Molecular Evaluation of Vitamin D_3 Receptor Agonists Designed for Topical Treatment of Skin Diseases[1]

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MC903 (calcipotriol) is a synthetic, low calcemic analog of the nuclear hormone 1α,25-dihydroxyvitamin D_3 and used in the treatment of psoriasis. The beneficial effects of MC903 on psoriasis are based on gene regulatory events. The genomic actions of 1α,25-dihydroxyvitamin D_3 and its analogs are primarily mediated by a complex of the vitamin D_3 receptor and the retinoid X receptor bound to a 1α,25-dihydroxyvitamin D_3 response element that can be considered as the molecular switch of 1α,25-dihydroxyvitamin D_3 signaling. In this study, the interaction of MC903 and two new analogs, GS1500 and EB1213, with this molecular switch was compared with that of 1α,25-dihydroxyvitamin D_3. In DNA-dependent limited protease digestion assays, ligand-dependent gel shift assays and mammalian-one-hybrid assays, all four ligands appeared to be equally sensitive VDR agonists that activated vitamin D_3 receptor–retinoid X receptor–1α,25-dihydroxyvitamin D_3 response element complexes at a concentration of approximately 0.2 nM. The analyzed VDR agonists, however, also showed individual molecular properties, such as a reduced sensitivity in HaCaT cells (MC903), a selectivity for DNA-bound vitamin D_3 receptor–retinoid X receptor heterodimers (GS1500) and a long-lasting stabilization of vitamin D_3 receptor–retinoid X receptor–1α,25-dihydroxyvitamin D_3 response element complexes (EB1213). This molecular evaluation demonstrated that the sensitivity in activating the vitamin D_3 receptor is already optimal for MC903, but the analog may not be ideal in keeping the receptor active and in selectively triggering 1α,25-dihydroxyvitamin D_3 signaling pathways. ***Key words: gene regulation/receptor conformation/vitamin D analogs/vitamin D receptor. J Invest Dermatol 116:785–792, 2001***

The nuclear hormone 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) is involved in the regulation of a variety of important biologic functions, such as calcium homeostasis (Mørk Hansen *et al*, 1996), as well as cellular growth, differentiation and apoptosis (Walters, 1992). These properties provide 1α,25$(OH)_2D_3$ with an interesting therapeutic potential against a variety of diseases, such as osteoporosis, cancer, and psoriasis (Pols *et al*, 1994). A more selective biologic profile of the hormone would be desired, however, as at superphysiologic (i.e., pharmacologic) concentrations the calcemic function of the hormone can cause side-effects, such as hypercalcemia, hypercalciuria, and soft tissue calcification (Vieth, 1990). Therefore, more than 2000 analogs of 1α,25$(OH)_2D_3$, which mainly contain modifications of the side chain, have been developed with the goal of improving the biologic profile of the natural hormone for a potential therapeutic application (Bouillon *et al*, 1995); however, only very few of these analogs have been chosen for clinical trials. Presently, the clinically most successful 1α,25$(OH)_2D_3$ analog, MC903 (calcipotriol), is topically applied against keratinocyte dysfunction in psoriasis. MC903 is a very low calcemic analog, mainly because systemically it is clearly more rapidly metabolized than the natural hormone (Kissmeyer and Binderup, 1991). Therefore, MC903 is active in keratinocytes and other dermal cells (Masuda *et al*, 1994), but has only very minor systemic effects; however, MC903 is still not a perfect drug, as it is not effective in all psoriasis patients and it may cause skin irritations in some. This leads to the question, if other analogs are more potent and selective in their action than MC903.

A very helpful and desired prerequisite to the rational design of 1α,25$(OH)_2D_3$ analogs is the detailed understanding of their molecular action. 1α,25$(OH)_2D_3$ and its analogs are specific ligands to the vitamin D_3 receptor (VDR) (Pike, 1991) that is a member of the nuclear receptor transcription factor superfamily (Mangelsdorf *et al*, 1995). The VDR acts preferentially as a heterodimer with the retinoid X receptor (RXR) (Carlberg, 1996) on specific DNA sequences in promoter regions of 1α,25$(OH)_2D_3$ target genes, referred to as 1α,25$(OH)_2D_3$ response elements (VDRE) (Carlberg, 1995). Simple VDRE are formed by two hexameric binding sites and VDR-RXR heterodimers bind preferentially to directly repeated binding site arrangements with three intervening nucleotides (DR3-type VDRE) or to inverted palindromes spaced by nine nucleotides (IP9-type VDRE). In addition, VDRE formed

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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; IP9, inverted repeat spaced by nine nucleotides; EC_{50}, half maximal activation; RXR, retinoid X receptor; LBD, ligand binding domain; VDR, 1α,25$(OH)_2D_3$ receptor; VDRE, 1α,25$(OH)_2D_3$ response element.

[1]The authors declared not to have conflict of interest

by direct repeats with by four or six intervening nucleotides have been described (Carlberg, 1997). Like all members of the nuclear receptor superfamily, the VDR contains a characteristic DNA-binding domain (DBD), that is formed by two zinc-finger motifs (Glass, 1994), and a ligand-binding domain (LBD) that consists of 12 α-helices (Moras and Gronemeyer, 1998). The most critical step in $1\alpha,25(OH)_2D_3$ signaling is the induction of a conformational change within the LBD of the VDR by interaction with $1\alpha,25(OH)_2D_3$ or its analogs. The three-dimensional structure of the seven presently analyzed, ligand-bound LBD (including that of the VDR; Rochel *et al*, 2000) suggested that there is a conserved agonistic conformation of all nuclear receptor LBD. In this agonistic conformation the activation function (AF) 2 domain within helix 12 is able to contact the interaction domain of coactivator proteins of the p160-family, such as SRC-1, TIF2, and RAC3 (Herdick *et al*, 2000a), and/or of the DRIP/TRAP family (Rachez *et al*, 1999).

