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Novel Gemini analogs of 1α,25-dihydroxyvitamin D_3 with enhanced transcriptional activity

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Abstract

The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), exerts its effects through regulation of target gene transcription. Configuration at C-20 of $1,25(OH)_2D_3$ is important in determining potency, as shown by the high potency of analogs with inverted configuration at C-20 (20-epi compounds). Gemini analogs of $1,25(OH)_2D_3$ contain two side chains, combining a C-20-normal with a C20-epi side chain. We studied the potency of analogs combining double (Gemini) side chains with a 23-triple bond and a C-26,27-hexafluoro substitution in either the 20-epi (analog 20R) or 20-normal (analog 20S) side chain. These novel Gemini analogs were 8–50-fold more potent than $1,25(OH)_2D_3$ in inducing U937, HL-60G, and THP-1 differentiation and 5–50-fold more potent in inducing transcription from the osteocalcin vitamin D response element or the 25-hydroxyvitamin D_3-24-hydroxylase (24OHase) promoter. *In vivo*, following i.p. injection in vitamin D-deficient mice, the 20S analog induced significantly higher levels of calbindin-D_{9K} mRNA in intestine, and 24OHase and calbindin-D_{28K} in kidney than $1,25(OH)_2D_3$ or analog 20R. Increased potency did not correlate with ligand-receptor binding affinity. In GST-pull down assays using *in vitro* translated VDR, Gemini analogs showed equivalent (or even attenuated) potency to $1,25(OH)_2D_3$ in recruiting cofactors DRIP205 and GRIP-1 to VDR. However, Gemini analogs were up to 15-fold more potent than $1,25(OH)_2D_3$ in recruiting the same cofactors to VDR in GST-pull down assays using equal amounts of VDR from nuclear extracts of VDR transfected and hormone treated (24 hr) COS-7 cells. Deletion of C-19 in either 20S or 20R Gemini analogs resulted overall in slightly less potent analogs compared to Gemini itself. We conclude that enhanced potency of the novel Gemini analogs is at least partly due to increased metabolic stability of the analogs, resulting in more cofactor binding and elevated levels of transcription.

Keywords: Vitamin D; Analogs; Gemini; Transcription; Cofactors; Leukemic cell differentiation

1. Introduction

Historically, the hormonally active form of vitamin D, $1,25(OH)_2D_3$, is known as a pivotal factor in maintaining calcium homeostasis through regulation of genes in intestine, kidney, and bone (see for recent reviews [1–3]). In recent years, $1,25(OH)_2D_3$ has also been identified as an important regulator of basic cellular processes such as proliferation and differentiation [4,5]. The potency of $1,25(OH)_2D_3$ to stimulate differentiation (and inhibit proliferation) of different types of neoplastic cells led to an increasing research effort directed at the design and development of $1,25(OH)_2D_3$ analogs that improve these characteristics (reviewed by [6,7]). Effects of $1,25(OH)_2D_3$ on proliferation and differentiation are thought to be transcriptional events mediated by the VDR. Upon binding to $1,25(OH)_2D_3$, VDR forms a heterodimer with RXR, recruits several cofactors (including DRIP-205 and GRIP-1 [8–10]), and binds $1,25(OH)_2D_3$ response elements (VDREs) in promoters of target genes and thus acts as a ligand-dependent transcription factor effecting target gene transcription (see for review [1,11]).

In search of $1,25(OH)_2D_3$ analogs that enhance transcriptional activity many side-chain modifications have

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Abbreviations: $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; DBP, vitamin D binding protein; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; RPMI, Roswell Park Memorial Institute medium; VDR, vitamin D receptor.

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been reported [6,7]. One of the most effective structural changes reported to date has been the inversion of configuration at C-20, resulting in the so called 20-epi analogs. These analogs have a 200–5000-fold increased antiproliferative capacity over $1,25(OH)_2D_3$ [12] and about 500 times enhanced transcriptional activity [13]. Several mechanisms contributing to this increase in potency have been reported, including increased stability of the analog itself [14], different conformation of the liganded VDR facilitating VDR action [13], protection of VDR against proteolytic degradation [15], and increased cofactor binding [16,17]. The potency of analogs with inverted configuration at C-20 led to the recent synthesis of an analog called Gemini that contains two six carbon side-chains, combining a C-20-normal with a C-20-epi side chain [18]. Subsequently, Gemini and its nor-19 derivative were shown to readily bind to VDR and generate unique conformational changes, however, without permitting superior gene transcription activity, despite the presence of the C-20-epi side chain [19,20]. Two potential positions for the extra side chain of Gemini have been proposed in the ligand binding domain of VDR [21], which might enable Gemini analogs to selectively activate vitamin D functions.

Here we report on the characterization of Gemini analogs of $1,25(OH)_2D_3$ that combine the Gemini characteristic of a double side chain with features of previously synthesized potent analogs [6,22,23], being a 23-triple bond and a 26,27-hexafluoro substitution in either the S (normal) or R (epi) side chain and a deletion of the C19 hydroxyl group (nor-19 variant).

