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Endogenously produced nonclassical vitamin D hydroxy-metabolites act as “biased” agonists on VDR and inverse agonists on ROR α and ROR γ

Andrzej T. Slominski^{a,b,c,*}, Tae-Kang Kim^a, Judith V. Hobrath^d, Allen S.W. Oak^a, Edith K. Y. Tang^f, Elaine W. Tieu^f, Wei Li^e, Robert C. Tuckey^f, Anton M. Jetten^g

^aDepartment of Dermatology, USA

^bComprehensive Cancer Center, Cancer Chemoprevention Program, University of Alabama at Birmingham, USA

^cPathology and Laboratory Medicine Service, VA Medical Center, Birmingham, AL 35249, USA

^dDrug Discovery Unit, College of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom

^eDepartment of Pharmaceutical Sciences University of Tennessee HSC, Memphis, TN 38163, USA

^fSchool of Chemistry and Biochemistry, University of Western Australia, Crawley, WA, Australia

^gCell Biology Section, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA

*Corresponding author:

Andrzej T. Slominski, MD, PhD, Department of Dermatology, University of Alabama at Birmingham, Birmingham, AL 35249. USA;
e-mail: aslominski@uabmc.edu; phone: 205.934.5245

Conflict of Interest:

The authors declare no conflict of interest

Highlights

- CYP11A1- derived hydroxyvitamin D derivatives are present in human skin and serum
- CYP11A1- derived hydroxyvitamin D derivatives are biologically active
- CYP11A1- derived hydroxyvitamin D derivatives act as partial/biased agonists on VDR
- CYP11A1- derived hydroxyvitamin D derivatives as “inverse” agonists on ROR α and ROR γ
- 20(OH)D₃, 20(OH)D₂ and 20,23(OH)₂D₃ are noncalcemic at pharmacological doses

Abstract

The classical pathway of vitamin D activation follows the sequence $D3 \rightarrow 25(OH)D3 \rightarrow 1,25(OH)_2D3$ with the final product acting on the receptor for vitamin D (VDR). An alternative pathway can be started by the action of CYP11A1 on the side chain of D3, primarily producing 20(OH)D3, 22(OH)D3, 20,23(OH)₂D3, 20,22(OH)₂D3 and 17,20,23(OH)₃D3. Some of these metabolites are hydroxylated by CYP27B1 at C1 α , by CYP24A1 at C24 and C25, and by CYP27A1 at C25 and C26. The products of these pathways are biologically active. In the epidermis and/or serum or adrenals we detected 20(OH)D3, 22(OH)D3, 20,22(OH)₂D3, 20,23(OH)₃D3, 17,20,23(OH)₃D3, 1,20(OH)₂D3, 1,20,23(OH)₃D3, 1,20,22(OH)₃D3, 20,24(OH)₂D3, 1,20,24(OH)₃D3, 20,25(OH)₂D3, 1,20,25(OH)₃D3, 20,26(OH)₂D3 and 1,20,26(OH)₃D3. 20(OH)D3 and 20,23(OH)₂D3 are non-calcemic, while the addition of an OH at C1 α confers some calcemic activity. Molecular modeling and functional assays show that the major products of the pathway can act as “biased” agonists for the VDR with high docking scores to the ligand binding domain (LBD), but lower than that of 1,25(OH)₂D3. Importantly, cell based functional receptor studies and molecular modeling have identified the novel secosteroids as inverse agonists of both ROR α and ROR γ receptors. Specifically, they have high docking scores using crystal structures of ROR α and ROR γ LBDs. Furthermore, 20(OH)D3 and 20,23(OH)₂D3 have been tested in cell model that expresses a Tet-on ROR α or ROR γ vector and a RORE-LUC reporter (ROR-responsive element), and in a mammalian 2-hybrid model that test interactions between an LBD-interacting LXXLL-peptide and the LBD of ROR α/γ . These assays demonstrated that the novel secosteroids have ROR-antagonist activities that were further confirmed by the inhibition of *IL17* promoter activity in cells overexpressing ROR α/γ . In conclusion, endogenously produced novel D3 hydroxy-derivatives can act both as “biased” agonists of the VDR and/or inverse agonists of ROR α/γ . We suggest that the identification of large number of endogenously produced alternative hydroxy-metabolites of D3 that are biologically active, and of possible alternative receptors, may offer an explanation for the pleiotropic and diverse activities of vitamin D, previously assigned solely to 1,25(OH)₂D3 and VDR.

Key words

Hydroxyvitamin D, CYP11A1, ROR α , ROR γ , VDR, 1,25D3-MARRS

1. Introduction:

1.1. Classical pathway of vitamin D activation

Vitamin D is generated from the photochemical transformation of 7-dehydrocholesterol (7DHC) that requires UVB energy ($\lambda=280-320$ nm) and represents the most fundamental reaction in photobiology, not requiring any enzyme [1-3]. After exposure to UVB, the B ring of 7DHC absorbs the electromagnetic energy leading to the breakage of the C9-C10 bond, opening the B-ring and thereby producing previtamin D3. The latter subsequently undergoes thermal isomerization to form D3, or with high doses of UVB produces lumisterol (L3) and tachysterol (T3) [1-4]. These reactions are dependent on the temperature and the UVB dose and are reversible.

It is well established that D3 can be activated by two sequential hydroxylations, the first at C25 (catalyzed by CYP2R1 and CYP27A1) and the second at C1 α (catalyzed by CYP 27B1) to generate biologically active 1,25(OH)₂D3 as a final product [4-7]. In addition, circulating 25(OH)D3 can be activated in target tissues by ubiquitously expressed CYP27B1 (reviewed in [6, 8, 9]). 1,25(OH)₂D3 is inactivated by CYP24A1 which initially hydroxylates it at C24 then catalyzes subsequent oxidations leading to shortening of side chain and the production calcitric acid [10-13].

1.2. Biological activity of 1,25(OH)₂D3

In addition to regulating calcium homeostasis, 1,25(OH)₂D3 displays a variety pleiotropic activities, which include inhibition of proliferation and stimulation of the differentiation program in cells of different lineage, anticancerogenic effects, and enhancement of innate, and attenuation of adaptive immune activities and inflammation [4, 8, 14-17]. Its effects are mediated via the vitamin D receptor (VDR), which after agonist activation and dimer formation with RXR binds to the VDR responsive element (VDRE) to influence expression of responsive genes [14, 17-19].

In the skin, 1,25(OH)₂D3 plays an important role in the regulation of skin barrier functions and in the regulation of hair follicle growth and cycling, and has anti-cancerogenic, anti-proliferative and anti-inflammatory effects [3, 17, 20]. Most recently, it was reported that it can inhibit skin cell death and DNA damage induced by exposure to UVR [20-23]. Because of the toxic effect secondary to calcemia, the pharmacological use of 1,25(OH)₂D3 is limited.

1.3 Analogs of 1,25(OH)₂D3 with reduced calcemic activity

Many analogs of 1,25(OH)₂D3 have been chemically synthesized with the aim of reducing calcemic activity, without the loss of therapeutically useful anticancer activities (reviewed in [14, 24-27] or immunoregulatory properties (reviewed in [28, 29]). Modification of the A-ring, CD ring and side chain have all produced analogs with reduced calcemic activity. Key changes include replacement of the C1 α -hydroxyl group with a 1 β -CH₂OH, C3-epimerization, removal of C19, epimerization at C20, addition of a second side chain at C20 (Gemini analogs), insertion of a double bond at C16 and a triple bond at C23, and insertion of an oxygen in place of C22. Many side chain modifications between C22 and C26 have also been aimed at reducing metabolism by CYP24A1 rather than reducing calcemic activity. The effects of isomerization of the two hydroxyl groups in the A-ring of 1,25(OH)₂D3 was reported by Fleet et al [30], and is of particular interest since one of the resulting diastereomers, 3-epi-25-dihydroxyvitamin D3, is a natural metabolite of 1,25(OH)₂D3. 1,25(OH)₂D3 (where hydroxyl groups are 1 α and 3 β) was compared to 1 β ,3 β ; 1 α ,3 α and 1 β ,3 α diastereomers. The 1 α ,3 α isomer (3-epi-1,25(OH)₂D3) is produced *in vivo* by the action of vitamin D 3-epimerase on 1,25(OH)₂D3 [31-33]. All three of these diastereomers showed reduced binding to the VDR with binding strength only partially correlating with their ability to stimulate calcium transport. The 3-epi-1,25(OH)₂D3 diastereomer stimulated calcium transport in excess of its relative ability to bind to the VDR. Other studies on 3-epi-1,25(OH)₂D3 indicate that its reduced binding to the VDR does generally correlates with its reduced biological activity [30, 32, 34, 35], but there are notable exceptions such as the maintenance of the ability to suppress

parathyroid hormone secretion by cultured parathyroid cells and enhancement of the ability to stimulate HL-60 cell apoptosis, relative to 1,25(OH)₂D₃ [36, 37].

While several of the low-calcemic synthetic analogs discussed above show some promise for the treatment of hyperproliferative and immunological disorders, hypercalcemia resulting from long-term high therapeutic doses remains a significant problem [24, 26, 29]. The studies with synthetic analogs also illustrate the possibility of designing specific analogs for specific therapeutic applications. It is well established that 1 α -hydroxylation of the A-ring of 25(OH) D₃ dramatically enhances its binding to the VDR and its calcemic activity. However, until our studies on CYP11A1-derived secosteroids that lack the 1 α -hydroxyl group (described below) little was done to explore the possibility that active metabolites might be synthesized that lack the 1 α -hydroxyl group (or equivalent), as it was generally thought to be indispensable for tight binding to the VDR.