In the last few years several *in vitro* and cell culture methods have been developed that can be used for a molecular evaluation of the efficacy of the VDR–ligand interaction. One of the most powerful methods is the limited protease digestion assay, in which interaction of the VDR with a ligand protects its LBD against protease digestion, which allows a characterization and quantitation of functional VDR conformations (Nayeri and Carlberg, 1997; Quack and Carlberg, 2000a). Recently, it was shown that this assay can also be performed in the presence of DNA and cofactors (Herdick *et al*, 2000a; Quack and Carlberg, 2000a). The ligand-dependent gel shift assay provides a quantitation of the ligand-dependent VDR-RXR-VDRE complex formation and monitors receptor dimerization, DNA binding, and ligand interaction at the same time (Quack *et al*, 1998; Quack and Carlberg, 2000b). The mammalian-one-hybrid assay is a most simplified version of a reporter gene assay, in which the functionality of the isolated VDR-LBD can be monitored in different living cells (Herdick *et al*, 2000a). The three methods together provide sufficient informations for a molecular evaluation of a VDR ligand. In this report, the three assays systems were used for the evaluation of MC903 in comparison with two new analogs, GS1500 and EB1213, and the natural hormone. All four ligands appeared to be equally sensitive VDR agonists, but also showed individual molecular properties, such as a reduced sensitivity in HaCaT cells (MC903), a selectivity for DNA-bound VDR-RXR heterodimers (GS1500), and a long-lasting stabilization of VDR-RXR-VDRE complexes (EB1213).

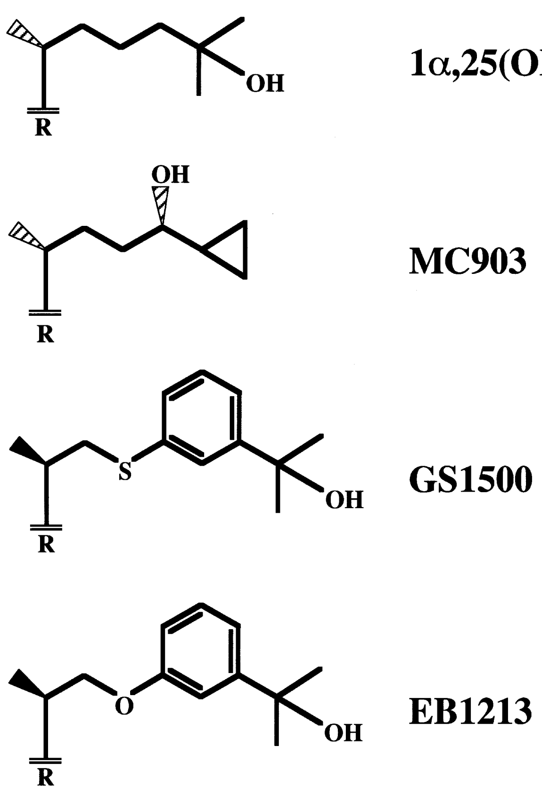


Figure 1. Structure of $1\alpha,25(OH)_2D_3$ and its analogs. The structure of the side chain is given.

MATERIALS AND METHODS

Compounds $1\alpha,25(OH)_2D_3$ and its analogs MC903 (Carlberg *et al*, 1994), GS1500 (Mathiasen *et al*, 1998), and EB1213 (Mørk Hansen *et al*, 1996) (for the structure of their side chains see **Fig 1**) were synthesized in the Department of Chemical Research at LEO Pharmaceutical Products (Ballerup, Denmark). Most characteristic are the cyclopropane ring in MC903 and the aromatic benzene ring in GS1500 and EB1213 combined with an altered stereochemistry at the C20 atom (20-epi). All VDR agonists were dissolved in 2-propanol; further dilutions were made in dimethyl sulfoxide (for *in vitro* experiments) or in ethanol (for cell culture experiments).

DNA constructs and *in vitro* protein translation The full-length cDNA 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). *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 RNA *in vitro* using rabbit reticulocyte lysate as recommended by the supplier (Promega, Mannheim, Germany). For mammalian-one-hybrid assays, 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). In reporter gene constructs the luciferase gene was driven either by three copies of the GAL4 binding site or four copies of the DR3-type VDRE of the rat ANF gene promoter (core sequence AG**AGGTCA**TGA**AGGACA**) (Kahlen and Carlberg, 1996).

Limited protease digestion assay *In vitro* translated, [^{35}S]-labeled VDR protein alone or in combination with *in vitro* translated RXR and 1 ng of unlabeled rat ANF DR3-type VDRE were incubated with graded or saturating concentrations of ligand for 15 min at room temperature in 20 μl binding buffer [10 mM HEPES, pH 7.9, 1 mM DTT, 0.2 μg per μl poly(dI-C) and 5% glycerol]. The buffer was adjusted to 150 mM of monovalent cations by addition of KCl. Trypsin (Promega, final concentration 8.3–16.6 ng per μl) was then added and the mixtures were further incubated for 15 min (or indicated times, see **Fig 4**) 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% sodium dodecyl sulfate, 0.025% bromophenol blue). The samples were denatured at 85°C for 3 min and electrophoresed through a 15% sodium dodecyl sulfate–polyacrylamide gel. The gels were dried and exposed to a Fuji MP2040S imager screen. The individual protease-sensitive VDR fragments were quantitated on a Fuji FLA2000 reader (Tokyo, Japan) using Image Gauge software.