2. Materials and methods

2.1. Compounds

$1,25(OH)_2D_3$ and analogs (obtained from Hoffmann LaRoche) were dissolved in ethanol. Structures and

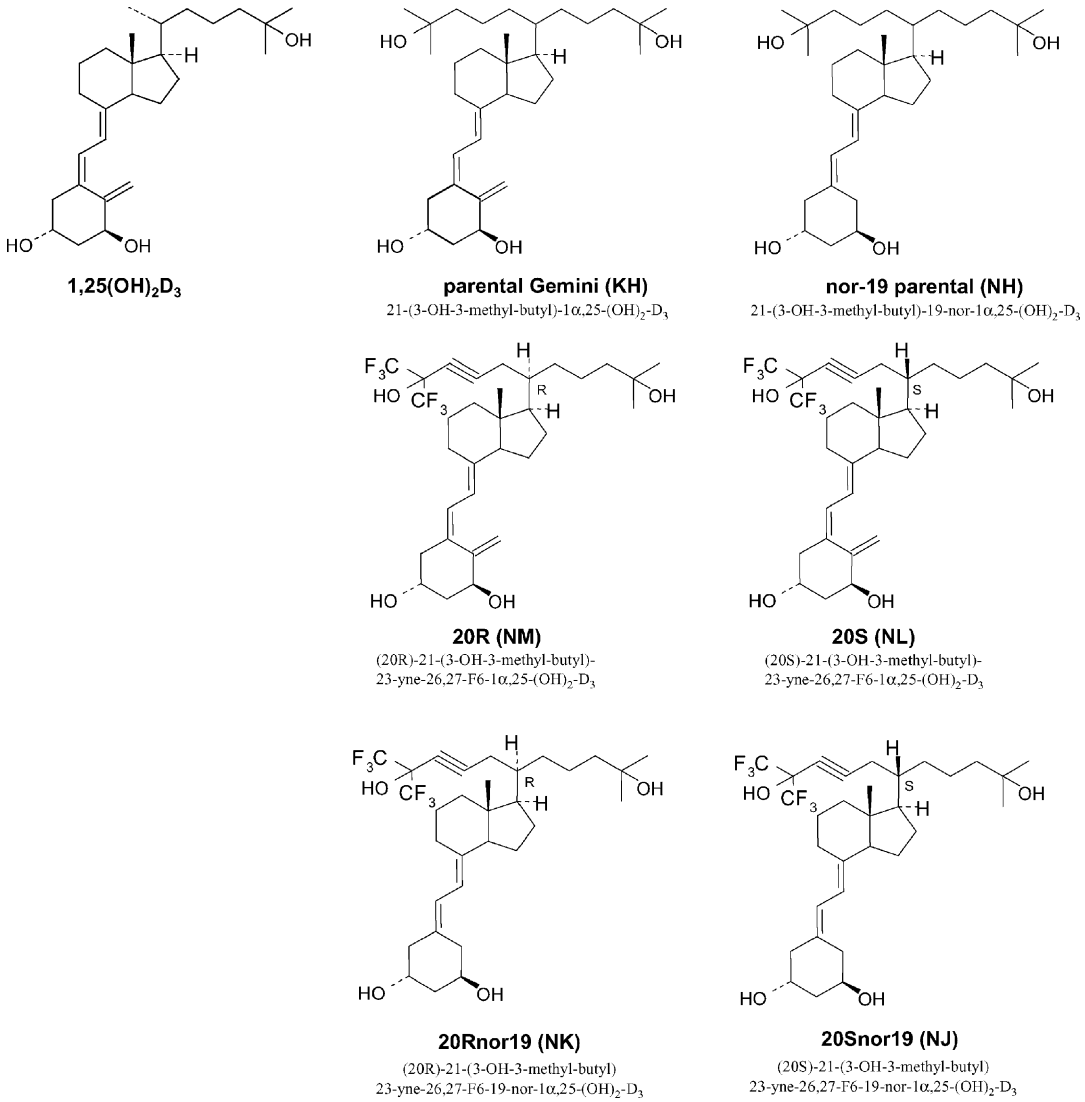

Fig. 1. Molecular structures and chemical names of ligands tested. The letter code was introduced by Bouillon *et al.* [6].

Table 1
Summary of relative competitive indices (RCI) for VDR and DBP binding of $1,25(OH)_2D_3$ and the Gemini analogs

	Letter code[a]	RO number	Relative comparative index	
			VDR	DBP
$1,25(OH)_2D_3$			100	100
Parental	KH	RO27-2310	38 ± 10.6	3 ± 1.1
20S	NL	RO43-83582	38 ± 12.0	21 ± 3.5
20R	NM	RO43-83586	35 ± 7.8	14 ± 8.5
Parental-nor-19	NN	RO27-5646	6 ± 2.3	1 ± 0.3
20S-nor-19	NJ	RO43-83561	15 ± 7.8	10 ± 4.2
20R-nor-19	NK	RO43-83562	15 ± 4.2	9 ± 1.8

Human DBP RCI and chick intestinal VDR RCI were determined from separate steroid competition assays as described in Section 2.

[a] Letter code as introduced by Bouillon *et al.* [6].

chemical names of the compounds studied are shown in Fig. 1. Roche identification numbers and the letter code as introduced previously [6] are listed in Table 1.

2.2. Binding affinity assays

Steroid competition assays were carried out to study the relative affinity (relative competitive index) of the Gemini analogs for chick intestinal VDR and human DBP according to our standard procedures [24]. In these assays increasing concentrations of the analog or non-radioactive $1,25(OH)_2D_3$ were incubated with a fixed amount of tritiated $1,25(OH)_2D_3$ and either VDR or DBP. After separation of bound and free ligand, binding affinity is calculated relative to $1,25(OH)_2D_3$. By definition the *r*elative *c*ompetitive *i*ndex (RCI) of $1,25(OH)_2D_3$ is set to 100% for both VDR and DBP.