1.4. New challenges in vitamin D biology.

The consensus conveyed by the majority of the literature is that all biologically relevant phenotypic effects of D₃ can be assigned to one molecule, 1,25(OH)₂D₃, and one receptor, VDR [3, 15, 17, 38]. This makes both 1,25(OH)₂D₃ and VDR a bioregulatory couple, which would regulate vastly unrelated or sometime contradictory effects, which is highly unusual for endogenous ligands and their respective receptor. The existence of an alternative membrane bound receptor for 1,25(OH)₂D₃, e.g., 1,25D₃-membrane-associated, rapid response steroid-binding protein (1,25D₃-MARRS), has been proposed by some authors [39, 40]. This review, supplemented by new data and molecular modelling, will offer an additional explanation for the pleiotropic phenotypic effects of D₃ by identifying both a family of novel bioactive D₃ hydroxy-derivatives and the retinoic acid-related orphan receptors (RORs) α and γ , which function as alternative nuclear receptors for these compounds in addition to the VDR.

2. New pathways of vitamin D activation.

Until recently, the traditional role of CYP11A1 was believed to be to initiate steroid synthesis, solely in steroidogenic organs using cholesterol as the substrate. This involved hydroxylations at C22 and C20 followed by oxidative cleavage of the bond between C20 and C22 to produce pregnenolone, a precursor to all steroids [41, 42]. However, the expression of CYP11A1 in peripheral tissues, albeit at low levels, has now been documented [43] and alternative substrates to cholesterol have been identified experimentally, such as 7DHC, vitamins D₂ and D₃, ergosterol and lumisterol [44-49]. Additionally, the possibility of other sterol/secosteroidal compounds serving as substrates has been predicted theoretically based on molecular modeling [50].

2.1. Hydroxylation of side chain of vitamin D by CYP11A1

An assumed concept that vitamins D_{3/2} are only activated through 25- and 1-hydroxylations [D_{3/2}→25(OH)D_{3/2}→1,25(OH)₂D_{3/2}] has been challenged by recent findings of CYP11A1-initiated metabolism of vitamin D: D₃→ **20(OH)D₃** + 22(OH)D₃ + 17(OH)D₃→(OH)_nD₃ and D₂→20(OH)D₂→17,20(OH)₂D₂→(OH)_nD₂ [47, 48, 51-61]. The main metabolite resulting from a single hydroxylation of D₃ by CYP11A1 is 20(OH)D₃, with 22(OH)D₃ and 17(OH)D₃ also being produced [47, 51, 61]. In the major pathway, 20(OH)D₃ is further hydroxylated by CYP11A1 to 20,23(OH)₂D₃, 20,22(OH)₂D₃, 17,20(OH)₂D₃ and 17,20,23(OH)₃D₃ [47, 51]. In addition the prodrug, 1 α (OH)D₃, is metabolized by CYP11A1 to 1,20(OH)₂D₃ [62] and 1,20,23(OH)₃D₃ (unpublished observation). A full list of the D₃ hydroxymetabolites produced by CYP11A1 and their stereochemistry is presented in Table 1.

2.2. Modification of CYP11A1 derived D₃-hydroxymetabolites by CYP27B1, CYP27A1, CYP24A1 and CYP3A4.

20(OH)D₃, 22(OH)D₃, 20,22(OH)₂D₃ and 20,23(OH)₂D₃ can be hydroxylated by CYP27B1 at C1 α to the corresponding di- or trihydroxyderivatives (Table 1) [54, 56, 58, 63, 64]. The main product of D₃ metabolism initiated by CYP11A1, 20(OH)D₃, can be hydroxylated on the side chain by CYP27A1, CYP24A1 and CYP3A4 to 20,24(OH)₂D₃, 20,25(OH)₂D₃ and/or 20,26(OH)₂D₃, which

are further 1α hydroxylated by CYP27B1 to the corresponding trihydroxyderivatives (Table 1)[55-57, 64-66]. In addition 20,23(OH)₂D3 is hydroxylated by CYP24A1 to 20,23,24(OH)₃D3 and 20,23,25(OH)₃D3 [55].

While originally produced by enzymatic synthesis in amounts enabling identification, chemical routes of synthesis for 20(OH)D3, 20,23(OH)₂D3 and 20,24(OH)₂D3 have now been established [64, 65, 67-69] which also confirmed that the enzymatically produced 20(OH)D3 is the 20*S* enantiomer[67]

2.3. *In vivo* production of novel D3-hydroxyderivatives

Many of the vitamin D3 hydroxymetabolites listed in Table 1, including 20(OH)D3, 22(OH)D3, 20,23(OH)₂D3, 20,22(OH)₂D3, 1,20(OH)₂D3, 1,20,23(OH)₃D3, and 17,20,23(OH)₃D3 can be made by epidermal keratinocytes, adrenals, placenta, or colorectal adenocarcinoma Caco-2 cells, tissues and cells known to express CYP11A1, following incubation with D3 [58, 70]. Production of 20(OH)D3, 22(OH)D3, 20,23(OH)₂D3, 20,22(OH)₂D3, 1,20(OH)₂D3 also occurs in human dermal fibroblasts incubated with D3 substrate [71]. Similarly, production of 20(OH)D2, 17,20(OH)₂D2 and 1,20(OH)₂D2 was observed in human placentas *ex-utero*, adrenal glands *ex-vivo*, and isolated human epidermal keratinocytes and colorectal Caco-2 cells incubated with D2 [59].

The final proof of the *in vivo* production of these CYP11A1-derived hydroxyderivatives is provided by our most recent report of the detection of not only 25(OH)D3 and 1,25(OH)₂D3, but also of 20(OH)D3, 22(OH)D3, 20,22(OH)₂D3, 20,23(OH)₂D3, 20,24(OH)₂D3, 20,25(OH)₂D3, 20,26(OH)₂D3, 1,20,23(OH)₃D3 and 17,20,23(OH)₃D3 in extracts of the human serum and epidermis, and of pig adrenals, with 1,20(OH)₂D3 being detected solely in the epidermis [72]. In addition, we detected 1,20,24(OH)₃D3, 1,20,25(OH)₃D3 and 1,20,26(OH)₃D3 in extracts of pig adrenal gland [72]. Interestingly, the serum concentrations of 20(OH)D3 and 22(OH)D3 were, respectively, 30- and 15-times lower than 25(OH)D3, but in the nM range consistent with them displaying biological activity [72]. Contingent upon firmly establishing a physiological role for these secosteroids, future monitoring of human serum samples for CYP11A1-derived D3-hydroxymetabolites may be warranted.

Detection of novel D3 hydroxymetabolites in human serum and epidermis poses new challenges in the field in relation to the biochemistry of the pathway and the attendant physiological consequences. Thus, a relationship between vitamin D levels and production of these compounds in the context of environmental factors and routes of delivery, and in a tissue/organ context, remains to be investigated. For example, since 25(OH)D3 is not metabolized by CYP11A1 [47], we can predict that oral intake of D3 will have a minor effect on the production of the novel hydroxyderivatives. However, D3 derived from skin or delivered through parenteral routes would be metabolized by tissues expressing high levels of CYP11A1 such as adrenals, gonads and placenta, with likely systemic effects. These important considerations should set a background for extensive future investigations.

3. RORs (retinoic acid-related orphan receptors), an overview

There are three members of the ROR subfamily of nuclear receptors, ROR α - γ (NR1F1-3) [73, 74]. The ROR transcription factors exhibit a domain structure containing an N-terminal domain, a highly conserved DNA-binding domain (DBD) with two C2-C2 zinc finger motifs, a ligand-binding domain (LBD), and a hinge domain between the DBD and LBD. Transcription regulation by RORs is mediated through monomeric interaction with ROREs (ROR response elements) in the regulatory regions of target genes [73, 75]. RORs are important players in the regulation of many physiological processes. They exhibit critical functions in embryonic development, differentiation, and in many immune and metabolic pathways in the adult [73, 74]. RORs have been implicated in a number of pathologies, including cancer, (auto)immune disease and metabolic syndrome.

Evidence has accumulated demonstrating that RORs act as ligand-dependent transcription factors [74, 76-78]. Cholesterol and cholesterol sulfate and small molecules can interact with the LBD of RORs to modulate their transcriptional activities [79]. Intermediates of the pathway of cholesterol biosynthesis can also act as endogenous agonists of ROR γ [80, 81], whereas vitamin D derivatives such as 20(OH)D3/2 exhibit ROR α and ROR γ inverse agonist activity as their binding to the receptor reduces the basal

activity of the receptor resulting in the opposite pharmacological responses to the agonist [81-83]. Therefore, ROR ligands may be of value in the development of novel therapeutic strategies for treatment of different inflammatory and metabolic disorders, and neuropsychiatric diseases and cancer, in which RORs are implicated.

4. Biological activity of CYP11A1-derived vitamin D hydroxyderivatives

4.1. An overview of biological activity

The biological activity of 20S(OH)D3 in the skin was the subject of a recent review [70], therefore the description below is brief. 20(OH)D3 and its hydroxymetabolites exert prodifferentiation, antiproliferative, and antiinflammatory activities on skin cells, comparable or better than that of 1,25(OH)₂D3 [53, 64, 65, 67, 70, 83-92]. 20(OH)D3 shows antifibrotic properties both *in vitro* [87-89] and in an *in vivo* mouse model of bleomycin induced scleroderma [89]. Most recently it was shown that both 20(OH)D3 and 20,23(OH)₂D3 enhance defense mechanisms against UVB-induced oxidative stress and DNA damage in cultured human keratinocytes [23] and murine skin *in vivo* [22]. 20(OH)D3 and its hydroxymetabolites also show anti-cancer properties that are cell-lineage dependent [53, 54, 64, 68, 69, 88, 90, 92-97].

We have now tested the effects of 20,25(OH)₂D3, 1,20,25(OH)₃D3, 20,26(OH)₂D3, 1,20,26(OH)₃D3, 20,24(OH)₂D3, and 1,20,24(OH)₃D3 on keratinocyte proliferation and found that they display similar potency (IC₅₀) and efficacy (maximal inhibition) to 1,25(OH)₂D3, both in the presence and absence of the 1 α -hydroxyl group, on the inhibition of cell proliferation (Fig. 1).