Gel shift assay *In vitro* translated VDR-RXR heterodimers were incubated with graded or saturating concentrations of ligands 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 the [^{32}P]-labeled rat ANF DR3-type VDRE (50,000 cpm) was 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 × TBE (45 mM Tris, 45 mM boric acid, 1 mM ethylenediamine tetraacetic acid, pH 8.3) and were quantitated on a Fuji FLA2000 reader.

Transient transfections and reporter gene assay HeLa human cervix carcinoma cells and HaCaT immortalized human

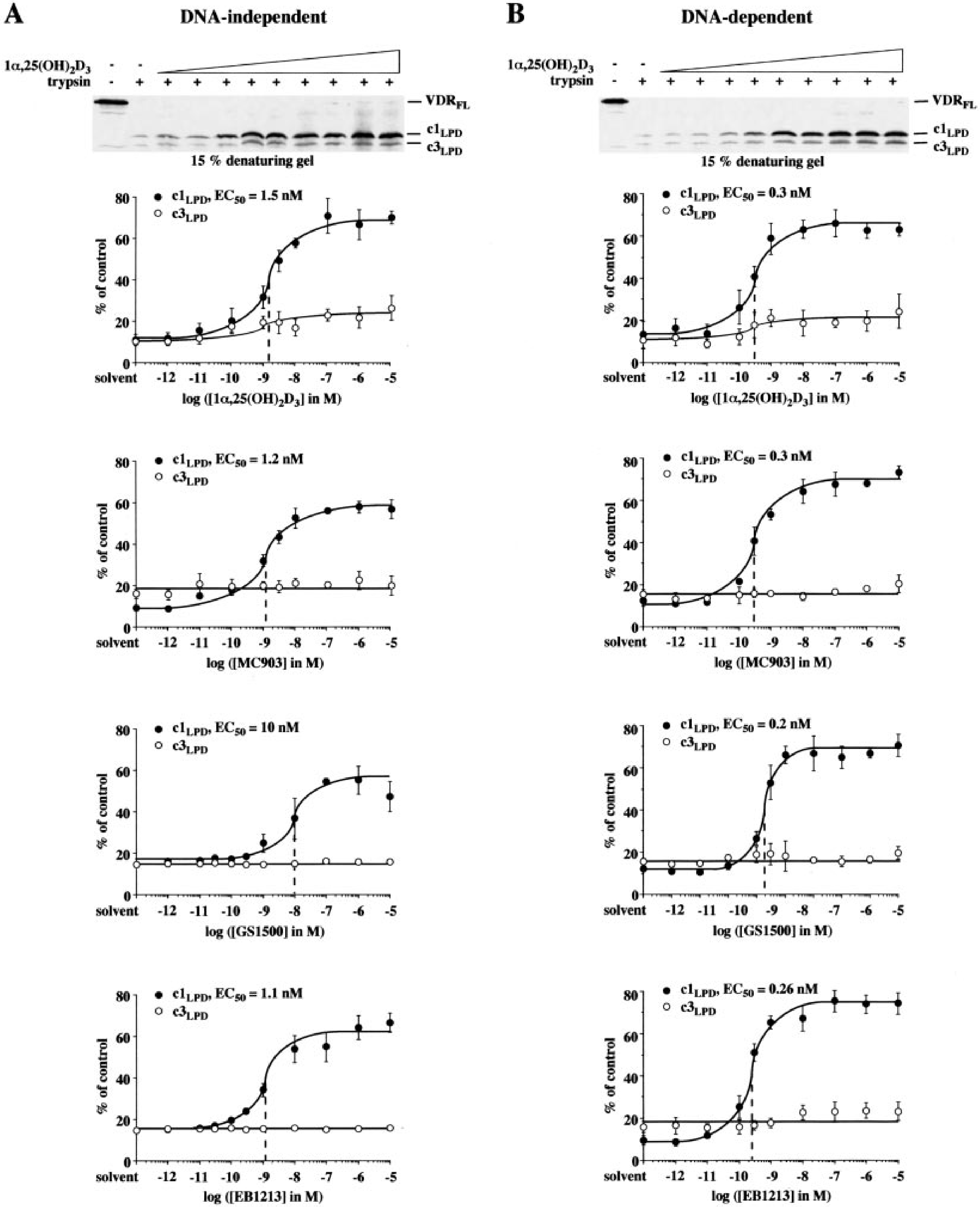
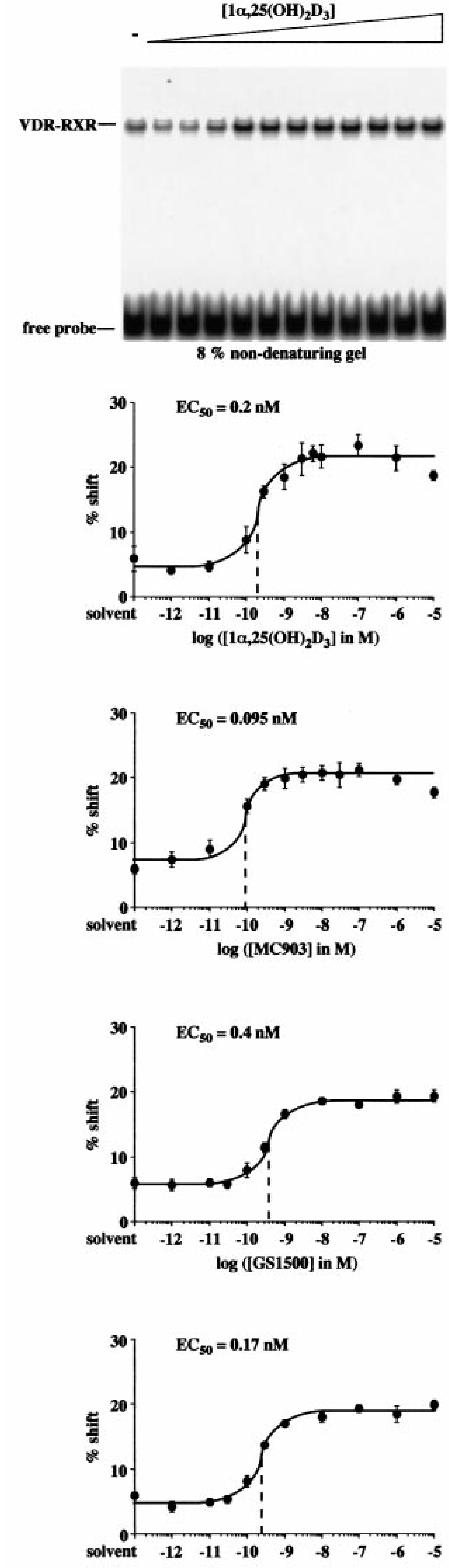


