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Ligand-mediated conformational changes of the VDR are required for gene transactivation☆

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Abstract

The central element of the molecular switch of nuclear 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) signaling is the ligand-binding domain (LBD) of the Vitamin D receptor (VDR), which can be stabilized by 1α,25$(OH)_2D_3$ or its analogues in to agonistic, antagonistic or inverse agonistic conformations. The positioning of helix 12 of the LBD is of most critical importance for these conformations, because it determines the distance between the charge clamp amino acids K246 and E420 that are essential for VDR–coactivator (CoA) interaction. Most VDR ligands have been identified as agonists and only a few (e.g., ZK168281 and TEI-9647) as pure or partial antagonists. Antagonists induce corepressor (CoR) dissociation from the VDR but prevent completely or partially CoA interaction and thus transactivation. Gemini is a 1α,25$(OH)_2D_3$ analogue with two identical side chains that despite its significantly increased volume binds to the VDR and acts under most conditions as an agonist. Interestingly, supramolar CoR concentrations shift Gemini from an agonist to an inverse agonist, which actively recruits CoR to the VDR and thus mediates repression of 1α,25$(OH)_2D_3$ target genes. Gemini is the first described (conditional) inverse agonist to an endocrine nuclear receptor (NR) and may function as a sensor for cell-specific CoA/CoR ratios.

Keywords: Vitamin D; Vitamin D analogues; Nuclear receptor conformations; Coactivator proteins; Protein–DNA interaction; Corepressor proteins

1. Introduction

The Vitamin D receptor (VDR) is the only nuclear protein that binds the biologically most active Vitamin D metabolite, 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), with high affinity. VDR is one of the 11 members of the nuclear receptor (NR) superfamily that function as classical endocrine receptors, such as the receptors for the nuclear hormones retinoic acid, thyroid hormone, estradiol, progesterone, testosterone, cortisol, and aldosterol, that bind their specific ligand with a K_d of 1 nM or lower [1]. The protein–DNA complex of a NR and its specific response element (RE) can be considered as a molecular switch for those genes that contain such a RE in their promoter region [2]. Like the very most members of the NR superfamily, VDR contains two zinc finger structures forming a characteristic DNA-binding domain (DBD) of 66 amino acids [3] and a carboxy-terminal ligand-binding domain (LBD) of approximately 300 amino acids, which is formed by 12 α-helices [4]. Ligand-binding causes a conformational change within the LBD, in which helix 12, the most carboxy-terminal α-helix, closes the ligand-binding pocket via a "mouse-trap like" intramolecular folding [5] (Fig. 1). Moreover, the LBD is involved in a variety of interactions with nuclear proteins, such as other NRs, coactivator (CoA) and corepressor (CoR) proteins [6]. These ligand-triggered protein–protein interactions are the central molecular events of nuclear 1α,25$(OH)_2D_3$ signaling.

An essential prerequisite for a direct modulation of transcription via 1α,25$(OH)_2D_3$-triggered protein–protein interactions is the location of activated VDR close to the basal transcriptional machinery. This is achieved through the specific binding of the VDR to a 1α,25$(OH)_2D_3$ response element (VDRE) in the regulatory region of a primary 1α,25$(OH)_2D_3$ responding gene [2]. The DBD of the VDR contacts the major grove of a hexameric sequence, referred to as core binding motif, with the consensus sequence RGKTCA (R = A or G, K = G or T). The affinity of monomeric VDR to a single binding motif is not sufficient for the for-

Abbreviations: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; CAR, constitutive androstane receptor; CoA, coactivator; CoR, corepressor; DBD, DNA-binding domain; ER, estrogen receptor; DR3, direct repeat spaced by three nucleotides; MD, molecular dynamics; NR, nuclear receptor; RE, response element; RXR, retinoid X receptor; LBD, ligand-binding domain; LPD, limited protease digestion; VDR, Vitamin D_3 receptor; VDRE, 1α,25$(OH)_2D_3$ response element

