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REVIEW

Vitamin D receptor 2016: novel ligands and structural insights

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ABSTRACT

Introduction: Vitamin D_3 activates via its hormonal form 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), the transcription factor vitamin D receptor (VDR). VDR is expressed in most human tissues and has more than 1,000 target genes. Thus, 1α,25$(OH)_2D_3$ and its synthetic analogs have a broad physiological impact. The crystal structures of the VDR ligand-binding domain (LBD), and its various ligands, allows further the understanding of the receptor's molecular actions.

Areas covered: We discuss the most important novel VDR ligands and the further insight derived from new structural information on VDR.

Expert opinion: There is an increasing appreciation of the impact of vitamin D and its receptor VDR not only in bone biology, but also for metabolic diseases, immunological disorders, and cancer. Detailed structural analysis of the interaction of additional novel ligands with VDR highlight helices 6 and 7 of the LBD as being most critical for stabilizing the receptor for an efficient interaction with co-activator proteins, i.e. for efficient agonistic action. This permits the design of even more effective VDR agonists. In addition, chemists took more liberty in replacing major parts of the 1α,25$(OH)_2D_3$ molecule, such as the A- and CD-rings or the side chain, with significantly different structures, such as carboranes, and still obtained functional VDR agonists.

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Bile acids; crystal structure; vitamin D; vitamin D analogs; vitamin D receptor

1. Introduction

Since more than 500 million years, phyto- and zooplanktons have been using energy provided by UV-B radiation (290–315 nm) and converting 7-dehydrocholesterol to pre-vitamin D_3, which further isomerizes to vitamin D_3. This UV-B-dependent, nonenzymatic reaction also takes place in the human skin [1]. While plankton uses vitamin D_3 only as a chemical sunscreen, in vertebrates it acts via its metabolite 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) as a hormone that controls the uptake of sufficient amounts of calcium for stabilizing bones [2]. In parallel to the evolution of the control of calcium homeostasis, 1α,25$(OH)_2D_3$ also became involved in the regulation of cellular differentiation and growth, such as the induction of cellular differentiation (e.g. in HL-60 leukemia cells), the induction of apoptosis (e.g. in MCF-7 breast cancer cells), and the inhibition of proliferation of a large variety of cell types (e.g. in Caco-2 colon cancer cells) [3]. The control of innate and adaptive immunity by vitamin D_3 is even more promising [4], since most responsive 1α,25$(OH)_2D_3$ target genes, such as *CD14* (encoding for a co-receptor of toll-like receptor 4) and *CAMP* (encoding for the antibacterial protein cathelicidin), are found in immune cells [5,6]. These actions indicate that 1α,25$(OH)_2D_3$ and its metabolic precursors also have preventive and therapeutic potential for extra-skeletal chronic diseases, such as cardiovascular and autoimmune diseases, cancer, diabetes, and infections [7]. Therefore, pharmaceutical companies developed over the past 30 years more than 3000 vitamin D analogs with in part selective properties [8].

The physiological functions of vitamin D are mediated by target genes of the vitamin D receptor (VDR), which is the only protein that binds 1α,25$(OH)_2D_3$ at sub-nanomolar concentrations [9]. The VDR gene displays highest expression in metabolic tissues, such as intestine, kidneys, and bone, but most of the other 400 tissues and cell types that form the human body also show some VDR expression [7]. VDR is an endocrine member of the nuclear receptor superfamily, the members of which can be specifically activated by low nanomolar concentrations of a small lipophilic molecule in the size of cholesterol [10]. This functional characteristic is found only with a few other transcription factors, such as the nuclear receptors for the steroid hormones estradiol, testosterone, progesterone, cortisol, and mineralocorticoids; the vitamin A derivative all-*trans* retinoic acid; and the thyroid hormone triiodothyronine [11]. All members of the nuclear receptor superfamily have a similar mode of action [12], but the physiological actions of their ligands are clearly distinct. Nuclear receptors contain a structurally conserved ligand-binding domain (LBD) [13], which accommodates in its lower part a ligand-binding pocket (LBP) providing 400–1400 $Å^3$ space for a specific binding of their respective ligands [14]. VDR carries a relatively small LBP

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Article highlights

- Vitamin D is essential for the maintenance of health, such as prevention of bone disorders, muscle weakness, cancer and autoimmune diseases. Thus synthetic vitamin D analogs have a broad therapeutic potential.
- The number of crystal structures of the VDR-LBD with different natural and synthetic ligands is constantly increasing. Detailed structural analysis of the latest VDR crystal structures indicated that helices 6 and 7 of the LBD are most critical for the stabilizing the receptor for an efficient interaction with co-activator proteins.
- Major parts of the 1α,25(OH)$_2$D$_3$ molecule, such as the A- and CD-rings or the side chain, were replaced with significantly different structures, such as carboranes, and still functional VDR agonists were obtained.

This box summarizes key points contained in the article.

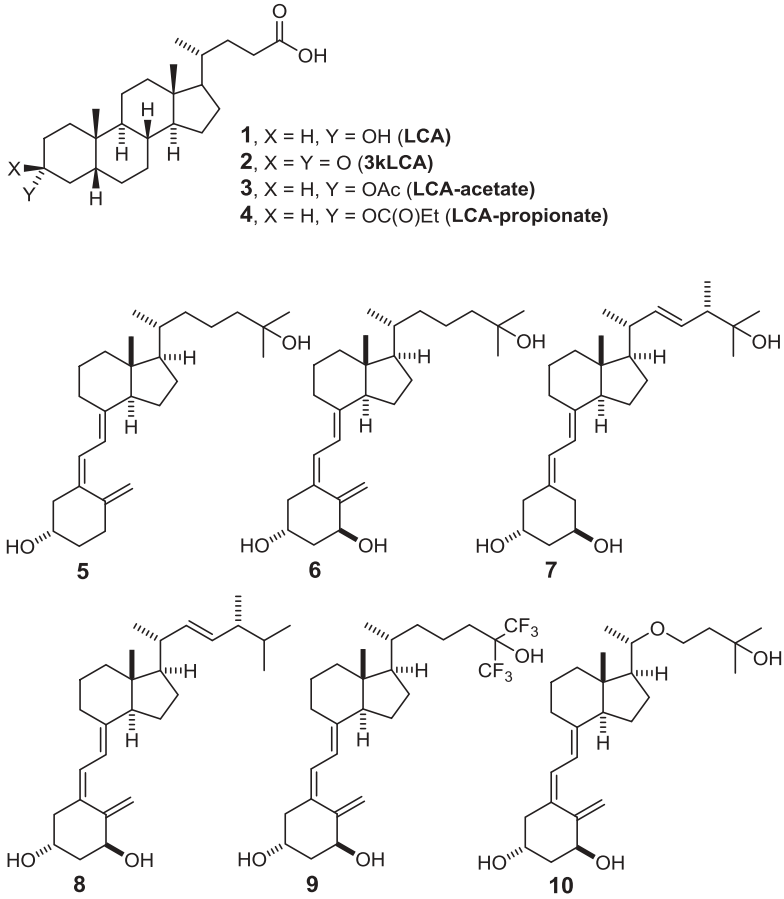
that is shaped optimally, in order to bind with high affinity and specificity 1α,25(OH)$_2$D$_3$ and many of its synthetic analogs.

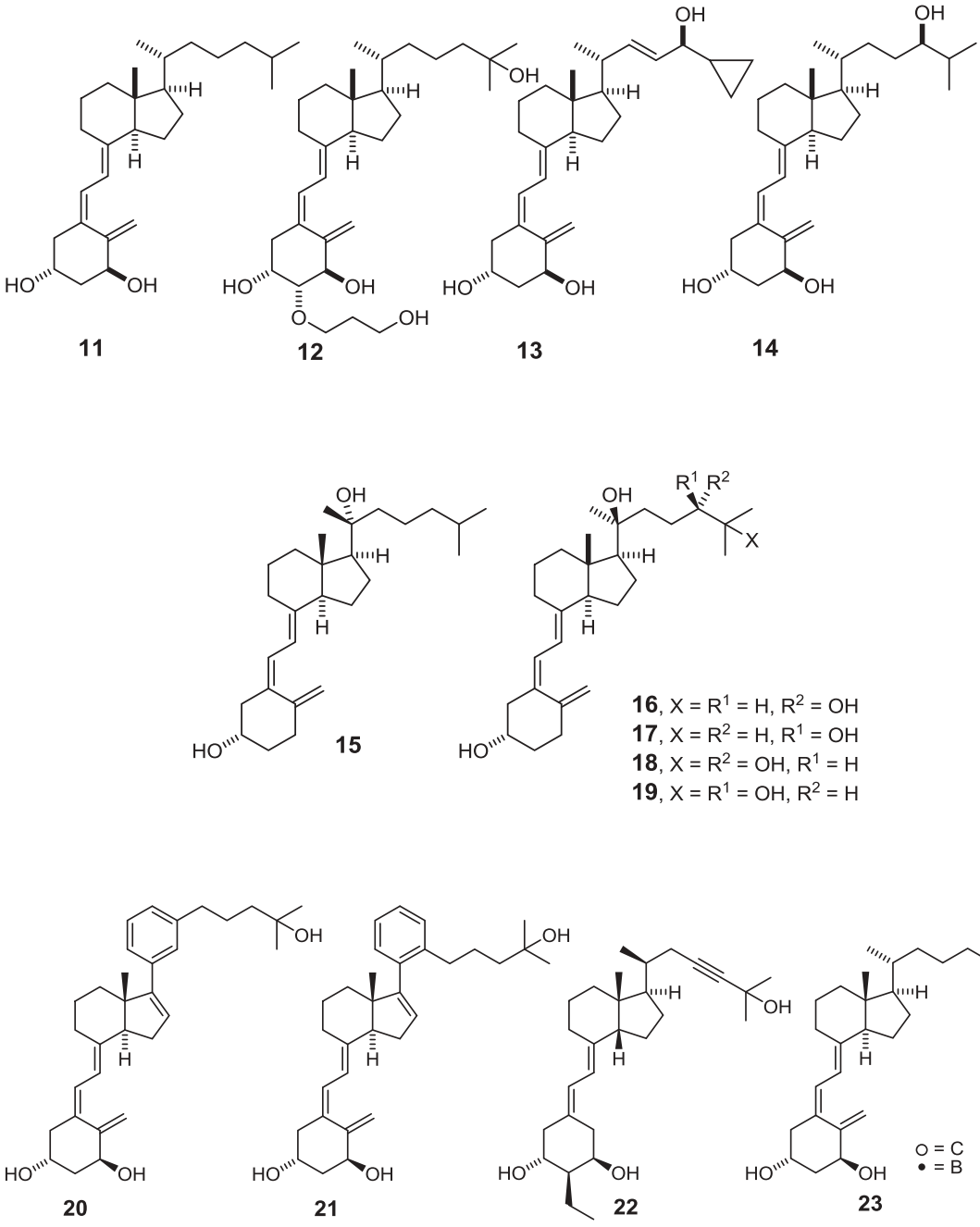
An essential condition for the transcription of a 1α,25(OH)$_2$D$_3$ target gene is that the genomic loci of both the gene's core promoter region containing the transcription start site and the VDR binding sites of distal enhancer regions are located within accessible chromatin [11]. VDR's interactions with nuclear proteins depend on the absence or presence of 1α,25(OH)$_2$D$_3$ within the receptor's LBD, i.e., most of these protein–protein interactions are ligand-dependent [15]. For example, the genome-wide pattern of accessible chromatin changes after stimulation with 1α,25(OH)$_2$D$_3$ [16]. When bound to its specific genomic binding sites, ligand-activated VDR is able to recruit co-activator proteins, such as those of the mediator complex, which build a bridge to the basal transcriptional machinery being assembled on the transcription start site of 1α,25(OH)$_2$D$_3$ target genes. In this way, the transcription of the VDR/1α,25(OH)$_2$D$_3$ target genes is stimulated and proteins are produced that change the functional profile of the respective vitamin D target tissues and cell types.

