

## Previtamin D<sub>3</sub> with a *trans*-Fused Decalin CD-ring Has Pronounced Genomic Activity\*

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Deletion of C19 in the structure of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] does not substantially alter the biological potency but prevents the conversion between the vitamin and the previtamin form. Hence, this modification allows the study of locked previtamin and vitamin forms. The locked 19-nor-1,25(OH)<sub>2</sub>-previtamin D<sub>3</sub> analog (19-nor-previtamin D) had a low biological activity and was a rather weak activator of the genomic signal transduction pathway. 19-Nor-*trans*-decalin-1,25(OH)<sub>2</sub>-vitamin D<sub>3</sub> (19-nor-TD-vitamin D), characterized by the presence of a *trans*-fused decalin CD-ring system, was 10-fold more potent than the parent compound and was a potent activator of the genomic signal transduction pathway. Surprisingly, the previtamin, 19-nor-*trans*-decalin-1,25(OH)<sub>2</sub>-previtamin D<sub>3</sub> (19-nor-TD-previtamin D), was as potent as 1,25(OH)<sub>2</sub>D<sub>3</sub> in inhibiting cell proliferation and inducing cell differentiation and represents the first previtamin structure with pronounced vitamin D-like activity. Furthermore, this compound interacted as efficiently as 1,25(OH)<sub>2</sub>D<sub>3</sub> with the vitamin D receptor (VDR), retinoid X receptor (RXR), coactivators, and DNA, which illustrated its potent ability to activate the genomic signal transduction pathway. Analysis of the transactivation potency of 12 VDR point mutants after stimulation with 19-nor-TD-previtamin D revealed that this analog used the same contact points within the receptor as did 1,25(OH)<sub>2</sub>D<sub>3</sub>. This could be confirmed by modeling analysis of this compound in the ligand binding pocket of VDR. In conclusion, a previtamin D<sub>3</sub> analog is presented with genomic activities equivalent to 1,25(OH)<sub>2</sub>D<sub>3</sub>.

1,25(OH)<sub>2</sub>D<sub>3</sub>, the active metabolite of vitamin D<sub>3</sub>, is an important regulator not only of calcium and phosphate metabolism but also cell proliferation and differentiation (1). It exerts its genomic effects through the vitamin D receptor, a ligand-dependent transcription factor. Conformational changes of VDR,<sup>1</sup> induced by the binding of 1,25(OH)<sub>2</sub>D<sub>3</sub>, allow the recep-

tor to dimerize with the retinoic X receptor and bind vitamin D-responsive elements (VDREs) in the promoter region of target genes. Interaction of the VDR-RXR-VDRE complex with coregulator molecules and the basal transcription machinery leads to alterations in gene transcription.

1,25(OH)<sub>2</sub>D<sub>3</sub> has a central rigid CD-bicyclic ring portion to which two flexible entities, the side chain and the seco-B,A-ring, are connected. The characteristic seco structure of vitamin D<sub>3</sub> originates from the photolytic cleavage of the provitamin 7-dehydrocholesterol. The resulting previtamin triene (previtamin D<sub>3</sub>) then reversibly thermo-isomerizes to the vitamin form in its less stable 6-*s-cis* conformation. Rapid rotation about the C6-C7 single bond eventually yields vitamin D<sub>3</sub> in its more stable extended 6-*s-trans* conformation (2, 3). Vitamin D<sub>3</sub> but also its hydroxylated metabolites are in constant equilibrium with their previtamin D<sub>3</sub> isomers. Previous studies with analogs of 1,25(OH)<sub>2</sub>D<sub>3</sub> that are either structurally blocked in the previtamin form (4, 5) or that only slowly isomerize to the vitamin form (6) indicated that the previtamin form of 1,25(OH)<sub>2</sub>D<sub>3</sub> is a poor activator of the genomic signaling pathway with minimal effects on cell proliferation and differentiation (7). However, the previtamin D<sub>3</sub> form is a potent activator of the non-genomic signal transduction pathway (5–7).

The aim of this study is to investigate the biological profile and the genomic signaling pathway of four different locked 19-nor-1,25(OH)<sub>2</sub>-(pre)-vitamin D<sub>3</sub> analogs, with or without a modified CD-ring. Indeed, the sigmatropic shift required for the previtamin-vitamin D conversion cannot occur in these analogs because of the deletion of C19 (4). First, the affinity to VDR was determined as well as the stability of VDR conformation after interaction with the ligand. Second, the potency of the analogs to induce interaction of the VDR-RXR heterodimer with DNA (vitamin D-responsive elements) and TIF2 (as a representative of the p160 family of coactivators; Ref. 8) was investigated. Third, the ability of 19-nor-1,25(OH)<sub>2</sub>-(pre)-vitamin D<sub>3</sub> analogs to induce VDR-dependent transcriptional activation was studied in transient transfection experiments and by determining the induction of vitamin D 24-hydroxylase (CYP 24) gene expression. Finally, docking of the analogs in the ligand binding domain (LBD) of VDR was studied by evaluation