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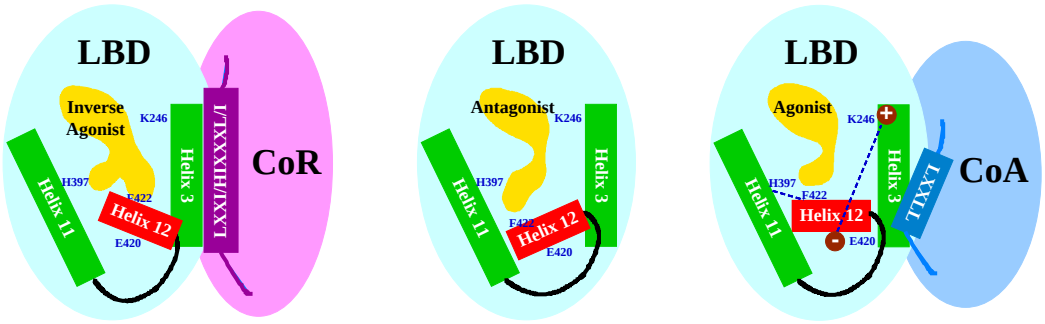

Conformation:	**inverse agonistic**	**antagonistic**	**agonistic**
Status of molecular switch:	**off**	**blocked**	**on**
Transactivation:	**repressed**	**not active**	**active**

Fig. 1. Conformations of ligand-bound VDR. Model of the inverse agonistic, antagonistic and agonistic conformation of the LBD of the VDR. The most important helices and their critical amino acids are indicated. Only agonists are able to stabilize via a H397-F422 interaction helix 12 in position that creates between the charge clamp amino acids E420 and K246 a distance of 19 Å that is critical for efficient interaction with the NR interaction box of CoA proteins. The exact position of the CoA box (LXXLL) and the CoR box (LXXI/HIXXXL/I) in relation the LBD has not yet been experimentally proven.

mation of a stable protein–DNA complex and thus VDR requires formation of homo- and/or heterodimeric complexes with a partner NR in order to allow efficient DNA binding [7]. In most cases the heterodimeric partner of VDR is retinoid X receptor (RXR) and simple VDREs are often formed by a direct repeat of two hexameric core binding motifs spaced by three nucleotides (DR3). However, strong DNA binding of VDR–RXR heterodimers is also observed to RGTTCA motifs as a direct repeat spaced by four nucleotides (DR4) or as an everted repeat with nine interfering nucleotides (ER9) [8].

CoR proteins, such as NCoR, SMRT, and Alien, link non-liganded, DNA-bound VDR–RXR heterodimers to enzymes with histone deacetylase activity that cause chromatin condensation [9]. This gives VDR intrinsic repression properties comparable to retinoic acid and thyroid hormone receptors. The conformational change within VDR's LBD after binding of $1\alpha,25(OH)_2D_3$ or one of its agonistic analogue results in replacing a CoR by a CoA protein of the p160-family, such as SRC-1, TIF2, and RAC3 [10]. These CoAs link the ligand-activated VDR to enzymes displaying histone acetyl transferase activity that cause chromatin opening. Ligand-activated VDR–RXR heterodimers seem to change rapidly between CoAs of the p160-family and those of the DRIP/TRAP family. The latter are part of a mediator complex of approximately 15 proteins that build a bridge to the basal transcription machinery [11]. In this way ligand-activated VDR–RXR heterodimers fulfill two tasks, opening chromatin and activating transcription.

2. Molecular evaluation of Vitamin D analogues

More than 2000 synthetic analogues of $1\alpha,25(OH)_2D_3$ are presently known and the majority of them carry a modification in the side chain. $1\alpha,25(OH)_2D_3$ analogues have been developed with the goal to improve the biological profile of the natural hormone for a therapeutic application either in hyperproliferative diseases, such as psoriasis and different types of cancer, or in bone disorders, such as osteoporosis [12]. Most analogues have been identified as agonists, a few are antagonists (e.g., ZK159222 and TEI-9647) and only Gemini and some of its derivatives act under restricted conditions as inverse agonists. Basically, all of the analogues interfere with the molecular switch of nuclear $1\alpha,25(OH)_2D_3$ signaling, i.e., they contact DNA-bound VDR–RXR heterodimers. Central element of this switch is the LBD of the VDR, which can be stabilized by $1\alpha,25(OH)_2D_3$ analogues either in its agonistic, antagonistic or inverse agonistic conformation.