4.2. CYP11A1-derived D3 hydroxymetabolites are non-calcemic

20S(OH)D3 is noncalcemic at pharmacological doses of 3 μ g/kg in rats that is in contrast to 1,25(OH)₂D3 and 25(OH)D3 [95]. However, C1 α hydroxylation confers limited calcemic activity, similar to that of 25(OH)D3 but still less than for 1,25(OH)₂D3 [95]. 20S(OH)D3 and its chemically synthesized epimer 20R(OH)D3 are also noncalcemic at extremely high doses of 30-60 μ g/kg in C57BL/6 mice, and do not show any signs of toxicity in serum chemistry, liver and kidney functions, and blood morphology or histopathology of the heart, liver, spleen and kidneys [68, 93]. In addition, 20,23(OH)₂D3 is noncalcemic at 3 μ g/kg in C57BL/6 mice [89] and 20(OH)D2 is noncalcemic at 4 μ g/kg in rats, again showing a lack of histopathological signs of kidney or heart damage [53]. Therefore, we suggest that 20(OH)D3, 20(OH)D2 and 20,23(OH)₂D3 can serve as therapeutic or adjuvant agents.

5. Receptors for CYP11A1-derived D3 hydroxymetabolites

5.1. CYP11A1-derived D3 hydroxymetabolites act as “partial/biased” VDR agonists

Our previous studies have documented that 20S(OH)D3 and 20,23(OH)₂D3 can act as “biased” agonists on the VDR (discussed in [70]). They may also be termed biased agonists, a term now commonly applied to some ligands for G-protein coupled receptors which are functionally selective (biased) for certain response pathways from a particular receptor[98,99]. The involvement of VDR in the regulation of differentiation, proliferation and immune functions of keratinocytes was demonstrated by experiments showing that silencing of the gene encoding the VDR significantly inhibits the phenotypic effects of either 20(OH)D3/2 or 20,23(OH)₂D3 [53, 84-86]. This was further substantiated by the ability of CYP11A1-derived D3 hydroxymetabolites to translocate VDR to the nucleus with high affinity [53, 61, 92]. However, they differ substantially from 1,25(OH)₂D3 in that these metabolites lack calcemic activity (as described above), act as very poor activators of CYP24A1 expression [53, 84, 86, 95] and have very poor activity on a synthetic VDRE promoter construct that was however improved by hydroxylation in position C1 α [64, 65].

The above findings led us to further test a number of the CYP11A1-derived secosteroids using the commercially available LanthaScreen TR-FRET Vitamin D receptor Coactivator kit assay (Fig. 2). The assay showed that only the 1 α (OH) derivatives of CYP11A1-derived D3 hydroxymetabolites increase the affinity of the co-activator peptide to the VDR-LBD suggesting that the conformational change induced by the secosteroids lacking a 1 α (OH) is different or less than that induced by 1,25(OH)₂D3. Therefore

we carried out ligand-induced VDR translocation assays using VDR-GFP (Fig. 3), which showed that CYP11A1-derived hydroxyderivatives, both with and without the $1\alpha(\text{OH})$ group, cause translocation of the VDR to the nucleus with high potency, however potency was greater with the $1\alpha(\text{OH})$ group present. This is in agreement with previous data demonstrating the high efficiency of D3 hydroxyderivatives with a full-length side chain for stimulating VDR translocation, with higher potency being seen for the $1\alpha(\text{OH})$ derivatives and is also consistent with our molecular modeling studies [92]. Thus, our conclusion that novel CYP11A1-derived D3 hydroxymetabolites act as partial and/or ‘biased’ agonists on the VDR has been correct, however, the nature of the interaction with the receptor requires further analyses with *in silico* modeling being presented below.

5.1.1. Molecular modeling utilizing the VDR crystal structure.

The effects of ligand – VDR interaction can translate into structural changes of functionally relevant regions of the VDR remote to the ligand binding site. To understand better the structural basis of the binding of hydroxyvitamin D3 metabolites to the VDR we performed docking studies into the genomic and non-genomic pockets in the VDR crystal structure (PDB code 1DB1).

5.1.1.1. Docking results utilizing the genomic site of VDR.

After preparation of the VDR crystal structure (PDB code 1DB1), the series of hydroxyl-vitamin D3 metabolites listed in Table 2 were docked into the genomic site of VDR using the Glide XP docking method [84] (Schrödinger software), as detailed in the Supplemental file. Table 2 lists Glide XP docking scores of the resulting poses, which are a measure of the predicted interaction energy between the docked ligand and the receptor. Glide XP scores in the series are generally slightly less favorable compared to that of $1,25(\text{OH})_2\text{D}_3$, with five exceptions showing only slightly better scores (Table 2). As expected, docked poses of the hydroxyl-D3 series approximately overlap with the co-crystallized $1,25(\text{OH})_2\text{D}_3$ (Fig. 4A). Docked poses show excellent complementarity between non-polar ligand atom groups and the large hydrophobic surface of the pocket. Interactions formed by analogs in this series are schematically summarized in Figure 4B. While $23(\text{OH})$ and $24(\text{OH})$ groups may interact with either His305 or His397 in the series, hydroxyl groups in other positions show preference for one of the two histidines. Hydrogen bonding interactions formed by $1\alpha(\text{OH})$ and $3(\text{OH})$ groups are analogous to those formed by the co-crystallized ligand, $1\alpha,25(\text{OH})_2\text{D}_3$. Compounds containing a $1\alpha(\text{OH})$ group tend to score more favorably than the corresponding analogs lacking this group due to the additional hydrogen bonding of the $1\alpha(\text{OH})$ group with Arg274 and Ser237. For example, Figure 5A illustrates the docked pose of $1\alpha,20S(\text{OH})_2\text{D}_3$, in comparison with the structure of the co-crystallized ligand in VDR (PDB code 1DB1). Figure 5B shows polar interactions predicted by two possible docked poses of $1\alpha,20S,23R(\text{OH})_3\text{D}_3$ that mainly differ in the interactions formed with the histidine residues 305 and 397. One of the poses is very similar to that obtained for the parent $20S,23R(\text{OH})_2\text{D}_3$ lacking the $1\alpha(\text{OH})$ group.

Docked poses of D3 and $17(\text{OH})\text{D}_3$ differ from those of other secosteroids in having only a single anchor, the $3(\text{OH})$ group, to participate in hydrogen bonding interaction, while non-specific, hydrophobic contacts constitute the rest of the interactions with VDR. These poses likely would not represent stable, populated conformers or true bound structures although they can be accommodated in the binding site. Compared to D3, alfacalcidol ($1\alpha(\text{OH})\text{D}_3$) has an additional hydrogen bonding anchor, the $1\alpha(\text{OH})$ group and it is a known VDR partial agonist. While calcitric acid has a lower docking score consistent with low affinity binding, its docked pose suggests that in addition to hydrogen bonding through $1\alpha(\text{OH})$ and $3(\text{OH})$ groups it may also form water-mediated polar interactions with His305/His397, and thus may potentially bind to the VDR.

Docking results predict specific ligand – receptor interactions, the effects of which can propagate from local to more distant regions playing a role in modulating VDR functional activity. Hydrogen/Deuterium exchange (HDX) studies demonstrated that ligand interactions in the VDR genomic pocket modulates the flexibility/dynamics of functionally relevant regions in VDR including helix H12, crucial for cofactor interactions, as well as the VDR – retinoid X receptor α (RXR α) heterodimer interface [85]. Further regions remote to the binding site may show altered kinetics as well. A molecular dynamic simulation study of VDR with bound $1,25(\text{OH})_2\text{D}_3$

and an antagonist demonstrated distinct effects of these ligands on stabilizing the conformation of key residues in the ligand binding domain of VDR [86]. HDX analysis provides evidence that upon binding of $1\alpha(\text{OH})\text{D}_3$ (alfacalcidol) that lacks a $25(\text{OH})$ group, helix H12 remains highly dynamic [85]. The lack of the $25(\text{OH})$ is likely essential for the partial agonist activity of this D_3 analog. The agonist $1,25(\text{OH})_2\text{D}_3$ co-crystallized in the VDR structure forms hydrogen bonding interaction with His305 through its $25(\text{OH})$ group. While most analogs in Table 2 interact with His305, it is unclear whether analogs that may bind but lack this interaction would have any functional effects on VDR. Further, C24 enantiomers of the top scoring compound, $1\alpha,24,25(\text{OH})_3\text{D}_3$, as well as enantiomers of its analog, $24,25(\text{OH})_2\text{D}_3$, are predicted to form hydrogen bonding interactions with both His305 and His397. While docking suggests that these analogs can bind VDR in the genomic site, whether simultaneous interaction with both histidines would result in VDR activation or not is presently not known.

The presented docking results compare predicted poses of the new analog series with the binding mode of the co-crystallized agonist $1,25(\text{OH})_2\text{D}_3$. Docked ligand poses and their Glide XP scores (where the more negative the score, the more favorable the binding) suggest that compounds in Table 2 can bind VDR in the genomic pocket analogously to the co-crystallized ligand except for D_3 and $17(\text{OH})\text{D}_3$. Non-polar contacts are important for the binding of this series. Analogs containing a $1\alpha(\text{OH})$ group bind more favorably than their counterparts lacking this group. Docked poses predict interactions common within this series, as well as distinct interactions with His305/His397.

