gemini and its 19-nor derivative. Since Gemini and its derivatives show only a very low affinity to the vitamin D binding protein (Norman et al., 2000; and data not shown) these results indicate that the natural hormone, and not the Gemini compounds, were metabolized in HeLa cells. Similar results were obtained from reporter gene assays in VDR- and RXR-overexpressing Cos-7 cells, in which the luciferase reporter gene was driven by four copies of the DR3-type VDRE of the rat ANF promoter (Fig. 5B). In this system, EC₅₀-values of 0.04 nM for Gemini, of 0.8 nM for Ro27-

5646, of 1.8 nM for the natural hormone, of 3 nM for Ro27-5840, and Ro27-6462, and of 5.5 nM for Ro27-3752 were obtained. The induction factor at maximal concentrations was found to be for 1α,25(OH)₂D₃ clearly higher than for Gemini and its derivatives. Ro28-1909 showed to be not active in this system.

DISCUSSION

The effects of 1α,25(OH)₂D₃ and its analogues on bone mineralization as well as on cell cycle regulation, i.e., their therapeutic potential in quite different diseases such as osteoporosis, psoriasis, and breast cancer, are mediated through the regulation of specific genes by the nuclear receptor VDR. This indicates that the analysis of the gene regulatory potential of a VDR agonist is essential for its evaluation as a

