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Alternative Binding Sites at the Vitamin D Receptor and their Ligands

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Abstract

In recent decades, the majority of ligands developed for the vitamin D receptor (VDR) bind at its deeply buried genomic ligand binding pocket. Theses ligands can be categorized into agonists and partial agonists/antagonists. A limited number of ligands, most of them peptides, bind the VDR–coactivator binding site that is formed in the presence of an agonist and inhibit coactivator recruitment, and therefore transcription. Another solvent exposed VDR–ligand binding pocket was identified for lithocholic acid, improving the overall stability of the VDR complex. Additional proposed interactions with VDR are discussed herein that include the alternative VDR–ligand binding pocket that may mediate both non-genomic cellular responses and binding function 3 that was identified for the androgen receptor. Many VDR ligands increase blood calcium levels at therapeutic concentrations *in vivo*, thus the identification of alternative VDR–ligand binding pockets might be crucial to develop non-calcemic and potent ligands for VDR to treat cancer and inflammatory disease.

Keywords

vitamin D receptor; ligand; alternative VDR–ligand binding pocket; binding function 3; VDR–coactivator inhibitor; $1,25(OH)_2D_3$

1. Introduction

The vitamin D receptor (VDR) is a ligand-activated transcription factor and belongs to the superfamily of nuclear receptors, as recently reviewed (Pike et al., 2018). The receptor is expressed primarily in the epithelia of endocrine organs (e.g. parathyroid gland, mammary gland), digestive system, bronchi, kidneys, and thymus (Wang et al., 2012). In addition, VDR can be found in leukocytes and bone cells. Many natural occurring VDR ligands have been identified including vitamin D metabolites with a secosteroid structure and bile acids that partially control VDR function in the intestine. For a recent review see (Makishima and

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Yamada, 2018). In the absence of a genomic agonist, VDR can be found in the cytosol or attached to the cell membrane (Huhtakangas et al., 2004). Ligand binding will induce nuclear localization of VDR and promote VDR–DNA complexation in conjunction with the retinoid X receptor (RXR) (Figure 1) (Orlov et al., 2012). Specific VDR response elements have been identified in the promoter sequences of genes that are induced or repressed by VDR (Haussler et al., 2011). VDR-mediated gene regulation is dependent on interactions with coregulators (coactivators and corepressors). These proteins are part of the transcriptional complex that interacts with RNA polymerase II and other binding proteins in addition to DNA, as reviewed by (White et al., 2018). Chromatin remodeling and specific gene transcription are nuclear functions of these transcriptional VDR complexes, which have been summarized in (Nurminen et al., 2018).

2. VDR and its genomic ligand binding pocket

VDR can be divided into the following domains: N-terminus, DNA binding domain (DBD), a hinge region that enables flexibility for dimerization, DNA binding domain, and a ligand binding domain (LBD) (Figure 2a). The LBD consists of 12 helices and forms a compact three dimensional structure, especially when bound to a ligand (Figure 2b). The ligand binding pocket is buried deeply in the receptor, which enables very specific interactions with natural ligands, most prominently 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) (Figure 2c). Thousands of VDR ligands have been synthesized in recent decades that bind at this particular ligand binding pocket (reviewed by (Verlinden et al., 2018). It has been shown that these ligands influence the shape of the binding pocket, which was elegantly shown with the Gemini ligand bearing two alkyl side chain substituents (Norman et al., 2000). The occupation of an additional 25% volume in comparison to $1,25(OH)_2D_3$ resulted in higher transcriptional activity and pronounced anticancer activity of Gemini. Other recently reviewed VDR ligands include (Belorusova and Rochel, 2018) and antagonists (Saitoh, 2018). Furthermore, VDR ligands with multiple aromatic rings have been developed that, in contrast to $1,25(OH)_2D_3$, did not elevate blood calcium levels (Stites et al., 2018). Commercialized VDR ligands include tacalcitol and calcipotriol for psoriasis, doxercalciferol, falecalcitriol, and maxacacitol for secondary hyperparathyroidism, and eldecalcitol for osteoporosis (Leyssens et al., 2014). During the last three decades, anti-cancer properties of vitamin D analogs have been demonstrated *in vivo*, however, one of the most promising vitamin D analogs, seocalcitol, showed insufficient efficacy in phase II clinical trials (Dalhoff et al., 2003). The biochemical changes induced by VDR ligands in cancer cells include gene regulation, activation and inhibition of enzymatic pathways, and overall changes in cell differentiation and proliferation (Van Driel et al., 2018). The specific changes for different cancers were recently reviewed for leukemia (Studzinski et al., 2018), breast cancer (Beaudin and Welsh, 2018), prostate, renal, and bladder cancer (Trump, 2018), colon cancer (Barbchano et al., 2018), skin cancer (Ransohoff et al., 2018), and lung cancer (Shaurova et al., 2018). In addition, some vitamin D analogs have anti-inflammatory properties that have prompted detailed investigations on the role VDR and its ligands play in inflammation and immunity summarized by (Mann et al., 2018).