5.1.1.2. Docking results utilizing the non-genomic site of VDR.

Since VDR is also present in the membrane caveolae and has an alternative $1,25(\text{OH})_2\text{D}_3$ binding A-pocket where occupation induces non-genomic rapid responses [100-103], we included this site in the analysis. Tyr295 in the non-genomic site of the VDR crystal structure (PDB code 1DB1) penetrates deep into the pocket region and interferes with ligand binding. The side chain of Arg158 is also in a conformation that is not compatible with accommodating ligands into this site. Therefore, prior to docking the rotamer conformations of Tyr295 and Arg158 were changed and the structure was relaxed with the known non-genomic agonist, $1\alpha,25$ -hydroxylumisterol modelled into the binding site. Preparation of the VDR non-genomic site structure prior to docking is described in Appendix A. Glide XP docking of hydroxy-vitamin D_3 compounds listed in Table 2 into the non-genomic site resulted in reasonable poses for some of the analogs with $23(\text{OH})$, $24(\text{OH})$ or $25(\text{OH})$ - substitutions, which form hydrogen bonding interactions in a highly polar region of the pocket. Docked poses are closely overlapping, suggesting a common binding mode, as illustrated in Figure 6. Interactions with non-polar residues mapping the non-genomic pocket contribute significantly to the binding of these metabolites. The $3(\text{OH})$ group in each docked pose forms hydrogen bonding interactions with Arg274, Ser237 while $1\alpha(\text{OH})$ -containing analogs interact with the carbonyl backbone of Ile271, in close proximity to Ser275. Superposition of other D_3 analogs from Table 2 onto these poses suggests unfavorably close contacts involving hydroxyl groups and VDR residues in this site, although it is possible that some of these analogs can induce further conformational changes of the non-genomic site to adjust to their binding. Calcitric acid shows a similar orientation to D_3 analogs, while its carboxylate group is oriented toward Arg158 and Arg296.

Thus, we explored *in silico* the possible binding of our analog series in the non-genomic site of VDR. The crystal structure of this pocket was slightly modified to simulate conformational changes induced by the binding of the known non-genomic agonist, $1\alpha,25$ -hydroxylumisterol. Docking results suggest that it is possible to accommodate most hydroxy- D_3 analogs with 23 -, 24 - or 25 -(OH) substitutions, as well as calcitric acid in the ligand-induced conformation of the VDR non-genomic pocket.

5.1.2. Concluding remarks on VDR

Thus, both functional studies and *in silico* modeling support that the novel CYP11A1-derived D_3 hydroxymetabolites with and without a $1\alpha(\text{OH})$ group act on the VDR, the vast majority at the genomic site. $1\alpha(\text{OH})$ derivatives generally have better docking scores in agreement with their higher efficiency to translocate VDR to nucleus. Furthermore, interaction with VDRE [53, 64, 65, 84, 86, 95] or with specific coactivators (Fig. 2) is greatly enhanced or requires, respectively, the presence of the $1\alpha(\text{OH})$ group in the

novel secosteroids. On the other hand, VDR silencing [53, 84-86] emphasizes the importance of the VDR for the phenotypic effects in skin cells. It is interesting that antiproliferative and prodifferentiation or immunomodulatory effects of compounds with and without the $1\alpha(\text{OH})$ group, on human skin cells or immune cells, are either similar or show cell type and phenotypic trait-dependent differences (Fig. 1)[64, 65, 90, 92, 95]. Only a few secosteroids may potentially interact with the non-genomic site including $1\alpha,25(\text{OH})_2\text{D}_3$, $23\text{S}(\text{OH})\text{D}_3$ (which can potentially be derived from CYP11A1 action on vitamin D3), and the products of $1,25(\text{OH})_2\text{D}_3$ catabolism, $24\text{R},25(\text{OH})_2\text{D}_3$ and calcitroic acid..

We suggest that the interaction of CYP11A1-derived secosteroids with the VDR and downstream transcriptional activity is co-activator specific for VDR in the proper cellular context, with agonistic activity modified by the presence of a $1\alpha(\text{OH})$ group. The mechanism may be similar to those proposed for classical vitamin D ligands [16-20, 38, 104]. It must also be noted that $20(\text{OH})\text{D}$ is a relatively poor substrate for CYP27B1, however, its hydroxylation by CYP27A1 or CYP24A1 at either the 24, 25 or 26 position make the resulting dihydroderivative an excellent substrate for 1α hydroxylation [53, 54]. Thus, the challenge posed by the novel hydroxyderivatives on the nature of their activation of the VDR can be properly answered using CYP27B1^{-/-} mice. Furthermore, the nature of their interactions with the VDR-signaling pathway will have to be elucidated using chromatin immunoprecipitation sequencing (ChIP-seq) techniques with a bioinformatics approach. Such experiments represent our future goal and the studies will include testing of membrane bound signaling pathways with selected compounds that may interact with the non-genomic site [100, 103, 105, 106].

5.2. Retinoic acid orphan receptors (ROR) α and γ

5.2.1. Functional studies

Using cultured CHO cells expressing a TET-On ROR α or γ vector and RORE-LUC reporter we have shown that $20(\text{OH})\text{D}_3$ and $20,23(\text{OH})_2\text{D}_3$ inhibit the transactivation of the reporter by ROR α and ROR γ , with these secosteroids being significantly more potent than $20(\text{OH})\text{D}_2$, D_2 and $1,25(\text{OH})_2\text{D}_3$ [83]. The latter showed the lowest effect on transactivation activity. This antagonistic activity was further supported by mammalian two-hybrid analysis examining the agonist-dependent interaction of the LBD with an LBD-interacting LXXLL-peptide [83]. $20(\text{OH})\text{D}_3$ repressed the interaction between the LBD of ROR and the LXXLL-peptide in a dose-dependent fashion. Moreover, $20(\text{OH})\text{D}_3$ and $20,23(\text{OH})_2\text{D}_3$ were able to attenuate the promoter activity of ROR α and ROR γ target genes, including *Bmal1* and *G6pase*, respectively [83]. Again they were more potent than $20(\text{OH})\text{D}_2$ and D_2 , with $1,25(\text{OH})_2\text{D}_3$ having no effect. Finally, $20(\text{OH})\text{D}_3$ and $20,23(\text{OH})_2\text{D}_3$ repressed *IL17* promoter activity in cells overexpressing ROR γ or ROR α , with $20,23(\text{OH})_2\text{D}_3$ showing the higher potency [83]. Also, $20(\text{OH})\text{D}_3$, $20,23(\text{OH})_2\text{D}_3$, and $1,25(\text{OH})_2\text{D}_3$ inhibited RORE-LUC reporter activity in human melanoma cells and keratinocytes in a similar manner [83]. We have repeated these assay with an extended list of CYP11A1-derived secosteroids which demonstrates that they inhibit RORE activity in keratinocytes (Fig. 7), and supports the results of molecular modeling that predicts that they are ligands for RORs (see below).

5.2.2. Molecular modeling utilizing the ROR α , ROR γ crystal structures.

Movement of activation function helix 2 (AF2) is associated with the activation of ROR α and ROR γ . This helix is stabilized through a network of hydrogen bonding interactions including the His479 – Tyr502 (ROR γ) interaction. Inverse agonists can act by disrupting this interaction. Among D3 metabolites the antagonism/inverse agonism of $20\text{S}(\text{OH})\text{D}_3$, $20\text{S},23\text{S}(\text{OH})_2\text{D}_3$ and $1\alpha,25(\text{OH})_2\text{D}_3$ has been demonstrated. We therefore performed docking of secosteroids listed in Table 2 to better understand interactions formed by CYP11A1-derived hydroxy-metabolites of D3 with ROR α and ROR γ and to predict if other natural metabolites in this series may also bind these nuclear receptors. We utilized the Glide XP docking method (implemented in Schrödinger software) to dock the D3 series into refined crystal structures of ROR α (PDB code 1N83) and ROR γ (PDB code 3KYT), as described in Appendix A. Glide XP docking scores of top ranking poses are listed in Table 2. Most hydroxy-D3 metabolites show comparable or

slightly less favorable docking scores compared to cholesterol, 20(OH)-cholesterol and the three confirmed hydroxy-D3 inverse agonists/antagonists listed above. The hydrophobic moiety of D3 is accommodated favorably in the largely non-polar pocket of RORs, approximately overlapping with co-crystallized cholesterol ligands. The 7 α -methyl-octahydroindene moiety common in the ring structures between cholesterol and D3 metabolites is closely overlapping between docked poses of the series (Table 2) and the co-crystallized 20(OH)-cholesterol in ROR γ and cholesterol in ROR α . The aliphatic chains of the docked hydroxylated D3 analogs occupy the same region as the corresponding chain of the co-crystallized cholesterol ligands. The selected docking poses contain cyclohexyl in the α -chair conformation; however, analogous and comparably scoring poses were additionally obtained that mainly differ from those presented in that they adopt a β -chair conformation where the 3(OH) group is hydrogen bonding with (ROR α) Gln289 and Arg367 (water-bridged interaction).

20*S*,23*R*(OH)₂D3 docked into ROR α and ROR γ , shown in Figures 8A and 8B, respectively, illustrates a binding mode that is common of the hydroxylated vitamin D3 analog series in Table 2. Also, poses docked into ROR α and ROR γ show similar orientation and analogous interactions. Analogs in this series form favorable non-polar contacts with a number of residues mapping the ROR α , γ binding sites as illustrated for 20*S*,23*R*(OH)₂D3 in Figures 8A, B. Common to all analogs is hydrogen bonding interaction formed by the 3(OH) group with Arg370 and the backbone carbonyl of Arg367 in ROR α , and the corresponding residues in ROR γ : Arg367 and the backbone carbonyl of Arg364. The 1 α (OH) group in most analogs containing this group hydrogen bonds with the backbone carbonyl of Tyr380(ROR α)/Phe377(ROR γ). In nearly all cases 20*S*(OH) participates as hydrogen bond acceptor in a hydrogen bond with Cys323(ROR α)/Cys320(ROR γ). Docked poses at both ROR α and ROR γ predict that the 22(OH) group can form the analogous interaction with this cysteine. As shown in Figure 8, the 23(OH) is in proximity of His484(ROR α)/His479(ROR γ) (heavy atom distances: 5.4 Å, 4.5 Å, respectively), which is consistent with possible water-bridged hydrogen bonding with the histidine. Analogously, the position of the 23(OH) as well as the 24(OH) in most docked poses is compatible with potential water-bridged hydrogen bonding with this histidine and/or with Tyr507(ROR α)/Tyr502(ROR γ). Both 25(OH) and 26(OH) groups tend to form hydrogen bonding interaction with Tyr507(ROR α)/Tyr502(ROR γ) and in case of several ROR γ docked analogs also with His479. Interestingly, the *R* configuration for 20(OH) is much less favorable than natural *S*, due to desolvation of the 20*R*-hydroxyl group in a non-polar environment, which is more pronounced in case of ROR α (Glide XP score > 3 kcal/mole less favorable for *R* than *S*). Favorable interactions and Glide XP scores comparable to natural ligands suggest that the series of secosteroids listed in Table 2 can fit favorably into the active site of ROR α and ROR γ .