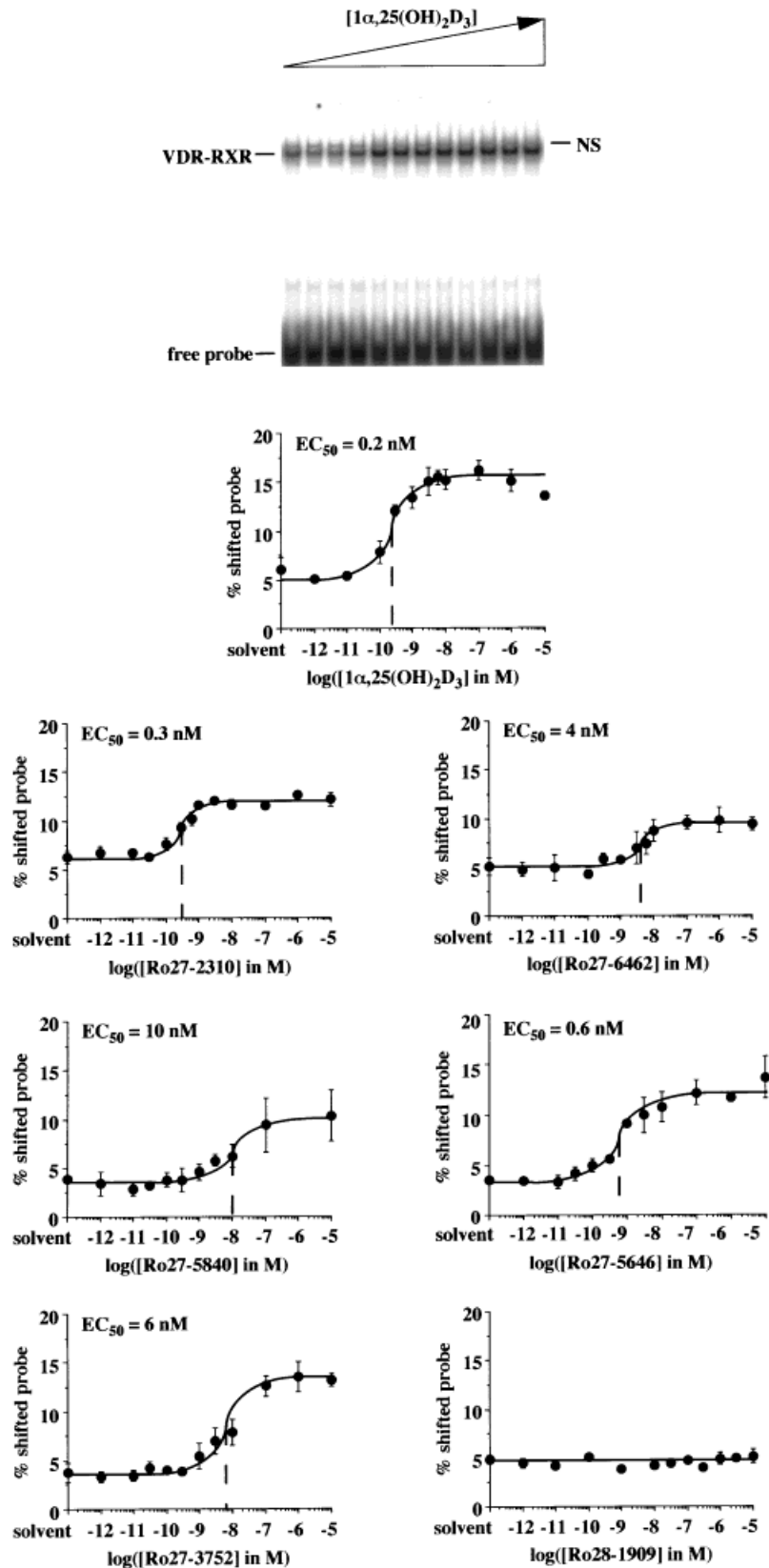


Fig. 4. Stabilization of VDR–RXR heterodimers on DNA. Gel shift experiments were performed with in vitro translated VDR–RXR heterodimers, that were preincubated at room temperature with graded concentrations of the indicated ligands and the [^{32}P]-labelled DR3-type VDRE from the rat ANF gene promoter.

The amount of VDR–RXR–VDRE complexes in relation to free probe was quantified by phosphorimaging. A representative experiment for $1\alpha,25(\text{OH})_2\text{D}_3$ is shown. Data points represent the mean of triplicates and the bars indicate standard deviation.

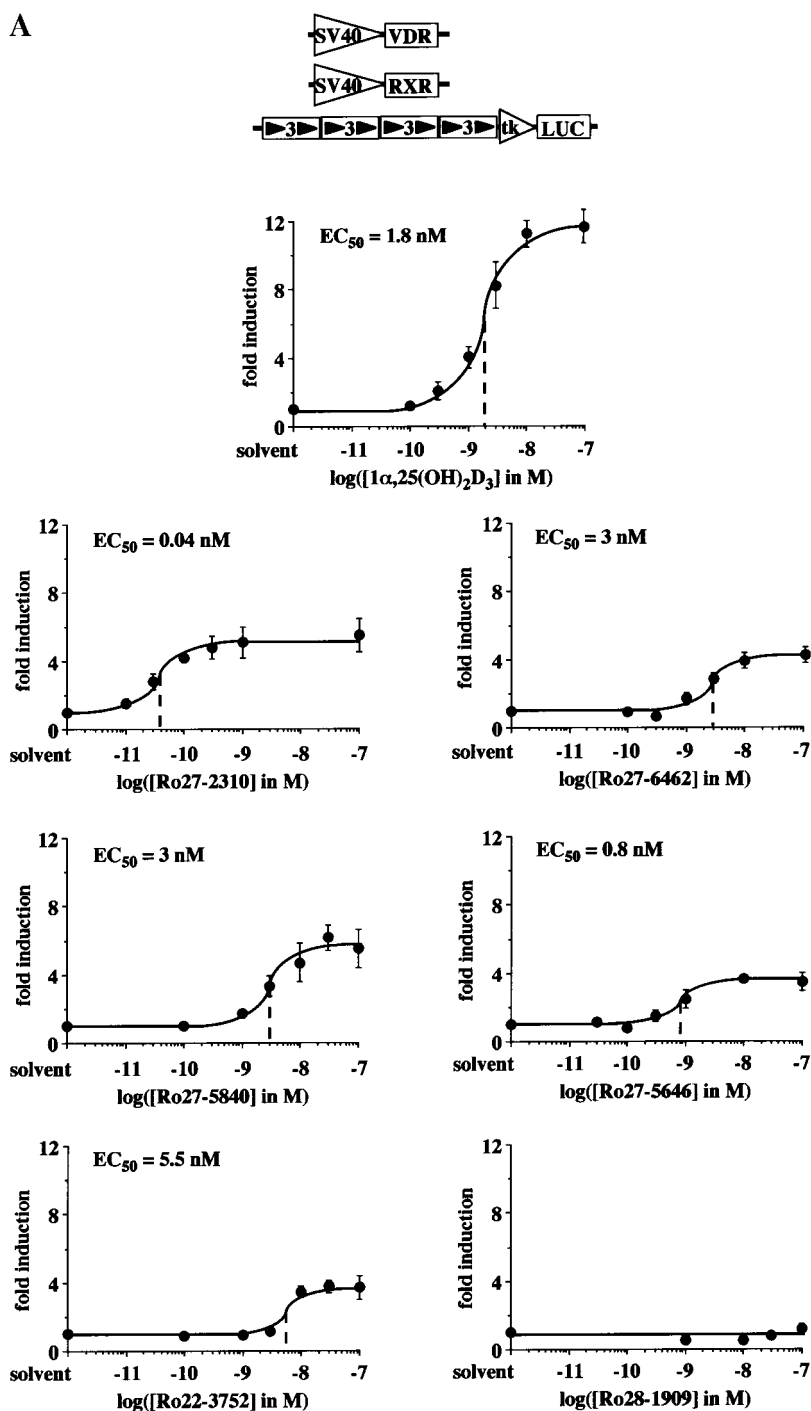


Fig. 5. Functional activity ex vivo. Luciferase reporter gene assays were performed with extracts from HeLa (A) or Cos-7 (B) cells that were transiently transfected with a reporter gene construct-driven by three copies of the GAL4 binding site and an expression vector for a GAL4_{DBD}VDR_{LBD} fusion protein (A) or with a luciferase reporter gene construct-driven by four copies of the DR3-type VDRE from the rat ANF gene promoter together