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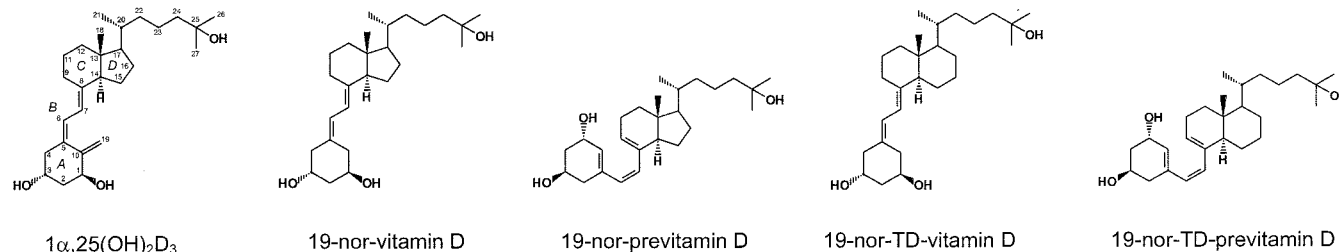
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<sup>1</sup> The abbreviations used are: VDR, vitamin D receptor; VDRE, vita-

min D-responsive element; RXR, retinoid X receptor; 19-nor-vitamin D, 19-nor-1,25(OH)<sub>2</sub>-vitamin D<sub>3</sub>; 19-nor-TD-vitamin D, 19-nor-*trans*-decalin-1,25(OH)<sub>2</sub>-vitamin D<sub>3</sub>; 19-nor-previtamin D, 19-nor-1,25(OH)<sub>2</sub>-previtamin D<sub>3</sub>; 19-nor-TD-previtamin D, 19-nor-*trans*-decalin-1,25(OH)<sub>2</sub>-previtamin D<sub>3</sub>; DBP, vitamin D-binding protein.

TABLE I  
Chemical structures and biological activities of 19-nor-(TD)-(pre)-vitamin D analogs

Summary of the *in vitro* effects of 19-nor-(TD)-(pre)-vitamin D analogs of 1,25(OH)<sub>2</sub>D<sub>3</sub>. The binding of 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs to pig VDR and human DBP is expressed by their dissociation constants. The antiproliferative effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> and analogs on MCF-7 cells were expressed as the concentrations required for the half-maximal inhibition of [<sup>3</sup>H]thymidine incorporation. The prodifferentiating effects were determined in HL-60 cells (NBT reduction) and were expressed as the concentrations necessary for the half-maximal response in the differentiation assay. The calcemic activity of 1,25(OH)<sub>2</sub>D<sub>3</sub> and its 19-nor-(TD)-(pre)-vitamin D analogs was determined in mice by intraperitoneal injections during 7 consecutive days. This activity was expressed as the maximal dose that could be administered without exceeding a serum calcium concentration observed when mice were treated with 0.1 μg/kg/day 1,25(OH)<sub>2</sub>D<sub>3</sub>. All values represent mean and S.D. of at least two independent experiments.



Compound	Binding studies		Antiproliferative/prodifferentiating effects		Calcemic effects
	VDR K <sub>D</sub>	DBP K <sub>D</sub>	MCF-7 EC <sub>50</sub> -value for [ <sup>3</sup> H]thymidine incorporation	HL-60 EC <sub>50</sub> -value for NBT-reduction	Maximal applicable dose (μg/kg per day)
1,25(OH) <sub>2</sub> D <sub>3</sub>	(8.9 ± 1.9) × 10 <sup>11</sup> M <sup>-1</sup>	(4.4 ± 0.8) × 10 <sup>8</sup> M <sup>-1</sup>	(2.5 ± 0.2) × 10 <sup>-8</sup> M	(2.7 ± 0.8) × 10 <sup>-8</sup> M	0.1
19-nor-vitamin D	(2.5 ± 0.1) × 10 <sup>10</sup> M <sup>-1</sup>	(3.5 ± 0.7) × 10 <sup>7</sup> M <sup>-1</sup>	(2.3 ± 0.3) × 10 <sup>-8</sup> M	(2.0 ± 1.0) × 10 <sup>-8</sup> M	5
19-nor-previtamin D	(8.6 ± 1.0) × 10 <sup>9</sup> M <sup>-1</sup>	(8.2 ± 0.6) × 10 <sup>7</sup> M <sup>-1</sup>	(2.3 ± 0.3) × 10 <sup>-7</sup> M	(3.8 ± 0.3) × 10 <sup>-7</sup> M	20
19-nor-TD-vitamin D	(1.7 ± 0.5) × 10 <sup>10</sup> M <sup>-1</sup>	(4.4 ± 1.2) × 10 <sup>6</sup> M <sup>-1</sup>	(4.8 ± 0.8) × 10 <sup>-10</sup> M	(3.8 ± 0.3) × 10 <sup>-9</sup> M	0.1
19-nor-TD-previtamin D	(2.6 ± 0.1) × 10 <sup>9</sup> M <sup>-1</sup>	(3.9 ± 0.5) × 10 <sup>6</sup> M <sup>-1</sup>	(5.3 ± 0.8) × 10 <sup>-9</sup> M	(3.0 ± 0.1) × 10 <sup>-8</sup> M	5

of the transactivating capacity of 12 different point mutants of human VDR, and a docking model for 19-nor-TD-previtamin D was proposed that is concordant with the results of the mutational analysis.

#### EXPERIMENTAL PROCEDURES

**1,25(OH)<sub>2</sub>D<sub>3</sub> and Analogs**—1,25(OH)<sub>2</sub>D<sub>3</sub> was a gift of J. P. van de Velde (Duphar, Weesp, The Netherlands). The analog 19-nor-1,25(OH)<sub>2</sub>-vitamin D<sub>3</sub>, originally synthesized by DeLuca and co-workers (9, 10), was resynthesized under the name 19-nor-vitamin D. The other analogs studied in this report are 19-nor-*trans*-decalin-1,25(OH)<sub>2</sub>-vitamin D<sub>3</sub> (19-nor-TD-vitamin D), 19-nor-1,25(OH)<sub>2</sub>-previtamin D<sub>3</sub> (19-nor-previtamin D), and 19-nor-*trans*-decalin-1,25(OH)<sub>2</sub>-previtamin D<sub>3</sub> (19-nor-TD-previtamin D) (Table I) (11).

**Biological Profile of Compounds**—The affinity of 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs to pig VDR and human DBP were determined as described previously (12–14). Inhibition of MCF-7 cell proliferation and the induction of HL-60 cell differentiation by 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs as well as their calcemic effects were measured as described previously (14).

