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Inhibitors for the Vitamin D Receptor–Coregulator Interaction

Kelly A. Teske, Olivia Yu, and Leggy A. Arnold*

Department of Chemistry and Biochemistry and Milwaukee Institute of Drug Discovery (MIDD),
University of Wisconsin-Milwaukee, 3210 N. Cramer Street, Milwaukee, WI, 53211, USA

Abstract

The vitamin D receptor (VDR) belongs to the superfamily of nuclear receptors and is activated by the endogenous ligand 1,25-dihydroxyvitamin D₃. The genomic effects mediated by VDR consist of the activation and repression of gene transcription, which includes the formation of multi-protein complexes with coregulator proteins. Coregulators bind many nuclear receptors and can be categorized according to their role as coactivators (gene activation) or corepressors (gene repression). Herein, different approaches to develop compounds that modulate the interaction between VDR and coregulators are summarized. This includes coregulator peptides that were identified by creating phage display libraries. Subsequent modification of these peptides including the introduction of a tether or non-hydrolysable bonds resulted in the first direct VDR–coregulator inhibitors. Later, small molecules that inhibit VDR–coregulator interactions were identified using rational drug design and high throughput screening. Early on, allosteric inhibition of VDR–coregulator interactions was achieved with VDR antagonists that change the conformation of VDR and modulate the interactions with coregulators. A detailed discussion of their dual agonist/antagonist effects is given as well as a summary of their biological effects in cell-based assays and *in vivo* studies.

Keywords

Vitamin D receptor; VDR; coregulator; coactivator; corepressor; coregulator peptides; VDR–coregulator inhibitor; VDR antagonist

1. Introduction

1,25-Dihydroxyvitamin D₃ (1,25-(OH)₂D₃) is a highly active metabolic product of vitamin D₃, which is produced from cholesterol via a light-induced rearrangement in skin cells or absorbed from various food sources in the intestine. The phase I metabolism of vitamin D₃ is mediated by P₄₅₀ enzymes, especially CYP2R1 (1) and CYP27B1 (2), which are mainly expressed in the liver and kidney. The hydroxylation of vitamin D₃ to 1,25-(OH)₂D₃ increases its affinity for the vitamin D receptor (VDR), which is a member of the nuclear receptor superfamily. VDR, like many nuclear receptors, binds DNA as a heterodimer with retinoid X receptor (RXR), which is promoted by 1,25-(OH)₂D₃ (3). Although ligand binding is essential for gene regulation, many other proteins have been identified that mediate the process of gene transcription. These include coregulatory proteins or

*Corresponding author: arnold2@uwm.edu, Tel. 414-229-2612, Fax 414-229-3029.

coregulators that bridge the gap between the nuclear receptor dimer and RNA polymerase II. Currently more than 250 coregulators have been identified, but their exact functions and interactions with DNA-bound VDR are only partially understood (4). Coregulators can be categorized as either coactivators or corepressors depending on their influence on gene expression. Coactivators may stabilize the transcriptional machinery; have endogenous histone acetyl transferase (HAT) activity or other enzymatic functions that alter chromatin structure, or are involved in mRNA maturation (Figure 1). In contrast, corepressors may reduce the basal activity of nuclear receptor-mediated transcription by means of disrupting the DNA-bound multi-protein transcriptional complex or by recruitment of histone deacetylases (HDAC) to induce a tighter chromatin structure. Herein, we will discuss coregulators relevant to VDR and small molecules that can inhibit these interactions, with an emphasis on VDR–coactivator interactions. We will also summarize the physiological effects of these small molecules *in vitro* and *in vivo*.

1.1. VDR Coactivators

The binding between VDR and 1,25-(OH)₂D₃ induces a conformational change that enables the interaction between VDR and coactivators; thus, agonist binding is important for most VDR–coactivator interactions. On the molecular level, this includes the repositioning of helix 12 (or the C-terminus of VDR) to form a new hydrophobic pocket that enables coactivator interaction. The molecular interaction between VDR and steroid coactivator 1 (SRC1) has been elucidated by x-ray crystallography with a SRC1 peptide that bears the nuclear interaction domain (NID) with a central LXXLL motif (L = leucine and X = any amino acid) (5). Although coactivator mutation studies have identified essential amino acids and a minimal amino acid sequence for nuclear receptor binding, crystal structures confirmed the presence of hydrogen bonding with the E420 and K246 residues of VDR, as well as hydrophobic interactions with NID leucine residues. SRC1 and its related coactivators SRC2 and SRC3 exhibit three NIDs (6). Binding studies with different SRC NIDs have demonstrated the preference of VDR for the third NID of all SRCs (7). Once bound to the DNA/VDR/RXR complex, SRCs are able to transfer acetyl groups to histones to weaken the interaction between negatively charged DNA phosphate backbone and the usually positively charged histone (8, 9). In addition, SRCs recruit other proteins with HAT activity such as CREB-binding protein/p300 (10) and p300/CBP-associated factor (11). Further relaxation of chromatin may be achieved with the recruitment of methyltransferase 1 and protein arginine N-methyltransferase 1 (12, 13). The vitamin D receptor interacting protein (DRIP) complex includes coactivator DRIP205 (14), which directly interacts with VDR and is important for the recruitment of RNA polymerase II (15). DRIP205 and other partners of this coactivator complex lack HAT activity (16), supporting the model of a sequential recruitment of DRIP205 after the dissociation of VDR and SRCs (17). Other coactivators have been identified that interact with VDR, such as PGC-1 alpha (18), NCoA62 (19), Smad3 (20), Ets-1 (21), WINAC (22), and CCAAT displacement protein (23).

1.2. VDR Corepressors

Similar to other nuclear receptors, VDR interacts with corepressors in the absence of ligand or in the presence of antagonists (24). Crystal structures reveal that this interaction includes

a different orientation of helix 12 and requires multiple and longer NIDs (25). The most studied corepressors for VDR are the nuclear receptor corepressor (NCoR) (26) and the silencing mediator for retinoid or thyroid-hormone receptors (SMRT) (27). In complex with VDR, they recruit HDAC3 which in turn improves chromatin packing close to the promotor site and represses transcription (24). Other corepressors interacting with VDR are Hairless (28), and Alien (29).