Figure 2. Stabilization of VDR conformations by $1\alpha,25(OH)_2D_3$ and its analogs. Limited protease digestion assays were performed by preincubating *in vitro* translated [^{35}S]-labeled VDR alone (*A*) or in combination with nonlabeled *in vitro* translated RXR and the rat ANF DR3-type VDRE (*B*) with graded concentrations of $1\alpha,25(OH)_2D_3$ or its analogs. After digestion with trypsin, samples were electrophoresed through 15% sodium dodecyl sulfate–polyacrylamide gels. The amount of ligand-stabilized VDR conformations 1 ($c1_{LPD}$, *filled circles*) and 3 ($c3_{LPD}$, *open circles*) in relation to VDR input was quantitated by phosphorimaging. Representative experiments are shown for $1\alpha,25(OH)_2D_3$. Data points represent the mean of triplicates and the bars indicate SD. The EC_{50} values for the stabilization of $c1_{LPD}$ were determined from the respective dose–response curves.



keratinocytes were seeded on to six-well plates (10^5 cells per ml) and grown overnight in phenol red-free Dulbecco's minimal Eagle's medium supplemented with 10% charcoal-treated fetal bovine serum. 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 both cell lines) or 1 μg of the rat ANF DR3-type VDRE-driven reporter plasmid and 1 μg each of pSG5-based receptor expression vectors for VDR and RXR (for HaCaT cells) with 15–20 μg *N*-[1-(2,3-Dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (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 Dulbecco's minimal Eagle's medium, the liposomes were added to the cells. Phenol red-free Dulbecco's minimal Eagle's medium supplemented with 30% charcoal-treated fetal bovine serum (500 μl) was added 4 h after transfection. At this time, graded concentrations of VDR agonists were also added. The cells were lyzed 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 to protein concentration and induction factors were calculated as the ratio of luciferase activity of ligand-stimulated cells to that of solvent controls.

RESULTS

Stabilization of VDR conformation The interaction of 1α,25$(OH)_2D_3$ and its analogs MC903, GS1500, and EB1213 (for structure of their side chain see **Fig 1**) with the VDR in solution (**Fig 2*A***) or within VDR-RXR-VDRE complexes (**Fig 2*B***) was analyzed in DNA-independent and DNA-dependent limited protease digestion assays, which were performed with *in vitro* translated, [^{35}S]-labeled VDR protein alone or in combination with *in vitro* translated unlabeled RXR and unlabeled rat ANF DR3-type VDRE, respectively. This assay displays a concentration-dependent stabilization of two VDR fragments, $c1_{LPD}$ (28 kDa) and $c3_{LPD}$ (23 kDa), that contain major parts of the LBD [from the trypsin cutting site after arginine 173 to either the carboxy-terminus at position 427 ($c1_{LPD}$) or to arginine 391 ($c3_{LPD}$)] and represent the agonistic and the nonagonistic conformation of the VDR-LBD (Herdick *et al*, 2000a; Herdick and Carlberg, 2000), whereas a reasonable amount of the VDR fragment $c2_{LPD}$ is observed only with VDR antagonists (Bury *et al*, 2000; Herdick *et al*, 2000b). All four ligands showed the typical profile of VDR agonists, i.e., a stabilization of 60–80% of all VDR molecules in $c1_{LPD}$ and only 10–20% in $c3_{LPD}$ (Herdick *et al*, 2000a). MC903 and EB1213 appeared to be indistinguishable from the natural hormone as they stabilized VDR in solution with an EC_{50} value of 1.2–1.5 nM, which is approximately five times higher than the concentration needed for a stabilization of the VDR within VDR-RXR-VDRE complexes (EC_{50} value of 0.2–0.3 nM). Interestingly, GS1500 showed the same sensitivity for the stabilization of the VDR within VDR-RXR-VDRE complexes

Figure 3. 1α,25$(OH)_2D_3$ and its analogs stabilize VDR-RXR heterodimer complex formation on DNA. Gel shift experiments were performed with *in vitro* translated VDR-RXR heterodimers, which were preincubated at room temperature with graded concentrations of 1α,25$(OH)_2D_3$ or its analogs and the [^{32}P]-labeled rat ANF DR3-type VDRE. 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 quantitated by phosphorimaging. A representative experiment is shown for 1α,25$(OH)_2D_3$. Data points represent the mean of triplicates and the bars indicate SD. The EC_{50} values for VDR-RXR-VDRE complex formation were determined from the respective dose–response curves.

than the three other VDR agonists (EC_{50} value of 0.2 nM), but 50 times higher concentrations (EC_{50} value of 10 nM) were needed to stabilize the VDR in solution.