**Plasmids**—The expression vectors for human VDR (pSG5-VDR) and human RXRα (pSG5-RXR) together with the luciferase reporter plasmid, which contains the rat ANF DR3-type VDRE, were kind gifts of C. Carlberg (Kuopio, Finland) (15). pCMX-expression vectors encoding wild type VDR and 12 different VDR point mutants (L233A, V234A, I268A, I271A, R274A, S275A, S278A, W286A, V300A, H305A, H397A, and Y401A) were used in transfection experiments (16). The pRSETA vector in which TIF2.5 (the nuclear interaction domain of TIF2; Ref. 17) was cloned was a kind gift of K. Hofman (Leuven, Belgium) (18).

**Limited Proteolytic Digestion of VDR**—<sup>35</sup>S-labeled human VDR was synthesized *in vitro*, preincubated with vehicle (ethanol), 1,25(OH)<sub>2</sub>D<sub>3</sub>, or analogs and subsequently digested at room temperature with a limited amount of trypsin (0–150 μg/ml) for 15 min (Roche Applied Science) (19).

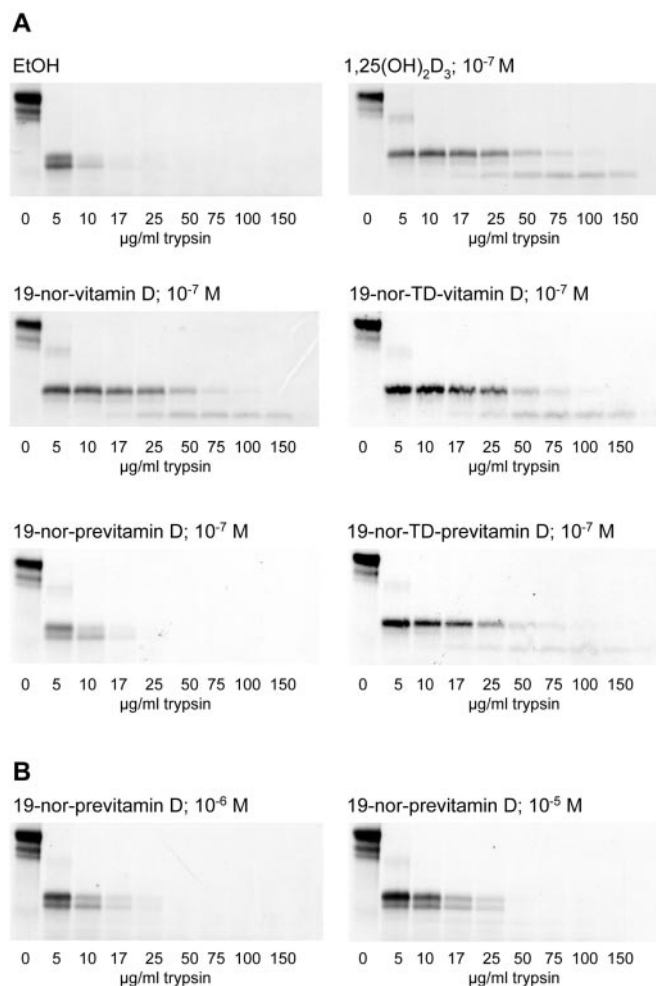
**Ligand-dependent Gel Shift Assay**—<sup>32</sup>P-labeled DR3-type VDREs were added to equal amounts of *in vitro* translated VDR and RXR proteins, which were preincubated with vehicle (ethanol), 1,25(OH)<sub>2</sub>D<sub>3</sub>, or analogs (19). The potency of the analogs of 1,25(OH)<sub>2</sub>D<sub>3</sub> to induce

interaction of the VDR-RXR heterodimer with TIF2.5 was investigated with supershifts. Therefore, BL21(DE3)pLysS competent cells (Invitrogen) were transformed with the pRSETA-TIF2.5 vector, and the recombinant TIF 2.5 protein was isolated with TALON (BD Biosciences) according to the instructions of the manufacturer.

**Transactivation Potency**—COS-1 cells (American Type Culture Collection, Manassas, VA) were maintained as described previously (19). COS-1 cells (30,000) were seeded in 24-well dishes. The next day, cells were transfected with FuGENE (Roche Applied Science) with 400 ng of reporter plasmid, 200 ng of pCMX-based expression vectors for wild type or mutant VDR, and 200 ng of pSG5-RXR. The day after transfection, cells were stimulated with vehicle or dilutions of 1,25(OH)<sub>2</sub>D<sub>3</sub> or analogs. The next day, cells were lysed with the reporter lysis buffer (Roche Applied Science), and luciferase activity was measured with the luciferase assay system (Promega, Madison, WI). Luciferase activity in each well was normalized for its protein content and expressed relative to transfected vehicle-stimulated cells.

**Quantitative Real Time PCR**—MC3T3-E1 mouse osteoblasts (RIKEN Cell Bank, Tsukuba, Japan) were cultured in α-minimum Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biochrom KG, Berlin, Germany), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamax™-I (Invitrogen). MC3T3-E1 cells (80,000) were seeded in 6-well dishes. The next day, cells were stimulated with vehicle (ethanol), 10<sup>-9</sup> M 1,25(OH)<sub>2</sub>D<sub>3</sub>, or analogs. Twenty-four hours after stimulation, RNA was isolated with the RNeasy kit (Qiagen, Gilden, Germany). Quantitative real time PCR and calculation of the amount of target cDNA in the sample was done as described previously (20). β-Actin-normalized CYP24 expression after treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> or analogs was expressed relative to normalized CYP24-levels in vehicle-stimulated cells. Primers (forward, 5'-GCG GAA GAT GTG AGG AAT ATG C-3'; reverse, 5'-AAG ACT GTT CCT TTG GGT AGC GT-3'); and probe (5'-FAM-AAG GCC TGT CTA AAG GAG TCC ATG AGG CTT A-TAMRA-3') for CYP24 were purchased from Eurogentec (Seraing, Belgium).