3. VDR and its predicted alternative (non-genomic) binding site

In addition to genomic actions mediated by VDR, many non-genomic fast cellular processes are triggered by vitamin D analogs and have been reviewed by (Hii and Ferrante, 2016). This includes activation of phospholipases (Bourdeau et al., 1990), phosphatases (Bettoun et al., 2004), kinases (de Boland and Norman, 1998), and voltage-gated ion channels (Zanello and Norman, 2004). VDR ligands such as $1,25(OH)_2$ lumisterol D_3 (Figure 3B) weakly inhibit the VDR–1,25(OH)2D3 interaction, but potentiate chlorine ion channels in Sertoli cells at 1 nM, more effectively than $1,25(OH)_2D_3$ (Menegaz et al., 2010). This prompted the hypothesis of an alternative VDR ligand binding pocket (A pocket) mediating non-genomic processes in cells (Mizwicki et al., 2004). Molecular modeling identified additional hydrophobic space in VDR-LBD by reversing the donor-acceptor interaction of H229 and Y295 (Figure 3A). Subsequent docking revealed that $1,25(OH)_2$ lumisterol D_3 (Figure 3B) has a high affinity for the VDR "A pocket", possibly supporting non-genomic pathways at very low concentrations. Molecular modeling also confirmed binding of a high energy β-chair conformation of $1,25(OH)_2D_3$ to the "A pocket", providing a possible explanation for non-genomic effects of $1,25(OH)_2D_3$ in addition to its genomic effects (Mizwicki et al., 2004). The ability of VDR to bind different biomolecules, such as lipids (Jurutka et al., 2007), supported the hypothesis that the VDR "A pocket" might accommodate phosphatidyl-inositol (3,4,5)-trisphosphate and mediate the attachment of VDR to the cell membrane. Structural evidence for nuclear receptor–lipid binding was observed for nuclear receptor SF-1 (steroidogenic factor 1) and LRH-1 (liver receptor homolog 1) (Krylova et al., 2005). High affinity genomic agonists are expected to compete for VDR binding and enable nuclear localization. To support this hypothesis, specific binding assays are needed to identify and confirm molecular binding to the "A pocket" of VDR.

4. VDR and its coactivator binding site

The VDR coactivator binding site is formed when a VDR ligand is bound to the ligand binding pocket (genomic pocket or G pocket) of VDR. The agonistic ligand forms hydrogen bond interactions with VDR's H305 located on the loop between helix 6 and helix 7 and H397 located on helix 11 (Figure 4A). This triggers a conformational change whereby helix 12 moves towards VDR-LBD and forms hydrogen bond interactions with I414, T415 and E420 (Figure 4A). F422, located on helix 12, interacts with H305 via a π-H interaction. The movement of helix 12 provides a hydrogen bond acceptor (E420) in the relative vicinity of hydrogen bond donor K246 (Figure 4B). These "charge clamp" residues form hydrogen bond interactions with coactivators and enable activation of VDR-mediated transcription by recruitment of RNA polymerase II to the transcription start site. Details of this process have been summarized by (Pike et al., 2018).

5. Peptide-based ligands that bind the VDR coactivator pocket

Crystal structures of VDR-LBD in the presence of agonists and short coactivator peptides reveal the possibility of developing peptide-based ligands to inhibit the interaction between coactivators and VDR (Figure 5) (Vanhooke et al., 2004). To bind the predominately hydrophobic VDR coactivator binding site, successful peptide ligands have hydrophobic

residues such as leucine. Proteomic mutation identified the most effective spacing of leucine residues to be LxxLL (L = leucine and X = any amino acid) with flanking residues to stabilize its helical structure (McInerney et al., 1998). Binding between synthetic peptides derived from coactivator amino acid sequences and VDR was determined by fluorescence polarization and alpha screen (Teichert et al., 2009). Peptides with strong VDR affinity have a general sequence of A/QLLRYLLDK/R. Furthermore, interactions between VDR and coactivator peptides were influenced by the structure of the VDR agonist (Zhang et al., 2010). To further improve VDR binding, especially with the support of flanking residues, a phage library (1.5×10^8 members) was created in *E. coli* and the corresponding peptides were pulled-down with $1,25(OH)_2D_3$ liganded VDR (Chang et al., 1999). Peptides C33 and D47 interacted tightly with VDR as determined by a mammalian two-hybrid assay (Table 1, entries 2 and 3).

Investigations with other nuclear receptors indicated that the binding between C33 and D47 with VDR is not selective. Cell-based assays showed a reduction of $1,25(OH)_2D_3$-induced transcription for a luciferase gene under control of a bone gamma carboxyglutamate protein (*BGLAP*) promoter in the presence of transcriptionally expressed C33 and D47 (Pathrose et al., 2002). In addition to VDR, further research identified peptides EBIP-37, EBIP-51, EBIP-70, and EBIP-96 interacted with estrogen receptor β (ERβ) in the presence of estradiol (Table 1, entries 4-7) (Hall et al., 2000). Identified by a similar method, peptides 3, 4, and 5, introduced by the Pike group, exhibited stong VDR binding in the presence of $1,25(OH)_2D_3$ (Table 1, entries 8-10) (Zella et al., 2007). All three peptides were able to inhibit transcription induced by $1,25(OH)_2D_3$ of a luciferase gene under control of an *BGLAP* promoter when transcriptionally expressed in cells. Based on these results, the initial LxxLL motif can be better described as a PL/MLxxLL motif for peptides with strong VDR binding. The investigation of focused phage peptide libraries has confirmed that specific flanking residues adjacent to the LxxLL motif promote VDR binding. Interestingly, the random approach identified peptides with sequences very similar to natural occurring coactivators such as DRIP205 (Table 1, entry 1). When expressed in cells, these peptides can inhibit $1,25(OH)_2D_3$-induced transcription, demonstrating that coactivator binding is essential for VDR-meditated transcription. Although these peptides found applications as fluorescent probes to identify new ligands for VDR (Nandhikonda et al., 2013), they lack the ability to cross cell membranes.

Recently, the Kurihara group introduced stapled, helical peptides that bind liganded VDR (Demizu et al., 2013). The peptides have a central LLxxLL motif and a covalent crosslinker in the *i* and *i*+3 position (Figure 6). Furthermore, 2-aminoiso-butyric acid was introduced to promote peptide helicity (Demizu et al., 2016). The introduction of hydrophilic hydroxyl groups attached to the linker significantly increased the affinity to VDR, as shown for DPI-06 and DPI-07, with IC_{50} values for inhibiting the VDR–coactivator interaction at 220 μM and 3.2 μM, respectively. The introduction of α-hydroxymethylserine, rather than 2-aminoiso-butyric acid, also increased the affinity to VDR, which lead to DPI-10 with an IC_{50} of 20 μM (Misawa et al., 2015).