2. Peptide-based Inhibitors of the VDR–Coregulator Interaction

The identification of the central coactivator LXXLL motif as essential to mediating nuclear receptor binding prompted investigations to develop peptide-based VDR–coactivator inhibitors to evaluate the function of this protein–protein interaction. Pioneered by McDonnell et al. for the estrogen receptor, a phage display library of synthetic LXXLL peptides was generated and screened with two hybrid assays against a panel of nuclear receptors including VDR (30–32). Two peptides, C33 and D47, were identified to bind VDR (Figure 2). Further investigations of this library identified two more peptides, EBIP41 and EBIP44, with moderate affinity for VDR. Importantly, when C33, D47, EBIP41 and EBIP44 peptides were expressed as Gal4 DBD (DNA binding domain) fusions in cells, they inhibited the VDR-mediated transcription in a reporter assay under control of an osteocalcin (OC) promoter (33). In addition, RXR-selective peptide F6 was able to inhibit VDR-mediated transcription, demonstrating transactivation between RXR and VDR. A more exhaustive phage display library identified three more LXXLL peptides (Figure 2, compounds 3, 4, and 5) that not only bind VDR in a two hybrid assay but also inhibit VDR-mediated transcription when expressed in cells (34). These peptides exhibit a consensus sequence of (H/F)P(L/M)LXXLL. Importantly, the binding of these peptides to VDR was more pronounced in the presence of VDR agonists than VDR antagonists. However, the limitation of these peptides for further investigation is their inability to regulate endogenous VDR target genes. Overall, phage display studies contributed to the validation of VDR–coactivator interactions as a target to modulate transcription. In addition, they produced the first potent and selective VDR–coactivator inhibitors. Currently, coregulator peptides are used as fluorescent probes for high throughput assays (HTS) to identify new nuclear receptor modulators (35).

Different strategies have been developed to overcome the limitations of peptide reagents in cell-based assays, such as inactivity when transfected as a fusion peptide or limited cell permeability and stability (36). For the thyroid receptor and estrogen receptor–coactivator interactions, cyclic LXXLL peptides were generated by means of a lactam bridge, resulting in inhibitors with IC₅₀ values of less than 100 nM (37, 38). Improvements on these peptide inhibitors were made by introducing a non-redox-sensitive thioether bridge (39) and unnatural amino acids such as neopentyl glycine instead of leucine. The most active inhibitors, known as PERMS (peptidomimetic estrogen receptor modulators) had IC₅₀ values as low as 70 pM (40). Cellular activity for these peptide inhibitors was not reported until (41) developed them with a nona-arginine tag (R₉) that was earlier introduced by (42) as an intracellular delivery tag. PERMs with a R₉ tag were reported recently with cellular activities as low as 3 μM for the regulation of the pS2 gene expression in MCF-7 cells (43). Recently, new cyclic peptide VDR–coactivator inhibitors were introduced by (44, 45)

bearing hydrocarbon linkers that have been reported to increase stability and oral bioavailability (46). A dramatic IC_{50} value change from 220 μM to 3.2 μM was observed by changing the nonfunctional linker (Figure 3, DPI-06) to a functionalized linker (Figure 3, DPI-07). Prior to this study, it was reported that all hydrocarbon linkers can result in multiple binding modes of cyclic peptides and, in the case of stapled peptide ER-coactivator inhibitors, they did not significantly increase their potency (47). The strong influence of the linker on VDR binding makes the design of these molecule unpredictable and without cell-based data, uncertain of their permeability and stability.

3. Small Molecule Inhibitors of the VDR-Coregulator Interaction

During the last decade, many direct inhibitors of various nuclear receptor-coactivator interactions have been developed (36, 48). In regard to VDR, (49) reported the first reversible inhibitor of the VDR-coactivator interaction in 2010. Using a rational design approach, a benzodiazepine scaffold was substituted with branched hydrophobic groups to mimic the *i*, *i*+3, and *i*+4 positions of leucine in coactivator DRIP205 (Figure 4) (5). Docking studies revealed that compound 2 might form hydrogen bonds with rat VDR clamp residues Glu416 and Lys242. The inhibition activity of compound 2 (IC_{50} = 17 μM) was recapitulated in cells with a reporter gene assay. In addition, compound 2 inhibited estrogen receptor β (ER β) mediated transcription at a similar concentration, whereas ER α mediated transcription was not modulated. Prompted by these results, a more exhaustive structure-activity relationship (SAR) study was reported in 2013 by the same group (50). Despite the large number of analogs with various substituents in the 7- and 8- position, only marginal improvement (IC_{50} = 14 μM) was observed for compound 35 (Figure 4). However, the aniline function in the 8-position was confirmed to be important for binding, probably interacting with Glu417 of VDR.

The first irreversible VDR-coactivator inhibitors were identified in 2012 using high throughput screening (51). Among 275,000 compounds, 140 inhibitors with cellular activity were identified, including a group of 3-indolylmethanamines. A comprehensive SAR study around the 3-indolylmethanamine scaffold identified compound 31B as the most active VDR-coactivator inhibitor in cells (IC_{50} = 4.2 μM [Figure 5, A]). In addition, a linear free energy relationship between inhibition rates of 3-indolylmethanamines bearing different electronic substituents confirmed irreversibility. Due to the unique mode of binding, a high selectivity of 31B toward VDR with respect to other nuclear receptors was observed. In addition, 31B is selective towards the interaction between VDR and coregulator peptide SRC2-3 above other LXXLL coregulator peptides. Importantly, down-regulation of VDR target gene *TRPV6* by 31B was observed in the presence of 1,25(OH) $_2$ D $_3$ for DU145 cancer cells as well as anti-proliferation at higher concentration. Inhibition of VDR-mediated transcription and anti-proliferation in the presence of 31B was also observed for ovarian cancer cells OVCAR8 and SKOV3 and endometrial cancer cells ECC-1. In cis-platinum resistant SKOV3 cells, other biomarkers of anti-proliferation and apoptosis were upregulated in the presence of 31B, such as activation of caspase 3, phosphorylation of MAP kinases p38 and SAPN/JNK, up-regulation of P21, and cell-cycle arrest. In a cisplatin-resistant SKOV3 xenograft tumor model, 31B treatment delivered 5 times a week at a dose of 5 mg/kg led to suppressed tumor growth after two weeks. In addition, reduced tumor