This review is an update and extension of our previous summaries in this series [17,18] and describes aspects about the VDR and its natural and synthetic ligands that have not been covered by Takada and Makishima [19]. A reasonable number of new 1α,25(OH)$_2$D$_3$ analogs have been published and/or patented within the last 4 years. In addition, the list of VDR crystal structures has significantly grown. Thus, we will discuss the most important novel VDR ligands and further insight derived from new structural information.

2. Evolutionary oldest VDR ligands: bile acids

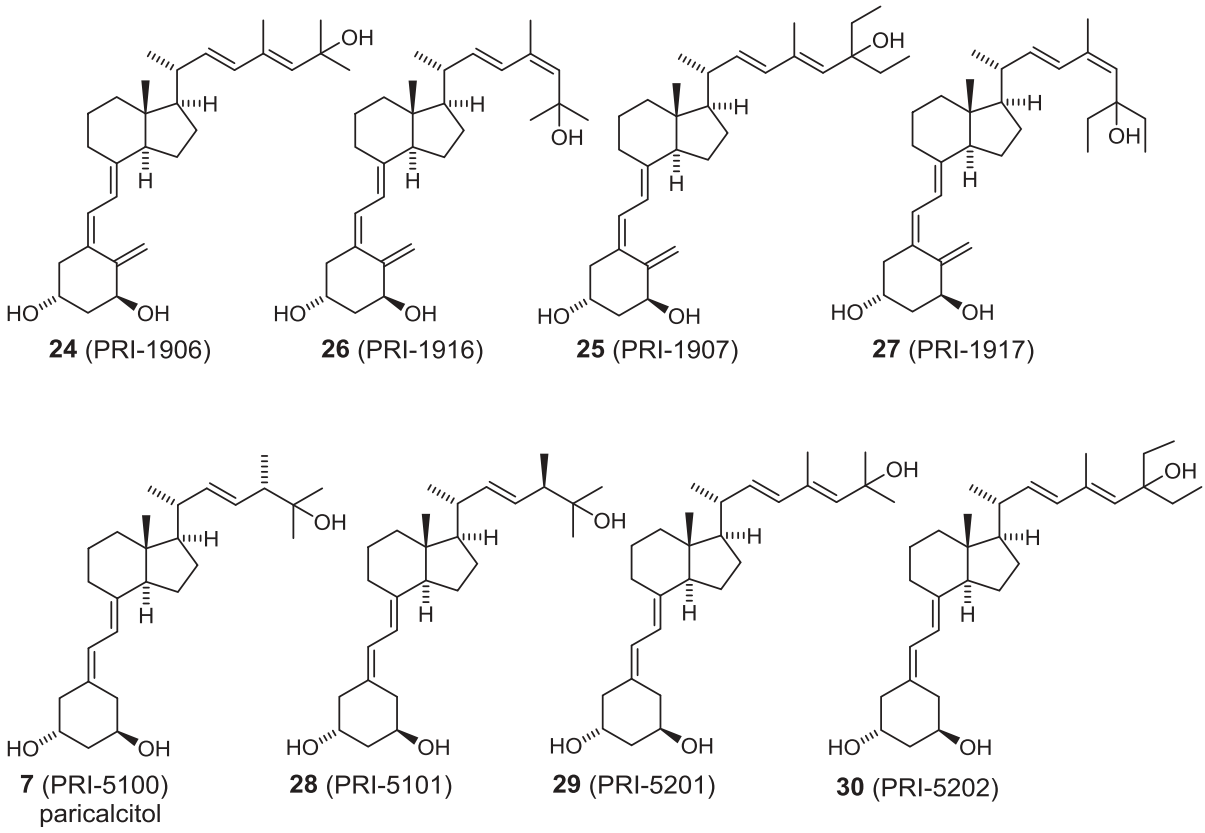
Early in evolution, nuclear receptors were ligand-independent transcription factors that primarily controlled cellular metabolism [20]. In a multistep process, the precursor of VDR learned to bind and be activated by bile acids and their metabolites [21]. Some of them, such as the secondary bile acid, lithocholic acid (LCA, **1**), bind VDR in rather high concentrations, but the natural bile acid 3-ketolithocholic acid (3kLCA, **2**) is more potent. Other derivatives of LCA, such as LCA-acetate (**3**) and





LCA-propionate (**4**), are 10- to 30-times more potent than LCA depending on the assay used. The four available crystal structures of the rat VDR-LBD with LCA, 3kLCA, LCA-acetate, and LCA-propionate show the mechanism of bile acid agonism [22]. The overall fold of the VDR-LBD is maintained, but since the bile acids are weak agonists compared to $1\alpha,25(OH)_2D_3$, some regions show higher fluctuations, such as the N-terminal region of the LBD (A116 to Q122), the region flanking the insertion domain (D160 to G164 and S212 to L217), the C-terminal end (S423), and two residues binding mediator protein 1 (Q421 and I422).

Bile acids bind to the VDR-LBP in the opposite orientation as $1\alpha,25(OH)_2D_3$ (Figure 1(a)), i.e., their 24-carboxyl group points toward the β-turn region of the LBD. The β-part of the sterol backbone directs to helices H6-7 and H11 of the LBD, which is similar to the binding of the bile acid 6α-ethyl-chenodeoxycholic acid (obeticholic acid) within the LBD of the nuclear receptor farnesoid X receptor [23]. One of the oxygen atoms from the carboxyl-moiety of the bile acids directly interacts with amino acids S274 (S278 in human) and Y143 of VDR, while the other oxygen is bridged through a water molecule to amino acids S233 (S278 in human) and R270 (R274 in human). These interactions are conserved in all four structures (Figure 1). The difference in the binding affinity of the four bile acids most likely derives from the interaction of their 3-OH group, such as a complex water-mediated hydrogen bond (H-bond) network present in LCA (no direct contact with histidine residues, Figure 1(c)), 3kLCA (one direct contact with H301 (H305 in human), Figure 1(d)) and LCA-acetate and LCA-propionate directly contacting VDR residues (Figures 1(e,f).



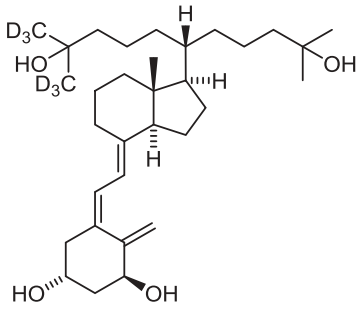
The complex of the zebrafish VDR-LBD with LCA shows a very unique interaction pattern, such as the simultaneous binding of two LCA molecules to the receptor [24] (Figure 2(c)). One LCA molecule binds to the LBP in an analogous fashion as in the rat VDR-LBD (Figure 2(b)), but the other LCA molecule contacts the VDR surface close to the H1-3 loop and helix H3 (Figure 2(d)). The orientation of the first LCA molecule is supported by H-bonds, while the second LCA molecule is stabilized and oriented by mostly hydrophobic interactions with the amino acids D181, R184, F185 of helix H2; D260, S263, Y264, Q267 of helix H3; and L443 of helix H12 as well as additional H-bond interactions of its 3-OH group with S263 and Q267 (Figure 2(d)). The 24-carboxyl group of the ligand has indirect water-mediated interactions with amino acids D181 and K268. The structural and functional data support a two-step LCA binding that assists co-activator protein interactions and stabilizes the H11-12 loop and helix H12. The binding of the second LCA molecule may further stabilize the VDR-LBD and is required for the full activation of the receptor by bile acids [24].

3. Design of vitamin D analogs

Synthetic analogs of $1\alpha,25(OH)_2D_3$ have been developed with the goal of improving the biological profile of the natural hormone for a therapeutic application either in hyper-proliferative diseases, such as psoriasis and different types of cancer, or in bone disorders, such as osteoporosis [8]. Although in the last years the majority of new VDR ligands were direct derivatives of the natural hormone $1\alpha,25(OH)_2D_3$, an increasing number of mimics have been described. The modifications of $1\alpha,25(OH)_2D_3$ were done in the side chain, in the A-ring (often in combination with changes in the side chain), in the CD-rings, and in the triene system. Since many years, the modifications follow two main strategies, which are either (1) to increase the affinity of the compounds for binding to the VDR-LBP or (2) to modulate the metabolic stability of the molecules [17].

The central step in the action of VDR ligands is the conformational shift of the VDR-LBD and the resulting exchange of protein–protein interaction partners, such as co-activator and co-repressor proteins [25]. Only those VDR ligands that cause both an efficient dissociation of co-repressor proteins from the receptor as well as the specific binding of co-activator proteins finally lead to transcriptional activation, i.e., act as agonists. The nature of the physiological actions of $1\alpha,25(OH)_2D_3$ and the respective potential therapeutic application of its synthetic analogs make the development of VDR agonists more interesting than that of VDR antagonists.