Stabilization of VDR-RXR-VDRE complexes In order to confirm the DNA-dependent sensitivity of the four VDR agonists, 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 $1\alpha,25(OH)_2D_3$ and its three analogs (**Fig 3**). A comparable amount (approximately 20% shifted probe) of concentration-dependent VDR-RXR heterodimer complex formation on the VDRE was observed with all four compounds and provided EC_{50} values between 0.095 and 0.4 nM. This ligand sensitivity is comparable with that observed in DNA-dependent limited protease digestion assays (**Fig 2***B*). Moreover, this confirms that all three analogs show a sensitivity for the stabilization of VDR-RXR-VDRE complexes that is very close to that of $1\alpha,25(OH)_2D_3$.

The kinetics of VDR-ligand dissociation within VDR-RXR-VDRE complexes was analyzed by DNA-dependent limited protease digestion assays, which were performed with *in vitro* translated VDR-RXR heterodimers bound to the rat ANF DR3-type VDRE and saturating concentrations of $1\alpha,25(OH)_2D_3$ and the three analogs and solvent as a control (**Fig 4**). The incubation time with trypsin varied between 15 min and 24 h. It is important to note that trypsin was found to be still active even after 24 h of incubation (data not shown). The amount of ligand-stabilized VDR was quantitated and plotted over time, which allowed a

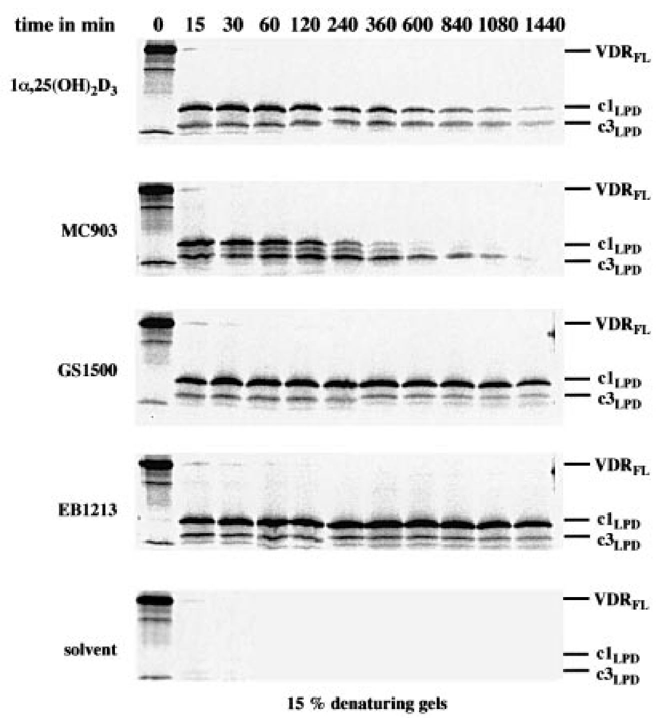


Figure 4. Different half-lives of VDR-ligand conformations. Limited protease digestion assays were performed by preincubating *in vitro* translated [35S]-labeled VDR together with nonlabeled *in vitro* translated RXR and the rat ANF DR3-type VDRE and saturating concentrations (10 μM) of $1\alpha,25(OH)_2D_3$, its analogs or solvent (as a control). The samples were incubated for indicated times with trypsin and were then electrophoresed through 15% sodium dodecyl sulfate–polyacrylamide gels. The amount of ligand-stabilized VDR conformations (sum of $c1_{LPD}$ and $c3_{LPD}$) in relation to VDR input was quantitated by phosphorimaging. Representative experiments are shown for all four VDR agonists and solvent control. Data points represent the mean of triplicates and the bars indicate SD. The half-lives ($t_{1/2}$) were determined from the respective time-course curves.