2.3. In vitro transcription

COS-7 and CV-1 African green monkey cells obtained from the American Type Culture Collection (ATCC) were maintained in DMEM (COS-7) or MEM with Earle's buffered salts and non-essential amino acids (CV-1) supplemented with 10% heat-inactivated FBS at 37° and 5% CO_2/95% air. Cells were transfected at 70% confluency in 100 mm tissue culture dishes using the calcium phosphate precipitation method [25] for COS-7 or DEAE-dextran (Sigma) for CV-1 [26]. COS-7 cells were transfected with a chloramphenicol acetyltransferase (CAT) reporter driven by the rat 24OHase promoter (−1367/+74 see [27]) and CV-1 cells were transfected with a human osteocalcin VDRE driven alkaline phosphatase reporter [26]. Both cells were cotransfected with a human VDR expression plasmid (a gift from J.W. Pike, University of Wisconsin). After 16 hr of transfection medium was changed to DMEM with 2% charcoal-treated FBS and cells were treated with $1,25(OH)_2D_3$ or analogs. After 24 hr of treatment CAT activity was determined in COS-7 cell lysates normalized to total protein level according to Gorman *et al.* [28]. Secreted alkaline phosphatase activity in the culture medium of CV-1 cells was determined as described previously [26].

2.4. In vivo treatment and Northern blots

Six weeks old, male B57/BL6 mice were put on a strontium diet (Harlan Teklad) for a week to induce a vitamin D_3-deficient state as described earlier [29]. Mice (20 g) were given a single intraperitoneal injection of 45 ng (2 μg/kg bodyweight) of $1,25(OH)_2D_3$ or analog in 100 μL 90% 1,2 propanediol, 10% ethanol 16 hr before sacrifice. Controls were injected with vehicle only. The proximal 6 cm of intestine and the kidneys were collected and snap frozen in liquid nitrogen until RNA isolation. Serum was collected and serum calcium concentrations were determined using a Perkin Elmer Atomic Absorption Spectrophotometer 4000 (Perkin Elmer). For RNA isolation, tissues were homogenized in RNA_{BEE} (Tel-Test Inc.). Northern blots for 24OHase, calbindin-D_{9K}, and calbindin-D_{28K} on kidney and for calbindin-D_{9K} on intestinal RNA samples were performed as described earlier [30]. Probe hybridization was quantified using a Typhoon 9410 variable mode phospho imager (Amersham Biosciences).

2.5. Surface and enzyme markers of differentiation

U937, HL-60, and THP-1 human leukemia cells were obtained from ATCC. The well-differentiating HL60-G cells were subcloned from HL60 cells as previously reported [31]. Cells were routinely cultured at 37° in RPMI 1640, supplemented with 10% heat-inactivated, iron-enriched FBS (HyClone). For experiments, cells were seeded into fresh culture medium at 0.5–2 × 10^5 cells/mL in 25 cm^2 tissue culture flasks and incubated with test agents for 96 hr. Following this treatment, aliquots of 1 × 10^6 cells were washed twice with phosphate-buffered saline (PBS) and incubated with 0.5 μL MY4-RD1 (Coulter) at room temperature for 45 min, to determine the expression of surface cell marker CD14. Following three washes with ice-cold PBS, cells were suspended in 0.5 mL PBS and analyzed using an Epics Profile II instrument (Coulter Electronics). For assessment of differentiation by monocyte-serine esterase (MSE), also known as non-specific esterase (NSE), smears were made by re-suspending 2 × 10^6 cells in 100 μL PBS and spreading on slides. The air-dried smears were fixed in formalin–acetone mixture buffer for 30 s, then washed with distilled H_2O and stained for 45 min at room temperature with the following solution: 67 mM phosphate buffer, pH 7.6, 8.9 mL, hexazotized pararosaniline, 0.6 mL, 10 mg alpha-naphthyl acetate, and 0.5 mL ethylene glycol monomethyl ether. MSE-positive cells were determined by counting 500 cells in each group. ED_{50} is the concentration of a compound required to achieve 50% differentiation of cells following exposure for 96 hr, and was obtained by testing each

compound at three concentrations in duplicate. Results shown are mean values from three experiments.

2.6. GST-pull downs

GST–DRIP205 (527–970) and GST–GRIP-1 plasmids [16] were obtained from Dr. L.P. Freedman (Merck and Co. Inc.). Fusion proteins were isolated by growing the constructs in *Escherichia coli* DH5α cells for 3–4 hr at 37° and induction of protein expression with 0.1 M IPTG for 3.5 hr at 30°, followed by harvesting and sonication (3 × 10 s) of the bacteria in PBS containing 1 mM PMSF and 1 mM DTT. Cell debris was pelleted at 9000 *g* twice for 10 min. Glutathione-Sepharose beads (750 μL, Amersham Biosciences) were washed with PBS containing 1 mM PMSF and 1 mM DTT and allowed to bind the fusion proteins in the bacterial supernatant for 2 hr under gentle agitation at 4°. Beads were then washed three times in PBS containing 1 mM PMSF and 1 mM DTT and the volume reconstituted to 750 μL. Bound proteins were analyzed and quantified by SDS–PAGE. ^{35}S-VDR was transcribed and translated using the TNT Quick Coupled Transcription/Translation System from Promega. Four microliters of ^{35}S-VDR was pre-incubated with increasing concentrations of $1,25(OH)_2D_3$ or analog at room temperature for 15 min in GST-binding buffer (20 mM Hepes pH 7.4, 50 mM NaCl, 75 mM KCl, 1 mM EDTA, 0.05% Triton X-100, 10% glycerol, 1 mM DTT). Next, 20 μg of GST–DRIP or GST–GRIP-1 fusion proteins were added and allowed to bind to ^{35}S-VDR while rotating at 4° for 2 hr. Beads were washed 3× with GST-binding buffer before elution in SDS–PAGE sample buffer by boiling for 5 min. Samples were subjected to electrophoresis on 12% SDS–PAGE gels. Dried gels were exposed to light sensitive films.