The most detailed information about the molecular mechanisms of $1\alpha,25(OH)_2D_3$ analogs can be obtained from VDR-LBD crystal structures. The structure of the VDR-LBD complexed with the natural hormone has been known since 16 years [26], but since then VDR has also been crystallized with a large number of synthetic analogs [27]. A comparison of these crystal structures suggests that in general the different analogs act like the natural hormone, i.e., they stabilize the VDR-LBD in a conformation that is not significantly different to that of the $1\alpha,25(OH)_2D_3$-VDR complex. Most important in this context is the position of the three OH groups (at C1α, C3β, and C25), which, in most vitamin D compounds, occupy a very similar position. This also implies that there is only one agonistic VDR conformation that is characterized by a contact between the OH group at C25 of the ligand and amino acids H305 and H397 of the LBD.



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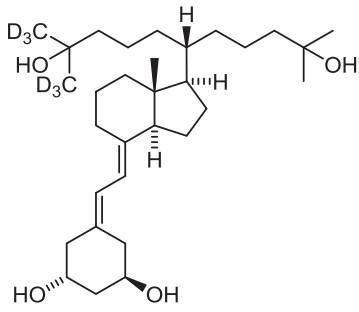

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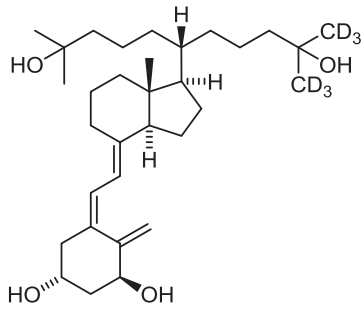

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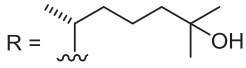

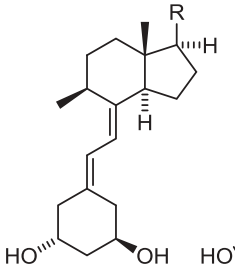

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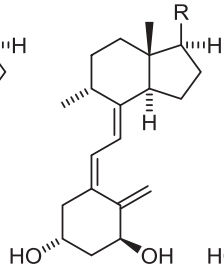

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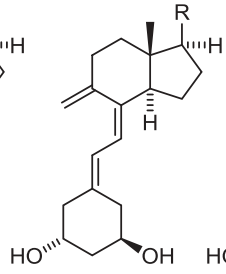

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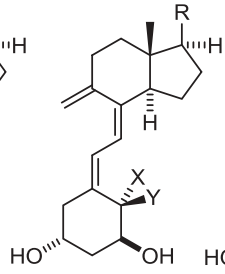
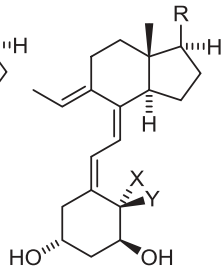


37, X= Me, Y = H
38, X= H, Y = OH



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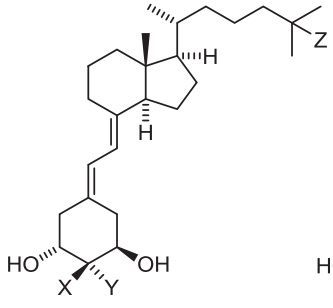
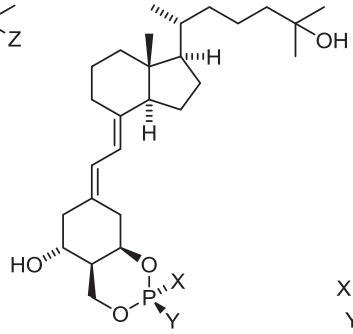
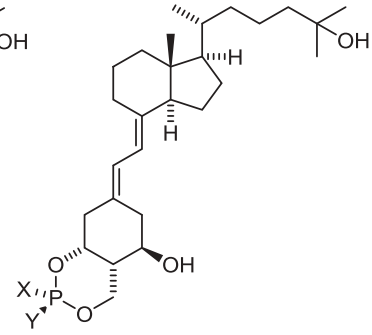


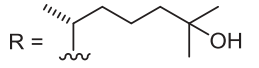
40, X = Y = Z= H
41, X = CH_2OH, Y= H, Z=OH
42, X = H, Y= CH_2OH, Z=OH

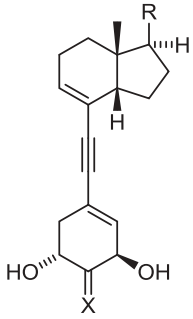


43, X= OPh, Y= =O
44, X= =O, Y = OPh

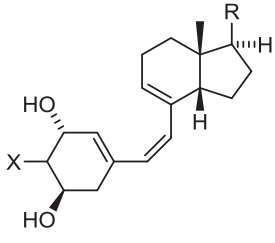


45, X= OPh, Y= =O
46, X= =O, Y = OPh

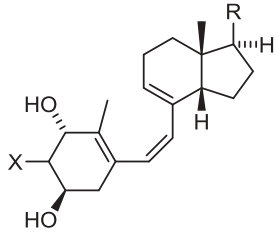




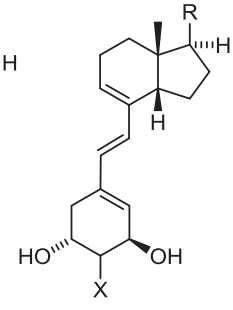
47, X= CH_2
48, X= H, α-CH_3
49, X= H, β-CH_3



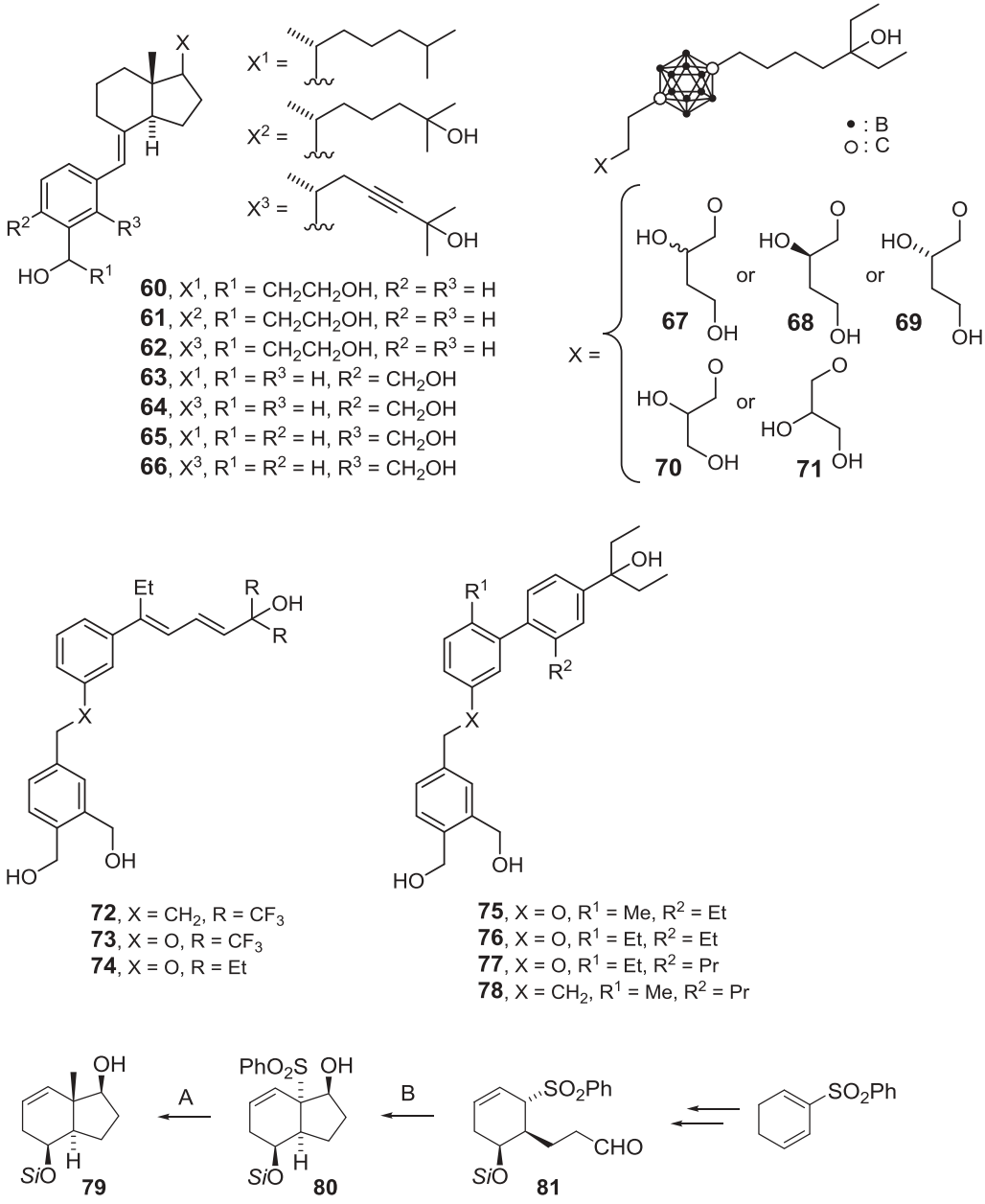
50, X= H
51, X= α-CH_3
52, X= β-CH_3



53, X= H
54, X= α-CH_3



55, X= H
56, X= CH_2
57, X= α-CH_3
58, X= β-CH_3
59, X= α-$OCH_2CH_2CH_2OH$



Si = Protecting group. A) $AlMe_3$, *n*BuLi, tol. B) $Ba(OH)_2$, $THF\text{-}H_2O$.

In the following chapters, different classes of 1α,25$(OH)_2D_3$ analogs are discussed and, where applicable, insight from VDR crystal structures is highlighted.

4. Marketed vitamin D analogs

Despite the synthetic effort made on the synthesis of analogs of 1α,25$(OH)_2D_3$, only few analogs have reached the market [28,29]. A short summary including their applications [commercial name (company)] is listed:

Calcidiol (**5**, 25OHD_3) is used in the treatment of chronic hypocalcemia, renal osteodystrophy [*Calderol* (Upjohn), *Hidroferol* (Faes Farma)], and rickets [*Dedrogyl* (Roussel), *Hidroferol* (Faes Farma)].