Crystal structures of ROR γ with small molecule antagonists co-crystallized in a novel allosteric binding pocket has been recently published [92]. In order to explore whether hydroxy-metabolites of D3 may bind this allosteric site we attempted docking our series into the allosteric pocket. Unsuccessful docking suggests that the allosteric site is not suitable to accommodate D3 analogs.

Thus, docking results predict that most of the presented hydroxy-D3 metabolites can bind in the active site of ROR α and ROR γ in poses that approximately overlap with co-crystallized cholesterol ligands. Docked compounds form extensive non-polar contacts, common in the series, while hydrogen bonding interactions involve specific hydroxyl group substituents in analogs.

5.3. Future directions in identifying receptors for vitamin D metabolites

The functional assays and molecular modeling clearly indicate that novel CYP11A1-derived D3 hydroxymetabolites can act both as partial/biased agonists on VDR and inverse agonists for ROR α and ROR γ . Their activities on the VDR can be modified by hydroxylation of their side chain and additional hydroxylation at C1 α , setting a background for further studies as indicated in 5.1.2 that are necessary to dissect which phenotypic effects, aside of calcemia and CYP24A1 activation, are dependent on the 1 α (OH) group. Since it is clear that novel D3 hydroxyderivatives can interact with RORs, it must be clarified which effects are strictly or partially dependent on RORs using ROR α / γ ^{-/-} and VDR^{-/-} cells. It is interesting that 20(OH)D3 and 20,23(OH)₂D3 show higher docking scores on ROR γ than previously established natural ligands represented by cholesterol and 20-hydroxycholesterol (Table 2),

consistent with their inverse agonist activity reported previously [83]. This suggests a potential beneficial use of these compounds in inflammatory and autoimmune diseases with other secosteroids serving as additional candidates.

The list of compounds potentially acting on the non-genomic site of the VDR is shorter and aside of 1,25(OH)₂D₃ includes 1 α ,24S,25(OH)₃D₃, 23S(OH)D₃ and surprisingly calcitroic acid. The *in silico* modeling with the novel secosteroids indicates that they are not perfect ligands for 1,25D₃-MARRS with predicted candidates (1,20,24(OH)₃D₃, 1,24,25(OH)₃D₃, 17,20,23(OH)₃D₃) probably acting as low affinity ligands for this receptor that could be better or similar to the classical 1,25(OH)₂D₃. A surprising finding is the prediction of cholesterol and 20(OH)cholesterol binding to the genomic VDR pocket, with respective Glide XP scores of -9.99 and -9.78, . It suggests that further careful studies on the role of cholesterol and its metabolites on VDR activity are required, since these are abundant in cells, and related bile acid metabolites can act on the VDR [107-109].

6. Concluding remarks

Over 12 years we have documented the existence of new pathways of vitamin D₃ metabolism started by the action of CYP11A1 and further modified by the actions of CYP27B1, CYP27A1, CYP24A1 and CYP3A4, generating at least 21 hydroxymetabolites with additional ones still to be experimentally defined (Table 1) [43, 50]. At least 13 of them are endogenously produced [72]. These metabolites display biological activity by acting both as “biased” agonists of the VDR and/or inverse agonists of ROR α and ROR γ . A subset of these compounds have the potential to act on the non-genomic VDR site or less likely on 1,25D₃-MARS as suggested by molecular modeling.

We propose that the identification of a number of hydroxymetabolites of D₃ may offer an explanation for the pleiotropic and diverse activities of vitamin D that have previously been assigned to 1,25(OH)₂D₃. We suggest that these diverse phenotypic effects are also mediated by interactions with, in addition to the VDR, receptors including ROR α and ROR γ . Also, for selected compounds one may propose action on the non-genomic site of VDR with the possibility that just a few of the secosteroids act as low affinity agonists on 1,25D₃-MARS.

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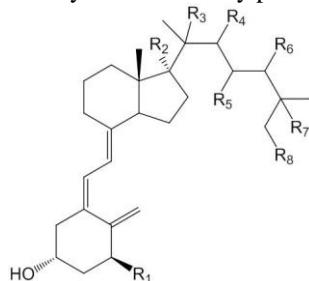
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Table 1. Hydroxyvitamin D3 metabolites produced by the action of CYP11A1 on vitamin D3 and further hydroxylated by other vitamin D-metabolizing cytochromes P450.

CYP11A1 Metabolite	Secondary Metabolite	CYPs Involved	References
20S(OH)D3			[44, 47, 51, 58, 60, 61, 67, 71, 72]
	1 α ,20S(OH) ₂ D3	27B1	[54, 58, 63, 71, 72]
	20S,24R(OH) ₂ D3	24A1 or 3A4	[55, 57, 65, 66, 72]
	20S,24S(OH) ₂ D3	24A1 or 3A4	[55, 65, 66, 72]
	20S,25(OH) ₂ D3	24A1 or 27A1 or 3A4	[55, 57, 66, 72]
	20S,26(OH) ₂ D3	27A1	[57, 72]
	1 α ,20S,24R(OH) ₃ D3	24A1, 27B1	[54, 65, 72]
	1 α ,20S,24S(OH) ₃ D3	24A1, 27B1	[65]
	1 α ,20S,25(OH) ₃ D3	24A1 or 27A1 or 3A4, 27B1	[54, 72]
	1 α ,20S,26(OH) ₃ D3	27A1, 27B1	[54, 72]
17 α (OH)D3			[51, 61]
22(OH)D3			[58, 61, 71, 72]
	1 α ,22(OH) ₂ D3	27B1 (low activity)	[54]
20R,22(OH) ₂ D3			[47, 58, 61, 71, 72]
	1 α ,20S,22(OH) ₃ D3	27B1	[54]
20S,23S(OH) ₂ D3			[51, 58, 60, 61, 64, 71, 72]
	1 α ,20S,23S(OH) ₃ D3	27B1	[54, 56, 58, 64]
	20S,23S,24(OH) ₃ D3	24A1	[55]
	20S,23S,25(OH) ₃ D3	24A1	[55]
17 α ,20S(OH) ₂ D3*			[51, 60, 61]
17 α ,20S,23S(OH) ₃ D3*			[51, 58, 60, 61, 72]

*Not acted on by CYP27B1 (9).

Table 2. Glide XP docking scores of compounds docked into the ROR α , ROR γ and VDR crystal structures. All R groups not specified are hydrogens. Some of the compounds listed are isomers of naturally produced D3 hydroxyderivatives and their synthesis by human P450s has not been established. NP: not the preferred isomer if the other enantiomer shows a significantly more favorable pose. NI: not included among structures shown. ND: Not successfully docked or only poorly scoring poses obtained.



Compounds	Structure	ROR α	ROR γ	VDR (genomic site)	VDR (non-genomic site)	1,25D3- MARRS
D3		-10.74	-11.22	-12.69 ^a	ND	ND
1 α ,25(OH)2D3	R1=R7=OH	-10.30	-11.88	-15.07	-13.56	-7.47
20S(OH)D3	R3=OH	-10.99	-12.60	-12.77	ND	ND
20R(OH)D3	R3=OH	NP	-10.99	-12.85	ND	ND
20S,22R(OH)2D3	R3=R4=OH	-10.56	-11.79	NP	ND	ND
20S,22S(OH)2D3	R3=R4=OH	-10.60	-12.18	-12.29	ND	ND
20S,23R(OH)2D3	R3=R5=OH	-10.60	-12.87	-14.05	ND	-7.98
20S,23S(OH)2D3	R3=R5=OH	-10.41	-12.65	-13.94	ND	-7.20
20S,24R(OH)2D3	R3=R6=OH	-10.70	-11.82	-13.52	ND	ND
20S,24S(OH)2D3	R3=R6=OH	-10.81	-12.29	-13.87	ND	ND
20S,25(OH)2D3	R3=R7=OH	-10.02	-10.33	-13.35	ND	ND
20S,26(OH)2D3	R3=R8=OH	-10.82	-11.10	-13.34	ND	ND
1 α ,20S(OH)2D3	R1=R3=OH	-10.15	-11.00	-13.88	ND	ND
1 α ,20S,22R(OH)3D3	R1=R3=R4=OH	-11.47	-11.64	NP	ND	ND
1 α ,20S,22S(OH)3D3	R1=R3=R4=OH	-11.67	-12.99	-14.30	ND	ND
1 α ,20S,23R(OH)3D3	R1=R3=R5=OH	-9.96	-10.81	-15.37 -15.27	ND	-7.53
1 α ,20S,23S(OH)3D3	R1=R3=R5=OH	-10.36	-11.22	-15.03	ND	-7.90
1 α ,20S,24R(OH)3D3	R1=R3=R6=OH	-10.79	-11.77	-14.95	ND	-8.70
1 α ,20S,24S(OH)3D3	R1=R3=R6=OH	-10.97	-10.42	-15.80	ND	-7.97
1 α ,20S,25(OH)3D3	R1=R3=R7=OH	-11.23	-11.22	-14.83	ND	ND
1 α ,20S,26(OH)3D3	R1=R3=R8=OH	-10.54	-12.15	-14.92	ND	ND
22R(OH)D3	R4=OH	-11.23	-11.27	-12.10	ND	ND
22S(OH)D3	R4=OH	-11.32	-12.16	-13.40	ND	ND
23R(OH)D3	R5=OH	-10.19	-10.82	-13.00	NP	ND
23S(OH)D3	R5=OH	-10.47	-11.76	-12.72	-12.77	ND
25(OH)D3	R7=OH	-10.46	-10.26	-13.36	-12.65	ND
17(OH)D3	R2=OH	-10.84	-10.99	-12.53 ^a	ND	ND
23R,25(OH)2D3	R5=R7=OH	-10.19	NP	14.00	-14.01	-7.99
23S,25(OH)2D3	R5=R7=OH	NP	-10.74	-13.21	-13.26	-8.66
1,23R,25(OH)3D3	R1=R5=R7=OH	-10.35	NP	-15.01	-14.54	-8.40
1,23S,25(OH)3D3	R1=R5=R7=OH	-11.08	-11.34	-16.02	-15.05	-9.11
24R,25(OH)2D3	R6=R7=OH	-10.00	-12.34	-14.64	-13.85	-8.43
24S,25(OH)2D3	R6=R7=OH	NP	NP	-14.12	-14.74	-8.83
1 α ,24R,25(OH)3D3	R1=R6=R7=OH	-10.07	-12.32	-16.21	NP	-8.34
1 α ,24S,25(OH)3D3	R1=R6=R7=OH	-10.12	NP	-15.81	-14.66	-8.30
17,20S(OH)2D3	R2=R3=OH	-10.99	-12.66	-13.70	ND	ND
17,20S,23R(OH)3D3	R2=R3=R5=OH	-11.06	-11.84	-14.67 -14.46	ND	-8.11
17,20S,23S(OH)3D3	R2=R3=R5=OH	-10.45	-10.75	-14.44	ND	-7.81
Calcitroic acid	NI	-8.72 -8.64	-8.96	-9.96	-12.11	
20(OH)-cholesterol	NI	-11.60	-12.17	-9.78	-10.01	
Cholesterol	NI	-11.92	-12.49	-9.99	-10.31	