Traditional ligand-binding assays use radio-labeled ligand and provide an idea of the receptor–ligand interaction affinity, but do not visualize the action of the molecular switch, i.e., conformational changes of the receptor. Therefore, in vitro assay systems, such as limited protease digestion (LPD), ligand-dependent gel shift and supershift, were developed to get a more detailed understanding of the response of VDR–RXR heterodimers to $1\alpha,25(OH)_2D_3$ and its analogues [13]. In the LPD assay the interaction of the VDR with a ligand protects its LBD in a characteristic way against protease digestion and allows the discrimination and quantification of functional VDR conformations. The assay monitors, in which conformation the VDR was at the moment of the protease "snapshot." It is traditionally performed in a DNA-independent fashion, but more accurate results are obtained in presence of RXR, DNA, and cofactors (CoAs or CoRs) [14]. The ligand-dependent gel shift assay provides a quantification of the ligand-dependent VDR–RXR–VDRE complex formation and monitors receptor dimerization, DNA binding

and ligand interaction at the same time [15]. The supershift assay is a gel shift assay in the presence of CoAs or CoRs and demonstrates the ligand-triggered interaction between DNA-bound VDR–RXR heterodimers with cofactors [16].

3. VDR agonists

The central step in $1\alpha,25(OH)_2D_3$ signaling is the conformational change of VDR's LBD and the resulting exchange of protein–protein interaction partners. Only those VDR ligands that cause both an efficient dissociation of CoRs from the receptor as well as a specific binding of CoAs finally lead to transcriptional activation, i.e., act as agonists. In fact, most known $1\alpha,25(OH)_2D_3$ analogues show an agonistic potential but they differ greatly in their efficiency. Some of these agonists have been shown to be superagonists, i.e., they act in living cells more potent than $1\alpha,25(OH)_2D_3$. A comparison of some prominent superagonists, such as MC1288, KH1060, EB1089, CB1093, and others with $1\alpha,25(OH)_2D_3$ in ligand-dependent gel shift assays on DR3-type VDREs showed that all of them have an EC_{50}-value of approximately 0.1 nM for the complex formation of VDR–RXR heterodimers on DNA [8,16,17]. Moreover, a comparison of all presently known DR3-type VDREs demonstrated that they differ in their affinity for VDR–RXR heterodimers but show identical molecular action, i.e., they are all activated with an EC_{50}-value of approximately 0.1 nM [8]. This suggests that on classical DR3-type VDREs none of superagonists is significantly more potent in activating VDR–RXR heterodimers than the natural hormone. In GST pull-down assays, which are traditionally performed with monomeric receptor in solution, the three members of the p160 CoA family showed identical binding to the VDR, i.e., VDR seems not to have any selectivity for the members of this CoA family [16]. Supershift assays provided with the natural hormone as well as with all tested superagonists EC_{50}-values in the order of 0.1 nM for the interaction of DNA-bound VDR–RXR heterodimers with CoAs. This demonstrates that ligand-dependent gel shift and supershift assays provide the same quality of information about the molecular switches of $1\alpha,25(OH)_2D_3$ signaling. This suggests that the concentration value of 0.1 nM seems to be a lower threshold for VDR activation, which even superagonists cannot pass.

The crystal structure of VDR's LBD has been solved with the natural agonist [4] or two superagonists [18], the 20-epi analogues MC1288 and KH1060, as ligands. Contrary to expectations the conformations of these three VDR-agonist complexes were found to be nearly identical. This suggests that there is only one agonistic conformation of the VDR. This agonistic conformation is characterized by a contact between the C25-hydroxyl group of $1\alpha,25(OH)_2D_3$ and H397 of the receptor [4] and is supported by an additional, less important hydrogen bond with H305 [19]. The direct ligand contact of H397 enables this amino acid to contact F422 of helix 12. Helix 12 forms the "lid" of the ligand-binding pocket and projects its inner hydrophobic surface towards the bound hormone (Fig. 1). Precise positioning of helix 12 via the H397–F422 bridge creates a distance of 19 Å between the negatively charged E420 on the surface of helix 12 and the positively charged K246 on the surface of helix 3. This charge clamp structure is essential for contacting the LXXLL motif of the NR interaction box of CoA proteins (Fig. 1).