formation was partially caused by a compromised *de novo* production of fatty acids due to lower expression of FASN in the tumor. Further SAR studies resulted in the discovery of 3-indolylmethanamine PS121912, a VDR–coactivator inhibitor that inhibited VDR-mediated transcription with an IC₅₀ of 590 nM (Figure 5A) (52). Similar to 31B, PS121912 is selective towards VDR and has a preference for the interaction between VDR and coregulator peptide SRC2–3. Importantly, ChIP studies revealed that in HL60 leukemia cells PS121912 was able to reduce the DNA occupancy of VDR and binding of SRC2. However, PS121912 promoted the recruitment of NCoR to the VDR–DNA complex (53). PS121912 reversed the regulation of VDR target genes in the presence of 1,25-(OH)₂D₃ at a concentration of 500 nM and modulated the transcription of many genes affiliated with the cell cycle control. Elevated levels of P21 protein levels were observed for the PS121912 in the presence and absence of 1,25-(OH)₂D₃ in HL60 cells as well as increased levels of pro-apoptotic serine protease HTRA. In a mouse HL60 xenograft model at 3 mg/kg five times a week, a significant change in tumor volume was observed after three weeks of treatment (Figure 5B)(54). The blood calcium levels and animal weight did not differ from the control group.

4. VDR Antagonists or Allosteric Inhibition of the VDR–Coregulators Interaction

The synthesis of new synthetic analogs of 1,25-(OH)₂D₃ resulted in the identification of new VDR ligands that initiate the recruitment of coactivators much like 1,25-(OH)₂D₃. However, a different class of VDR ligands were discovered that bind VDR and only weakly promote VDR–coactivator interactions. Usually, the biological effects of these antagonists have been determined in the presence of agonists like 1,25-(OH)₂D₃, giving results similar to the vehicle control. Interestingly, the degree of coactivator recruitment by VDR depends on the chemical structure of the VDR antagonist. Thus, the quality of a VDR antagonist can be defined by its residual agonistic activity. On the molecular level, this behavior is believed to be caused by the orientation of helix 12 (Figure 6). Depending on the structure, VDR antagonists may influence the equilibrium of VDR bound to coactivators, corepressors, or neither. Crystal structures of all three possible complexes have been reported for nuclear receptors. However, VDR prefers to crystallize solely with an agonist arrangement. Recently, VDR–antagonist structures showed some significant differences in their overall structure in comparison with the VDR–agonist complex. However, it is believed that these high energy structures are accompanied by less-ordered VDR–antagonist structures that don't crystallize. Herein, we will discuss the biological consequences of VDR antagonists in the presence and absence of 1,25-(OH)₂D₃.

4.1. TEI-9647

Early identification of antagonist effects of VDR ligands was based on their ability to inhibit the differentiation of promyelocytic leukemia cells. In the presence of 1,25-(OH)₂D₃, HL60 cells transition to monocytes, which is believed to be mediated by genomic effects of VDR, including the recruitment of coregulators (55). In the contrary, NB4 cell differentiation in the presence of 1,25-(OH)₂D₃ is considered a model for non-genomic 1,25-(OH)₂D₃ mediated effects (56). TEI-9647 and its diastereomer TEI-9648 (Figure 7) inhibited HL60

differentiation but not NB4 differentiation in the presence of 1,25-(OH)₂D₃ (57). The differentiation was quantified by the reduction of a redox-sensitive dye (nitro blue tetrazolium chloride or NBT) and modulation of cell surface marker expression of CD71 and CD11b (58). Cell differentiation can also be induced by retinoic acids,(59) promoting the transition of HL60 cells into granulocytes. TEI-9647 and TEI-9648 were not able to inhibit this transition in the presence of 1,25-(OH)₂D₃; thus both compounds are not interacting with RXR.(58) TEI-9647 and TEI-9648 bind VDR 10-fold less than 1,25-(OH)₂D₃ (58). TEI-9647 reduced the expression of VDR target genes *CYP24A1* and *P21* in HL60 cells treated with 1,25-(OH)₂D₃ (60, 61). Reporter assays carried out with different cells lines confirmed transcriptional inhibition by TEI-9747 in the presence of 1,25-(OH)₂D₃ – although to a different degree. In HeLa cells (human cervical carcinoma) TEI-9647 behaved like a very weak antagonist, which was partially caused by the inability of TEI-9647 to block VDR–RXR binding (62). However, TEI-9647 did not influence the VDR–DNA binding shown by gel shift. Importantly, TEI-9647 reduced the binding between VDR and coactivator SRC1 in Saos-2 cells but did not influence the effect of 1,25-(OH)₂D₃ with respect to translocation to the nucleus as shown with a GFP-labeled VDR in COS7 cells. The partial antagonist/agonist effect of TEI-9647 was also observed by (63) using gel shift experiments to quantify the binding between VDR and RXR. However, the antagonist activity of TEI-9647 was much greater than its agonist activity with an IC₅₀ value of 2.5 nM. TEI-9647 induces the release of coactivator SRC2 and corepressor NCoR *in vitro*, which was observed in the presence and absence of RXR (63, 64). In addition, TEI-9647 inhibited the recruitment of SRC3 and DRIP205 (65).