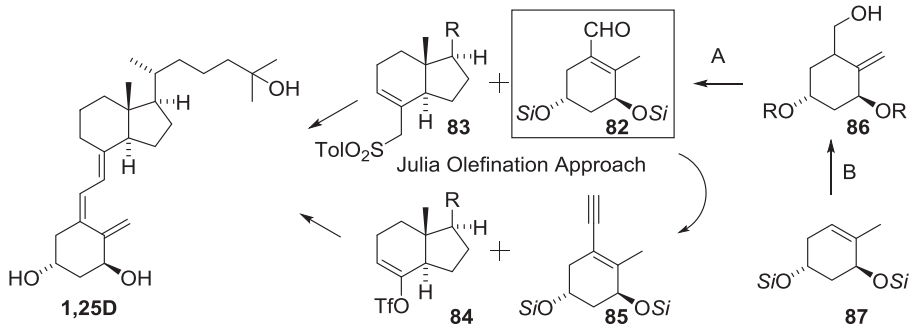
Calcitriol (**6**, 1α,25$(OH)_2D_3$) is prescribed for renal osteodystrophy [*Rocatrol* (Roche), Calcijex (Abbott)], osteoporosis [*Rocatrol*, Roche], and psoriasis [*Silkis* (Galderma)].

Paracalcitol (**7**, 19-nor-1α,25$(OH)_2D_2$, *Zemplar*, Abbott Laboratories) is used for secondary hyperparathyrodism.

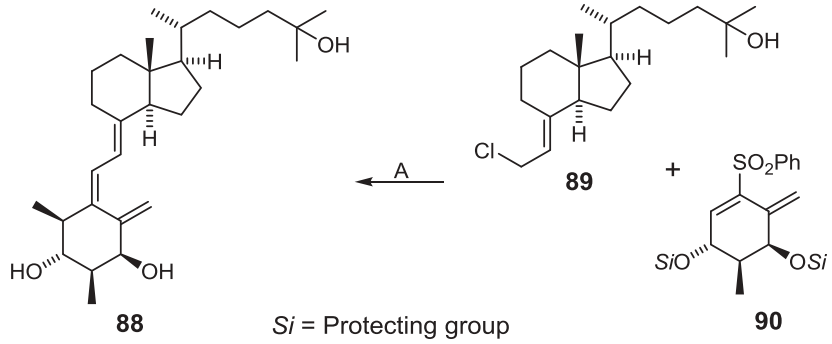
Doxercalciferol (**8**, 1αOHD_2, *Hectorol*, Bone Care International) is prescribed for secondary hyperparathyrodism.

Falecalcitriol (**9**, 1α,25$(OH)_2$–26,27-F_6-D_3, is prescribed for secondary hyperparathyrodism in Japan [*Hornel* (Taisho Pharmaceuticals and Sumitomo Pharmaceuticals), Fulstan (Kissei Pharmaceuticals)].

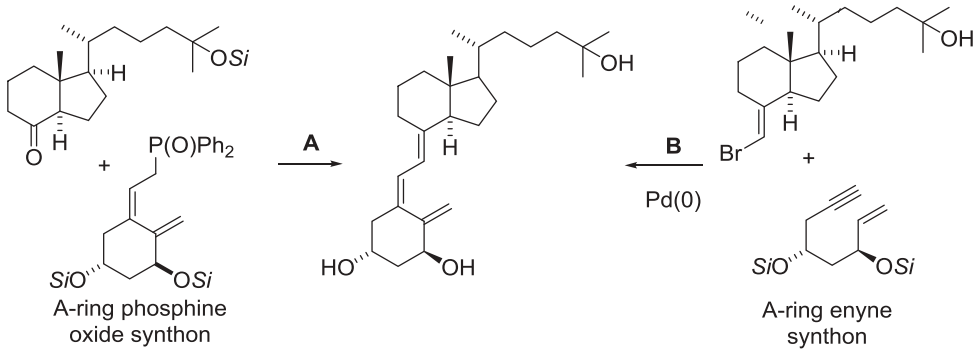
Oxacalcitriol (**10**, 22-oxa-1α,25$(OH)_2D_3$) is used for secondary hyperparathyrodism and psoriasis [*Oxarol* (Chugai Pharmaceuticals)] in Japan.



Key Steps: A) $AlMe_3$, trioxane, 2,6-diphenylphenol, CH_2Cl_2. B) PCC, CH_2Cl_2; DBU, CH_2Cl_2.



Key Steps: A) Methylation; sulfone coupling; deprotection; sulfone elimination.



A) Wittig-Horner approach; B) Trost approach. *Si* = Protecting group

Alfacalcidol (**11**, 1α,OHD_3) is used for renal osteodystrophy [*Alfarol* (Chugai Pharmaceutical), One-Alpha (Leo Pharmaceuticals)], secondary hyperparathyrodism [*Alfarol* (Chugai Pharmaceutical)], osteoporosis [*Alfarol* (Chugai Pharmaceutical), Alpha D_3 (Teva Pharmaceuticals)], and rickets [*Alfarol* (Chugai Pharmaceutical)].

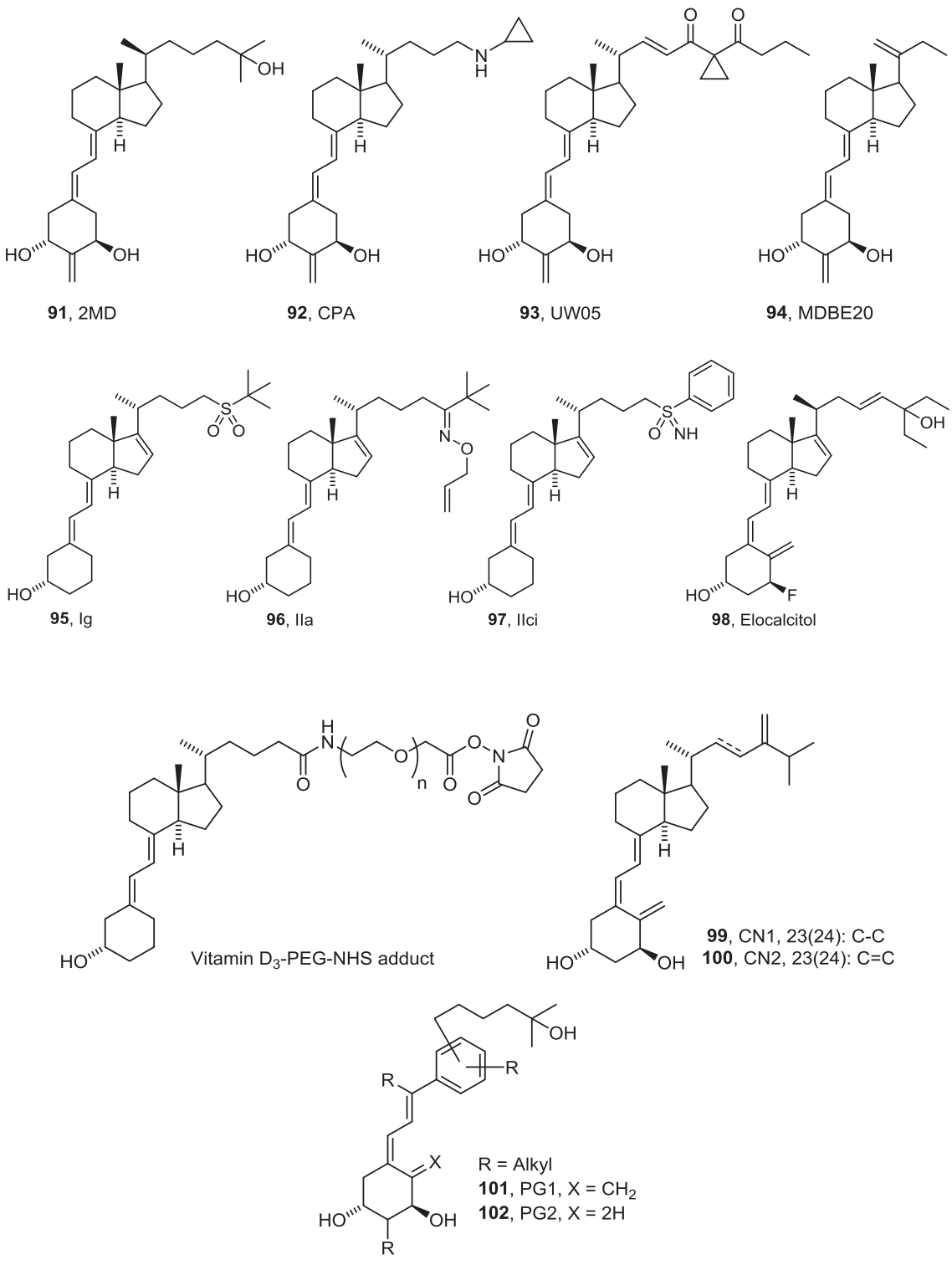
Eldecalcitol (**12**, ED-71, 2β-(3-hydroxypropoxy)-1α,25$(OH)_2D_3$) is prescribed for osteoporosis only in Japan [*Edirol* (Chugai Pharmaceutical)].

Calcipotriol (**13**, 22-ene-26,27-dehydro-1α,25$(OH)_2D_3$) is used for psoriasis [*Davionex* (Leo Pharmaceuticals), *Dovonex* (Warner Chilcott)].

Tacalcitol (**14**, 1α,24$(OH)_2D_3$) is prescibed for psoriasis [*Bonalfa* (Teijin), *Curatoderm* (Merck KgaA)].

5. Vitamin D side-chain analogs

The nonnaturally occurring vitamin D analog 20*R*-hydroxyvitamin D_3 (**15**) [30] has been synthesized by the classical route starting from pregnolone acetate. Three years later, the same research team [31] also reported similar syntheses of the hydroxylated vitamin D compounds 20*S*,24*S*$(OH)_2D_3$ (**16**), 20*S*,24*R*$(OH)_2D_3$ (**17**), 1α,20*S*,24*S*$(OH)_3D_3$ (**18**), and 1α,20*S*,24*R*$(OH)_3D_3$ (**19**). The structures of these analogs are identical to those prepared enzymatically. Biological studies show that the 24*R*-isomer is more potent than the 24*S*-isomer, regardless of whether the compound is 1α-hydroxylated or not. In contrast, the 20,24$(OH)_2D_3$ isomers lack the ability to activate VDR. However, the corresponding 1α-OH-derivatives activate VDR signaling better than 1α,25$(OH)_2D_3$



and similarly than 22-oxa-1α,25(OH)$_2$D$_3$. Interferon γ inhibition assays indicated that the 24*R*-isomer is more potent than the 24*S*-epimer with regard to anti-inflammatory activities. The enzyme CYP27B1 metabolizes compound **17** 5.5-fold faster than compound **16**.