^aSee discussion of potentially unstable poses.

Figure legends

Figure 1. Hydroxymetabolites of 20(OH)D3 inhibit keratinocytes proliferation. The cells were cultured for 48 h in the presence or absence of 1 α ,25(OH)₂D3, 20S,25(OH)₂D3, 1 α ,20S,25(OH)₃D3, 20S,26(OH)₂D3, 1 α ,20S,26(OH)₃D3, 20S,24R(OH)₂D3, or 1 α ,20S,24R(OH)₃D3 and cell proliferation was measured by the MTS assay as described in the supplemental file and in [90, 92]. Data represent means \pm SE (n=3-4). *, p<0.05; **, p<0.01; ***, p<0.001 at student t-test. #, p<0.05; ##, p<0.01; ###, p<0.001 at one-way ANOVA test.

Figure 2. Only the 1 α (OH)-derivatives of 20(OH)D3 hydroxymetabolites increase the affinity of the co-activator peptide to the VDR-LBD in the LanthaScreen TR-FRET Vitamin D receptor Coactivator kit assay (Thermo Fisher Scientific, Inc., Waltham, MA) (for details see supplemental file).

Figure 3. Hydroxymetabolites of 20(OH)D3 stimulate VDR-GFP translocation from the cytoplasm to the nucleus in the SKMEL-188 melanoma line stably overexpressing VDR-GFP [53]. The cells were incubated for 90 min in the presence of 20S,25(OH)₂D3, 1 α ,20S,25(OH)₃D3, 20S,26(OH)₂D3, 1 α ,20S,26(OH)₃D3, 20S,24R(OH)₂D3 or 1 α ,20S,24R(OH)₃D3, or vehicle (ethanol) and translocation to the nucleus was measured as described in [92] with modifications listed in the supplemental file Data represent means \pm SE (n=3-4). *, p<0.05; **, p<0.01; ***, p<0.001 at student t-test. #, p<0.05; ##, p<0.01; ###, p<0.001 at one-way ANOVA test.

Figure 4. Common binding mode predicted for hydroxy-D3 metabolites in the genomic site of the VDR crystal structure (PDB code 1DB1). (A) Docked poses of hydroxy-D3 metabolites (listed in Table 2) are shown with thin bonds and green colored carbons. For comparison, the co-crystallized 1,25(OH)₂D3 is shown with thick bonds and light brown colored carbons. All other atoms are colored by atom type (O: red, N: blue, S: yellow, H: white). Only VDR residues involved in polar interactions with the ligands are included. Hydrogen bonding interactions of 1 α (OH) and 3(OH) groups common in docked poses are indicated with dashed lines. (B) Schematic summary of interactions formed by docked analogs listed in Table 2 in the genomic site of VDR. Interactions formed between VDR residues and OH substituents in docked poses of the analog series are indicated with dashed lines, colored distinctly for each OH group. For example, 24(OH) in poses of some analogs interact with H397 while in case of other analogs with H305 (light blue dashed lines). VDR residue labels are color coded: those involved in polar interactions are dark blue; those contributing to non-polar interactions are dark brown.

Figure 5. Poses of selected hydroxy-D3 metabolites are shown in the genomic site of VDR (PDB code 1DB1). (A) Docked pose of 1 α ,20S(OH)₂D3 shown in comparison with the co-crystallized 1,25(OH)₂D3 in the VDR crystal structure (PDB code 1DB1). Carbon atoms in the docked pose are colored dark green, in the co-crystallized ligand light brown; all other atoms are colored by atom types (as in Fig. 4). Hydrogen bonding interactions are shown with dashed lines. In addition to residues involved in hydrogen bonding with shown ligands, all VDR residues that form non-polar contacts with the docked 1 α ,20S(OH)₂D3 are also displayed. (B) Two possible docked poses obtained for 1 α ,20S,23R(OH)₃D3 are shown simultaneously in the VDR genomic pocket, with their carbon atoms colored distinctly. Hydrogen bonding interactions are indicated with dashed lines. Only VDR residues involved in polar interactions are shown.

Figure 6. Docked poses of hydroxy-D3 metabolites in the non-genomic site of VDR, where the VDR crystal structure (PDB code 1DB1) was refined as described under Computational Methods (Supplemental file). VDR residues shown form polar or non-polar interactions with the docked ligands. Carbons in the docked poses are colored dark green; all other atoms are colored by atom type (as in Fig. 4). Hydrogen bonding interactions involving 1 α (OH) and 3(OH) groups shared in docked poses are indicated with dashed lines.

Figure 7. Effect of vitamin D3 hydroxyderivatives on ROR-RE activity in HaCaT cells. The cells were grown on 96 well white plates in DMEM media containing 5% charcoal-treated FBS. After transfection with ROR-RE and a Renilla luciferase construct, the cells were treated with the compounds for 48 h followed by luciferase assay. Methodological details are in supplemental file. *, p<0.05; **, p<0.01; ***, p<0.001 at student t-test. #, p<0.05; ##, p<0.01; ###, p<0.001 at one-way ANOVA test.

Figure 8. 20S,23R(OH)₂D3 docked in the active site of (A) ROR α – dark green colored ligand carbons, and (B) ROR γ – light brown colored ligand carbons. Atoms other than carbon are colored by atom type (as in Fig. 4). All residues contributing through polar or non-polar interactions to the binding of the docked ligand are displayed. Distances between hydrogen bonding atoms are as shown *via* dashed lines between the interacting atoms: (A) Pose at ROR α : 1. 2.1 Å, 2. 1.9 Å, 3. 2.5 Å; (B) Pose at ROR γ : 1. 1.8 Å, 2. 1.7 Å, 3. 2.0 Å.

Fig. 1.