GST-pull down assays were also performed using nuclear extracts of VDR-transfected and $1,25(OH)_2D_3$ or analog treated COS-7 cells. For these experiments COS-7 cells were transfected with a VDR expression plasmid using lipofectamin (In Vitrogen Life Technologies) and treated as described in Section 2.3. Nuclear extracts were isolated as described [32]. Equal amounts of VDR-containing nuclear extracts, as determined by Western blot, were rotated for 16 hr with 20 μg of GST–DRIP205 or GST–GRIP-1 at 4°. Beads were washed 3× with GST-binding buffer before elution in SDS–PAGE sample buffer by boiling for 5 min. Samples were run on 12% SDS–PAGE gels and proteins were blotted onto PVDF membranes which were subjected to VDR immunoblotting as described [33] using rat monoclonal antibodies from Affinity BioReagents Inc. Bands were quantitated using the Gene Genius Bio-imaging System (Syngene).

2.7. Statistics

Results are reported as mean ± SE. The Student's *t*-test was used to determine differences in potency between $1,25(OH)_2D_3$ and the analogs. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Reduced affinity of analogs for VDR and DBP

The relative affinity of the 20S and 20R Gemini analogs for VDR was lower than that of $1,25(OH)_2D_3$ (Table 1, 35% of that of $1,25(OH)_2D_3$) and even further reduced for the two nor-19 analogs (15% of that of $1,25(OH)_2D_3$). Relative DBP affinity was also reduced with relative competitive index values approximately 18% of that of $1,25(OH)_2D_3$ for the 20S and 20R Gemini compounds and approximately 10% for the two nor-19 analogs. No significant difference in affinities between 20R and 20S analogs was observed. Both parental compounds (without the hexafluoro substitution) had lower affinity for DBP than their hexafluorinated counterparts, whereas VDR affinity was not affected by the hexafluoro substitution.

3.2. Increased potency of Gemini analogs to induce leukemia cell differentiation

Differentiation of three leukemia cell lines (U937, HL-60G, and THP-1) following *in vitro* treatment of cells with the analogs was determined by assessing the ED_{50} of each analog to induce CD14 and MSE expression. Results for U973 are shown in Fig. 2, the responses of the two other cell lines were similar (not shown). All Gemini analogs tested were more potent (8–50-fold) than $1,25(OH)_2D_3$. The hexafluoro substitution induced a further 2–3-fold increase over the parental (non hexafluorinated) analogs. Analogs with 19-normal configuration were found to be 1.5–4-fold more potent than 19-nor analogs. No significant differences were observed in ED_{50}s between the 20S and 20R compounds (ED_{50} of 0.03 ± 0.01 nM vs. 0.03 ± 0.02 nM for CD14 and 0.10 ± 0.04 nM vs. 0.12 ± 0.03 nM for MSE, for 20S vs. 20R, respectively). It can also be observed in Fig. 2 that although the CD14 marker of differentiation was more sensitive than the MSE marker the relative potencies of the analogs on differentiation were very similar.

3.3. Increased potency on VDR-mediated gene transcription in vitro

All four analogs tested were more potent (5–50-fold) than $1,25(OH)_2D_3$ in inducing transcription from either the OC VDRE or the 24OHase promoter (Fig. 3). The 20S Gemini compound was more potent (2- and 7-fold, on 24OHase promoter and OC VDRE, respectively) than the 20R Gemini analog. 20S-nor-19 was 8-fold more potent than 20R-nor-19 in activating 24OHase transcription, no significant difference between the two was observed on the

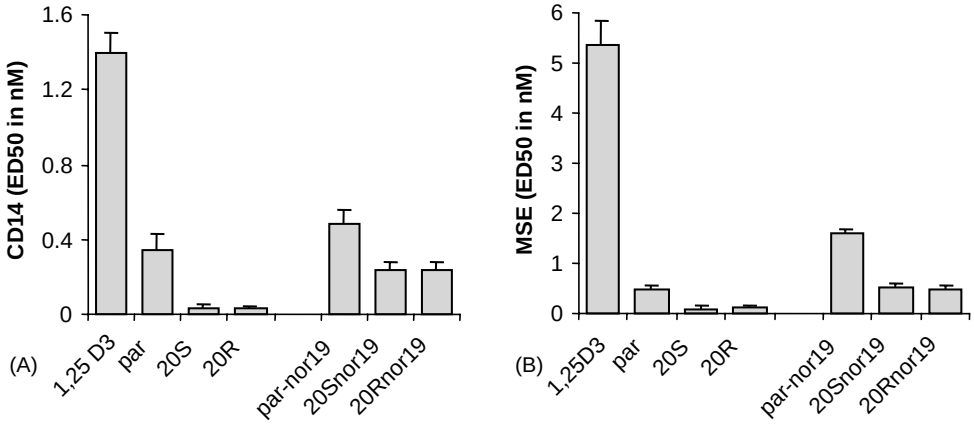

Fig. 2. Potency of $1,25(OH)_2D_3$ and analogs to induce expression of differentiation markers in human leukemia cells. U937 cells were cultured in the presence of increasing doses of analogs. ED_{50} is the concentration of required to achieve expression of the differentiation markers CD14 (A) or MSE (B) in 50% of exposed cells. Results shown are the mean ± 1 × SE from at least three experiments.

transcriptional activity on the OC VDRE. The non-hexafluorinated parental Gemini was as potent as the 20R hexafluoro modified Gemini in transcriptionally activating both OC VDRE and the 24OHase promoter, whereas the non-hexafluorinated nor-19 parental showed no increase in potency compared to $1,25(OH)_2D_3$.