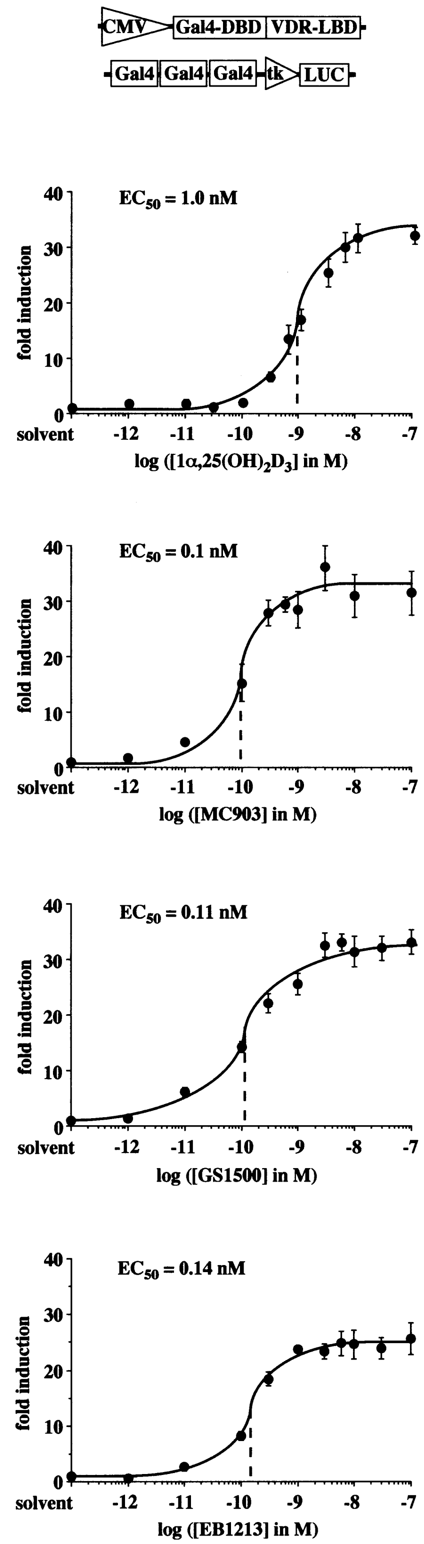


Figure 5. Agonistic effects of $1\alpha,25(OH)_2D_3$ and its analogs *in vivo*. Luciferase reporter gene assays were performed with extracts from HeLa cells that were transiently transfected with a reporter gene construct-driven by three copies of the GAL4 binding site and an expression vector for a $GAL4_{DBD}VDR_{LBD}$ fusion protein (as schematically depicted above). The cells were treated for 16 h with graded concentrations of $1\alpha,25(OH)_2D_3$ or its analogs. Stimulation of luciferase activity was calculated in comparison with solvent-induced controls. Data points represent the mean of triplicates and the bars indicate SD. The EC_{50} values for stimulation of VDR-driven gene activity were determined from the respective dose–response curves.