6. Small molecules that bind the VDR coactivator pocket

Rational design led to the development of a series of benzodiazepines in 2010 (Mita et al., 2010). Compound 1 is a bicyclic compound bearing three hydrophobic groups that mimics the *i*, *i+3*, and *i+4* leucine position of coactivators (Figure 7A). Structure-activity relationship studies revealed that the electron donating amine function in position 8 is preferable to diamines or a guanine structure. In the absence of a crystal structure, molecular modeling confirms the possibility that compound 1 binds both charge clamp residues E420 and K246 (Figure 7B). A later study exploring substituents in the 7 position identified compound 2, which inhibited the interaction between VDR and coactivator peptides at 14 μM (Figure 7B) (Mita et al., 2013). Compound 1 showed activity in cells and inhibited $1,25(OH)_2D_3$-induced transcription with an IC_{50} of 17 μM. Cellular activity for compound 2 was not reported.

High-throughput screening of 275,000 molecules identified a new class of VDR–coactivator inhibitors, 3-indolylmethanamines (Nandhikonda et al., 2012). These compounds have cellular activity and irreversibly inhibited the interaction between VDR and coactivator SRC2, with an IC_{50} of 4.2 μM (Figure 8). A free energy relationship study confirmed the slow formation of an electrophilic species in aqueous buffer (pH = 7.2). Binding studies in the presence of 2-mercaptoethanol confirmed the ability of 3-indolylmethanamines to react with nucleophiles in aqueous media. Because of the unique position of VDR cysteine residues, compounds such as 31B selectively inhibited VDR–coactivator interactions among other nuclear receptor–coactivator interactions. In respect to different coactivators, 31B preferably inhibited the interaction between VDR and SRC2. Interestingly, 31B induced apoptosis in cisplatin-resistant ovarian cancer cells SKOV3 (Guthrie et al., 2015). Further experiments *in vivo* confirmed the ability of 31B to reduce the growth of SKOV3-derived tumors. Mechanistic studies showed a reduction of glycose and lipid metabolism, which was mediated partially by VDR. Additional work resulted in the identification of PS121912 with sub-micromolar affinity for VDR (Figure 8) (Sidhu et al., 2014). PS121912 exhibited high selectivity toward VDR among other nuclear receptors. Chromatin immunoprecipitation studies showed the recruitment of NCoR to DNA bound VDR when treated with PS121912 (Sidhu et al., 2014). PS121912 induced apoptosis in leukemic HL-60 cell at 4.7 μM (EC_{50}) and regulated genes that are involved in the cell cycle and apoptosis, such as *CASP3* and *CASP7*. Like 31B, PS121912 was active *in vivo* and reduced rapid growth of HL-60 derived tumors in mice (Guthrie et al., 2015).

7. The VDR corepressor binding site

The VDR corepressor binding site is assumed to be formed either in the absence of VDR ligand or when bound to a VDR antagonist, which suppresses the relocation of helix 12 (Anami et al., 2016) (Figure 9). In contrast to other nuclear receptors, X-ray crystal structures of VDR bound to corepressor peptides have not been reported. However, *in vitro* studies have confirmed interactions between VDR and corepressors (Meyer and Pike, 2013). When bound to VDR, antagonist ML 3-452 decreased SRC3 binding to CYP24A1 promoter-bound VDR and increased the recruitment of corepressor NCoR (Lamblin et al., 2010). The conformational change of VDR helix 12 was confirmed by hydrogen/deuterium

exchange experiments when bound to antagonists/partial agonists (Zhang et al., 2010). The absence of helix 12 creates a large interaction surface between VDR and corepressors (Figure 9). Therefore, truncated corepressor peptides have very weak affinities for VDR-LBD (Teichert et al., 2009). Phage peptide libraries were used to identify novel peptide ligands for the VDR–antagonist complex (Zella et al., 2007). Although several peptides were isolated by interacting with VDR bound to antagonist/partial agonist ZK159222, none of them were able to bind VDR using a two-hybrid assay. Currently, no peptides or small molecules are known to bind the corepressor pocket of VDR.

8. A second binding site for lithocholic acid

Recent crystallization of zVDR and lithocholic acid (LCA) in the presence of coactivator peptide SRC2 resulted in two LCA molecules bound to zVDR (Figure 10) (Belorusova et al., 2014). Although this phenomenon is new for VDR, other nuclear receptors have been crystallized with two ligands bound, e.g. ERβ (Wang et al., 2006). The location, however, differs from ERβ in the sense that coactivator peptide SRC2 can bind VDR*2xLCA, whereas helix 12 blocks the coactivator binding site for the ERβ*2xhydroxytamoxifen complex. Thus, the chemical structure of the ligand influences the nuclear receptor conformation, which in turn influences the location and shape of the second binding site. For zVDR and LCA, the second LCA binding pocket is partially solvent exposed, has a relative large hydrophobic area, and offers interactions with S263 and K268 (Figure 10).

9. VDR and binding function 3

An alternative binding site for the androgen receptor (AR) called binding function 3 (BF3) was introduced in 2007 (Estebanez-Perpina et al., 2007). The analogous binding function 3 for VDR would be formed by helix 4 and helix 9 (Figure 11A). The first molecules that bound to AR-BF3 were identified by high throughput screening (Estebanez-Perpina et al., 2007). A later study applied virtual screening to identify additional molecules with high docking scores that inhibited AR-mediated transcription in cells (Lack et al., 2011). Interestingly, these molecules might also exhibit a good affinity for VDR-BF3, which is supported by the fact that molecular modeling give good docking scores for select molecules (Figure 11B).