3.4. Increased potency in vivo

The potency of the 20S analog to induce calbindin-D_{9K} mRNA levels in intestine following a single, 2 μg/kg, i.p. injection into vitamin D-deficient mice was significantly higher than that of $1,25(OH)_2D_3$ and of its 20R equivalent (Fig. 4). The 20S-nor-19 analog also tended towards a higher potency to induce intestinal calbindin-D_{9K} mRNA levels without reaching significance. In kidney, the 20S analog induced higher calbindin-D_{28K} and 24OHase mRNA levels than $1,25(OH)_2D_3$ following i.p. injection, whereas calbindin-D_{9K} mRNA levels were not significantly affected above the $1,25(OH)_2D_3$-induced levels by any of the analogs. Next to 20S, the 20R analog also induced 24OHase mRNA levels in kidney higher than those induced by $1,25(OH)_2D_3$. Significant differences between 20R and 20S analogs were observed for calbindin-D_{9K} and calbindin-D_{28K} in kidney. Serum calcium was not significantly affected by any of the treatments (not shown).

3.5. Cofactor recruitment

To test whether the increased potency of the novel Gemini analogs is due to increased cofactor recruitment to the liganded VDR, we performed GST-pull down assays using either *in vitro* translated ^{35}S-VDR (Fig. 5) or nuclear extracts from VDR transfected COS-7 cells (Fig. 6) and GST–DRIP205 or GST–GRIP-1. The interaction of VDR with each cofactor was dependent on the concentration of $1,25(OH)_2D_3$ or analog used, consistent with previous

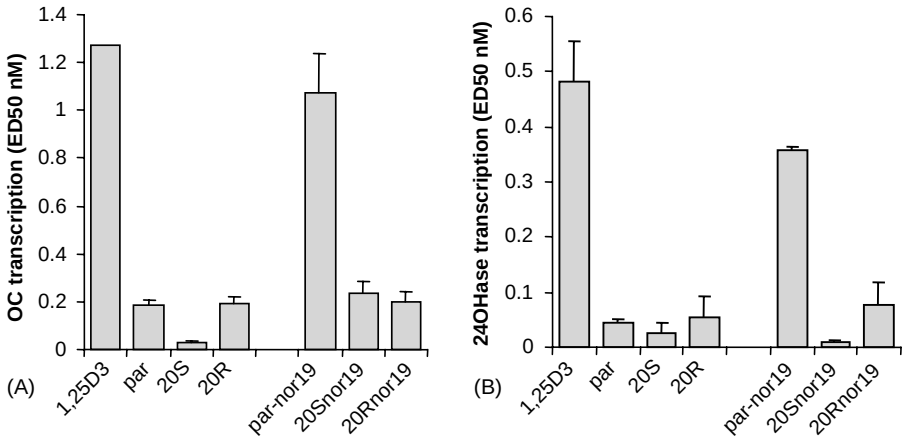

Fig. 3. Transcriptional activity of $1,25(OH)_2D_3$ and analogs. CV-1 cells were transfected with VDR and a reporter plasmid driven by the OC VDRE (A) and COS-7 cells were transfected with VDR and a reporter plasmid driven by the 24OHase promoter (B). Cells were treated with increasing doses of steroids and ED_{50} determined. Results shown are the mean ± 1 × SE from at least three experiments.

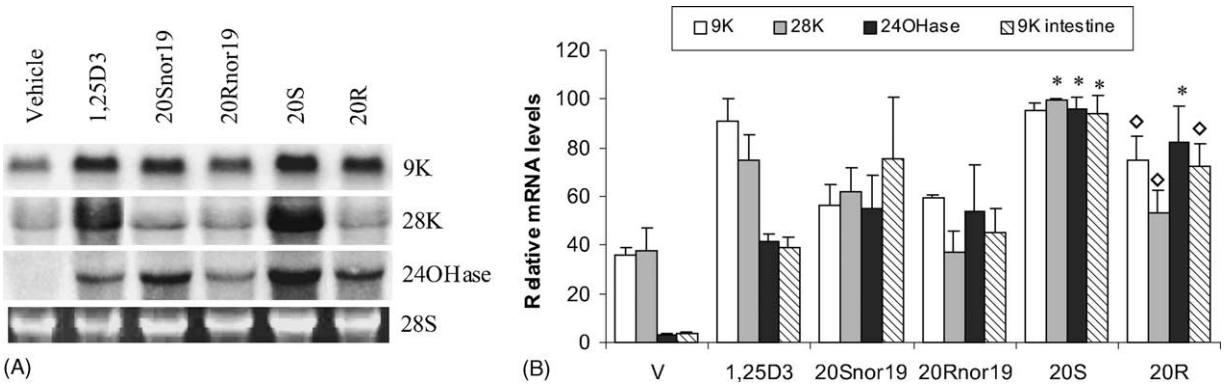

Fig. 4. Potency of $1,25(OH)_2D_3$ and analogs to induce mRNA levels *in vivo*. Vitamin D-deficient mice were given a single i.p. injection of 45 ng of one of the analogs, or vehicle (V) 16 hr before sacrifice. Intestine and kidney were isolated, total RNA isolated, and levels of calbindin-D_{9K} determined in intestine and 24OHase, calbindin-D_{9K}, and calbindin-D_{28K} in kidney using Northern blots. Representative blot of renal mRNA (A) and the mean of quantified blots for three mice $\pm$ 1 $\times$ SE (B) are shown. Asterisks represent significant differences from $1,25(OH)_2D_3$ treatment. Diamonds represent significant differences from 20S analog treatment.