An osteocalcin VDRE reporter assay in COS7 cells was used to investigate TEI-9647 in regard to a possible conjugate addition with VDR's nucleophilic residues H397 or H305 because analogs of TEI-9647 lacking the exocyclic double bond are very weak VDR binders (Figure 8) (66). Although TEI-9647 was fully reversible with 1,25-(OH)₂D₃, limited proteolysis assays showed a new 30 kDa band of VDR-LBD in the presence of TEI-9647. Further mechanistic studies using x-ray crystallography and mass spectrometry demonstrated that rat VDR but not human VDR showed a second mass peak after incubation with TEI9647 (67). The formation of a covalent adduct between VDR and TEI-9647 is supported by the fact that in contrast to rat VDR, human VDR has two cysteine residues (C403 and C410) in proximity to the unsaturated lactone of TEI-9647 (Figure 8). However, an agonist conformation with respect to the orientation of VDR's helix 12 was observed for crystal structures with TEI-9647 and rVDRwt, hVDR H305F, and hVDRH305F/H397F mutants. Although 1,25-(OH)₂D₃ interacts with both VDR histidine residues, TEI-9647 prefers H309 and interacts with H395 if H305 is mutated. The authors suggested that the antagonist action of TEI-9647 involves the interaction with H395 that positions the Michael acceptor (TEI-9647) toward C403 followed by alkylation. Further analysis of TEI-9647 using binding studies and molecular modeling demonstrated that TEI-9647 is a potent VDR agonist for hVDRH305F and hVDRH305F/H397F, reducing the ability of TEI-9647 to covalently interact with VDR (68). Therefore, TEI-9647 behaved like an antagonist in human cells but as a weak agonist in rat cells (69). Mutation of hVDR C403 and C410 residues eliminated the antagonistic effects of TEI-9647, and incorporation of cysteine residues into rVDR increased the antagonist activity of TEI-9647 (64). Interestingly, the

affinity of TEI-9647 for hVDR and hVDR C403S/C410N was similar, whereas the efficacy for hVDR was significantly higher for the double mutant. Importantly, TEI-9747 bound to hVDR was not able to recruit coactivator SRC2, in contrast to the hVDR double mutant.

Not surprisingly, the first analogs of TEI-9647, HLV and GC-3, behaved as agonists in a rat ROS17/2.8 osteoblast transcription assay and were able to increase calcium transport *in vivo* in D-deficient rats without antagonizing this action in the presence of 1,25-(OH)₂D₃ (Figure 9) (70). However, in COS7 monkey kidney cells, both compounds behaved as transcriptional antagonists in the presence of 1,25-(OH)₂D₃ (71, 72). Interestingly, only GC-3 was able to promote the binding between VDR and RXR, although both compounds reduced the VDR–RXR interaction in the presence of 1,25-(OH)₂D₃. In addition, both compounds reduced the recruitment of coactivators SRC1, SRC2, and SRC3 to VDR in the presence of 1,25-(OH)₂D₃ and inhibited the interaction with corepressors NCoR and SMRT in the absence of 1,25-(OH)₂D₃. The mRNA levels of VDR target genes such as *CYP3A4*, *E-Cadherin* and *CaT1* were repressed in the presence of 1,25-(OH)₂D₃ in SW480 cells (72). Further analogs of TEI-9647 include compounds with different substituents at the 2-position (Figure 10) (73–77). Compound 6a bearing a methyl substituent had 3 times greater affinity towards VDR than TEI-9647; however the ability to differentiate HL60 cells was only 32% of that of TEI-9647 (74). A 28-fold improvement of antagonist activity (HL60 differentiation) was observed for 5b in comparison to TEI-9647 exhibiting the same VDR affinity. The introduction of substituents in the 24-position resulted in epimers 16 and 17 that exhibited more than a 2-fold improvement of binding and antagonist activity. Further improvements were achieved with the introduction of two methyl groups at the 24-position, increasing the antagonist activity of 39 more than 13-fold. Finally, compound 39a has an IC₅₀ value of 93 pM and a VDR affinity of 67% in comparison with 1,25-(OH)₂D₃. QSAR analysis of these TEI-9647 analogs using a comparative molecular field analysis and a comparative similarity indices analysis resulted in the identification of the optimal spatial arrangement of substituents at the 2 and 24 position of TEI-9647 analogs (78–80).

In contrast to healthy patients, endogenous 1,25-(OH)₂D₃ strongly induced the formation of osteoclasts in patient with Paget's disease, leading to hyper bone resorption.(81) In bone marrow cells from patients with Paget's disease, TEI-9647 reduced the formation of osteoclasts in the presence and absence of 1,25-(OH)₂D₃ and in turn inhibited bone resorption induced by 1,25-(OH)₂D₃.(82) In addition, TEI-9647 inhibited the up-regulation of coregulator TAF_{II}-17 in the presence of 1,25-(OH)₂D₃, which is believed to mediate the hyperresponsiveness in Paget's disease patients towards 1,25-(OH)₂D₃ (83). TEI-9647 was also investigated in rats that were fed a vitamin D deficient, low calcium diet (-D rats) (84). The effect of 1,25-(OH)₂D₃ on intestinal calcium transport is biphasic with an earlier independent phase (85) and a later genomic driven process (86). TEI-9647 did not modulate the first phase but rather inhibited the effect of 1,25-(OH)₂D₃ in the later genomic response. *Parathyroid hormone (PTH)* is a negatively controlled gene by 1,25-(OH)₂D₃ (87). In -D rats, different doses of TEI-9647 acted as a weak agonist by decreasing PTH levels, stimulating calcium absorption, and mobilizing bone calcium. However, in combination with 1,25-(OH)₂D₃, TEI-9647 antagonized the action of the natural vitamin D hormone. In renal tissue, it was found that TEI-9647 inhibited the 1,25-(OH)₂D₃-induced relaxation of