Presently, the profile of more than 100 different VDR ligands has been compared by LPD assays. Most of these ligands predominantly stabilize a large fragment of the LBD of the receptor ($c1_{LPD}$, from R173 to the carboxy-terminus at position 427) [17]. This indicates that at the moment of the protease digestion "snapshot" most of the receptors were in the agonistic conformation. In the presence of RXR and a VDRE, $1\alpha,25(OH)_2D_3$ and its superagonists stabilize the agonistic VDR conformation with an EC_{50}-value of approximately 0.1 nM, i.e., with the same threshold concentration that was already observed in gel shift and supershift assays. All presently tested superagonists demonstrate the same high sensitivity for stabilizing VDR within DNA-bound VDR–RXR heterodimers, but with VDR monomers in solution they show individual EC_{50}-values in the order of 1–20 nM [17]. This suggests that VDR's LBD reaches its full ligand sensitivity only as a component of a DNA-bound VDR–RXR heterodimer.

Compared to the natural agonist some superagonists, such as EB1089, show RE selectivity [20] and others seem to differentiate more clearly between DNA-dependent and DNA-independent $1\alpha,25(OH)_2D_3$ signaling pathways than the natural hormone [16]. However, these relative differences in EC_{50}-values are in maximum of a factor of 10, so that promoter and pathway selectivities themselves are not sufficient to explain the improved in vivo profile of superagonists in relation to $1\alpha,25(OH)_2D_3$. Interestingly, the crystal structure of VDR's LBD bound by MC1288 or KH1060 showed that the modified side chain of both superagonists has more contact points with the ligand-binding pocket than the natural agonist [18]. Moreover, KH1060 was shown to stabilize the VDR against endogenous proteolytic degradation in living cells over a longer time period than $1\alpha,25(OH)_2D_3$ [21]. Using the LPD assay in vitro, several superagonists were described to stabilize the agonistic VDR conformation for a much longer time than the natural agonist, i.e., the agonistic conformation showed a significantly longer higher half-live due to binding of a superagonist [22]. This suggests that the stabilization of the ligand-activated VDR complex over time has a significant contribution to the in vivo profile of a superagonist.

4. VDR antagonists

NR ligands that bind with reasonable affinity to the LBD, but do not allow optimal positioning of helix 12 in its agonistic conformation, have the potential to act as antagonists,

i.e., they can block their specific receptor in its normal signal transduction process. Therefore, agonism and antagonism of natural and synthetic nuclear hormones are closely related processes. For most members of the NR superfamily several natural and synthetic agonists are known, but only for a few family members, such as the estrogen receptor (ER), the progesterone receptor and the retinoic acid receptor, synthetic antagonists are well characterized [23]. For the VDR two different types of antagonists have been described. These are the 25-carboxylic esters ZK159222 and ZK168281 [24,25] and the 26,23-lactone TEI-9647 [26]. Compared with the natural hormone, both types of compounds have relatively bulky ring structures in their side chains that are assumed to be the main structural basis of their antagonistic action. However, ZK159222 and ZK168281 carry a much longer side chain than TEI-9647 suggesting that there may be differences in the molecular mechanisms of their antagonistic action. ZK159222 and ZK168281 stabilize the complex formation of VDR–RXR heterodimers on a VDRE with the same potency and nearly the same sensitivity than $1\alpha,25(OH)_2D_3$ [24], whereas TEI-9647 shows both a reduced potency and a more than 10-fold reduced sensitivity [27]. This difference in sensitivity means that equimolar amounts of ZK159222 or ZK168281 are able to replace nearly half of the VDR-bound $1\alpha,25(OH)_2D_3$ molecules, whereas a more than 10-fold molar excess of TEI-9647 would be required for obtaining the same effect. This explains the different antagonistic efficacy of both types of VDR antagonists [27].