**Docking of 19-Nor-TD-previtamin D**—Low energy conformations of 19-nor-TD-previtamin D were obtained by systematic variation of the torsion around the C5-C6 and the C7-C8 bonds. The lowest energy



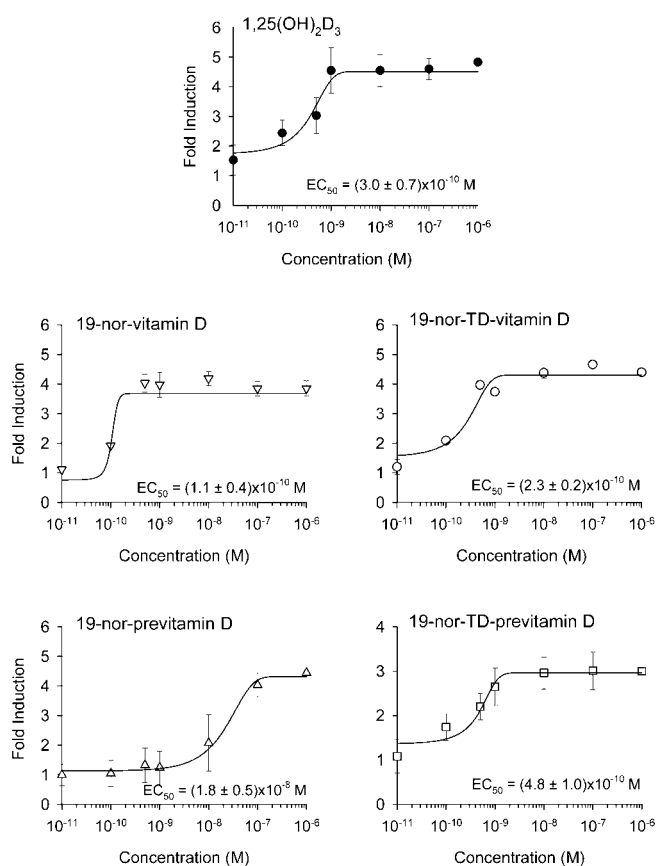
**FIG. 1. Limited proteolytic digestion of VDR.** A, VDR, *in vitro* translated in presence of [<sup>35</sup>S]methionine, was digested with graded concentrations of trypsin after incubation with vehicle (*EtOH*), 1,25(OH)<sub>2</sub>D<sub>3</sub>, or one of the 19-nor-(TD)-(pre)-vitamin D analogs (10<sup>-7</sup> M). Representative experiments are shown. B, *in vitro* translated VDR was incubated with higher concentrations of 19-nor-previtamin D (10<sup>-6</sup> and 10<sup>-5</sup> M) prior to digestion with increasing concentrations of trypsin. A representative experiment is shown.

conformations were manually docked in the ligand binding pocket of the VDR (protein data bank entry 1DB1, crystal structure of the VDR-LBD- $\Delta$ (165–215)) (21) so that the compound made similar interactions with VDR as did 1,25(OH)<sub>2</sub>D<sub>3</sub>. The complex of VDR and 19-nor-TD-previtamin D conformation that could be best fitted in the pocket was subjected to a molecular dynamics run followed by a minimization allowing the relaxation of the ligand and its surrounding amino acids. The molecular dynamics run was performed at 900 K for 100 ps. At 1-ps intervals the structures were minimized for 10 steps with the steepest descent method and for a subsequent 500 steps with the conjugate gradient procedure. The Insight II/Discover packages (Accelrys Inc., San Diego, CA) together with the CFF91 force field were used for all the calculations.

**Statistics**—Analysis of variance (ANOVA) followed by Bonferroni multiple comparison tests were used to determine significant differences.  $p < 0.05$  was accepted as significant. EC<sub>50</sub> values for the various dose-response curves were calculated with three different sigmoid regression equations (three- and four-parameter sigmoid regression equations and three-parameter Gompertz regression equation, Sigmaplot). The EC<sub>50</sub> values presented in the text and the figures represent the mean and S.E. of the EC<sub>50</sub> values obtained in those equations. The curves drawn in Fig. 2 represent the curve fitted by the four-parameter sigmoid regression equation.

## RESULTS

***In Vitro and in Vivo Biological Activity***—The *in vitro* biological potency of 19-nor-vitamin D was comparable with that of