10. Possible medical application of alternative VDR ligands

The clinical development of alternative VDR ligands is still underexplored, in contrast to ligands that bind the genomic VDR pocket (Figure 12). Ligands, including $1,25(OH)_2D_3$, bind cytosolic VDR and mediate non-genomic effects such as regulating ion-channel, phospholipase, and kinase activities. Anti-inflammatory effects have been shown to be mediated by direct interaction between liganded VDR and IKkβ (Chen et al., 2013). Thus, further ligand design might lead to the development of highly anti-inflammatory ligands. Inhibition of VDR–RXR dimerization and inhibition of VDR–coactivator interactions have antagonizing effects in transcription. Ligands that inhibit this interaction have been shown to exhibit anti-tumor activities (Guthrie et al., 2015). These ligand were non-calcemic, thus inhibiting VDR–coactivator interactions that mediate transcription of genes involved in

calcium homeostasis (Sidhu et al., 2014). The activation of VDR by minimizing interactions with corepressors has great potential because this mode would enhance vitamin D action, which in turn might be applicable to hyperparathyroidism. Unfortunately, ligands inhibiting this interaction have not yet been developed.

11. Conclusion and future directions

Protein crystal structures and molecular modeling have enabled the identification of new binding sites for small molecules (Figure 12). The majority of ligands bind the genomic VDR ligand binding pocket and induce small changes in the overall VDR structure. The alternative ligand binding pocket and BF3 pocket have been proposed for VDR, but have yet to be confirmed by X-ray crystallography. In contrast, peptides and a small molecule have been shown to bind the coactivator and the LCA second binding site, respectively. The VDR corepressor site is still elusive because of the absence of an apoVDR structure and the large number of naturally occurring VDR ligands that promote interactions with coactivators rather than corepressors. To accelerate the identification of alternative VDR binders, new assays have to be developed because current, commercially available binding assays only report the detection of small molecule binding to the genomic ligand and coactivator binding site. Many VDR ligands have been investigated for VDR binding and modulation of transcription. Rapid cellular effects were only investigated with a limited number of VDR ligands. A more concise battery of assays to characterize new VDR ligands would be helpful to identify classes of ligands with similar biological activity. This in turn will help to identify unique VDR ligands that interact with VDR at novel binding pockets.

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Abbreviations:

VDR	vitamin D receptor
RXR	retinoid X receptor
DBD	DNA binding domain
LBD	ligand binding domain
1,25(OH)$_2$D$_3$	1,25-dihydroxy vitamin D$_3$
SF-1	steroidogenic factor 1
LRH-1	liver receptor homolog 1
OC	osteocalcin
ERβ	estrogen receptor

DRIP205	vitamin D-interacting protein 205
SRC2	steroid receptor coactivator 2
NCoR	Nuclear receptor co-repressor 1
zVDR	zebrafish vitamin D receptor
LCA	lithocholic acid
AR	androgen receptor
BF3	binding function 3

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Highlights:

- Ligands with strong VDR interactions bind the genomic binding pocket
- The majority of biological effects mediated by VDR are based on gene transcription
- Ligands that bind alternative VDR pockets have a low affinity for VDR

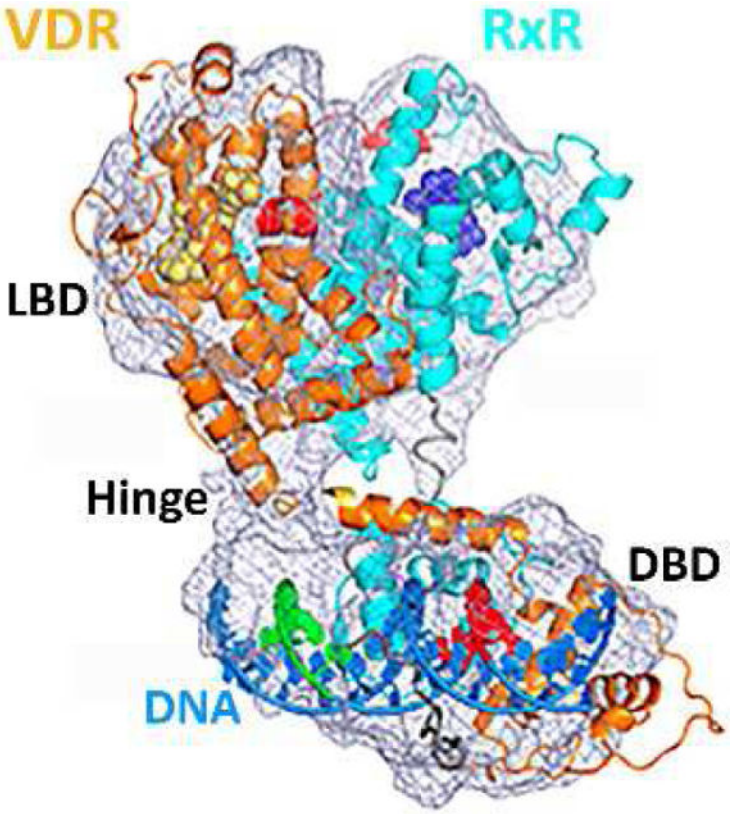

Figure 1.
Cryo-electron microscopy structure of liganded human VDR and RXR, bound to DNA and coactivator peptides. (Redrawn after Orlov et al., 2012)