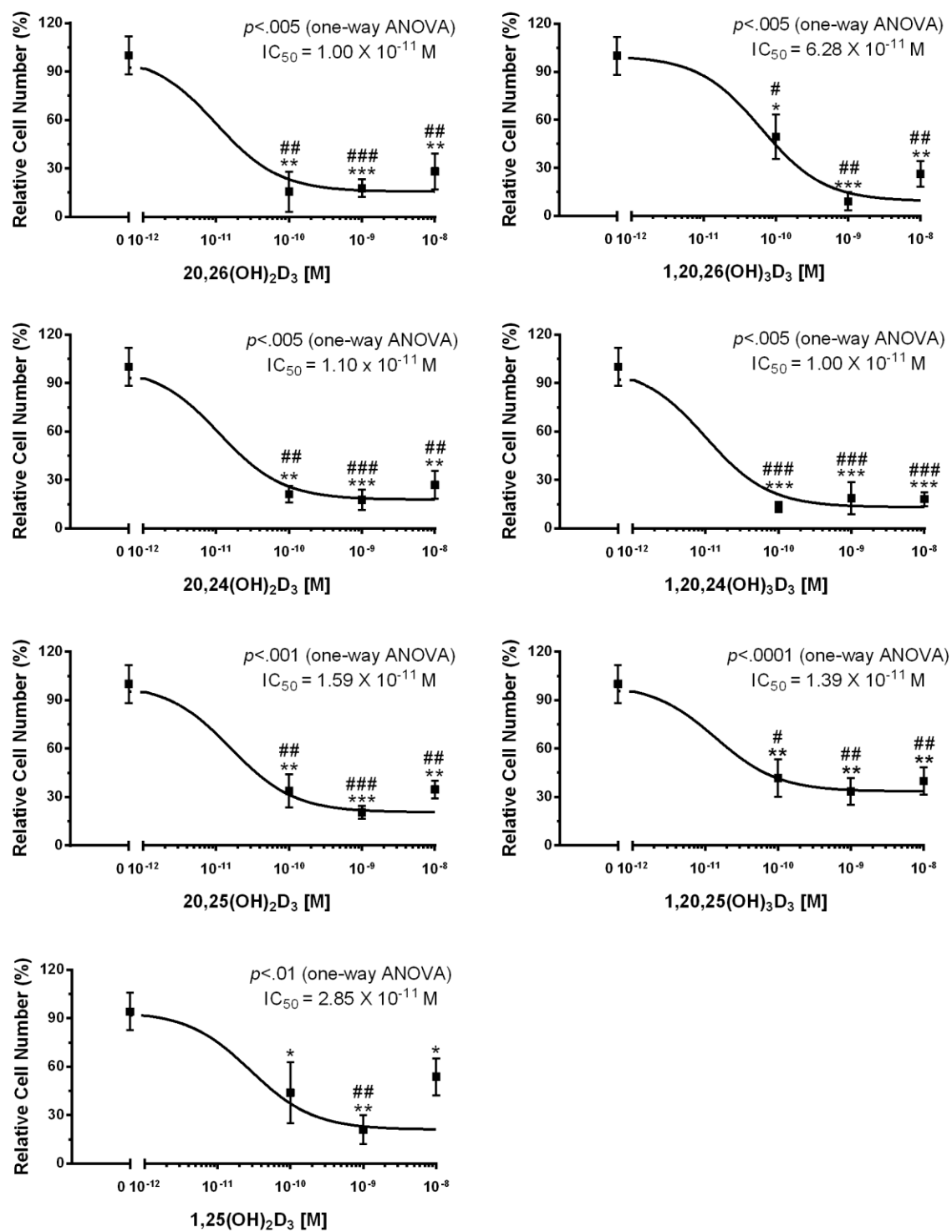


Fig. 2.

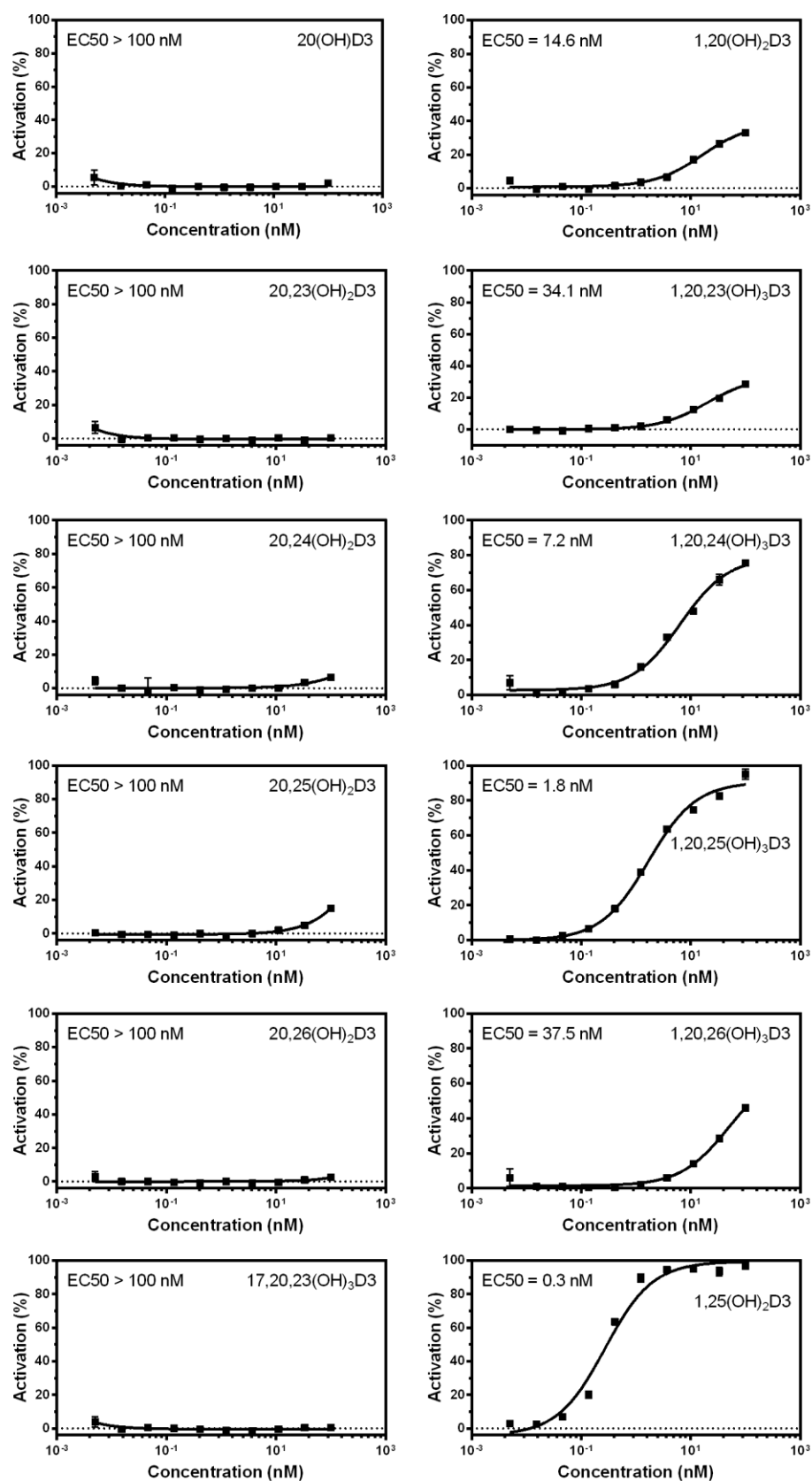


Fig. 3.

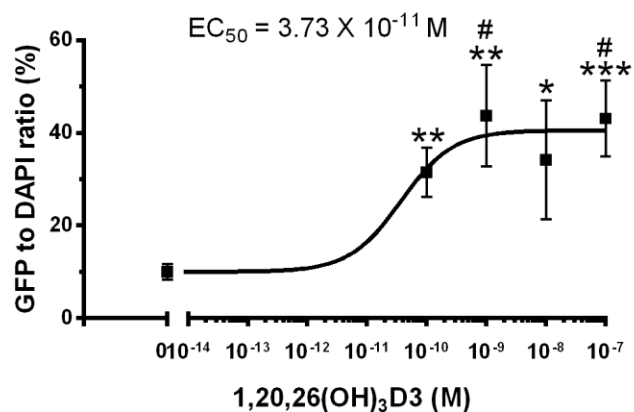
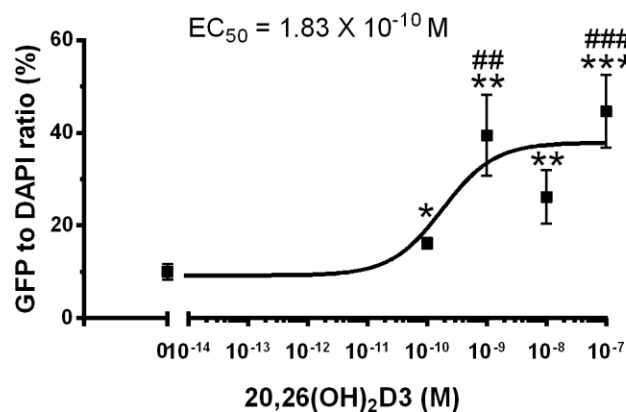
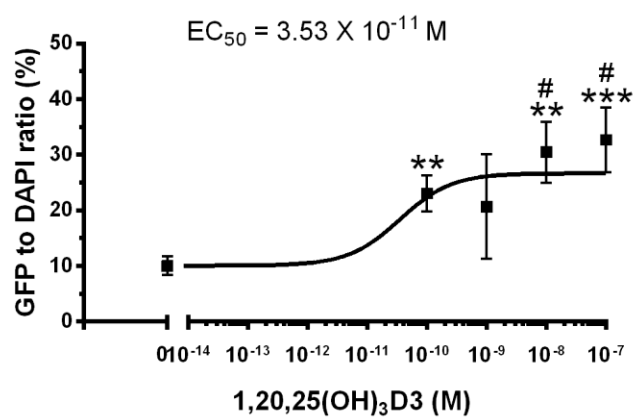
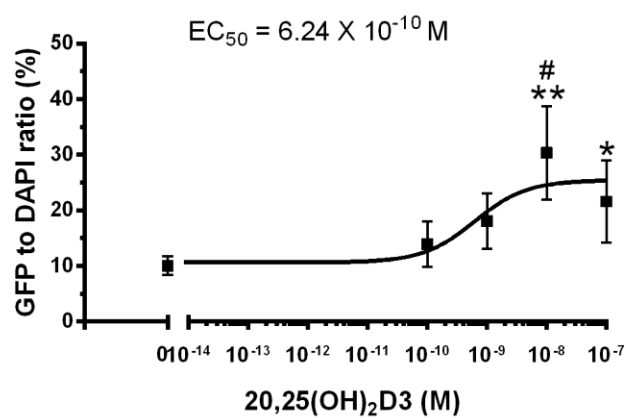
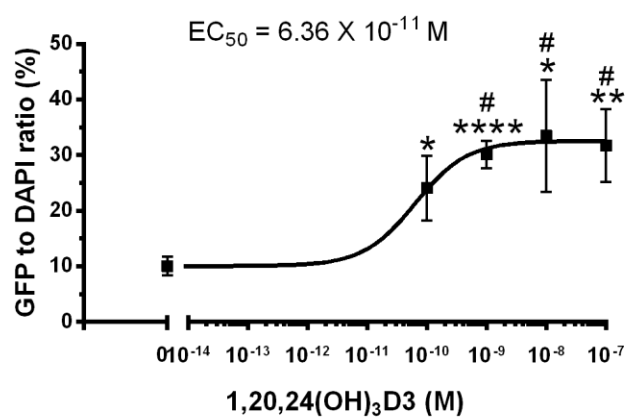
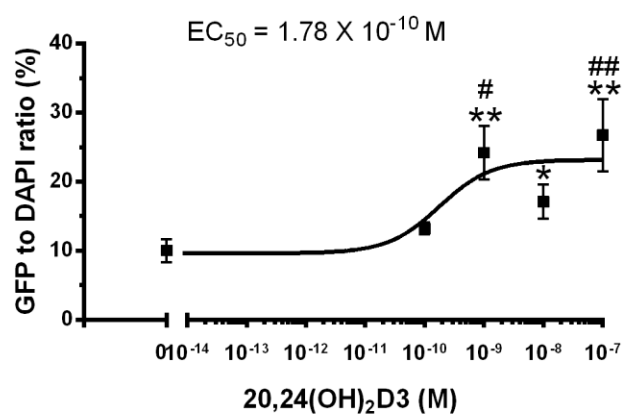


Fig. 4.