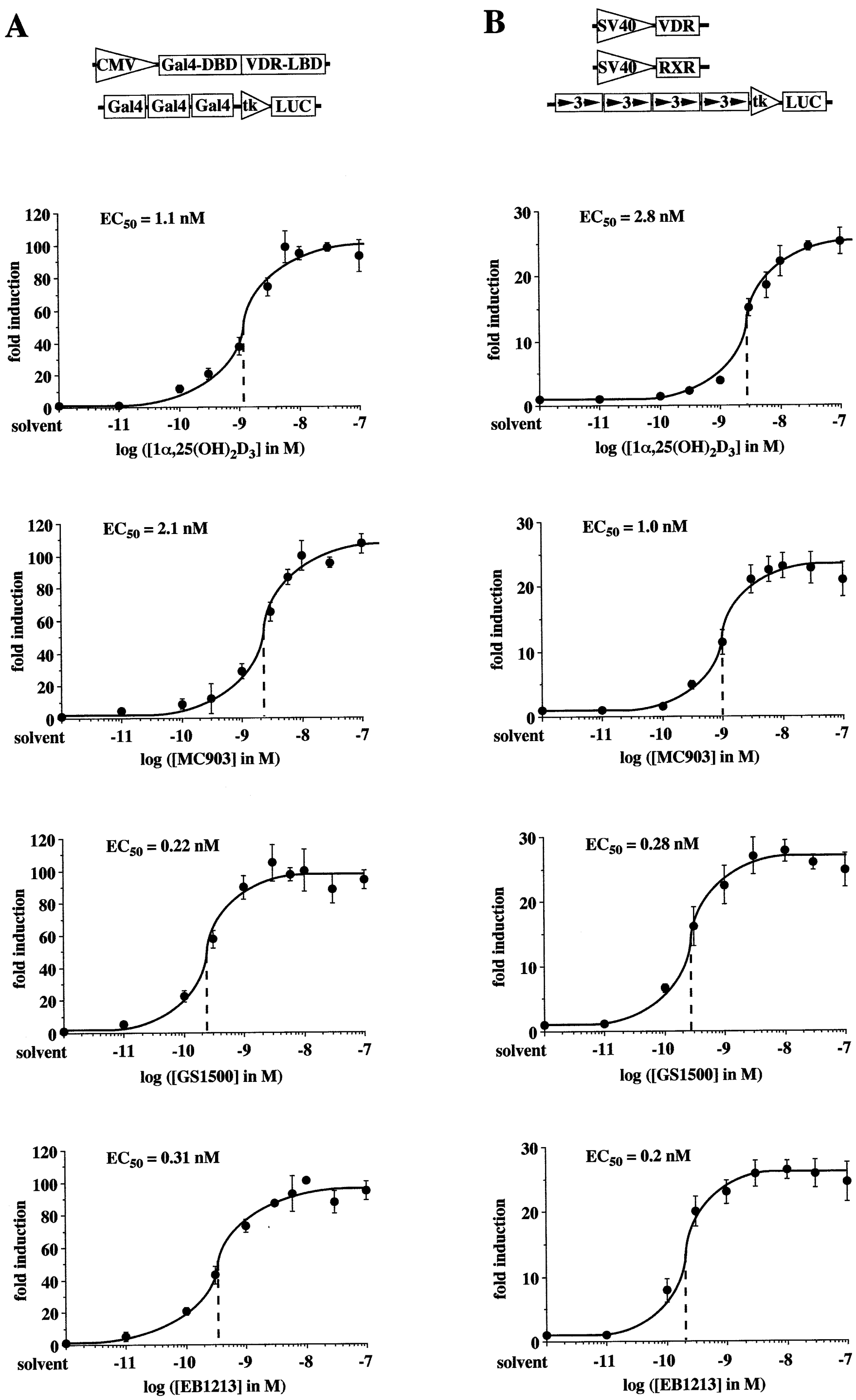


Figure 6. Agonistic effects of $1\alpha,25(OH)_2D_3$ and its analogs in HaCaT cells. Luciferase reporter gene assays were performed with extracts from HaCaT cells that were transiently transfected with a reporter gene construct-driven by three copies of the GAL4 binding site and an expression vector for a $GAL4_{DBD}VDR_{LBD}$ fusion protein (*A*) or with a luciferase reporter gene construct driven by four copies of the rat ANF DR3-type VDRE together with the expression vectors for VDR and RXR (*B*) as schematically depicted above the respective figures. The cells were treated for 16 h with graded concentrations of $1\alpha,25(OH)_2D_3$ or its analogs. Stimulation of luciferase activity was calculated in comparison with solvent-induced controls. Data points represent the mean of triplicates and the bars indicate SD. The EC_{50} values for stimulation of VDR-driven gene activity were determined from the respective dose–response curves.

determination of the half-life ($t_{1/2}$) of the VDR–ligand complex. Interestingly, the three analogs showed clearly different $t_{1/2}$ values, which were found to be lower (MC903, $t_{1/2}$ = 438 min) and higher (GS1500, $t_{1/2}$ = 1175 min and EB1213, $t_{1/2}$ = 2717 min) than that of the natural hormone ($t_{1/2}$ = 660 min). If only the stabilization of $c1_{LPD}$ is determined, the respective $t_{1/2}$ values are 472, 260, 1260, and 3375 min for $1\alpha,25(OH)_2D_3$, MC903, GS1500, and EB1213, respectively, i.e., no differences in the ranking of the VDR ligands.

Functional activity of VDR agonists in HeLa and HaCaT cells In order to test the functionality of $1\alpha,25(OH)_2D_3$ and the three selected analogs, classical mammalian-one-hybrid assays were performed in HeLa human cervix carcinoma 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 stimulation of the cells with graded ligand concentrations provided a 25–35-fold induction of reporter gene activity with EC_{50} values of 1.0 nM for $1\alpha,25(OH)_2D_3$, 0.1 nM for MC903, 0.11 nM for GS1500, and 0.14 nM for EB1213. This indicates that the *in vitro* sensitivity of the three analogs for the stabilization of VDR-RXR-VDRE complexes (**Figs 2** and **3**) translates well to their sensitivity in HeLa cells. In contrast, the natural hormone appears to be approximately 10 times less sensitive in living cells than *in vitro*.

Table I. Comparison of the four tested VDR ligands[a]

VDR ligand	$1\alpha,25(OH)_2D_3$	MC903	GS1500	EB1213
DNA-dependent stabilization of VDR conformations ($t_{1/2}$ in min)	660	438	1175	2717
DNA-dependent stabilization of $c1_{LPD}$ ($t_{1/2}$ in min)	472	260	1260	3375
DNA-independent stabilization of $c1_{LPD}$ (EC_{50} value in nM)	1.5	1.2	10	1.1
DNA-dependent stabilization of $c1_{LPD}$ (EC_{50} value in nM)	0.3	0.3	0.2	0.26
VDR-RXR-VDRE complex formation (EC_{50} value in nM)	0.2	0.095	0.4	0.17
Functional activity of VDR_{LBD} in HeLa cells (EC_{50} value in nM)	1.0	0.1	0.11	0.14
Functional activity of VDR_{LBD} in HaCaT cells (EC_{50} value in nM)	1.1	2.1	0.22	0.31
Functional activity of VDR-RXR in HaCaT cells (EC_{50} value in nM)	2.8	1.0	0.28	0.2
Anti-proliferative activity in HaCaT cells (IC_{50} value in nM)	50	32	0.11	0.28

[a]Anti-proliferative data from Mørk Hansen *et al* (1996).

The functional analysis of $1\alpha,25(OH)_2D_3$ and its analogs was extended to HaCaT immortalized human keratinocytes as a representative $1\alpha,25(OH)_2D_3$ target tissue. Mammalian-one-hybrid assays (**Fig 6*A***) as well as traditional reporter gene assays (**Fig 6*B***), that used overexpressed VDR and RXR proteins and a DR3-type VDRE-driven luciferase gene, were performed in this cell line. Interestingly, the mammalian-one-hybrid assay (**Fig 6*A***) provided for all four ligands more than 100-fold induction of gene activity, whereas the traditional assay (**Fig 6*B***) only showed an inducibility of 25-fold. Both types of reporter gene assays provided for GS1500 and EB1213 EC_{50} values of 0.2–0.31 nM, i.e., values that are very comparable with that obtained in HeLa cells (**Fig 5**). This demonstrates that mammalian-one-hybrid assays are as sensitive as traditional reporter gene assays. Moreover, the EC_{50} values that were obtained in both assays for $1\alpha,25(OH)_2D_3$ (1.1 and 2.8 nM) are in accordance with the results from HeLa cells (1.0 nM, see **Fig 5**). In contrast, in HaCaT cells the sensitivity of MC903 for activation of gene activity (EC_{50} values of 1.0 and 2.1 nM) was found to be 10–21-fold lower than in HeLa cells (**Fig 5**).