Compounds **20** and **21**, two new vitamin D analogs with aromatic side chains attached to C17, have been synthesized by Liu et al. starting from 1α-hydroxydehydroepiandrosterone [32]. The compounds bind weakly to the VDR (**20**, 0.01% and **21**, 0.015% compared to 1α,25(OH)$_2$D$_3$) and are slightly more potent than 1α,25(OH)$_2$D$_3$in inhibiting the proliferation of MCF-7 breast cancer cells (IC_{50}: **20**, 7.08 nM; **21**, 7.56 nM; 1α,25(OH)$_2$D$_3$, 12.5 nM). Both compounds induce HL-60 cell differentiation similar to 1α,25(OH)$_2$D$_3$.

The vitamin D analog **22**, a derivative of the low calcemic 19-nor and 14-epi-vitamin D analog inecalcitol [33], has been synthesized by De Clercq et al. by the Wittig–Horner approach [34]. Substitution at C2 enhances the affinity for the VDR. Compound **22** was 100-times less calcemic than 1α,25(OH)$_2$D$_3$ and 15-times more potent than 1α,25(OH)$_2$D$_3$ in inhibiting the

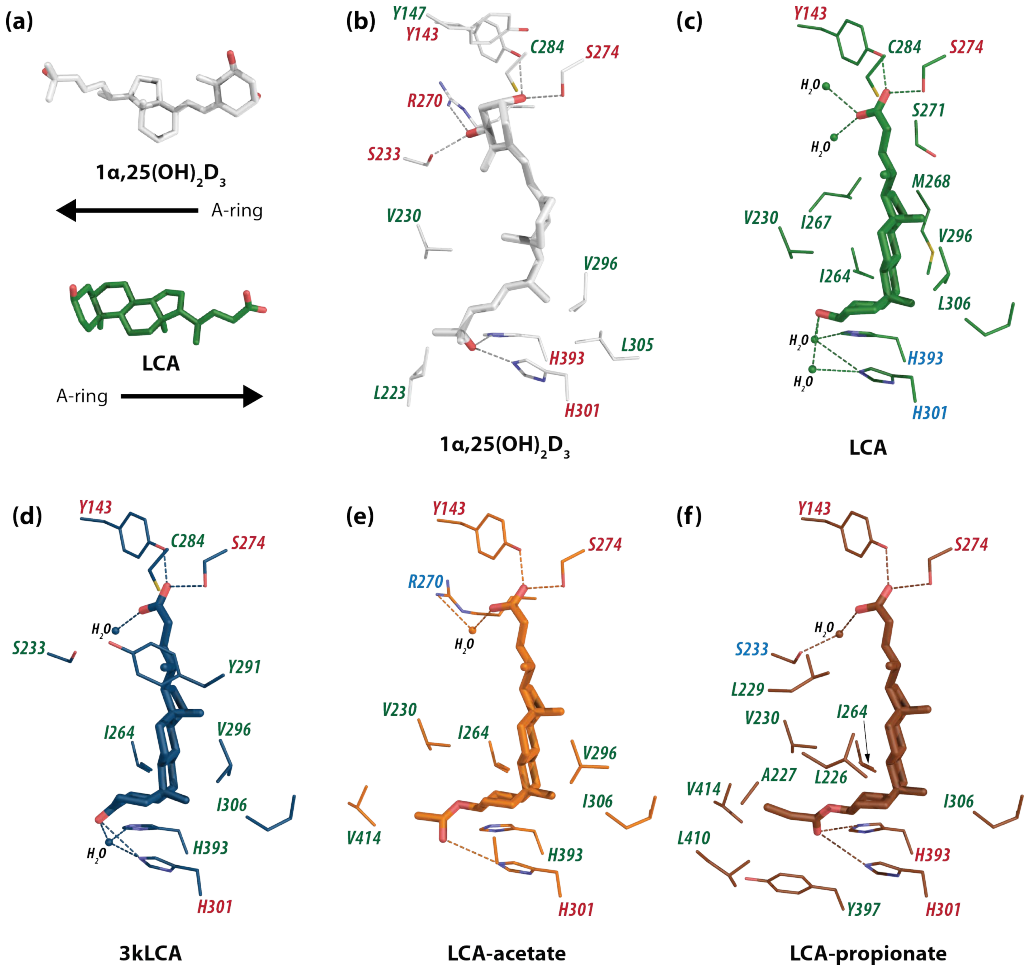


Figure 1. Mechanism of bile acid agonism in rat VDR-LBD complexes. The complexes of **1α,25(OH)$_2$D$_3$** and bile acids with the rat VDR-LBD are displayed with their surrounding interacting amino acid residues at a 3.8 Å distance cutoff. a. **1α,25(OH)$_2$D$_3$** and **LCA** bind in opposite orientation to the LBP. b. **1α,25(OH)$_2$D$_3$**-bound rat VDR-LBD (PDBID:1RK3). c. **LCA**-bound rat VDR-LBD (PDBID:3W5P). d. **3kLCA**-bound rat VDR-LBD (PDBID:3W5Q). e. **LCA-acetate**-bound rat VDR-LBD (PDBID:3W5R). f. **LCA-propionate**-bound rat VDR-LBD (PDBID:3W5T). The conserved anchoring amino acids in the **1α,25(OH)$_2$D$_3$** complex and as well as in all bile acid complexes are highlighted in red, the water-mediated amino acid contacts in blue and the differing residues in green. For the sake of clarity some of the conserved interacting residues, such as I267, S271 and W282, are not displayed. H-bonds are indicated by dashed lines. Important water molecules are displayed as small spheres. The illustration is based on the actual structural data and all bile acid-bound structures are shown in reference to that of **1α,25(OH)$_2$D$_3$** (**A**).

proliferation of MCF-7 cells. This vitamin D analog is a promising therapeutic agent.

1α-Hydroxy-25,26,27-trinor-24-*o*-carboranyl-vitamin D$_3$ (1,24cD$_3$, **23**) is a very recently published vitamin D analog, in which the tertiary OH group at C25 has been replaced with an *o*-carborane moiety (Figure 3) [35]. Despite the lack of this functional feature, the compound 1,24cD$_3$ is as effective as 1α,25(OH)$_2$D$_3$ in inhibiting the proliferation of MCF-7 cells and can induce the differentiation of HaCaT human keratinocytes. The binding of 1,24cD$_3$ to VDR, as determined by fluorescence polarization, is two times higher than that of 1α,25(OH)$_2$D$_3$ (IC$_{50}$ 2.9 versus 6.8 nM). Concerning induction of *CYP24A1* gene expression in MCF-7 cells, 1,24cD$_3$ is equally potent to 1α,25(OH)$_2$D$_3$, but does not show adverse calcemic effects in *in vivo* mouse models.

The complex of 1,24cD$_3$ with the VDR-LBD shows a highly similar conformation and overall the same protein topology as 1α,25(OH)$_2$D$_3$ (Figure 3(a)). However, small shifts are seen in the H6-7 loop (an important region for effective activation of VDR) and in the last part of helix H11, which is shifted by 0.6 Å. The differences are restricted to the area around the carborane-containing side chain, which is 2.4 Å longer than the side chain of 1α,25(OH)$_2$D$_3$. The hydrophobic nature of carborane favors the predominantly hydrophobic amino acid interactions in this part of the VDR-LBP. In addition, the carborane moiety forms unconventional H-bond (BH...HN) with amino acids H333 and H423 (H305 and H397 in human) (Figure 3(b)). All together, these small changes lead to higher stability of the VDR-LBD through increased stabilization of helices H3, H11, and H12.

A new series of four side-chain extended and branched analogs of 1α,25(OH)$_2$D$_2$, compounds **24** (PRI-1906) and **25** (PRI-1907), with methyl and ethyl groups at C25, respectively, were reported [36]. They were active, able to moderately inhibit proliferation, and significantly activated the expression of *CYP24A1* mRNA in PC-3 prostate cancer cells. A new generation of analogs of 1α,25(OH)$_2$D$_2$ was modified in two distinct parts of the molecule, combining side chains of **24** and **25** with the known 19-nor modification [37]. The known drug paricalcitol (**7**, PRI-5100) and its 21-epi analog (**28**, PRI-5101) were used as a reference. All of the tested analogs are less calcemic than 1α,25(OH)$_2$D$_3$. The analog **25**, and to lower extent **30** (PRI-5202), exerted some