In the LPD assay all VDR antagonists stabilize a clearly lower amount of the VDR molecule pool in the agonistic conformation than the natural hormone, whereas only antagonists stabilize a VDR fragment, $c2_{LPD}$ (from R173 to R402), that specifically represents the antagonistic conformation [24,25]. Interestingly, the antagonist-specific VDR fragment that is stabilized by TEI-9647 showed a slight migration difference compared to that is stabilized by ZK159222 [27,28] suggesting a difference between both antagonistic conformations. In contrast to the natural hormone and its agonistic analogues, none of the antagonists are able to mediate a significant interaction of the VDR with CoAs [27]. However, like $1\alpha,25(OH)_2D_3$, the binding of both types of antagonist to the VDR induces a dissociation of CoR proteins. This suggests that antagonists stabilize the VDR in a conformation blocking the interaction with CoAs but not preventing CoR dissociation [27] (Fig. 1). The potency of an antagonist depends on both its affinity to the LBD in relation to the natural ligand as well as its residual agonistic activity. Under standard conditions the remaining agonistic activity of ZK159222 and TEI-9647 showed to be approximately 20% of that of the natural ligand, whereas ZK168281 displayed an agonistic potential of only less than 5% [27,29]. This classifies ZK168281 as a true antagonist, whereas ZK159222 and TEI-9647 are only partial antagonists. However, the terms agonist and antagonists are often inappropriate for description of NR ligands, since many of them function as agonists in certain tissues and antagonists in others. For the ER, the term selective ER modulator (SERM) has been applied to compounds with mixed agonist and antagonist activity, such as tamoxifen and raloxifene [30]. Therefore, ZK159222 and TEI-9647 also could be referred to as selective VDR modulators.

Molecular dynamics (MD) simulations of VDR's LBD complexed with the natural agonist in comparison to ZK159222 and ZK168281 demonstrated that the extended side chain of both antagonists prevents the H397–F422 interaction [29]. Due to this disturbed interaction helix 12 is much more flexible and will be mostly in a position, in which the distance of the residues K246 and E420 deviates from the optimized value of 19 Å (Fig. 1). This decreases the affinity to CoAs or even makes interaction impossible. Although the side chains of both 25-carboxylic ester antagonists have the same number of atoms, the one of ZK168281 is more rigid. This results in a more effective disturbance of the H397–F422 interaction, drastically increases the K246–E420 distance and almost completely prevents CoA binding [29], i.e., it explains why ZK168281 is a true antagonist. The residual agonistic potential of the partial antagonist ZK159222 results from a less effective disturbance of the H397–F422 interaction, which still allows some CoA proteins to contact the VDR via the K246–E420 charge clamp. TEI-9647 has no extended side chain, so that it very unlikely that it directly disturbs the H397–F422 interaction. However, the side chain of TEI-9647 is rather bulky, so that it may disturb the correct positioning of helix 12 via other amino acid residues within the ligand-binding pocket. MD simulations suggest the existence of various antagonistic conformations of helix 12 [29], which fits with the slightly different antagonistic conformation that are stabilized by TEI-9647 and ZK159222 [27,28].

ZK159222 was shown to display tissue-specific agonism [31] and is the presently best-characterized selective VDR modulator, but the exact mechanisms of this specificity are presently not fully understood. However, it can be speculated that the direct interaction of the VDR with CoA and CoR proteins as well as with its partner receptor RXR might modulate the amount of agonism mediated by ZK159222. The relative amount of expression of these nuclear proteins differs between different cell types and could explain the cell-specific actions of ZK159222. There is no evidence that there are different VDR conformations in different VDR target tissues, but it is likely that VDR interacting proteins are differently effective in shifting VDR proteins from an antagonistic conformation to the agonistic conformation [31].