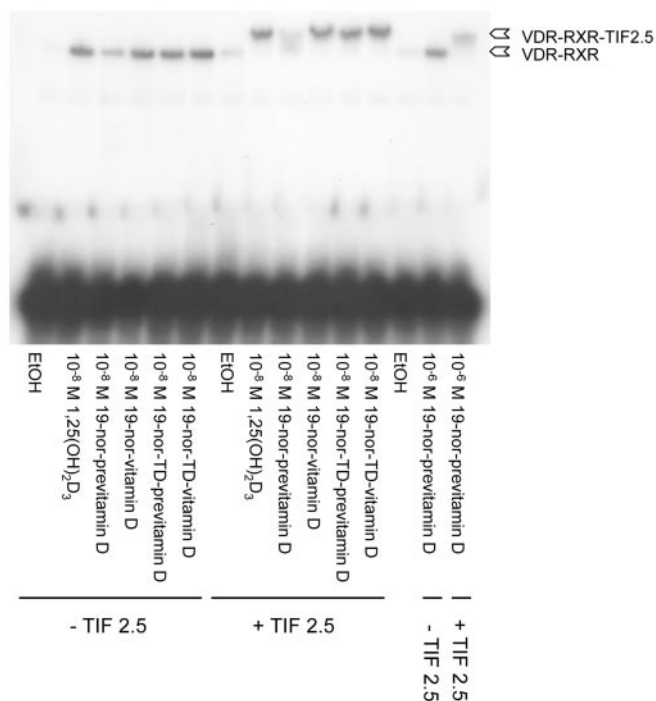


**FIG. 2. Ligand-dependent gel shift experiment.** Equal amounts of *in vitro* translated VDR and RXR were incubated with graded ligand concentrations prior to the addition of <sup>32</sup>P-labeled DR3-type VDREs. The amount of protein-complexed VDREs was quantified in relation to the free probe. Dose-response curves for 1,25(OH)<sub>2</sub>D<sub>3</sub> (●), 19-nor-vitamin D (▽), 19-nor-TD-vitamin D (○), 19-nor-previtamin D (△), or 19-nor-TD-previtamin D (□) have been performed. Each data point represents the mean of three independent experiments, and bars indicate S.E. In all individual experiments and for each ligand, the amount of shifted probe at each concentration was normalized to the average amount of probe that was shifted upon application of the highest concentration of that specific ligand. EC<sub>50</sub> values were calculated from dose-response curves.

1,25(OH)<sub>2</sub>D<sub>3</sub>, whereas its calcemic effects were 50-fold lower (Table I) (4, 9, 10). 19-Nor-previtamin D was 10-fold less potent than 1,25(OH)<sub>2</sub>D<sub>3</sub> in inhibiting proliferation and inducing differentiation and had low calcemic effects (4). 19-Nor-TD-vitamin D, characterized by a *trans*-fused decalin CD-ring, was 10 to 50 times more potent *in vitro* than 1,25(OH)<sub>2</sub>D<sub>3</sub> and, surprisingly, its previtamin form was as potent as the parent compound. The *in vivo* calcemic activity was comparable with that of 1,25(OH)<sub>2</sub>D<sub>3</sub> for 19-nor-TD-vitamin D and 50-fold lower for 19-nor-TD-previtamin D.

**Affinity for VDR and DBP**—The 19-nor-vitamin D analogs had slightly decreased VDR binding affinities, whereas both previtamin analogs bound very poorly to VDR with dissociation constants ( $K_D$ ) of only 1–3% when compared with 1,25(OH)<sub>2</sub>D<sub>3</sub>. The  $K_D$  of 19-nor-vitamin D for DBP was 10% of that of 1,25(OH)<sub>2</sub>D<sub>3</sub>, whereas the  $K_D$  values of the other analogs were <5% of that of the parent compound (Table I).

**Limited Proteolytic Digestion of VDR**—Incubation of radioactively labeled VDR with a limited amount of trypsin led to partial digestion of the receptor into two fragments (~34 and 32 kDa) (Fig. 1A). A 15-min incubation period in the presence of a final concentration of 17 µg/ml trypsin was sufficient to completely degrade the unliganded receptor. VDR, preincubated with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-7</sup> M), was more resistant to pro-



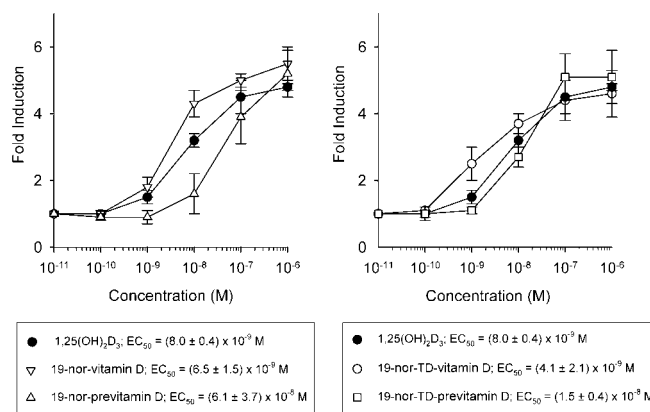
**FIG. 3. Interaction of the VDR-RXR heterodimer with TIF2.5.** Equal amounts of *in vitro* translated VDR and RXR were incubated with vehicle (*EtOH*), or with  $10^{-8}$  M  $1,25(\text{OH})_2\text{D}_3$ , 19-nor-previtamin D, 19-nor-vitamin D, 19-nor-TD-previtamin D, or 19-nor-TD-vitamin D with or without the nuclear receptor interacting domain of TIF2 (TIF2.5). For 19-nor-previtamin D, the same experiment was repeated in the presence of a higher ligand concentration ( $10^{-6}$  M).

teolytic breakdown, and a digestion profile of three fragments was observed ( $\sim 34$ , 32, and 28 kDa). Expectedly, similar digestion profiles were observed for 19-nor-vitamin D and 19-nor-TD-vitamin D, whereas 19-nor-previtamin D could only marginally stabilize VDR even when applied at higher concentrations (Fig. 1B). On the contrary, 19-nor-TD-previtamin D induced a VDR conformation that was as resistant to proteolytic breakdown as that induced by the parent compound (Fig. 1A).

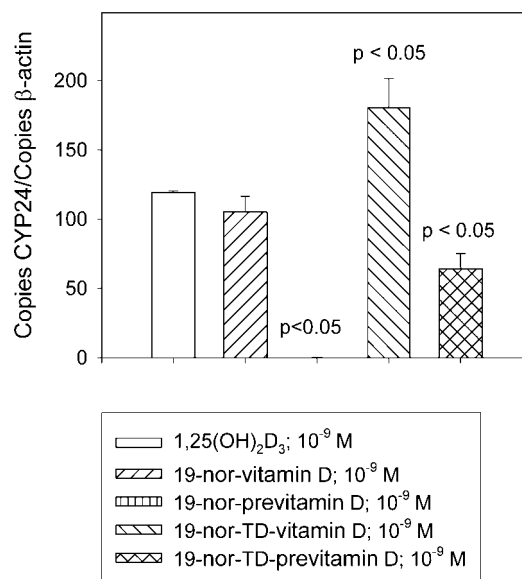
**Ligand-dependent Gel Shift Assay**—19-Nor-vitamin D and 19-nor-TD-vitamin D were equipotent to  $1,25(\text{OH})_2\text{D}_3$  in inducing VDR-RXR binding to DNA and inducing interaction with TIF2.5, whereas 19-nor-previtamin D required 60-fold higher concentrations to obtain half-maximal DNA-binding (Figs. 2 and 3). However, introduction of a *trans*-decalin CD-ring made 19-nor-TD-previtamin D almost as potent as  $1,25(\text{OH})_2\text{D}_3$  in inducing interaction of the VDR-RXR heterodimer with VDREs and TIF2.5.