renal arteries from hypertensive patients (88). Similar results were observed in *ex-vivo* rat renal arteries treated with U46619, a thromboxane A2 agonist. Here, TEI9647 reduced the phosphorylation of endothelial nitric oxide synthase and levels of NO that were elevated in the presence of 1,25-(OH)₂D₃ (89). In respect to cancer, TEI9647 reversed the down-regulation of *CYP21A2* in the presence of 1,25-(OH)₂D₃ in a reporter assay using mouse and human adrenocortical carcinoma cells (90). In MCF-7 cells, TEI-9647 reversed the 1,25-(OH)₂D₃-induced inhibition of aromatase enzyme, which is responsible for the conversion of testosterone to estradiol (91). In primary ER α -negative breast cancer cells, TEI-9647 reduced the induction of mRNA levels of *ER α* in the presence of 1,25-(OH)₂D₃ (92). In human acute lymphoblastic leukemia cells, TEI-9647 reduced the induction of proliferation by 1,25-(OH)₂D₃ in the presence of dexamethasone (93). Furthermore, in trophoblasts, TEI-9747 reversed the down-regulation of the *IL-10* mRNA in the presence of 1,25-(OH)₂D₃ (94) and antagonized the expression of *TNF- α* , *IL-6*, and *IFN- γ* in cultured trophoblasts in the presence of 1,25-(OH)₂D₃ (95). TEI-9647 also reversed the up-regulation of *prolactin* gene in human peripheral blood mononuclear cells in the presence of 1,25(OH)₂D₃ (96). In primary T-cells, TEI-9647 reversed the effects of 1,25-(OH)₂D₃ that induced IL-31 and oncostatin M production and reduced IL-22 expression. TEI-9647 also reduced the surface expression of VDR-target gene CCR10 in IL-21-induced terminal differentiation human B cells in the presence of 1,25-(OH)₂D₃. In human keratinocytes and neutrophils in the presence of 1,25-(OH)₂D₃, TEI-9647 inhibited the production of IL-37 as well as the induction of mRNA levels of *cathelicidin* (97).

4.2. ZK159222

ZK159222 exhibited a sub-nanomolar affinity for the VDR-RXR-VDRE (63, 98) (Figure 11). In the presence of 1,25-(OH)₂D₃, ZK159222 inhibited VDR-mediated transcription with an IC₅₀ value of 300 nM. SDS-PAGE demonstrated three different conformations of VDR–ZK159222 complex (69). These conformations may be responsible for the dissociation between liganded VDR and coactivators SRC1, SRC2, SRC3, and DRIP205 (34, 65). In addition, the interaction between corepressor NCoR and VDR was inhibited (63). The inhibitory effect was more pronounced in MCF-7 (breast) than HeLa (cervix) cells partially due to different expression levels of coactivators. ZK159222 also inhibited the interaction between VDR and corepressor SMRT as demonstrated with a pull-down assay (99). Furthermore, ZK159222 acted as a weak agonist (20% efficacy) in the absence of 1,25-(OH)₂D₃, which might be partially mediated by RXR. Indeed, gel shift assays have shown that 9-cis-RA was able to increase the agonist activity of ZK159222, enabling recruitment of SRC1, SRC2, and SRC3 (100). In the presence of 1,25-(OH)₂D₃, ZK159222 was able to inhibit the gene regulation mediated by RAR in the presence of retinoic acid (101). However, in serum-depleted media the antagonist effect of ZK159222 was not observed at a concentration of 100 nM. For rVDR, ZK159222 inhibited the interaction between VDR and SRC1 and DRIP205 but not between VDR and SRC2 or SRC3 at a concentration of 100 nM. For both rVDR and hVDR, ZK159222 antagonized the transcriptional activation by 1,25-(OH)₂D₃ in a reporter assay (64, 101). Mutation studies and molecular dynamics (MD) studies revealed that ZK159222 disturbed the H397-F422 interaction, while C403 and C410 VDR mutants did not influence the antagonistic effect of ZK159222, thus excluding covalent interactions as observed for TEI-9647 (69). In osteoblastic MC3T3-E1 cells,

osteopontin (*OPN*) but not *CYP24A1* mRNA levels were upregulated in the presence of ZK159222. ZK159222 induced a weak recruitment of VDR and RXR to the *CYP24A1* promoter but a more pronounced recruitment to the *OPN* promoter. Further studies revealed that the agonistic effect of ZK159222 at the *OPN* promoter was probably caused by fully acetylated histones that facilitate transcription. Coactivator recruitment was not promoted by ZK159222 at both promoter sites (102). In human fetal osteoblastic cells (SV-HFO) in the presence of 1,25-(OH)₂D₃, ZK159222 inhibited the expression of *osteocalcin*, alkaline phosphatase activity, and calcium contents (103). In osteoblastic ST2 cells, ZK159222 blocked the activation of the mRLD5 region of *mRANKL* in the presence of 1,25-(OH)₂D₃ (104). In addition, ZK159222 inhibited the up-regulation of *creatine kinase* in the presence of VDR agonist and estradiol in osteoblast-like ROS 17/2.8 cells (105). The calcemic activity of ZK159222 was 0.02% of that of 1,25-(OH)₂D₃ in mice after 5 days of 10 µg/kg/d (101).

ZK159222 also inhibited the differentiation of HL60 cells in the presence of 1,25-(OH)₂D₃ at a concentration of 6 nM (106). The process involves the up-regulation of *kinase suppressor of Ras-2* gene (*KRS-2*), which was demonstrated to be inhibited by ZK159222. (107) In addition, ZK159222 inhibited the phosphorylation of Raf-1 (108, 109) and the expression of *pRb* and *c/EBPβ* in the presence of 1,25-(OH)₂D₃. (110) ZK159222 inhibited the phosphorylation of phosphoinositide and Akt-mediated by phosphatidylinositol 3-kinase in the presence of 1,25-(OH)₂D₃ (111). Furthermore, ZK159222 inhibited the steroid sulphatase activity in the presence of 1,25-(OH)₂D₃ in HL60 cells (112). Interestingly, this effect was not observed in NB4 cells (113). In trophoblasts, ZK159222 reduced mRNA expression of *CYP24A1* in the presence of 1,25-(OH)₂D₃ and attenuated slightly the expression of *CYP27B1*. In the absence of 1,25-(OH)₂D₃, up-regulation of *CYP27B1* was more pronounced. ZK159222 was able to increase the concentration of cellular cAMP by itself. This effect was additive in the presence of 1,25-(OH)₂D₃. Similar results were obtained for hCG (human chorionic gonadotrophin) (114). Weak induction of *calbindin-D28K*, a cytosolic calcium binding protein, and *VDR* itself was observed in the presence of ZK159222 in choriocarcinoma derived cells (JEG-3). In the presence of 1,25-(OH)₂D₃, ZK159222 exhibited strong antagonist effects in these cells (115).