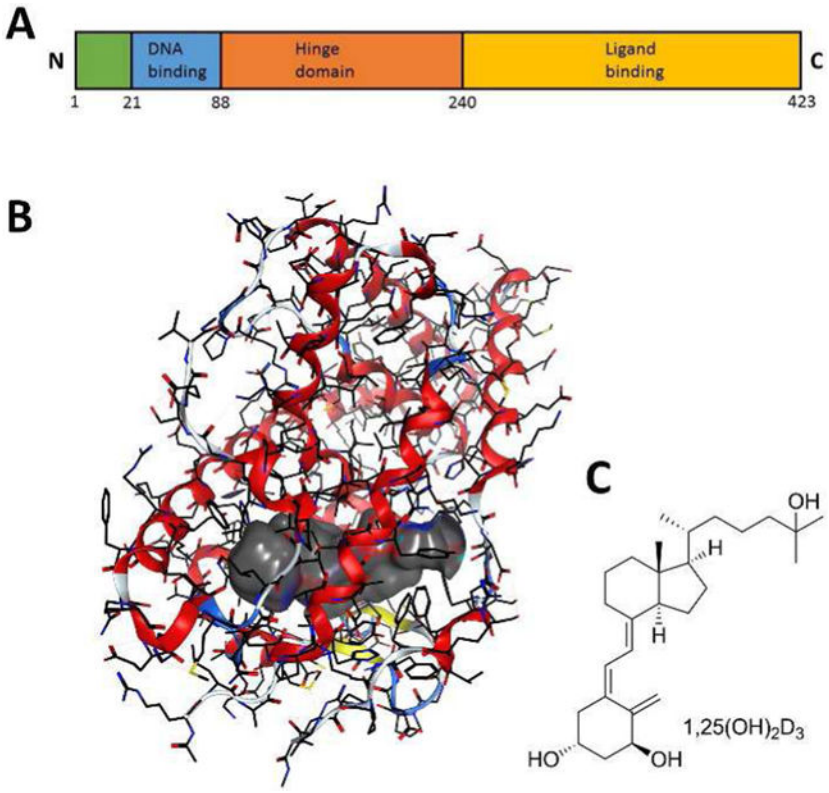

Figure 2.

A) VDR domains; B) X-ray crystal structure of VDR-LBD bound to $1,25(OH)_2D_3$ (gray surface) [1DB1]; C) Structure of $1,25(OH)_2D_3$.

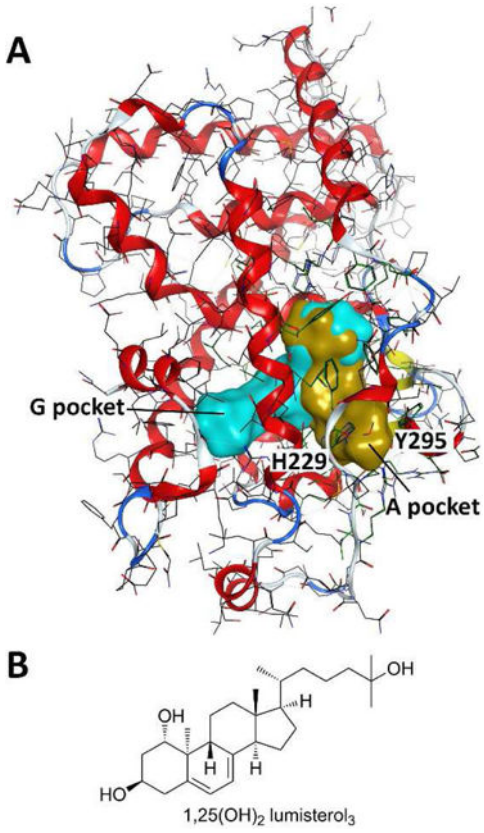

Figure 3.

A) VDR-LBD with genomic pocket (cyan) and alternative pocket (gold) recreated using crystal structure [1DB1] and instruction from (Mizwicki et al. 2004); B) Structure of $1,25(OH)_2$ lumisterol D_3

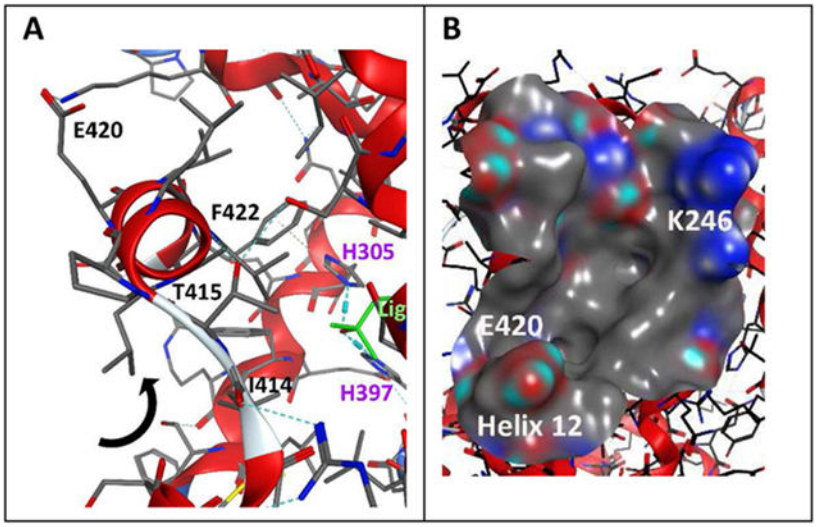

Figure 4.

A) Interaction between ligand and VDR-LBD with helix 12 that form the coactivator binding pocket; B) Surface of the coactivator binding site with clamp charged residues K246 and E420 [1DB1, crystal structure].