**Transactivation Potency**—The ability of 19-nor-vitamin D to transactivate a VDRE-containing reporter construct was slightly enhanced compared with  $1,25(\text{OH})_2\text{D}_3$ , and, again, introduction of a *trans*-fused decalin CD-ring further increased the transactivating capacity (Fig. 4). The transactivating capacity of 19-nor-TD-previtamin D was almost similar to that of  $1,25(\text{OH})_2\text{D}_3$  and increased substantially when compared with 19-nor-previtamin D.

19-Nor-vitamin D was as potent as  $1,25(\text{OH})_2\text{D}_3$  in inducing transcription of the vitamin D target gene CYP24 in mouse MC3T3-E1 osteoblasts, whereas 19-nor-TD-vitamin D was significantly more potent than the parent molecule (Fig. 5). When applied at  $10^{-9}$  M, 19-nor-TD-previtamin D substantially increased expression of CYP24 (Fig. 5), whereas 19-nor-previtamin D required higher concentrations to induce CYP24 gene transcription (data not shown).



**FIG. 4. Transactivating potency of analogs of  $1,25(\text{OH})_2\text{D}_3$  in COS-1 cells.** For each ligand, dose-response curves were performed, and luciferase activity was measured and expressed relative to the activity of transfected, vehicle-stimulated cells. Data represent the mean and S.E. of at least three independent experiments for  $1,25(\text{OH})_2\text{D}_3$  (●), 19-nor-vitamin D (▽), 19-nor-previtamin D (△), 19-nor-TD-vitamin D (○), or 19-nor-TD-previtamin D (□).  $\text{EC}_{50}$  values were calculated from dose-response curves and are displayed in the boxes.



**FIG. 5. Induction of CYP24 gene expression in MC3T3-E1 cells.** Twenty-four hours after stimulation with  $1,25(\text{OH})_2\text{D}_3$  or its analogs, CYP24 gene expression was assessed by quantitative real time PCR analysis and normalized to  $\beta$ -actin levels. Data represent the mean and S.E. of three independent experiments.  $p < 0.05$  indicates a significant difference between the expression of CYP24 in  $1,25(\text{OH})_2\text{D}_3$ -treated cells and expression in cells treated with one of the 19-nor-(TD)-(pre)-vitamin D analogs.

**Mutational Analysis of Transcriptional Activity of  $1,25(\text{OH})_2\text{D}_3$  and Analogs**—12 different point mutants of VDR were used to further investigate the interactions between the analogs and the amino acid residues lining the ligand binding pocket of VDR. The expression level and stability of these VDR mutants were evaluated by Western blot analysis and confirmed to be similar to those of wild type VDR (16, 22). To compare the transactivating ability of the various analogs, the  $\text{EC}_{50}$  concentration for transcriptional activation was used (concentrations as indicated in Fig. 4).

$1,25(\text{OH})_2\text{D}_3$  was unable to transactivate when cells were transfected with mutated VDRs carrying either the L233A, I271A, R274A, W286A, H397A, or Y401A mutation (Fig. 6). The mutants V234A, I268A, V300A, and H305A moderately

reduced the transactivation potency of  $1,25(\text{OH})_2\text{D}_3$  (50% of the activity of wild type VDR). The mutants S275A (70%) and S278A (80%) had little effect on the transactivation capacity of  $1,25(\text{OH})_2\text{D}_3$ . The transactivation profile for the different point mutants in cells incubated with 19-nor-vitamin D was almost identical to the profile for  $1,25(\text{OH})_2\text{D}_3$ . 19-Nor-TD-vitamin D had a partially different transactivation profile. Much like  $1,25(\text{OH})_2\text{D}_3$ , 19-nor-TD-vitamin D could not transactivate in cells transfected with the VDR mutants L233A, I271A, R274A, W286A, H397A, or Y401A. On the contrary, some other mutants became more potent in inducing transcription of the reporter gene when cells were incubated with 19-nor-TD-vitamin D instead of  $1,25(\text{OH})_2\text{D}_3$  (e.g. V234A, I268A, S275A, and H305A). The mutant receptor H305A was even as potent as

wild type VDR. The capacity of 19-nor-previtamin D to induce transcriptional activation of the mutant VDRs was completely abolished, except for the S278A mutant (110% compared with wild type VDR). On the contrary, the profile observed for 19-nor-TD-previtamin D was comparable with the profile for  $1,25(\text{OH})_2\text{D}_3$  and completely different from the one of 19-nor-previtamin D.