results showing the $1,25(OH)_2D_3$ dependency of cofactor binding to VDR [10]. Treatment of VDR transfected COS-7 cells with the analogs for 24 hr resulted in increased VDR–DRIP205 interaction compared to similar treatment with $1,25(OH)_2D_3$ (Fig. 6). Again, the 20S analog was most potent (15.3 ± 1.0-fold higher than $1,25(OH)_2D_3$). The 20S-nor-19 analog showed 12.4 ± 1.9-fold induction of DRIP205 recruitment over $1,25(OH)_2D_3$. The two 20S analogs were not significantly more potent than their 20R counterparts. Results obtained when GST–GRIP-1 was used to pull down VDR from the same nuclear extracts, were similar to results obtained with GST–DRIP205 (not shown).

In contrast, pull down assays using *in vitro* translated ^{35}S-labeled VDR and DRIP205 (Fig. 5) or GRIP-1 (not shown) in a cell free system indicated that the potency of the hexafluorinated analogs to induce cofactor interaction with VDR under these conditions was not increased compared to $1,25(OH)_2D_3$. Furthermore, pull down experiments following treatment of a VDR-containing nuclear extract (to mimic the nuclear environment *in vitro*) with $1,25(OH)_2D_3$ or analogs for 30 min also did not show a significant difference in potency between the natural hormone and the analogs (not shown).

4. Discussion

Research efforts over the last decade into the design, synthesis, and biochemical analysis of hundreds of $1,25(OH)_2D_3$ analogs have not only provided compounds which show a favorable profile for selective biological responses, but have also facilitated structure-function studies into VDR-mediated signaling events [6,7,34].

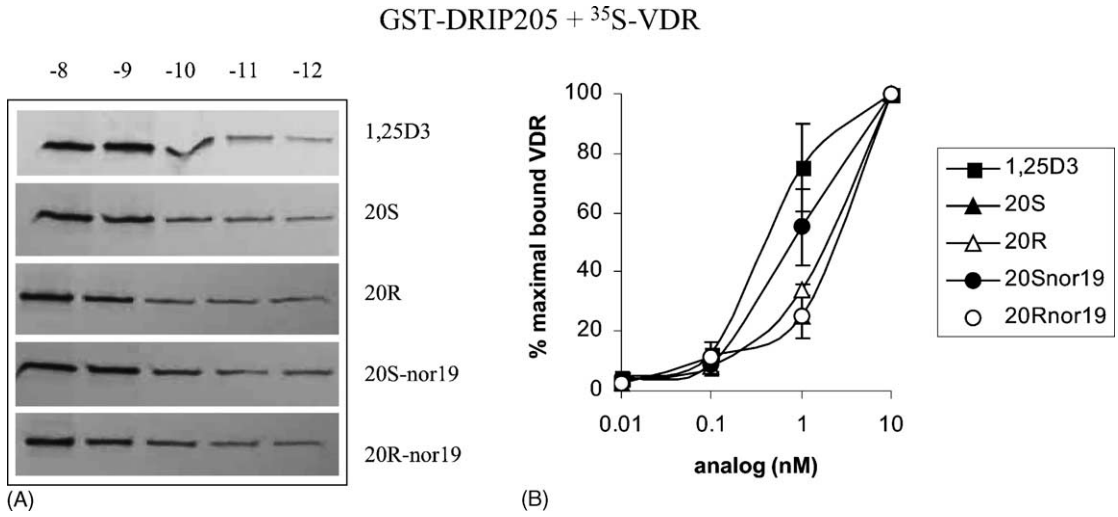

Fig. 5. Cofactor recruitment to VDR induced by $1,25(OH)_2D_3$ or analog in a cell free system. GST-pull down assays were performed using *in vitro* translated ^{35}S-VDR and GST–DRIP205 fusion protein in the presence of increasing doses of $1,25(OH)_2D_3$ or analog. A representative gel showing pulled down VDR using 10^{-8}–10^{-12} M $1,25(OH)_2D_3$ or analog (A) and the quantified means $\pm$ 1 $\times$ SE of three experiments (B) are shown.