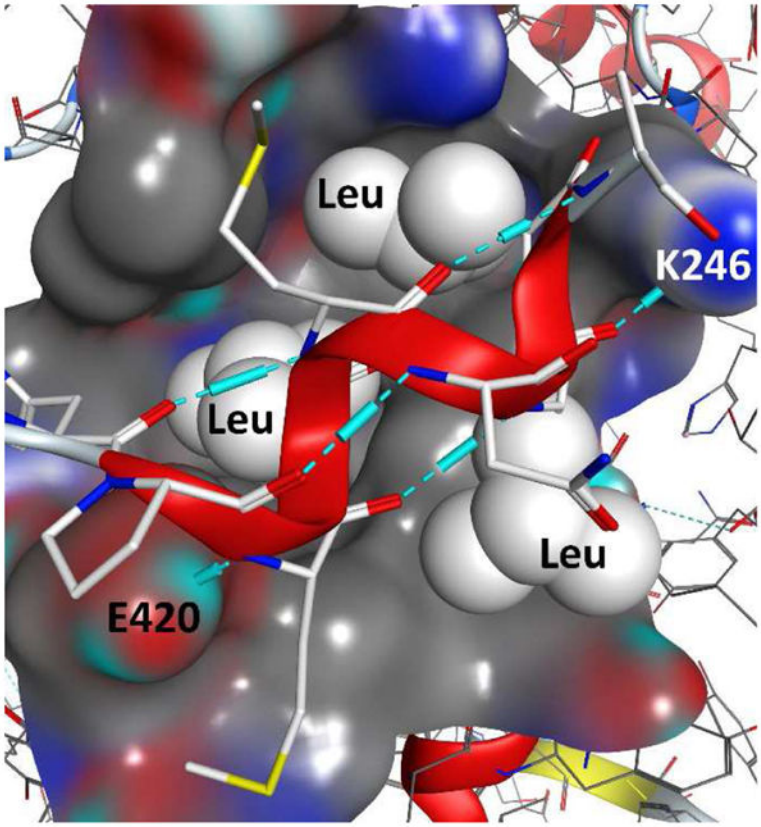

Figure 5.
Interaction between VDR-LBD and coactivator peptide DRIP2 (LxxLL) in the presence of $1,25(OH)_2D_3$.[1RKH, crystal structure]

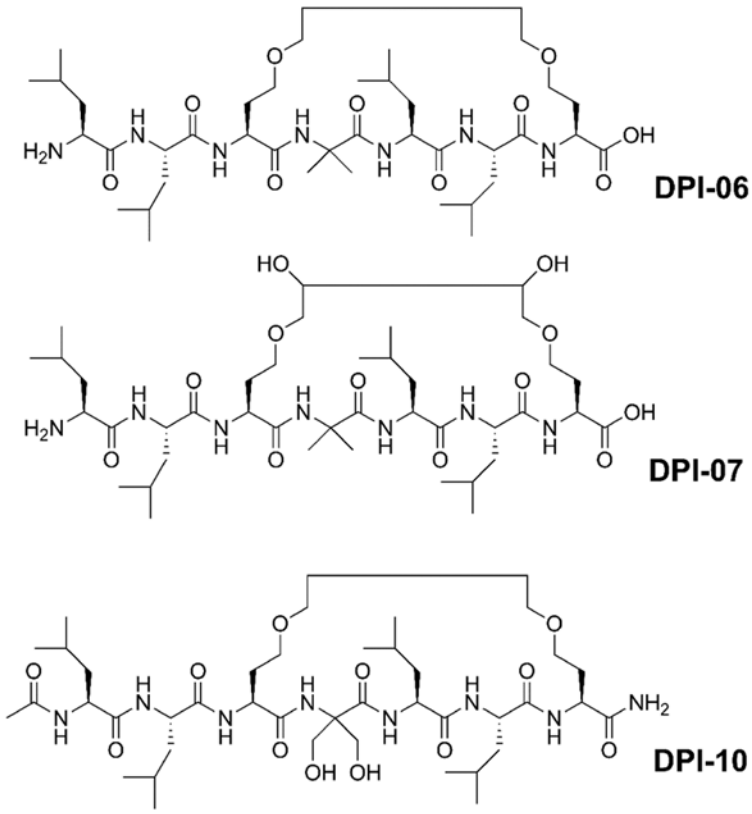

Figure 6:
Structures of cyclic, peptide-based VDR–coactivator inhibitors.

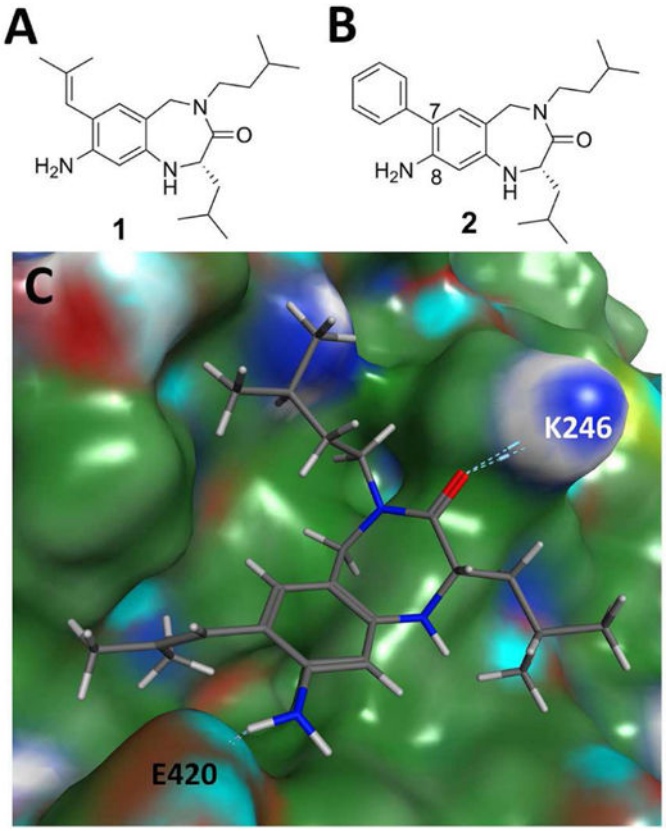

Figure 7.

A&B) Small molecule inhibitors of the VDR-coactivator interaction; C) docking structure of 1 bond to the VDR coactivator site using 1RKH crystal structure.