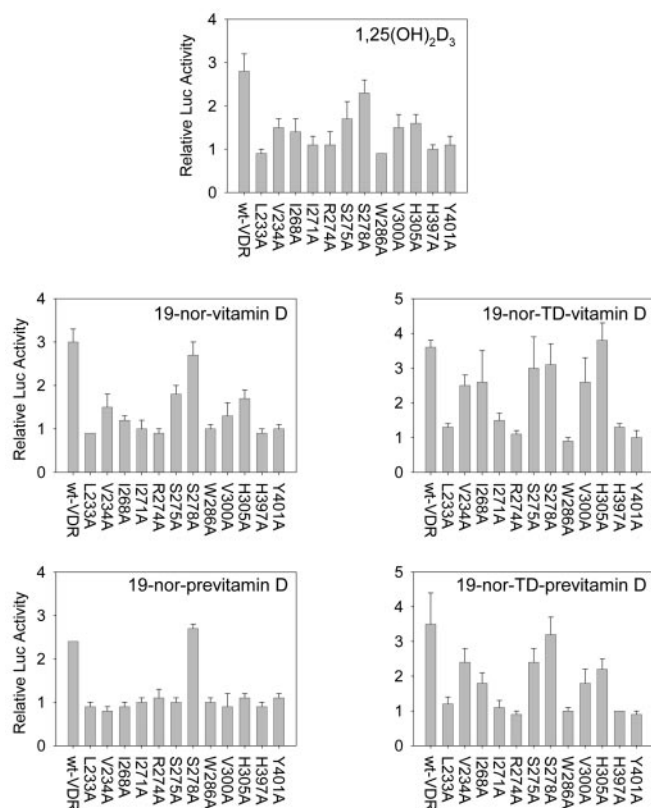
**Docking of 19-Nor-TD-previtamin D**—To investigate whether a compound in the previtamin conformation fitted in the ligand binding pocket of the VDR and made similar interactions with the VDR as  $1,25(\text{OH})_2\text{D}_3$ , some docking experiments were performed using 19-nor-TD-previtamin D and the crystal structure of the VDR-LBD ( $\Delta 165$ –215) (21). Based on the manual docking of different conformations of 19-nor-TD-previtamin D followed by molecular dynamics calculations, a possible conformation for 19-nor-TD-previtamin D in the VDR pocket was proposed (Fig. 7). In this conformation, 19-nor-TD-previtamin D made similar interactions with the VDR as  $1,25(\text{OH})_2\text{D}_3$ , i.e. the side chain of the analog had the same extended geometry, the 25-hydroxyl group made the same hydrogen bonds with H305 and H397, and even the  $1\alpha$ -hydroxyl and  $3\beta$ -hydroxyl groups made the same hydrogen bonds with S237 and Y143, respectively.

## DISCUSSION

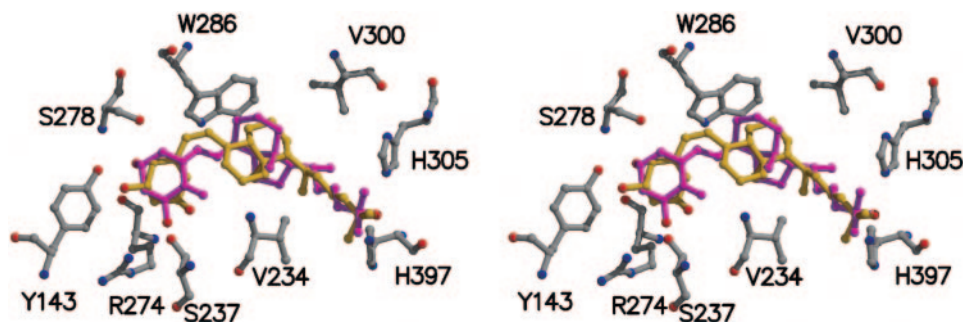
This paper describes the biological and underlying genomic activity of two different 19-nor-vitamin D analogs and their previtamin forms. 19-Nor-vitamin D was synthesized previously by DeLuca and co-workers (9, 10), and they and others (4) illustrated that the biological potency of this analog is comparable with that of  $1,25(\text{OH})_2\text{D}_3$  itself. In agreement with this, the interaction of 19-nor-vitamin D with VDR and its capacity to interact with VDREs and transactivate VDRE-containing reporter genes were similar to those of the parent compound.

19-Nor-previtamin D, on the contrary, had low biological potency, which confirms a previous study (4). Weak interactions with VDR, VDREs, and coactivators were probably the cause of its low genomic activity. Mutation of each of the different amino acids lining the ligand binding pocket of VDR, except for Ser-278, led to a reduction of the transactivating capacity of 19-nor-previtamin D to the level of vehicle-treated cells, which suggested that each of the various interactions were important for this compound to exhibit genomic activity.

Introduction of a *trans*-fused decalin CD-ring in 19-nor-vitamin D clearly enhanced the antiproliferative and prodifferentiating activity of 19-nor-TD-vitamin D. However, the interactions observed at the different steps in the pretranscriptional process were similar for this compound and  $1,25(\text{OH})_2\text{D}_3$ , which illustrated the importance of coregulatory molecules in



**FIG. 6. Transactivating potency of different point mutants of VDR in COS-1 cells.** Cells were stimulated with  $1,25(\text{OH})_2\text{D}_3$  or its analogs, each applied at their  $\text{EC}_{50}$  concentration (as determined in experiments presented in Fig. 4). Bars represent means and S.E. of at least three independent experiments. The overall transactivating profile obtained with  $1,25(\text{OH})_2\text{D}_3$  was significantly different from that obtained for 19-nor-TD-vitamin D and 19-nor-previtamin D.



**FIG. 7. Modeling of 19-nor-TD-previtamin D.** Stereo representation of the proposed conformation of 19-nor-TD-previtamin D in the ligand binding pocket of the VDR with  $1,25(\text{OH})_2\text{D}_3$  superimposed in its conformation in the crystal structure (PDB entry 1DB1). 19-nor-TD-previtamin D (yellow),  $1,25(\text{OH})_2\text{D}_3$  (purple), and some of the surrounding residues of the VDR are rendered as balls-and-sticks. The oxygen atoms of the hydroxyl groups of 19-nor-TD-previtamin D and  $1,25(\text{OH})_2\text{D}_3$  have a red color.