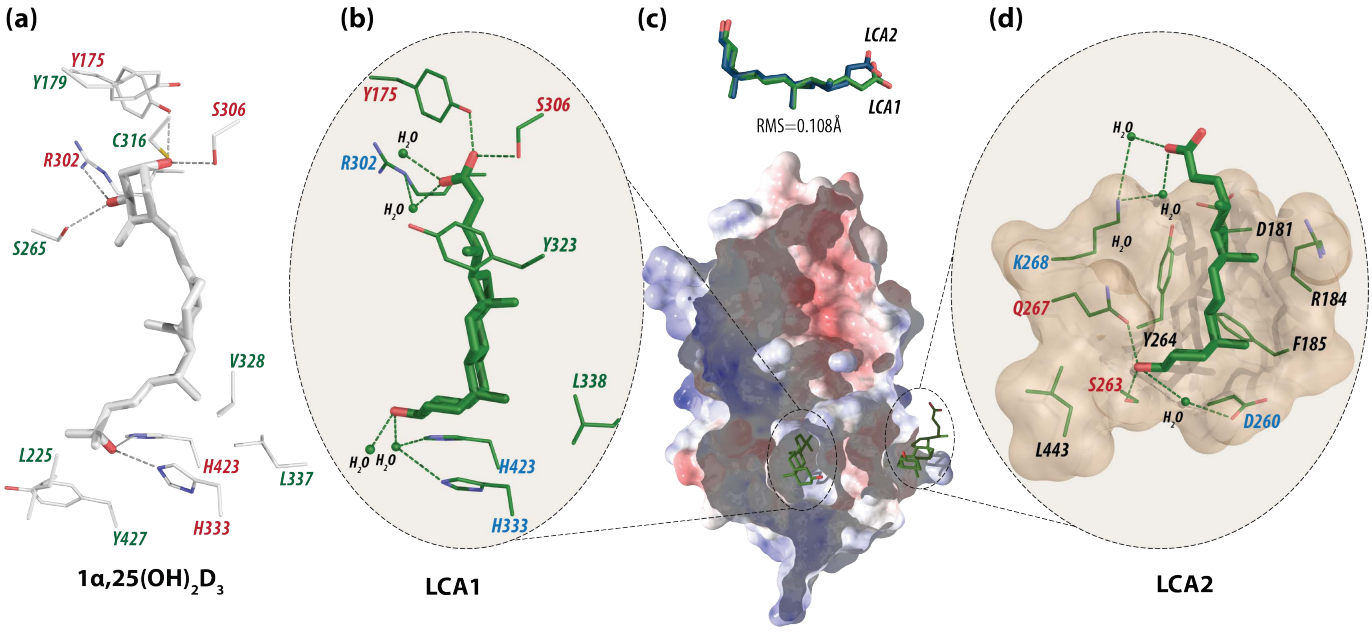


Figure 2. The unique interaction pattern of two bile acid molecules with the zebrafish VDR-LBD. The complex of **1α,25(OH)$_2$D$_3$** and two bile acid molecules with the zebrafish VDR-LBD are displayed with the surrounding interacting amino acid residues at 3.8 Å distance cutoff. a. **1α,25(OH)$_2$D$_3$**-bound zebrafish VDR (PDBID:2HC4). b. **LCA** binding inside the zebrafish VDR-LBD (PDBID:3W5P). c. The topology of the unique binding and the superimposition of the two bile acid molecules (PDBID:3W5P). d. **LCA** binding on the surface of the zebrafish VDR-LBD (PDBID:3W5P). The molecule inside of the LBD has conserved interactions compared to **LCA** in the rat VDR-LBD ortholog. The conserved anchoring amino acids in the **1α,25(OH)$_2$D$_3$** complex and well as in all bile acid complexes are highlighted in red, the water-mediated amino acid contacts in blue and the differing residues in green. For the sake of clarity some of the conserved interacting residues are not displayed. H-bonds are indicated by dashed lines. Important water molecules are displayed as small spheres. The illustration is based on the actual structural data and all bile acid-bound structures are shown in reference to that of **1α,25(OH)$_2$D$_3$** (**A**).

general toxicity, similar to 1α,25(OH)$_2$D$_3$, as they affected the weight gain of mice. It is noteworthy that mice receiving analog **29** (PRI-5201) showed no significant change in body weight when compared to vehicle-treated mice. The compounds of the first series (**24**, **25**, **26**, and **27**) bind with lower affinity to VDR than 1α,25(OH)$_2$D$_3$ (relative IC$_{50}$ ratio: 4, 37, 38, 3, respectively), but the VDR binding of the second series (**7**, **28**, **29**, and **30**) was better than that of 1α,25(OH)$_2$D$_3$ (relative IC$_{50}$ ratio: 414, 471, 194, 64, respectively). These compounds induce HL-60 cell differentiation more efficiently than 1α,25(OH)$_2$D$_3$.

6. Gemini-type vitamin D analogs

Double side-chain vitamin D analogs, termed 'Gemini', are structurally and functionally interesting compounds. In spite of their significantly increased volume (25%), they well fit into the VDR-LBD and bind the receptor with high affinity [38]. For most of the Gemini analogs that are based directly on the 1α,25(OH)$_2$D$_3$ backbone, two features, the 'diastereotopic' and the 'deutero' effects, have significant effects on their activity [39]. The diastereotopic effect concerns the topology of the side chain(s) orientation(s) at position C20. For example, 20*S*-epimer (threo) analogs, such as MC1288, show higher activity/stability compared to 20*R*-epimer (erythro) 1α,25(OH)$_2$D$_3$. Similarly, threo Gemini analogs are less calcemic and have higher transcriptional potency under many of the tested conditions. The so-called deuterium effect is the observation that the exchange of proto-methyl groups with their deutero substitutes in VDR analogs commonly increases their activity.

A number of Gemini analogs have been crystalized in complex with the zebrafish VDR-LBD and their diastereotopic and deuterium effects were investigated. Compared to 1α,25(OH)$_2$D$_3$, the accommodation of Gemini's second side chain induces a structural rearrangement where Cα atoms of some amino acids shift. In particular, movement of amino acid L309 (L337 in zebrafish, located in the H6-7 region) leads to an enlargement of the original L-shaped LBP to a Y-shape (Figure 4(a)) [10]. This is also the case for the other Gemini derivatives that had been solved in complex with zebrafish VDR-LBD, where amino acid L337 relocates accordingly [39–41]. All these Gemini compounds maintain comparable OH group anchoring interactions with the VDR-LBP, but for the 19-nor analogs 19-nor-threo-hexadeutero-Gemini (**32**) and 19-nor-erythro-hexadeutero-Gemini, there is no interaction with the amino acids L261, S265, and I299 (Figure 4(b)). The loss of the hydrophobic interaction with S265 may also account for the slightly lower biological activity of these compounds. Interestingly, in case of threo and erythro pairs, no preferred orientation for the proto- or deutero-methyl-containing chain has been noted. This suggests that VDR is unable to distinguish between the two methyl types [39]. The only difference seems to be the 0.3 Å shorter interaction of C26 of the ligands with the carbonyl group of amino acid A331 of the VDR-LBD for threo-hexadeutero-Gemini (**31**) (compared to erythro-hexadeutero-Gemini (**33**), Figures 4(c,d)).

7. Vitamin D analogs with modified CD-ring

9β-Methyl-19-nor-1α,25(OH)$_2$D$_3$ (**34**), 9α-methyl-1α,25(OH)$_2$D$_3$ (**35**), 9-methylene-19-nor-1α,25(OH)$_2$D$_3$ (**36**), 1α,25(OH)$_2$D$_3$-9-methylene-10(*S*),19-dihydrovitamin D$_3$ (**37**), 1α,25(OH)$_2$D$_3$-9-methylene-10(*R*),19-dihydrovitamin D$_3$ (**38**), and (9*E*)-9-ethylene-19-nor-1α,25(OH)$_2$D$_3$ (**39**) were synthesized by the

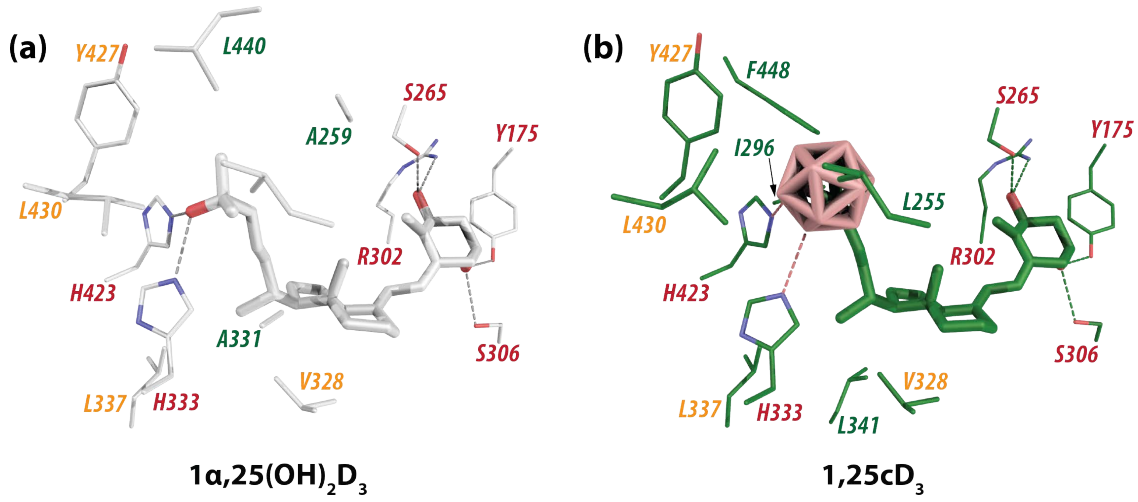


Figure 3. Recognition of analog with C24-*o*-carboranyl group bound to the zebrafish VDR-LBD. Details of the interactions mediated by the *o*-carboranyl group at C24 of **1α,25(OH)$_2$D$_3$** with residues of the zebrafish VDR-LBD at a 3.8 Å distance cutoff. a. **1α,25(OH)$_2$D$_3$**-bound zebrafish VDR-LBD (PDBID:2HC4). b. **1,24cD$_3$**-bound zebrafish VDR-LBD (PDBID:5E7V). Dihydrogen bonds between hydroboron groups and H333 and H423 are indicated by red dashed lines. The illustration is based on the actual structural data and **1,24cD$_3$**-bound structure is shown in reference to that of **1α,25(OH)$_2$D$_3$** (**A**).

teams of Mouriño and Sicinski [42]. The vitamin D-type trienes were introduced by thermal sigmatropic rearrangements on the corresponding pre-vitamin D intermediates, which in turn were accessed by the classical partial hydrogenation of dienynes or by a new Suzuki–Miyaura coupling method.

Competition binding assays revealed that the 9α-methyl group in **35** reduces the VDR binding affinity 200-fold compared to 1α,25(OH)$_2$D$_3$. Interestingly, the 19-nor-vitamin D compound **34** having a 9β-methyl substituent exhibits a VDR binding affinity similar to that of 1α,25(OH)$_2$D$_3$ (**34**, 50%; **35**, 0.5%; **36**, 0.5%; **37**, <0.001%; **38**, <0.001%; **39**, <0.001%). Compound **34** is also highly active in inducing differentiation and transcriptional activity in HL-60 cells, while compound **36** has a far weaker HL-60 cell differentiating potency compared to 1α,25(OH)$_2$D$_3$ (**34**, 20%; **35**, 0.05%; **36**, 0.1%; **37**, ~0.001%; **38**, ~0.001%; **39**, ~0.001%). With the notable exception of **34**, the compounds also exhibit, in reference to 1α,25(OH)$_2$D$_3$, low activities in activating the *CYP24A1* gene (**34**, 100%; **34**, 0.6%; **36**, 0.6%; **37**, ~0.001%; **38**, 0.3%, **39**: ~0.001%).