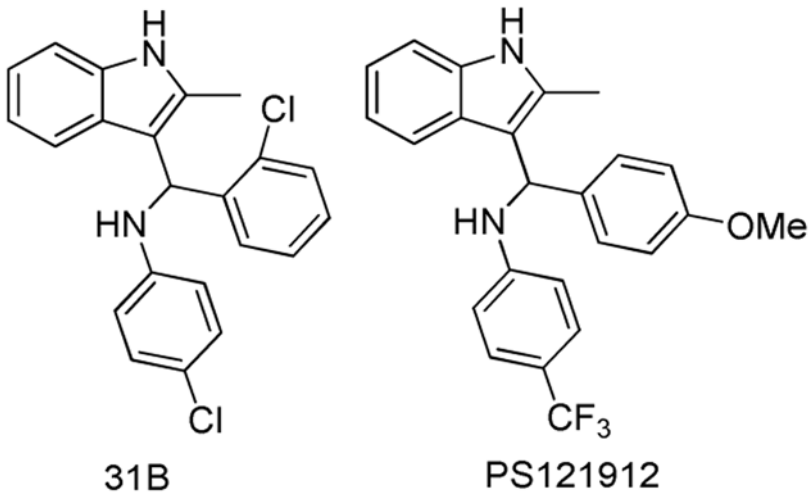

Figure 8:
Structure of VDR–coactivator inhibitors 31B and PS121912.

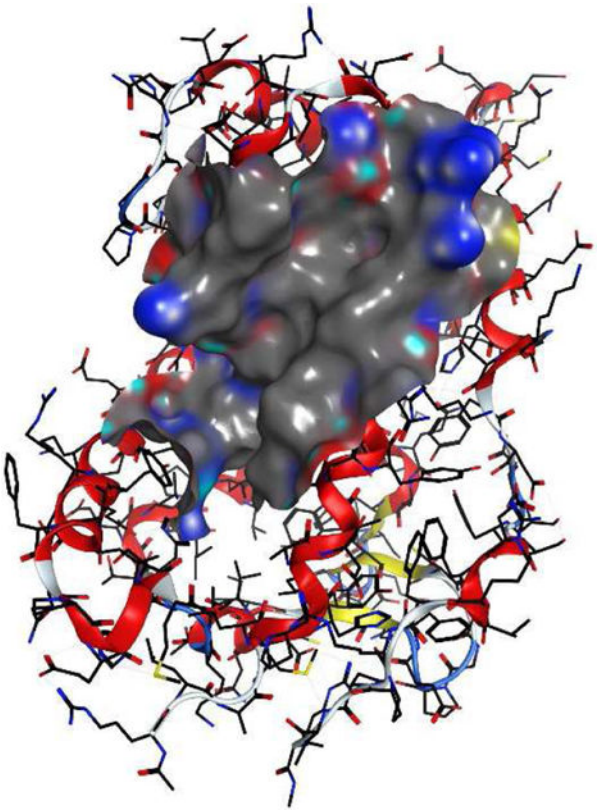

Figure 9.
VDR corepressor binding site is visible when H12 was deleted from the crystal structure 1RKH.

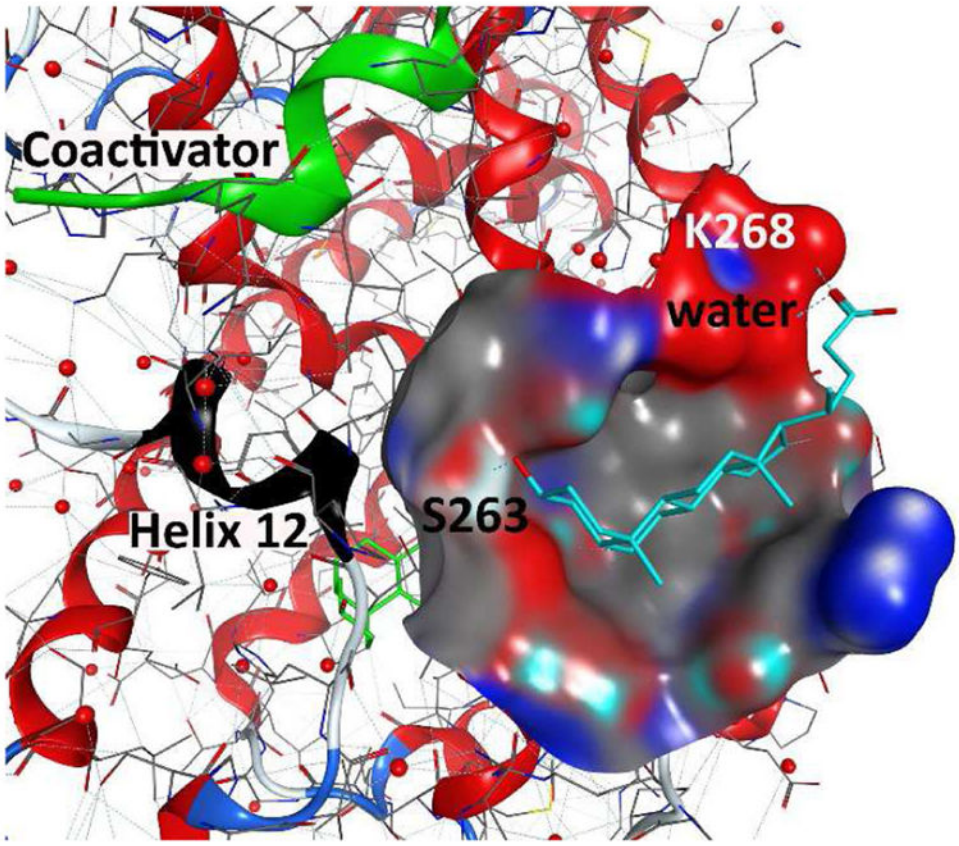

Figure 10.
Novel binding site for lithocholic acid in addition to the genomic VDR–ligand binding pocket based on crystal structure [4Q0A].