Antimicrobial response in monocytes pretreated with ZK159222 reduced the elevated *cathelicidin* mRNA levels when stimulated with a synthetic 19-kD *M. tuberculosis*-derived lipopeptide (116). In addition, ZK159222 reduced the up-regulation of *CYP24A1*, *CCL22*, and *CD300LF* in the presence of 1,25-(OH)₂D₃ in CD14⁺ monocytes (117). Furthermore, pretreatment with ZK159222 reduced the elevated *DEFB4* mRNA levels when stimulated with rIFN-γ and reversed the up-regulation of *CD14* and down-regulation of (toll-like receptors) *TRL2* and *TRL4* in the presence of 1,25-(OH)₂D₃ (118). In differentiated macrophages from human blood, addition of the VDR antagonist ZK159222 inhibited the induction of *human cationic antimicrobial protein-18* in the presence of 25-vitamin D₃ and TLR2/6 ligand Pam2CSK4 (119). ZK159222 also reversed the effect of 1,25-(OH)₂D₃ in *M. tuberculosis*-stimulated peripheral blood mononuclear cells (PBMC), which had decreased the protein and mRNA expression of *TLR2*, *TRL4*, *Dectin-1*, and *mannose receptor* (120).

ZK159222 also reduced the production of IL-10 in stimulated B-cells (121) and inhibited the differentiation of Langerhans cells (122).

In primary HUVEC cells, ZK159222 reduced the production of nitric oxide in the presence of 1,25-(OH)₂D₃ and reduced the phosphorylation of eNOS, p38, Akt, and ERK1/2 promoted by 1,25-(OH)₂D₃ (123). Overall, ZK159222 inhibited the growth of HUVEC cells in the presence of pro-proliferative 1,25-(OH)₂D₃ (124). In addition, ZK159222 reduced 3D cell migration stimulated by 1,25-(OH)₂D₃ and reduced mRNA levels of *MMP-2*. In keratinocytes, ZK159222 weakly activated the expression of *cathelicidin* in a luciferase reporter assay and slightly increased the production of cathelicidin in primary human keratinocytes (125). When stimulated with 25-vitamin D₃ and TGF-β1, ZK159222 inhibited the expression of *cathelicidin*, *CD14*, and *TLR2* (126). In cancer cells such as cervical carcinoma cells, ZK159222 restored mRNA levels of *human ether a-go-go-1 (EAG1)* that was down-regulated by 1,25-(OH)₂D₃ (127). In HeLa cells, a reporter assay under control of a human immunodeficiency virus type I long terminal repeat HIV-1 LTR promoter demonstrated the antagonist effects of ZK159222 in the presence of 1,25-(OH)₂D₃ (128). ZK159222 also reduced the production of P-glycoprotein, a member of the ABC transporter family, in LS174T colon adenocarcinoma cells in the presence of 1,25-(OH)₂D₃ (129). In LNCaP cells, ZK159222 reduced the mRNA and protein levels of the *prostate derived factor* stimulated 1,25-(OH)₂D₃ at 10 nM (130). Finally, ZK 159222 blocked the stimulatory effects of a VDR agonist on DNA synthesis in epithelia E304 cells (131) and prevented the decrease of *ceramide kinase* expression elicited by 1,25-(OH)₂D₃ in neuroblast-like SH-SY5Y cells (132).

4.3. ZK168281

ZK168281 has a picomolar affinity for the VDR-RXR-VDRE complex (Figure 12) (133). In addition, ZK168281 is less agonistic and three times more potent as an antagonist than ZK159222. Limited digestion studies demonstrated the formation of different VDR structures in the presence of ZK168281 in comparison to 1,25-(OH)₂D₃. The residual agonist effect of ZK168281 was 5% that of 1,25-(OH)₂D₃ (134). ZK168281 promoted the recruitment of NCoR (135) and inhibited the interaction with DRIP205 and SRC1 in two hybrid assays (34). ZK168281 behaved like a pure antagonist in five different cell lines, (69, 136) and C403S/C410N mutation did not alter the binding of ZK168281. Importantly, ZK168281 did not promote the complexation between VDR-RXR-SRC2-VDRE (137). In contrast to ZK159222, ZK168281 antagonist activity was not influenced by truncation of the VDR helix 12 or RXR helix 12. Mutation studies and molecular dynamics (MD) studies revealed that ZK168281 severely disturbs the H397 and F422 interaction of VDR (137). In addition, the agonistic effects of ZK168281 increased with VDR H302A and H397A mutations (138). Finally, ZK168281 inhibited the phosphorylation of phosphoinositide mediated by phosphatidylinositol 3-kinase in the presence of 1,25-(OH)₂D₃ (111).

4.4. ZK191784

ZK191784 has a structure similar to ZK159222 with a bioisosteric replacement of the ester functionality (Figure 12). The relative VDR binding is 33% that of 1,25-(OH)₂D₃ (139). ZK191784 inhibited the differentiation of HL60 cells in the presence of 1,25-(OH)₂D₃ and