with the expression vectors for VDR and RXR (B) as schematically depicted above the respective figures. Cells were treated for 16 h with graded concentrations of the indicated ligands. Stimulation of luciferase activity was calculated in comparison to solvent-induced controls. Each data point represents the mean of triplicates and the bars indicate standard deviations.

B

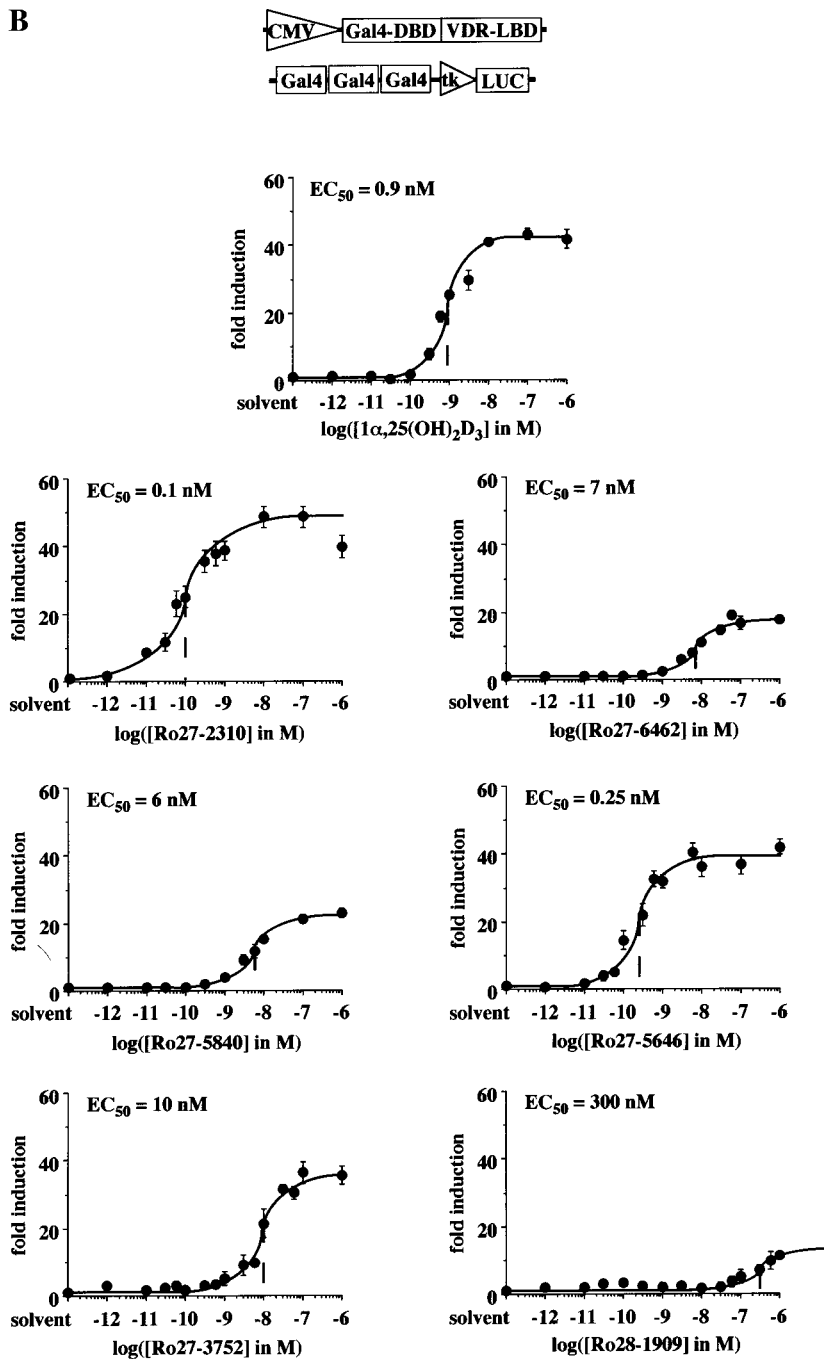


Fig. 5. (Continued)

candidate drug. Recent understanding of the mechanisms of $1\alpha,25(\text{OH})_2\text{D}_3$ signalling made clear that conformational changes of the VDR-LBD induce the dissociation of nuclear receptor-corepressor complexes and facilitate the interaction with coactivator proteins which consequently results in stimulation of transcriptional activity via various additional protein-protein interactions. Conformational

changes of DNA-bound VDR-RXR heterodimers are therefore the core of the molecular switch of $1\alpha,25(\text{OH})_2\text{D}_3$ signalling.