DISCUSSION

The nuclear receptor superfamily contains a series of transcription factors that are of high impact because they can be specifically regulated in their activity by small lipophilic compounds of natural or synthetic origin. The protein–DNA complex of nuclear receptor and its specific response element can be considered as a molecular switch for those genes that contain such a response element in their promoter region. The VDR appears to be an ideal member of the nuclear receptor superfamily, as apart from hypercalcemia no significant side-effects of its specific natural ligand, $1\alpha,25(OH)_2D_3$, are known. This makes prevention of hypercalcemia a primary target for the development of therapeutically important VDR agonists. In this study, the molecular action of the natural hormone was compared with that of three analogs that had been identified by biologic screenings to be low calcemic. Interestingly, on the level of the activation of RXR- and DNA-complexed VDR, i.e., on the level of the molecular switch, the sensitivity of all three analogs (EC_{50} values of approximately 0.2 nM) was found to be not significantly different to that of $1\alpha,25(OH)_2D_3$ (**Table I**). This identity was observed by two different methods, DNA-dependent limited protease digestion assays and ligand-dependent gel shift assays that appear to be very accurate tools for an *in vitro* evaluation of nuclear receptor–DNA–ligand interactions. Similar results have been obtained recently with other potent (but higher calcemic) $1\alpha,25(OH)_2D_3$ analogs, such as 20-epi-$1\alpha,25(OH)_2D_3$, 20-methyl-$1\alpha,25(OH)_2D_3$ and an analog with two side chains, referred to as Gemini (Herdick *et al*, 2000a). In that report, in addition to the above-mentioned methods, supershifts with coactivator proteins and gel shift clipping assays were used, but in all cases the EC_{50} value for the activation of the VDR by any of these ligands was found to be approximately 0.1 nM (Herdick *et al*, 2000a). This indicates that there appears to be a threshold of VDR activation of 0.1–0.2 nM that even optimized synthetic VDR agonists may not be able to exceed. This means that there is probably no synthetic VDR agonists having an affinity for the VDR that is significantly higher than that of the natural hormone. This observation is supported by traditional ligand binding assays (Binderup *et al*, 1994; Bouillon *et al*, 1995; van den Bemd *et al*, 1995).

Interestingly, the $1\alpha,25(OH)_2D_3$ analogs GS1500 and EB1213 showed the same EC_{50} values in the functional assays in HeLa and HaCaT cells as in the *in vitro* assays (approximately 0.2 nM). This indicates that the *in vitro* assays represent well the ligand sensitivity of the VDR in living cells and in turn suggests that both analogs are not significantly metabolized or absorbed by cellular or serum proteins. This conclusion appears to hold true for MC903 in HeLa cells, but not in HaCaT cells, where the EC_{50} value was found to be 22-fold higher. The reason for this difference is yet unknown and apparently not related to a fast metabolism of MC903 in HaCaT cells, in which the analog was shown to be rather stable (Løgsted Nielsen and Kissmeyer, unpublished results). In contrast, $1\alpha,25(OH)_2D_3$ showed higher EC_{50} values in the functional assays in HeLa and HaCaT cells compared with the *in vitro* assays. The functional assays are performed in the presence of serum and thereby also vitamin D binding protein. Therefore, it is likely that the binding of $1\alpha,25(OH)_2D_3$ to vitamin D binding protein has suppressed the free entry $1\alpha,25(OH)_2D_3$ into the cells and thereby increased the EC_{50} value (Vanham *et al*, 1988). As most $1\alpha,25(OH)_2D_3$ analogs have a reduced affinity for vitamin D binding protein compared with the natural hormone (Kissmeyer *et al*, 1995) the same difference in activity between functional and *in vitro* assays is, as observed, not expected.

On first glance, the functionality of MC903, GS1500, and EB1213 *in vitro* and in HeLa cells appears to be identical (**Table I**); however, in proliferation assays in HaCaT cells GS1500 and EB1213 are known to be more potent than MC903 and $1\alpha,25(OH)_2D_3$ (Mørk Hansen *et al*, 1996). This may be due to important differences in stabilizing either the VDR in solution or

VDR-RXR-VDRE complexes over time. Compared with the three other tested VDR agonists, GS1500 showed a clear preference for DNA-bound VDR-RXR complexes (**Table I**). A selectivity for DNA-bound VDR-RXR heterodimers means that DNA-independent actions of the VDR, such as the inhibition of IL-2 gene expression via the prevention of DNA binding of the transcription factor NF-AT (Alroy *et al*, 1995), will not be stimulated (or only at clearly higher concentrations) by the respective ligand. Moreover, GS1500 stabilized DNA-bound VDR-RXR complexes nearly twice as long as $1\alpha,25(OH)_2D_3$, whereas MC903 kept the complex stable only for a shorter time period than the natural hormone; however, EB1213 appears to be most potent as it stabilizes VDR-RXR-VDRE complexes more than four times longer than $1\alpha,25(OH)_2D_3$. An increased ligand-dependent stabilization of VDR-RXR-VDRE complexes would then result in longer-lasting effects of the respective ligand on gene activation.

In conclusion, the molecular evaluation of the analogs MC903, GS1500, and EB1213 in comparison with the natural hormone has indicated that they are all potent VDR agonists that show a very similar sensitivity in stabilizing the molecular switches of nuclear $1\alpha,25(OH)_2D_3$ signaling, i.e., DNA-bound VDR-RXR heterodimers. Individual properties of the compounds, however, could also be identified, of which the skin cell-specific reduced sensitivity of MC903, the DNA selectivity of GS1500 and the long-lasting stabilization of VDR-RXR-VDRE complexes by EB1213 are the most remarkable.

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