8. Vitamin D analogs with A-ring modifications

A novel synthetic approach to A-ring 19-nor-modified analogs has been reported by Fuchs et al. [43] and provided 19-nor-1α,25(OH)$_2$D$_3$ (**40**), 2α-hydroxymethyl-19-nor-1α,25(OH)$_2$D$_3$ (**41**), 2β-hydroxymethyl-19-nor-1α,25(OH)$_2$D$_3$ (**42**) and the corresponding cyclic phosphates **43**, **44, 45**, and **46**. Phosphate analogs were found to be less active than 1α,25(OH)$_2$D$_3$ in the human colon cancer cell lines Caco-2 and SW480-ADH [43].

Further structure–function relationships of A-ring-modified analogs have been reviewed by Glebocka and Chiellini [44].

9. 14-epi-Analogs with nonnatural polyene system

Kittaka et al. have synthesized by Pd-catalyzed methods the 14-epi-vitamin D analogs **47**–**59** possessing different polyene systems [45,46]. The VDR binding affinities in reference to 1α,25(OH)$_2$D$_3$ were **47**, 53%; **48**, 21%; **49**, 9%; **50**, 1%; **51**, 1%; **52**, 3%; **53**, 0.5%; **54**, 83%; **55**, 15%; **56**, 83%; **57**, 71%; **58**, 48%; and **59**, 4%. In dienynes **47**, **48**, and **49**, the methylene substitution improves binding to VDR. With the exception of **52**, the pre-vitamin D intermediates display low VDR binding affinity. Substitution at C2 of tachysterol analogs improved the affinity, and the methylene-substituted compound **58** was the most effective vitamin D analog.

Crystal structure analysis showed that compounds **57** and **58** fit well into the VDR-LBP when the C7,8 configuration is *s-trans* and the three OH groups overlap with those for 1α,25(OH)$_2$D$_3$. The C5-C8 linkers are successfully placed between amino acids S275 and W288 on one side and L233 on the other side, stabilizing the conformation of the ligand within the VDR-LBP.

Other vitamin D analogs with a nonnatural triene system (**34**–**39**) [42] were discussed above.

10. VDR ligand mimics

1α,25(OH)$_2$D$_3$ hybrids (**60**–**66**) possessing an aromatic ring linked to C7 have been synthesized by Peyrat et al. using a Negishi Pd-catalyzed method [47]. Compared to 1α,25(OH)$_2$D$_3$, the VDR affinities of the compounds were **60**, 13%; **61**, 23%; **62**, 9%; **63**, 12%; **64**, 74%; **65**, 30%; and **66**, 21%. Interestingly, the alkynyl analog **64** bearing CH$_2$OH groups at *meta*- and *para*-positions exhibits similar affinity for VDR as 1α,25(OH)$_2$D$_3$. In reference to 1α,25(OH)$_2$D$_3$, these analogs are quite weak in inducing HL-60 cell differentiation (**60**, 0.01%; **61**, 4%; **62**, 0.006%; **63**, 0.001%; **64**, 9%; **65**, 0.002%; and **66**, <0.001%).

Kagechika et al. [48] used bimolecular nucleophilic substitution in order to synthesize VDR agonists containing the hydrophobic 1,12-dicarba-*closo*-dodecaborane (*p*-carborane) unit. This carborane cage replaces the CD-rings of the natural hormone. However, compared to 1α,25(OH)$_2$D$_3$, the carborane-based VDR ligands show only very weak affinity for VDR (**68**, 0.008%; **69**, 0.001%). In reference to 1α,25(OH)$_2$D$_3$, these mimics are weakly active in inducing HL-60 cell differentiation (**67**, 5%; **68**, 8%; **69**, 2%; **70**, 0.05%; **71**, 0.001%). The racemic mixture of *S*- and *R*-isomers of compound **67** exhibits about 20% potency in HL-60 cell differentiation, i.e., its activity is comparable with 19-nor-1α,25(OH)$_2$D$_3$ [49]. Compared to the

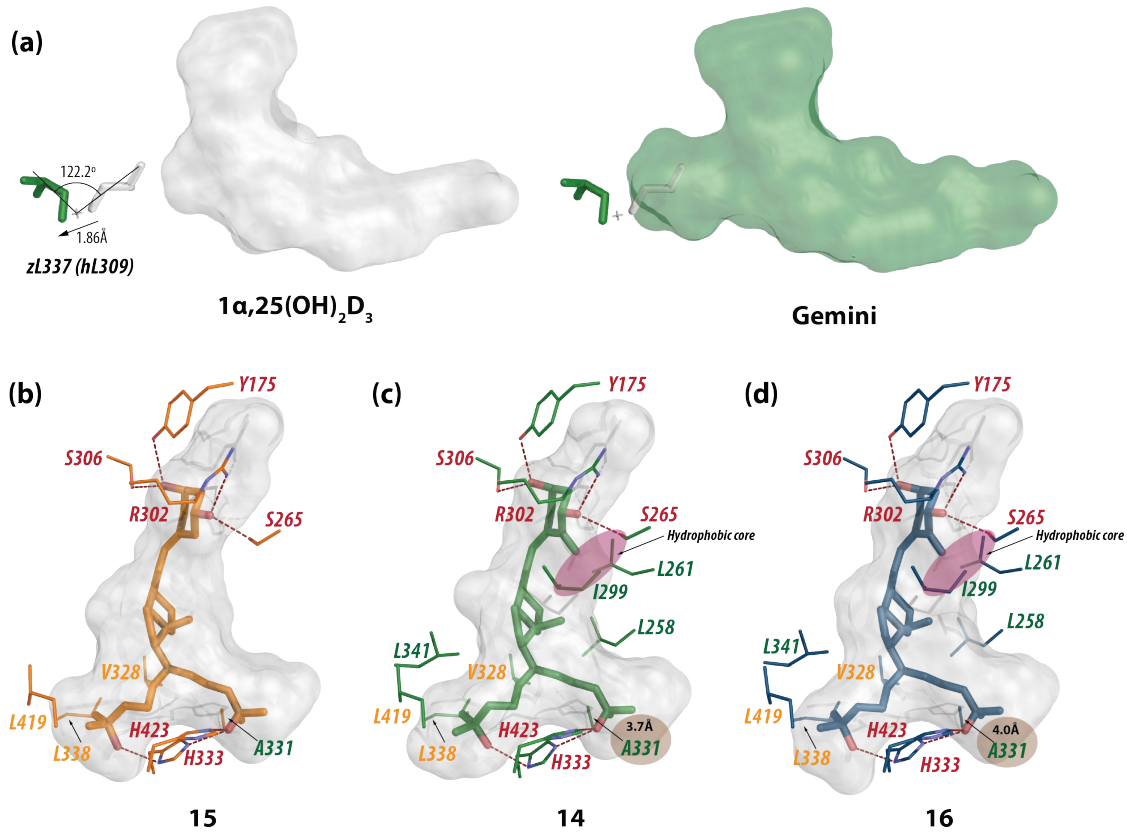


Figure 4. Diastereotopic and deuterium effects of Gemini analogues. The complexes of the zebrafish VDR-LBD with **1α,25(OH)$_2$D$_3$** and **Gemini** analogs are displayed with the surrounding interacting amino acid residues at a 3.8 Å distance cutoff. a. Location and flexibility of amino acid L337 that helps to accommodate the second side chain of **Gemini** analogs by adapting the shape of the LBPs. The **1α,25(OH)$_2$D$_3$**-bound zebrafish VDR-LBD (PDBID:2HC4) has a L-shaped LBP (grey), whereas the **Gemini**-bound (PDBID:2HCD) contains a Y-shaped LBP (green). b. Binding mode of **32** in the zebrafish VDR-LBD (PDBID:4IA1). c. Binding mode of **31** in in the zebrafish VDR-LBD (PDBID:4IA2). d. Binding mode of **33** in the zebrafish VDR-LBD (PDBID:4IA7). The hydrophobic interactions of amino acids L261, S265 and I299 create a small hydrophobic core that is present only in **31** and **33** but not in 19-nor analogs. The conserved anchoring points are shown in red. For the sake of clarity some of the conserved interacting residues are not displayed, other important ones are shown in orange color. H-bonds are indicated by red dashed lines. The illustration is based on the actual structural data and all **Gemini** analog-bound structures are shown in reference to that of **1α,25(OH)$_2$D$_3$**.

R-enantiomer, the *S*-isomer shows higher biological activity in cell differentiation assays as well as in competitive binding assays using ^{3}H-labeled 1α,25(OH)$_2$D$_3$ and the bovine thymus VDR-LBD (IC_{50} values of 0.64 and 4.1 μM).

For both isomers (*S*- and *R*-) of compounds **67**–**71**, the terminal primary OH group of the 1,3-diol corresponds to the 1α-OH group of 1α,25(OH)$_2$D$_3$ and the secondary OH group to the 3-OH group of the natural hormone. The location of the tertiary OH group in the aliphatic chain of the compounds is analogous to the 25-OH group of 1α,25(OH)$_2$D$_3$ (Figure 5). Crystal structure analyses of compounds **69** (Figure 5(b)) and **70** (Figure 5(c)) in complex with the rat VDR-LBD showed that the mimics adopt a position similar to 1α,25(OH)$_2$D$_3$ within the LBP, i.e., the anchoring interactions of the three OH groups are conserved in all structures. Although the carbon backbones with the terminal diols take different positions compared to 1α,25(OH)$_2$D$_3$, this difference allows the correct positioning of the primary and secondary OH groups for the conserved interactions. This demonstrates the flexible nature of the mimics and allows VDR to force their optimal conformation into its LBP.

Ciesielski et al. reported the structure–function analysis of the non-secosteroidal VDR ligands **72**–**78** that contain various aromatic units [50]. Compared to 1α,25(OH)$_2$D$_3$, these compounds are low-calcemic and induce potently transcriptional activity in HeLa human cervix adenocarcinoma cells (**72**, 200%; **73**, 500%; **74**, 5%; **77**, 120%; and **78**, 500%). These high transcriptional activities are a result of (1) enthalpic effects through additional and tighter intermolecular contacts and (2) entropic effects with a large contribution of solvation/desolvation.

11. New developments in vitamin D synthesis

Sikevar and Fuchs have reported a novel enantio-selective synthesis of vitamin D CD-rings [51]. The key step involves the formation of the *trans*-hydrandane alcohol **79** by an alcohol-directed intramolecular methylation of the enantiopure allyl sulfone **80** using $AlMe_3$.

A novel approach to the key A-ring aldehyde **82** from *(R)*-carvove has been reported by Chen and Ju [52]. Compound **83** was prepared by an ene-reaction on alcohol **87**. The strategy is of potential value for the synthesis of vitamin D analogs substituted at C2.