exerted only weak agonist effects for inducing the expression of *CD14*. Like 1,25-(OH)₂D₃, ZK191784 reduced the proliferation of stimulated lymphocytes and inhibited the expression of surface maker HLA-DR, B7.1, and ICAM-1, although to a lesser degree than 1,25-(OH)₂D₃. In addition, IL-12, IL-10, and TNF α secretion were reduced by ZK191784. In mice, ZK191784 reduced the hyperresponsiveness induced by 2,4-dimethylfluorobenzene without increasing the concentration of calcium in urine. For chronic intestinal inflammation, ZK191784 reduced the expression IFN- γ and IL-6 in mesenteric lymph node cells and lowered the numbers of activated CD11c+ dendritic-cells in the colon (140). In TRPV5^{-/-} mice, ZK191784 normalized the calcium hyperabsorption and expression of intestinal calcium transport proteins. Furthermore, ZK191784 reduced 1,25-(OH)₂D₃-dependent calcium uptake by Caco-2 cells (intestine). In WT mice, ZK191784 increased renal *TRPV5* and *calbindin-D28K* expression and decreased urine calcium excretion. In rat osteosarcoma cells, ZK191784 and 1,25-(OH)₂D₃ enhanced bone *TRPV6* mRNA levels and secretion of osteocalcin (141). However, *CYP24A1* was down-regulated in femoral bone for mice treated with ZK191784. The phosphate homeostasis was unaffected by ZK191784 (142). For HUVEC cells, ZK191784 increased the formation of nitric oxide and cell viability in the presence of 1,25-(OH)₂D₃ in the presence and absence of hydrogen peroxide. ZK191784 improved the expression of *beclin 1* and the phosphorylation of ERK1/2 increased as well as the repression of *BAX* in the presence of 1,25-(OH)₂D₃. Furthermore, 1,25-(OH)₂D₃ alone or in combination with ZK191784 was able to prevent the loss of mitochondrial potential and the consequent cytochrome C release and caspase activation (143). In keratinocytes, ZK191784 exhibited weak agonist behavior, reducing *HBD2* expression and increasing the expression of *cathelicidin* and *hCAP18* (125). In DU145 and LNCaP cells, ZK191784 reduced *MMP-9* and *MMP-2* as well as the surface expression of ICAM-1 (144).

4.5. Amide-based VDR Antagonists

ML-3-452 binds VDR with an EC₅₀ of 107 nM (Figure 13) (145). In CCS25 cells, ML-3-452 did not increase the expression of *CYP24A1* and *TSLP* but reduced their expression in the presence of 1,25-(OH)₂D₃. ML-3-452 reduced the recruitment of SRC3 to the VDR–DNA complex and increased the interaction between VDR and NCoR on the *CYP24A1* promoter.

The first compound in the lactam series was DLAM-01 exhibiting a VDR binding affinity of 10.2 nM (Figure 13). DLAM-1P binding affinity was 1.9 nM and inhibited the differentiation of HL60 cell at 1 μ M (146). DLAM-1P inhibited VDR-mediated transcription in a reporter assay in the nanomolar range, without showing any agonist activity in the absence of 1,25-(OH)₂D₃ (147). In contrast to TEI-9647, hVDR and rVDR were inhibited by DLAM-1P at the same concentration; thus hVDR cysteine residues did not influence this binding. DLAM-1P also suppressed the differentiation of bone marrow cells induced by 1,25-(OH)₂D₃ as well as the up-regulation of *RANKL* mRNA in mouse primary osteoblast (148). In addition, calcium reabsorption induced by 1,25-(OH)₂D₃ in mouse calvarial organ culture was reduced by DLAM-1P. For LNCaP cells, DLAM-1P promoted proliferation reduced by 1,25-(OH)₂D₃ and further decreased the up-regulation of *P21* (149). The introduction of substituents in the meta-position resulted in DLAM-1P-3,5(OEt)₂ with a 3.5

times higher affinity to VDR than 1,25-(OH)₂D₃ and an IC₅₀ of 90 nM for the inhibition of differentiation of HL60 cells (150, 151). DLAM-2P, bearing an ethylbenzene amide substituent reversed the decreased induction of *DMP-1* mRNA levels in the presence of 1,25-(OH)₂D₃ in cementoblasts and osteocytes-like cells (152).

4.6. Adamantane-based VDR antagonists

AD47 exhibited weak agonist activity in reporter assays employing an *osteopontin*, *CYP24A1*, and repeated VDRE as promoters at 10 nM (Figure 14) (72, 138). However, in the presence of 1,25-(OH)₂D₃, AD47 inhibited transcription at 100 nM. In the absence of 1,25-(OH)₂D₃ a weak interaction between VDR and RXR was observed. In the presence of 1,25-(OH)₂D₃ this interaction was significantly reduced by AD47. Similarly, AD47 was able to weakly promote the interaction between VDR and coactivator SRC1, but inhibited this interaction in the presence 1,25-(OH)₂D₃. Interestingly, AD47 strongly promoted the interaction of VDR with DRIP205 and no inhibition was observed in the presence of 1,25-(OH)₂D₃. In regard to corepressors, AD47 inhibited the recruitment of NCoR and SMRT but did not suppress the dissociation of the VDR–corepressor complex induced by 1,25-(OH)₂D₃. For reporter assays carried out with different cell lines, AD47 reduced the expression of *CYP24A1* in the presence of 1,25-(OH)₂D₃, except for HCT116 intestine cells. In addition, AD47 behaved as an agonist in the absence of 1,25-(OH)₂D₃ except for HEK293 kidney cells. Other genes such as *CaT1*, *CYP3A4* and *E-cadherin* were up-regulated by AD47 in SW480 intestine cells and reduced in the presence of 1,25-(OH)₂D₃. Four analogs, called ADMI1-4 with different stereochemical configurations, were all able to bind VDR and inhibited transcription in the presence of 1,25-(OH)₂D₃ in a luciferase reporter assay (Figure 14) (153, 154). *CYP24A1* expression was reduced in the presence of 1,25-(OH)₂D₃ for ADMI1 and ADMI3. ADTT exhibited a weak agonistic activity and did not promote the dimerization of VDR and RXR in the absence of 1,25-(OH)₂D₃ in HEK293 cells (Figure 14) (155). However, in the absence of 1,25-(OH)₂D₃, ADTT induced a weak VDR recruitment of SRC1 and dissociation of SMRT. Similar to AD47, ADTT behaved like an agonist in various cells except HEK293 kidney cells. In those cells, ADTT reduced VDR–DNA binding. In HCT116 colon cancer cells, however, ADTT induced recruitment of RXR and surprisingly SMRT to the DNA-bound VDR. In THP-1 cells, ADTT and ADMI3 reduced the mRNA levels of *CYP1A1* in the presence of 1,25-(OH)₂D₃ and benzo[a]pyrene (an aryl hydrocarbon agonist) (156). A recent series of ADTK1-4 analogs exhibited significantly less antagonist activity than ADTT (157).