determining the eventual biological activity in different cell types (8, 23, 24). Mutational analysis suggested that the orientation of this compound in the ligand binding pocket of VDR is different from the orientation of the parent compound. Indeed, the transactivating ability of several mutant VDRs (V234A, I268A, S275A, and H305A) was enhanced after incubation with 19-nor-TD-vitamin D when compared with 1,25(OH)<sub>2</sub>D<sub>3</sub>. A transactivating profile similar to that for 19-nor-TD-vitamin D was obtained for the analogs 20-*epi*-1,25(OH)<sub>2</sub>D<sub>3</sub> and KH 1060 (16). Crystallization studies of VDR, together with 20-*epi*-1,25(OH)<sub>2</sub>D<sub>3</sub> or KH 1060, revealed that the VDR protein conformation is identical whether it is complexed to 1,25(OH)<sub>2</sub>D<sub>3</sub> or its analogs, indicating that the ligands adapt to the binding pocket and not *vice versa* (25). The A- to D-ring moieties of 20-*epi*-1,25(OH)<sub>2</sub>D<sub>3</sub> or KH 1060 adopt the same conformation as in 1,25(OH)<sub>2</sub>D<sub>3</sub>, and it is only the side chain that can adapt its conformation in order to anchor the 25-hydroxyl group. A different transactivating potency obtained with the mutants V234A, I268A, and H305A can therefore probably be explained by a different position of the side chain of 19-nor-TD-vitamin D or the disappearance of some steric hindrance from, for example, His-305.

Most surprisingly, introduction of the *trans*-fused decalin CD-ring in 19-nor-previtamin D resulted in a previtamin compound that was as potent as 1,25(OH)<sub>2</sub>D<sub>3</sub> in inhibiting cell proliferation and inducing cell differentiation. To our knowledge, 19-nor-TD-previtamin D is the first previtamin structure with pronounced antiproliferative and prodifferentiating effects. Previously studied previtamin D<sub>3</sub> analogs, which were either locked in the 6-*s-cis* conformation or modified so that they only slowly isomerize to the vitamin conformation, have little effects on cell proliferation and differentiation (and underlying genomic effects) but are as potent as 1,25(OH)<sub>2</sub>D<sub>3</sub> in evoking rapid, non-genomic effects (5–7). On the other hand, lithocholic acid, a secondary bile acid with an intact steroid skeleton such as is present in provitamin D<sub>3</sub> binds to VDR and regulates gene transcription, but only at much higher (1000×) concentrations (26).

Despite its low binding affinity to VDR, 19-nor-TD-previtamin D was able to induce a VDR conformation that was as resistant to proteolysis as the conformation induced by 1,25(OH)<sub>2</sub>D<sub>3</sub>. The discrepancy between the low binding affinity of 19-nor-TD-previtamin D (determined at 4 °C) and its ability to induce a stable VDR conformation (determined at room temperature) is not well understood. Dissociation constants determined at room temperature were similar to those obtained at 4 °C, which excludes the suggestion that this discrepancy is due to the reaction temperature (data not shown). A possible explanation could be that 19-nor-TD-previtamin D dissociates less quickly from the receptor compared with 1,25(OH)<sub>2</sub>D<sub>3</sub>. However, this possibility cannot be investigated, as radioactively labeled 19-nor-TD-previtamin D is not available.

19-Nor-TD-previtamin D proved to be equipotent to 1,25(OH)<sub>2</sub>D<sub>3</sub> in inducing interaction with VDREs and the co-activator TIF2. Also, its transactivating capacity was similar to that of the parent compound. The genomic activity was confirmed in MC3T3-E1 mouse osteoblasts in which, after treatment with 10<sup>-9</sup> M 19-nor-TD-previtamin D, a substantial increase of CYP24-mRNA levels was observed. The transactivating as well as the antiproliferative capacity of 19-nor-TD-previtamin D were determined in cell culture medium containing 10% fetal bovine serum. Because of its lower DBP affinity, the free concentration of 19-nor-TD-previtamin D in this medium might be higher when compared with 1,25(OH)<sub>2</sub>D<sub>3</sub> and could partially underlie its relatively potent genomic activity.

However, despite its equally low affinity for DBP, the relative potency of 19-nor-TD-vitamin D as measured on primary human keratinocytes cultured in serum-free medium remained similar (10-fold more potent than 1,25(OH)<sub>2</sub>D<sub>3</sub>; data not shown).

Mutational analysis revealed that the overall transactivating profile obtained with 19-nor-TD-previtamin D did not differ significantly from the one obtained with 1,25(OH)<sub>2</sub>D<sub>3</sub>. These findings suggested that the general architecture of 19-nor-TD-previtamin D in the ligand binding pocket of VDR was similar to that of the parent compound. A docking model for this previtamin was proposed in which 19-nor-TD-previtamin D made similar interactions with VDR as did 1,25(OH)<sub>2</sub>D<sub>3</sub>. According to this model, the general position of the different hydroxyl groups of 19-nor-TD-previtamin D was maintained when compared with the parent secosteroid. Based on the crystal structure, these hydroxyl groups represent anchoring points that must be maintained to obtain an active conformation (21). Although this conformation of 19-nor-TD-previtamin D can explain the transactivation profile obtained from mutational analysis, only a crystallographic study can render the exact structure of the VDR-19-nor-TD-previtamin D complex.

In conclusion, introduction of a *trans*-fused decalin CD-ring in 19-nor-1,25(OH)<sub>2</sub>D<sub>3</sub> clearly enhanced the biological profile of both the vitamin and previtamin forms. 19-Nor-TD-previtamin D was as potent as 1,25(OH)<sub>2</sub>D<sub>3</sub> in inhibiting cell proliferation and inducing cell differentiation and represents the first previtamin structure with pronounced vitamin D-like activity. The potency of 19-nor-TD-previtamin D to induce functional interactions with VDR, RXR, and VDREs was very similar to the parent hormone. Mutational analysis and manual docking suggested that this previtamin analog can make similar contacts with the ligand binding domain of the receptor as does 1,25(OH)<sub>2</sub>D<sub>3</sub>.

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