In this report a novel class of $1\alpha,25(\text{OH})_2\text{D}_3$ analogues, Gemini and its derivatives, that contain two side chains at their carbon 20 atom, were investigated. A very interesting property of the Gemini compounds is their ability to preferentially stabilize monomeric VDR in a

different conformation, $c3_{LPD}$, than the natural hormone and other analogues that stabilize the VDR in $c1_{LPD}$ [Nayeri et al., 1996b; Liu et al., 1997; Nayeri and Carlberg, 1997]. However, when the VDR is complexed with RXR and a VDRE, i.e., in the context of the molecular switch the Gemini compounds also stabilized reasonable amounts of $c1_{LPD}$. Interestingly, the addition of a coactivator resulted in a preferential stabilization of $c1_{LPD}$ by Gemini compounds, whereas the addition of a corepressor reversed the effect, i.e., again a preferential stabilization of $c3_{LPD}$. Conformation 1 is considered as the activated form of the VDR, in which helix 12 of the LBD closed the ligand binding cleft and allowed an interaction of its AF-2 domain with coactivator proteins [Moras and Gronemeyer, 1998]. In contrast, when the LBD is in conformation 3, helix 12 is displaced and no interaction with coactivator proteins is possible, i.e., the VDR is in its inactive form. In contrast to the natural hormone and all presently investigated analogues, Gemini compounds appear to differentiate between DNA-bound and non-DNA-bound VDR molecules. There is growing evidence that the VDR not only acts as a DNA-bound transcription factor, but also as a DNA-independent modulator of other nuclear signalling pathways. One example is the repression of IL-2 gene expression by $1\alpha,25(OH)_2D_3$, for which it was suggested that the VDR inhibits the complex formation of the T-cell transcription factor NF-AT on its specific binding site in the IL-2 promoter [Alroy et al., 1995]. The selectivity of Gemini compounds for DNA-dependent $1\alpha,25(OH)_2D_3$ signalling pathways suggests that they would not be able to regulate IL-2 gene expression.

The ligand sensitivity of a VDR agonist in a DNA-dependent *in vitro* assay, such as the gel shift assay should be identical to that in functional *ex vivo* assays, such as the mammalian one-hybrid assay or the VDRE-driven reporter gene assay. However, if the agonist is metabolized during the relative long stimulation period of the *ex vivo* assay, the respective EC_{50} -values should be higher. This appears to be the explanation for the 5–9-times lower sensitivity of the natural hormone in the reporter gene assays when compared to the gel shift assay. In turn this comparison indicates that at least in HeLa and Cos-7 cells, the Gemini compounds are not significantly metabolized. Consequently, Gemini has a nearly 10-fold

higher sensitivity to mediate gene regulation in living cells than $1\alpha,25(OH)_2D_3$. An analysis of physiological effects of the VDR agonists, such as their antiproliferative and apoptosis inducing potential, would involve an incubation with cultured cells for 5 days or longer. In this case, the high metabolism rate of $1\alpha,25(OH)_2D_3$ in comparison to the relatively low rate of Gemini and its derivatives would create even more drastic differences in potency than the reporter gene assays. Accordingly, Gemini and its 19-nor derivative showed an up to 100-fold higher sensitivity in the inhibition of the proliferation of various tumor cells than $1\alpha,25(OH)_2D_3$ [Hisataka et al., 2000; Norman et al., 2000].

In the *in vitro* and *ex vivo* assays Gemini and its derivatives provided a very similar ranking. The 19-nor modification of Gemini (Ro27-5646) decreased the sensitivity only by a factor of 2–3, whereas the 5,6-*trans* (Ro27-6462), 3-*epi* (Ro27-5840), and 1 α -fluoro (Ro27-3752) derivatives were equal to each other but approximately 30-times less sensitive than Gemini. The des-C,D derivative of Gemini (Ro28-1909) showed only residual activity at maximal concentrations and cannot be considered as a VDR agonist. Taken together, the five obvious chemical modifications of the A-ring of Gemini were not able to improve the sensitivity of this novel type of analogues. Future modifications of Gemini have to address the side chains as it was done for most other $1\alpha,25(OH)_2D_3$ analogues [Bouillon et al., 1995].

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