4.7. Branched VDR Antagonists

Further development of VDR agonist GEMINI(158) resulted in VDR super-agonists (159) with higher affinity towards VDR than endogenous ligand 1,25-(OH)₂D₃ and VDR antagonists 4, 6, 7, 8, and 10 (Figure 15) (160). The agonistic activity of these ligands in the presence of 1,25-(OH)₂D₃ was very weak when measured by luciferase reporter assay. *CYP24A1* expression in the presence of 1,25-(OH)₂D₃ was reduced as well. No change in differentiation of HL60 cells was observed in the absence of 1,25-(OH)₂D₃, but inhibition of cell differentiation in the presence of 1,25-(OH)₂D₃ was demonstrated for compound 4 in conjunction with occupancy of a new binding pocket for the butyl substituent. Compound 4 was weakly able to support the binding between VDR and RXR but not between VDR and

SRC1. However, in the presence of 1,25-(OH)₂D₃, both interactions were inhibited by compound 4 at a concentration of 1 μM (161). Elongation of compound 4's carbon chain by one CH₂ unit resulted in a slightly more potent antagonist (162). Introduction of a methyl or an ethyl substituent in the 24 position increased the agonist activity of these ligands determined by a luciferase reporter assay (163). The substitution of a butyl by an ethyl substituent resulted in strong antagonistic behavior of VDR ligands that bear the hydroxyl functionality in the 24 position or have no alkyl substituents in the 25 position (164).

High throughput screening also identified PPARδ agonist GW0742 as a novel VDR antagonist (Figure 16) (165). GW0742 did not only bind VDR, but also other nuclear receptors. In the presence of GW0742, nuclear receptor target gene mRNA levels were suppressed in the presence of their endogenous ligands. The conversion of GW0742 acid functionality to an alcohol increased its agonistic activity in cells (166). Virtual screening using a nuclear receptor ligand database in conjunction with a stringent pharmacophore model for 1,25-(OH)₂D₃ identified many nuclear receptor ligands that might interact with VDR (167). For selected NR ligands such as H6036 VDR-coactivator inhibition was demonstrated.

5. Conclusion and Future Directions

The development of VDR-coregulator inhibitors has allowed us to identify different biological functions of VDR. VDR antagonists in particular have been invaluable to verify the involvement of VDR for biological effects induced by 1,25-(OH)₂D₃. However, the development of VDR antagonists as novel therapeutics has been met with caution partially because of the fear that antagonizing VDR will cause dysfunction and partially because VDR antagonists exhibit various degrees of agonism. ZK191784 is an excellent example of a situation where these obstacles can be overcome by careful ligand design. The regulation of VDR-coregulator interactions with VDR antagonists has been more challenging because recent discoveries have shown many factors that influence nuclear receptor-mediated transcription, such as position of DNA promoter and activation sites, cell type and expression of coregulators, the differentiation state of cells and their circadian rhythms, and many other factors. However, the development of metabolically stable VDR antagonists will enable us to characterize the *in vivo* effects mediated by VDR due to the presence of endogenous 1,25-(OH)₂D₃. Direct small molecule inhibitors of the VDR-coregulators interaction have been very challenging to develop as well, due to the fact that the interaction between VDR and coregulators is relatively weak and represents protein-protein interactions with large interaction surfaces. Nevertheless, the first scaffolds have been identified by rational design and high throughput screening. The optimization of these inhibitors in respect to affinity and selectivity among different nuclear receptors and their coregulators is still at an early stage. Small molecule VDR-coregulator inhibitors do not only represent research tools to dissect the transcriptional multi-protein complex that governs transcription but also drug candidates for diseases that deregulate VDR due to abnormal coregulators binding.

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Figure 1.

Simplified cartoon of VDR-mediated transcription. Repressed basal transcription of VDR as a heterodimer in the absence of ligand and presence of corepressor followed by the activation of transcription in the presence of 1,25-(OH)₂D₃ (VD3) and coactivator leading to histone acetylation and recruitment of RNA polymerase II.

																					Target
C33		H	V	E	M	H	P	L	L	M	G	L	L	M	E	S	Q	W	G	A	VDR
D47		H	V	Y	Q	H	P	L	L	L	S	L	L	S	S	E	H	E	S	G	VDR
EBIP41		R	R	D	D	F	P	L	L	I	S	L	L	K	D	G	A	L	S	Q	VDR
EBIP44		Y	G	L	K	M	S	L	L	E	S	L	L	R	E	D	I	S	T	V	VDR
F6		G	H	E	P	L	T	L	L	E	R	L	L	S	G	T	S	V	A	E	RxR
3		L	S	E	T	H	P	L	L	W	T	L	L	S	S	E	G	D	S	M	VDR
4		M	Q	E	R	F	P	M	L	W	D	L	L	D	L	P	S	P	T	S	VDR
5		L	G	E	S	H	P	L	L	M	Q	L	L	T	E	N	V	G	T	H	VDR

Figure 2.
Sequences of coactivator peptides that inhibit the interaction between VDR and coactivators

Figure 3.
Structures of cyclic peptide-based VDR-coactivator inhibitors.

Figure 4. Overlay between a crystal structure of VDR and coactivator peptide DRIP205 and docked conformation of compound 2 as well as structures of compound 2 and 35.

Figure 5.
A) Structures of 31B and PS121912; B) Anti-proliferative effect of PS121912 in a HL60 xenograft model.

Figure 6.
Possible equilibrium structures of VDR in the presence of antagonist.

Figure 7.
Structures of TEI-9647 and TEI-9648.

Figure 8.
Nucleophilic addition between TEI-9647 and VDR (Nu).

Figure 9.
Structures of HLV and GC-3.

Figure 10.
Structures of TEI-9647 analogs.

Figure 11.
Structure of ZK159222.

Figure 12.
Structures of ZK168281 and ZK191784.

Figure 13.
Structures of ML-3-452, DLAM-01, DLAM-1P, DLAM-1P-3,5(OEt)₂.

Figure 14.
Structures of AD47, ADI1-4, and ADTT.

Figure 15.
Structures of branched VDR antagonists.

Figure 16.
Structure of GW0742.