5. Inverse VDR agonists

Humans have 37 orphan NR superfamily members and for some of them in the past years low affinity ligands have been identified (K_d-value of 1 μM or higher) [1]. One of these "adopted" orphan NRs is the constitutive androstane receptor (CAR), which is an unusual member of the fam-

ily, since it interacts in the absence of ligand with CoAs and displays constitutive activity [32]. Furthermore, the natural CAR ligand androstanol acts as an inverse agonist by decreasing CoA interaction [33] and increasing CAR–CoR complex formation [34]. This opens the question, whether inverse agonists only exist for constitutively active NRs or inverse agonists can be developed for other ligand-activated members of the NR superfamily, such as VDR.

In the LPD assay most ligands stabilize a minor portion of the VDR molecule pool in the fragment $c3_{LPD}$ (from R173 to R391) [35], which is now interpreted as the representative of the inverse agonistic VDR conformation (previously also called non-agonistic conformation). Interestingly, analogues with two side chains at C20, such as Gemini and its derivatives, were found to stabilize VDR even preferentially in this conformation, if they are analyzed in the absence of RXR, VDRE and CoA [36]. MD simulations of the Gemini–VDR complex demonstrated that one of the two side chains of Gemini has the same location as the natural hormone, whereas for the second side chain two approximately equal positions were identified [37]. Receptor mutagenesis, CoA interaction studies in vitro and functional assays in living cells confirmed that Gemini uses both possible positions. Interestingly, in the presence of RXR, VDRE, CoAs and only low levels of CoRs Gemini behaves as a superagonist and under these condition the analogue stabilizes primarily the agonistic VDR conformation [16,36]. However, in functional assays it could be demonstrated that CoR excess shifts Gemini from an agonist to an inverse agonist that actively recruits CoR proteins to the VDR and mediates super-repression [35,38]. Amino acid F422 of helix 12 was shown to have a critical role in this process as confirmed by in vitro interaction studies with NCoR and conformational analysis by LPD. MD simulations indicated that the second side chain of Gemini creates tension within the LBD of VDR, which in excess of CoR proteins can be released by shifting helix 12 into an inverse agonistic position (Fig. 1). Gemini, therefore, seems to be a conditional inverse agonist of the VDR [38].

In the inverse agonistic conformation helix 12 has not moved in comparison to the apo-form of the VDR, so that the receptor is not able to contact CoA proteins (Fig. 1). The main difference between the antagonistic and the inverse agonistic conformation is that in the latter case CoR proteins do not dissociate from the receptor, i.e., that interaction with a CoR protein blocks the binding of a CoA protein [35]. An antagonists, such as ZK168281, can never convert to a superagonist, because its bulky side chains causes steric hindrance to helix 12. In contrast, the conditional inverse agonist Gemini turns into an agonist or even a superagonist, when VDR is exposed to lower CoR levels, binds as a heterodimer with RXR to DNA and is contacted by CoAs [16]. Therefore, Gemini seems to be able to discriminate between the well-characterized signaling via DNA-bound VDR–RXR heterodimers and less well-understood DNA-independent regulatory actions of the VDR (e.g., in the cytoplasma). However, even in situations when Gemini acts as an agonist, it is much more affected by higher CoR levels than other agonists, i.e., its potency decreases in the presence of CoRs [35].

6. Conclusion

DNA-bound VDR–RXR heterodimers are the molecular switches in $1\alpha,25(OH)_2D_3$ signaling. The agonistic, antagonistic and inverse agonistic conformation of VDR's LBD within this molecular switch explains well the functional profile of all three types of VDR ligands. The most critical issue in this aspect is the positioning of helix 12 and the resulting interaction with either CoR or CoA proteins. Therefore, analyzing the stabilization of VDR conformations by $1\alpha,25(OH)_2D_3$ analogues appears to be the most informative way for their in vitro evaluation. The presently most interesting analogue is Gemini, since it can switch from an inverse agonist to a superagonist and may function as a sensor for the cell-specific CoA/CoR ratio. Finally, for a whole evaluation of the profile of a VDR ligand its pharmacokinetic profile, such as cellular uptake, transport and in particular metabolic stability, should not be neglected.

Acknowledgements

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