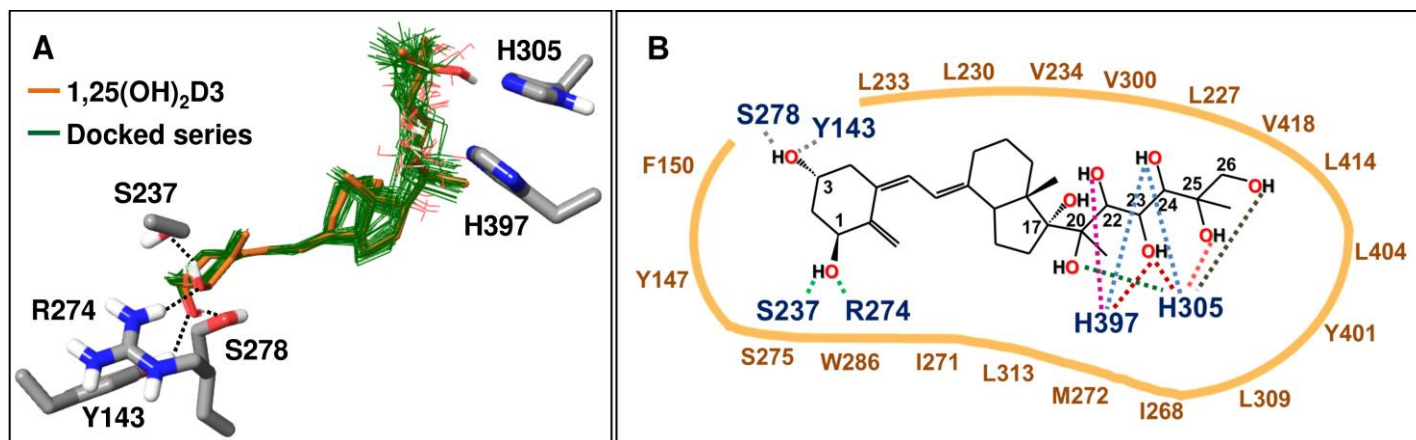


Fig. 5.

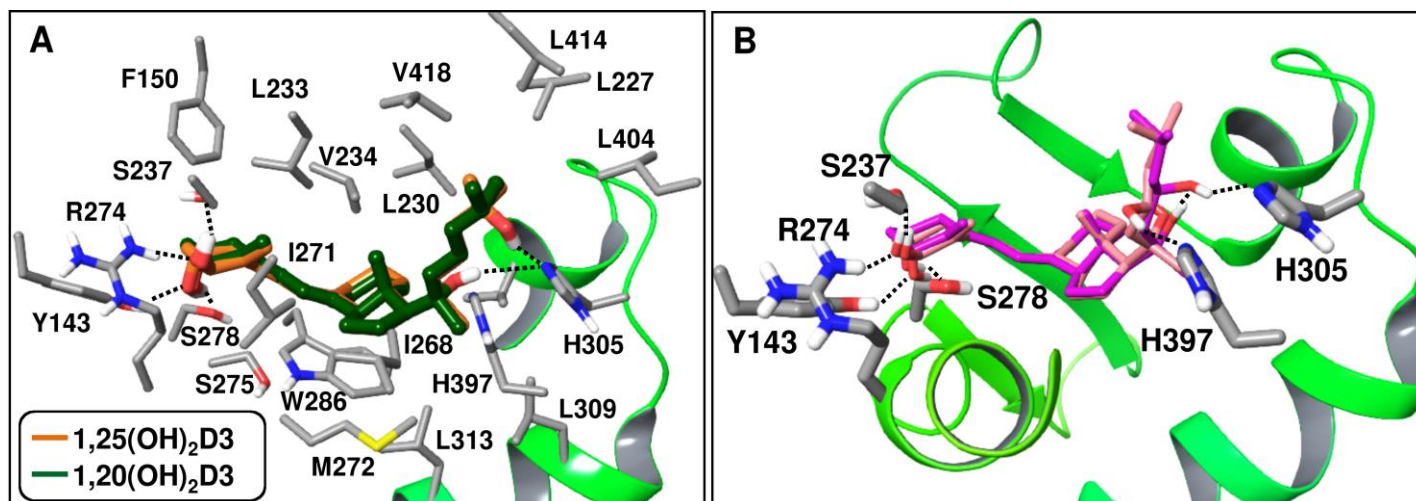


Fig. 6.

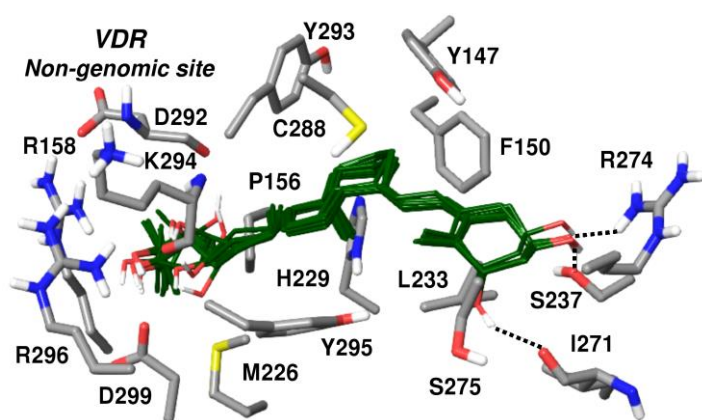


Fig. 7.

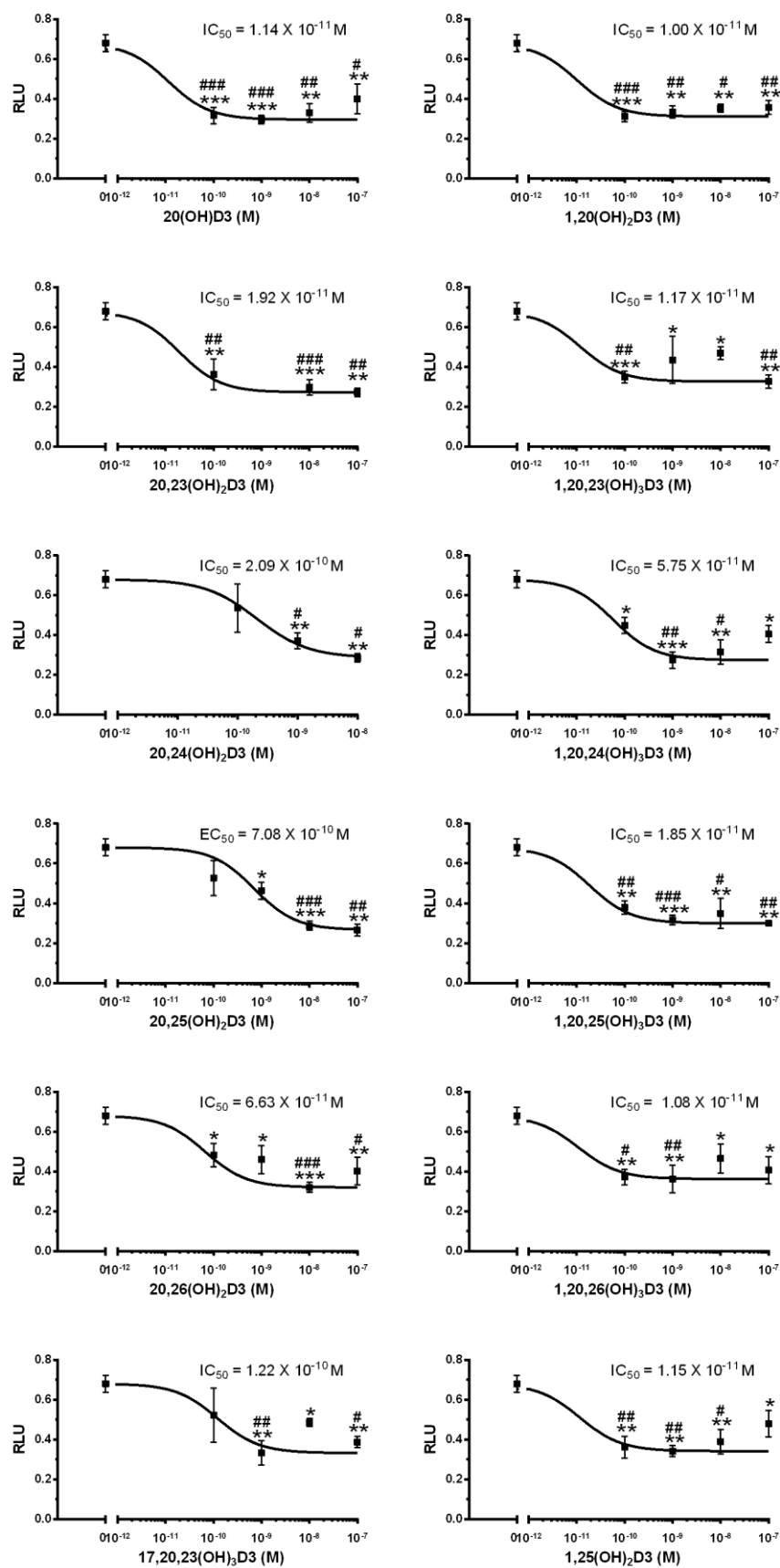


Fig. 8.