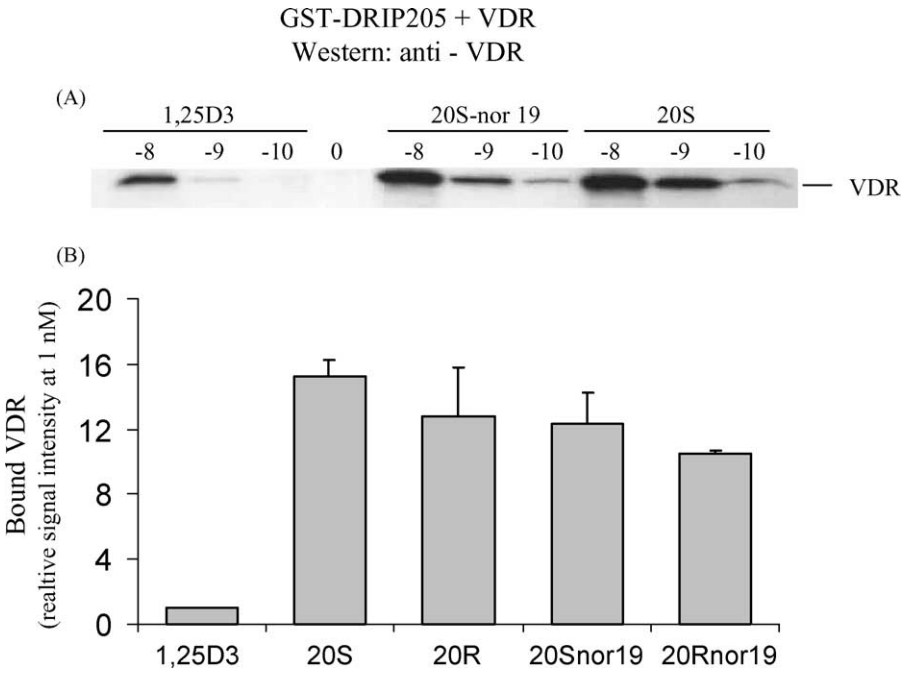

Fig. 6. Cofactor recruitment to VDR induced by $1,25(OH)_2D_3$ or analog *in vitro*. COS-7 cells were transiently transfected with VDR and treated for 24 hr with increasing doses of $1,25(OH)_2D_3$ or analog. Equal amounts of VDR containing nuclear extracts were used in GST-pull down assays with GST–DRIP205. A representative Western blot for VDR from pull down assays using 10^{-8}–10^{-12} M $1,25(OH)_2D_3$ or analog (A) and the mean ± 1 × SE of quantified VDR signals from three pull down assays using 1 nM of steroid (B) are shown.

With Gemini, a $1,25(OH)_2D_3$ analog was synthesized combining a 20-normal with a 20-epi side chain [18]. The rationale for design of Gemini was the unexpected high potency of an $1,25(OH)_2D_3$ analog resulting from a change of orientation on its C-20 atom (20-epi-$1,25(OH)_2D_3$). 20-Epi compounds are up to 500-fold more potent than $1,25(OH)_2D_3$ in triggering antiproliferative effects on human lymphoma cells and also induce differentiation and inhibit lymphocyte proliferation in response to IL-1 at much lower concentrations than $1,25(OH)_2D_3$ [12]. Transcriptionally, 20-epi analogs are reported to be 50–200-fold more potent than $1,25(OH)_2D_3$ [13,17]. Gemini was found to readily bind to VDR but, despite the presence of the 20-epi side chain, did not facilitate superior gene transcription activity [18,19].

To further enhance the potency of Gemini and its equally potent derivative 19-nor Gemini [20] (see also Fig. 1), we synthesized Gemini analogs containing a 23-triple bond and a 26,27-hexafluoro substitution. This modification has been shown earlier to enhance potency of the one side-chained natural hormone, $1,25(OH)_2D_3$ (see for review [6]). The side chain modifications were present in either the 20-normal (20S) or in the 20-epi (20R) side chain to study potential stereo selectivity of the asymmetric Gemini analogs. We report that Gemini analogs of vitamin D, modified with a 23-triple bond and a 26,27-hexafluor substitution, exhibit enhanced transcriptional activity *in vitro* as well as enhanced activity *in vivo* and increased potency to induce neoplastic cell differentiation compared to $1,25(OH)_2D_3$ or the parental Gemini.

A first step in generating VDR-mediated responses is binding of the ligand to VDR. Introduction of a 23-triple bond and a 26,27-hexafluor substitution in the side chain did not affect the relative competitive index, a measure for relative ligand affinity, for VDR. Affinity of the analogs was between 15 and 38% of that of $1,25(OH)_2D_3$, identical to the parental Gemini and slightly higher than the parental nor-19 Gemini. This shows that the introduced modifications did not dramatically impair or enhance Gemini's ability to bind VDR and that the LBD of VDR can accommodate Gemini independent of the side chain modifications that were introduced. In contrast to binding to the LBD of VDR, which is relatively unaffected by the presence of two side chains, the LBD of another important vitamin D binding protein, DBP, is practically inaccessible (*RCI* reduced to 1–3% of that of the natural hormone) for the Gemini parentals [18]. The difference in accessibility of both LBDs is not surprising, since there is no structural homology between the LBD of VDR and the LBD of DBP [35]. Introduction of the side chain modifications reported here resulted in an up to 10-fold higher affinity for the LBD of DBP compared to the parental Geminis, although the RCI is still notably lower (5–10-fold) than $1,25(OH)_2D_3$. Affinity for the VDR or DBP LBD did not differ between 20S or 20R modified Geminis. The role of DBP, through binding of its LBD, is to transport vitamin D and its metabolites through the circulation. Targeted disruption of DBP showed that DBP maintains stable serum stores of vitamin D and its metabolites modulating its bioavailability [36]. The low DBP binding affinity of the modified

Gemini analogs may affect their potency *in vitro* as well as *in vivo* as has been shown earlier for other analogs [37]. A weak affinity for DBP will result in an increased metabolic clearance rate, less hypercalcemia, and selective tissue distribution [7].