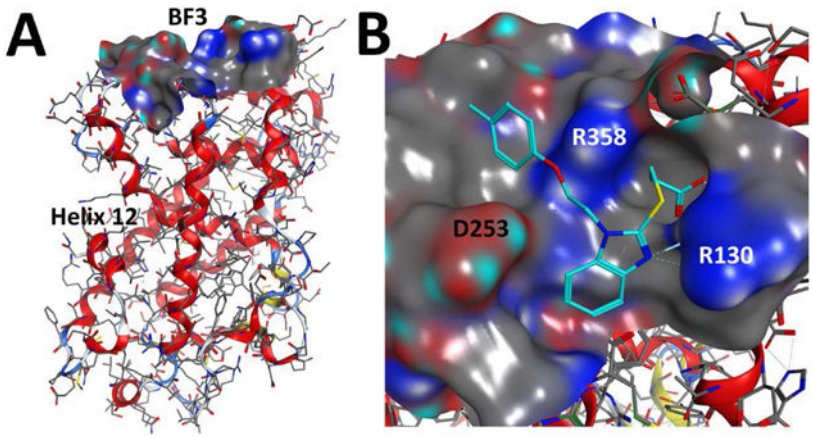

Figure 11.

A) Proposed binding function 3 (BF3) of VDR [1DB1]. B) Molecular docking of an AR-BF3 binder to VDR-BF3 using 1DB1 structure.

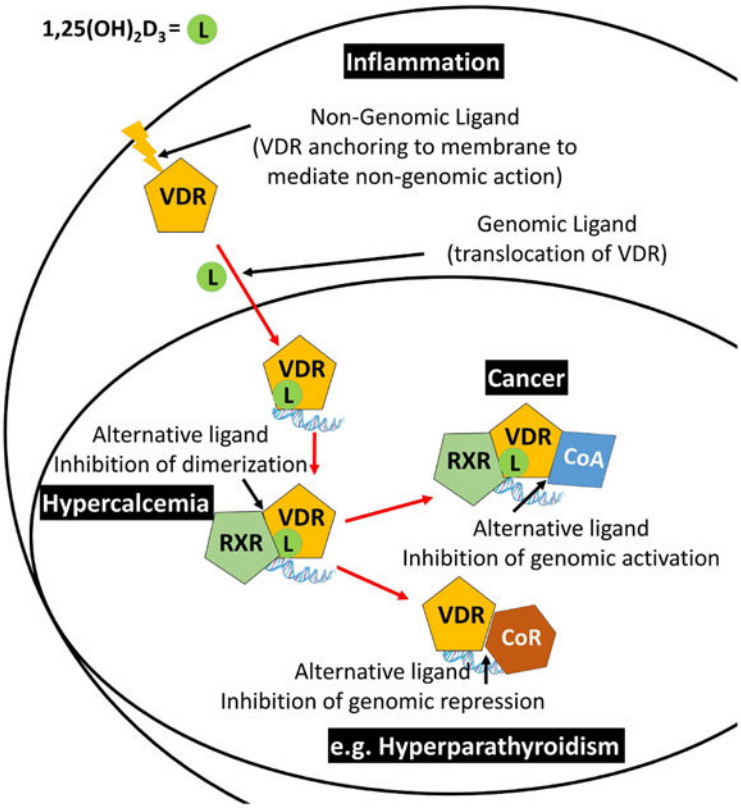

Figure 12.

Cellular interaction of VDR with endogenous ligand $1,25(OH)_2D_3$ and proteins that enable interception by alternative VDR ligands and their possible therapeutic applications.

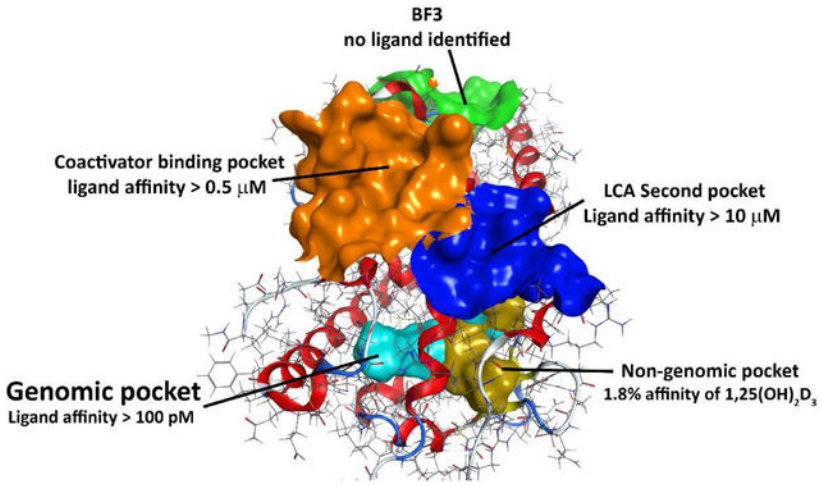

Figure 13.
Currently identified and proposed binding sites for VDR ligands.

Table 1.

Amino acid sequences of peptides that inhibit the interaction between VDR and coactivators.

Entry	name	Amino acid sequence																		
1	DRIP2	N	T	K	N	H	P	M	L	M	N	L	L	K	D	N	P	A	Q	D
2	C33	H	V	E	M	H	P	L	L	M	G	L	L	M	E	S	Q	W	G	A
3	D47	H	V	Y	Q	H	P	L	L	L	S	L	L	S	S	E	H	E	S	G
4	EBIP-37	T	G	G	G	V	S	L	L	L	H	L	L	N	T	E	Q	G	E	S
5	EBIP-51	F	P	A	E	F	P	L	L	T	Y	L	L	E	R	Q	G	M	D	E
6	EBIP-70	V	M	G	N	N	P	I	L	V	S	L	L	E	E	P	S	E	E	P
7	EBIP-96	V	E	S	E	F	P	Y	L	L	S	L	L	G	E	V	S	P	Q	P
8	3	L	S	E	T	H	P	L	L	W	T	L	L	S	S	E	G	D	S	M
9	4	M	Q	E	R	F	P	M	L	W	D	L	L	D	L	P	S	P	T	S
10	5	L	G	E	S	H	P	L	L	M	Q	L	L	T	E	N	V	G	T	H