The Fuchs team [53] reported the synthesis of the new vitamin D analog **88** by a convergent approach that involves the methylation of the A-ring vinyl sulfone **90** and *in situ* trapping of the corresponding allyl sulfonyl anion with the allyl chloride **89**. TBAF-promoted elimination of the sulfone group and concomitant desilylation gives compound **88**. In comparison with 1α,25(OH)$_2$D$_3$, compound **88** was less active in Caco-2 cell lines at concentrations of 1 and 10 nM but

showed the same activity at 100 nM. It was also found to be twice as active as 1α,25(OH)$_2$D$_3$in the cell line SW480-ADH.

The synthesis of A-ring intermediates for the synthesis of vitamin D analogs has been reviewed. One review deals with the preparation of 1,7-enynes as precursors of A-ring synthons for the Trost Pd-catalyzed approach [54] and the other refers to the preparation of A-ring phosphine oxide for the Lythgoe and Hoffman–Roche Wittig–Horner approach [55].

12. Patents related to VDR ligands

The *Wisconsin Alumni Research Foundation* has claimed patents on 2MD (**91**) [56] second-generation compounds. CPA (**92**) [57], UW05 (**93**) [58], and MDBE20 (**94**) [59] are new 2MD-derivatives that show, in comparison to 1α,25(OH)$_2$D$_3$, the following properties: affinity for VDR (**91**, 35%; **92**, 1.5%; **93,** 300%; **94,** 270%), HL-60 cell differentiation (**91**, 1000%; **92**, 70%; **93**, 50%; **94**, 1%), transcriptional induction of the *CYP24A1* gene (**92**, 15%; **93**, 100%; **94**, 5%), intestinal calcium transport (**92**, 15%; **93**, 65%; **94**, 55%), and calcium mobilization (**92**, 74%; **93**, 75%; **94**, 45%).

92 exhibits relatively high VDR binding activity and pronounced activity in arresting the proliferation of undifferentiated HL-60 cells and induces their differentiation to monocytes, thus evidencing use as an anticancer agent, especially for the treatment or prevention of leukemia, colon cancer, breast cancer, skin cancer, or prostate cancer.

93 and **94** both exhibit relatively high transcriptional activity as well as pronounced activity in arresting the proliferation of undifferentiated cells and inducing their differentiation to monocytes, thus evidencing their use as anticancer agents and for the treatment of skin diseases, such as psoriasis, as well as skin conditions such as wrinkles, slack skin, dry skin, and insufficient sebum secretion. The compounds also show lower activity *in vivo* on bone calcium mobilization and have lower *in vivo* intestinal calcium transport activity as compared to 1α,25(OH)$_2$D$_3$. Therefore, the compounds might be used to treat autoimmune disorders or inflammatory diseases, secondary hyperparathyroidism, renal osteodystrophy, and obesity.

Cytochroma, Inc. and *John Hopkins University* have claimed a patent [60] on 1-deoxy prohormones of activated vitamin D$_3$ compounds, i.e., analogs of calcidiol [**95** (Ig), **96** (IIa) and **97** (IIci)] were designed as prodrugs to have one or more beneficial properties, such as selective inhibition of the enzyme CYP24A1, low calcemic activity, and antiproliferative activity.

Celus Pharmaceuticals, Inc., has claimed a patent on vitamin D analogs, such as Elocalcitol (**98**), [61] for the treatment of neurological disorders; they can be administered at therapeutic doses over prolonged periods of time without affecting calcium levels or bone structure. Elocalcitol shows antiproliferative and anti-inflammatory effects. Derivatives of elocalcitol have been developed for the treatment of neurological disorders that are associated with inflammatory events or activation of a subject's immune system.

Extended Biosciences, Inc., has a patent on vitamin D$_3$-ghrelin conjugates, i.e., vitamin D$_3$-PEG-NHS adducts [62]. This invention provides carriers that enhance the absorption, stability, half-life, duration of the effect, and potency of bioavailability of ghrelin peptides. The carriers compromise targeting groups that bind the vitamin D binding protein.

Nanjing University of Science and Technology published a patent on new vitamin D analogs [**99** (CN1) and **100** (CN2)] based on active steroidal natural marine compounds [63].

Finally, a spanish team (Universidade de Santiago, Universidade da Coruña, and Servicio Galego de Saúde) have claimed a patent on highly active and low-calcemic des-CD-1α,25(OH)$_2$D$_3$ analogs possessing an aromatic D-ring [**101** (PG1) and **102** (PG2)] [64]. The vitamin D triene system was synthesized under mild conditions by a Pd-catalyzed cyclization–Suzuki–Miyaura process.

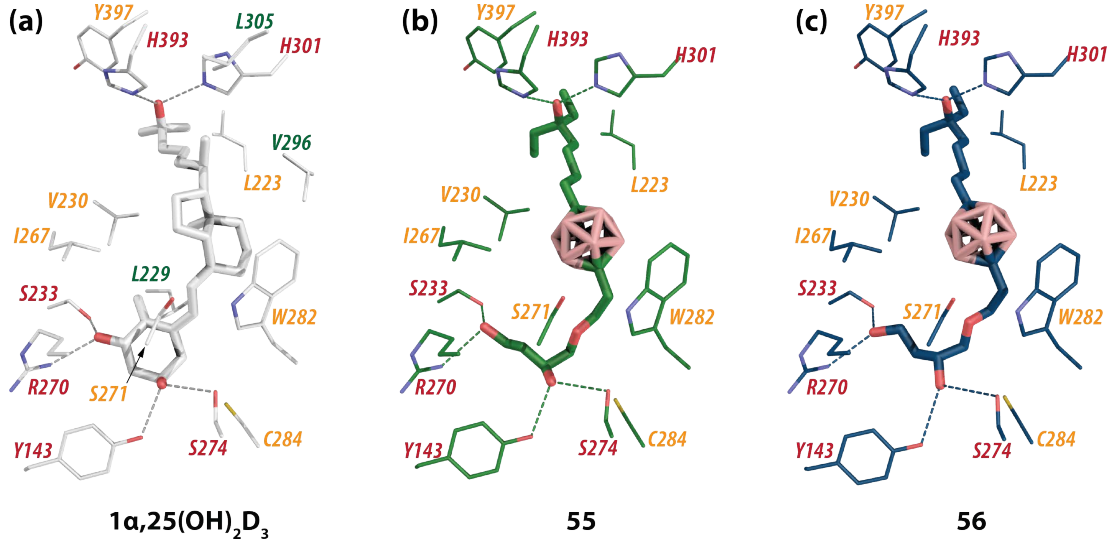


Figure 5. Recognition of mimics with an *o*-carborane center. Details of the interactions mediated by non-secosteroid analogs with a *p*-carboranyl group at the center are shown with residues of the zebrafish VDR-LBD at a 3.8 Å distance cutoff. a. **1α,25(OH)$_2$D$_3$** bound to the rat VDR-LBD (PDBID: 2ZLC). b. Analog **68** bound to the rat VDR-LBD (PDBID: 3VJS). **c.** Analog **69** bound to the rat VDR-LBD (PDBID: 3VJT). The conserved anchoring points in the **1α,25(OH)$_2$D$_3$** complex and in analogs **68** and **69** are shown in red. Conserved residues are annotated in orange and the differing residues in green. H-bonds are indicated by dashed lines. The illustration is based on the actual structural data and both *p*-carboranyl-bound structures are shown in reference to that of **1α,25(OH)$_2$D$_3$** (**A**).

13. Conclusion

The last 5 years showed a trend where various vitamin D chemists carried out more than just subtle modification of the natural hormone. The presence of the 25-OH group was considered essential, but recent advances in analog design led to molecules, such as the *o*-carborane analogs, that completely lack this OH group, but are still able to activate the VDR. In addition, it is now evident that it is possible to create analogs that completely lack A- and/or CD-rings, such as the *p*-carborane analogs, but still bind to VDR. Some of them show very high potency combined with very low *in vivo* calcemic effects. Thus, it is expected that the trend of creating nonsteroidal analogs and mimics will continue to rise in the near future.

14. Expert opinion

Although the structure of the VDR-LBD is already known since 16 years [26], every new VDR-LBD crystal structure provides some additional structural insight. More than a decade ago, it was assumed that the binding of a potent agonist should induce a significant structural change to the VDR-LBD, such as change of the position of helix H12 [65]. Moreover, it had been expected that the selective functional profile of some prominent vitamin D analogs can be observed on the level of significantly different VDR conformations [25]. However, all new structural data confirm that these expectations had been too high. In the case of adopted orphan nuclear receptors, such as peroxisome proliferator-activated receptor, we already described that only minor but important changes in the orientation of helix H12 [66] lead to a change in the co-factor interaction profile of the receptor. We can now confirm this observation also for the VDR, i.e., also for this endocrine member of the nuclear receptor superfamily, minor differences in the structure of the LBD are the basis of the selective functional profile of the different VDR agonists.

The detailed analysis of the additional novel crystal structures identified an interesting hydrophobic core region within helices H6 and H7 that is located on the opposite side of the VDR-LBD than helix H12. This region seems to sense the nature of the VDR ligand and thereafter increases or decreases its amino acid fluctuations. In case of Gemini analogs, the Cα atoms of some amino acids, such as L309, can simultaneously shift and rotate, allowing the opening of the additional space (up to 40%) in order to accommodate the second side chain of the analogs and to change the shape of the LBP from L- to Y-conformation. This region may serve as an allosteric effector in order to further stabilize helices H3, H4-5, H11, and 12, thereafter promoting the recruitment of co-activator proteins.

Since several years, the public awareness of the health impact of the natural compound vitamin D_3 has been experiencing a clear boost [67]. This response is due to increasing evidences that a sufficient vitamin D status is critical for the prevention of a number of non-musculoskeletal diseases, such as cancer, diabetes, and multiple sclerosis [68]. While in the healthy status, for prevention of disease, the vitamin D index of each human individual should be determined and personalized regimes of supplementation with the natural prohormone vitamin D_3 are advisable [69], in the case of disease, the use of more selective and potent synthetic vitamin D analogs is needed. Nevertheless, we were surprised about the large number of novel vitamin D analogs that were published and/or patented within the last few years. The most remarkable strategy for new vitamin D analogs is the introduction of carborane groups as replacement of the secosteroid structure as well as of the side chain. Thus, VDR agonists stay an active field research both in academia and industry.

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Declaration of interest

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