The modified Gemini compounds are up to 12-fold more potent in inducing differentiation of leukemia cells (HL60, U937, THP-1) than the parental, non-modified Gemini and more than 50-fold more potent than $1,25(OH)_2D_3$. Potency to induce neoplastic cell differentiation did not differ between 20S or 20R modified Geminis. Overall this correlates well with the *in vitro* transcriptional activity of the Gemini compounds on either the OC VDRE or the 24OHase promoter, except for a tendency of 20S analogs to be more potent than their 20R counterpart in the transcriptional assay. This difference between 20S and 20R is more pronounced *in vivo*, where mRNA levels of calbindin-D_{9K} in intestine and 24OHase and calbindin-D_{28K} in kidney were significantly induced only in 20S and not in 20R injected, vitamin D deficient, mice. Selective degradation of 20R vs. 20S analogs may contribute to the differences found between 20S and 20R modified Gemini analogs in transcriptional potency. Originally, the 23-triple bond combined with the 26,27-hexafluor substitution was designed to inhibit 24OHase-mediated degradation of $1,25(OH)_2D_3$-like compounds (reviewed in [38]). It is possible that the presence of this modification in the 20-normal side chain renders the 20S analogs poor substrates for 24OHase (similar to $1,25(OH)_2D_3$ carrying this modification), whereas the analog containing the 24OHase blocking modification in the 20-epi side chain (20R) is potentially a better substrate for the enzyme. Although the single injection protocol we used did not significantly increase serum calcium levels, the modified Gemini compounds may rise serum calcium with more long-term treatment.

To investigate whether differences in recruitment of cofactors to the liganded VDR, contribute to the increased potency of the modified Gemini analogs, GST-pull down experiments were performed. DRIP205 and GRIP-1 recruitment to *in vitro* translated VDR was not enhanced by the analogs compared to $1,25(OH)_2D_3$. This indicates that the enhanced potency of the modified Gemini analogs over $1,25(OH)_2D_3$ is not due to a Gemini-induced change in conformation of the liganded VDR resulting in increased recruitment of DRIP205 or GRIP-1. These results corroborate those of Herdick *et al*. [19], who found that Gemini did not enhance TRC-1, TIF2, or RAC3 recruitment to VDR over levels induced by $1,25(OH)_2D_3$. In fact, like in our experiments with DRIP205 and GRIP-1, analog-induced cofactor recruitment was attenuated, which may relate to the lower VDR affinity or steric hindrance by the second side chain of Gemini. Mimicking the nuclear environment *in vitro* (performing DRIP205 or GRIP-1 pull downs from a VDR-containing nuclear extract following *in vitro* treatment with $1,25(OH)_2D_3$ or the modified Gemini analogs) did not result in a change in cofactor recruitment to VDR confirming that the (lack of) nuclear environment is not a limiting factor in our *in vitro* pull down experiments. The lack of increased cofactor recruitment by Gemini differs from mechanisms reported to contribute to the increased potency of the 20-epi analogs, which seem to converge at the VDR ligand binding domain (LBD) having different points of contact with a ligand depending on its conformation at C-20 [39]. This is thought to result in a more stable activated complex [40] including enhanced heterodimer and cofactor binding [16,39]. Increased cofactor binding to 20-epi $1,25(OH)_2D_3$-liganded VDR was also reported by Issa *et al*. [34]. Interestingly, in the same study the 26,27-hexafluorinated $1,25(OH)_2D_3$ analog combined a decrease in cofactor recruitment with an increase in transcriptional activity, similar to the modified Gemini analogs. We have not done experiments to directly assess conformation of the VDR complex liganded with the novel Gemini analogs, such as protease sensitivity. However, cofactor recruitment is a functional consequence of the liganded VDR conformation and is assumed to regulate transcriptional activation. Cofactor recruitment was not increased by the analogs in a cell free system over levels induced by $1,25(OH)_2D_3$. Therefore, we feel that a potential difference in liganded VDR conformation, if present for the novel Gemini analogs, does not contribute to enhanced cofactor recruitment and is thus unlikely to explain the enhanced transcriptional activity of the analogs.

In pull down experiments using VDR in nuclear extracts of transiently transfected COS-7 cells that were treated with analogs for 24 hr, DRIP205 and GRIP-1 recruitment was notably induced over levels induced by $1,25(OH)_2D_3$ (up to 15-fold). Combined with a lack of induced cofactor recruitment in a cell free system these results indicate that stability of the analogs or the analog-liganded VDR complex is increased over $1,25(OH)_2D_3$, explaining the increased potency of the modified Gemini analogs in *in vitro* and *in vivo* experiments. The parental Gemini has been reported to enhance stabilization of the liganded-VDR–RXR complex in the presence of DNA, which may contribute to its increased potency [19].

Gemini analogs modified with a 23-triple bond and a 26,27-hexafluoro substitution possess enhanced differentiational and transcriptional potency. Deletion of C-19 in the 19-nor compounds attenuates the potency of the modified Geminis, which may be explained by the lower VDR affinity compared to the 19-normal compounds. Increased potency compared to $1,25(OH)_2D_3$ is not due to increased VDR affinity or increased ability of the liganded VDR to recruit cofactors in a cell free system. Based on the origin of the modifications introduced (designed to block 24OHase metabolization) in combination with the higher potency of the 20S vs. the 20R compound *in vivo*, we conclude that ligand stability is most likely the main contributor to the observed increase in potency. The more stable Gemini compounds reported on here, may facilitate further studies into the potential of Gemini to selectively

affect genomic responses. Crystallization of VDR with the parental Gemini showed that VDR can harbor the second side-chain in an extra 'pocket' (Moras, 12th Workshop on Vitamin D, Maastricht, 2003). This may enable the non-symmetric side-chains of the modified Gemini analogs to bind VDR in more than one conformation, potentially enabling separation of analog actions. Furthermore, it will of interest to study the metabolization of the modified analogs by 24OHase, which our data suggest may be dependent on the conformation at C-20.

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