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Molecular Basis of the Selective Activity of Vitamin D Analogues

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Abstract More than 2,000 synthetic analogues of the biological active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), are presently known. Basically, all of them interfere with the molecular switch of nuclear 1α,25$(OH)_2D_3$ signaling, which is the complex of the vitamin D receptor (VDR), the retinoid X receptor (RXR), and a 1α,25$(OH)_2D_3$ response element (VDRE). Central element of this molecular switch is the ligand-binding domain (LBD) of the VDR, which can be stabilized by a 1α,25$(OH)_2D_3$ analogue either in its agonistic, antagonistic, or non-agonistic conformation. The positioning of helix 12 of the LBD is of most critical importance for these conformations. In each of the three conformations, the VDR performs different protein–protein interactions, which then result in a characteristic functional profile. Most 1α,25$(OH)_2D_3$ 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 non-agonists. The functional profile of some 1α,25$(OH)_2D_3$ analogues, such as EB1089 and Gemini, can be modulated by protein and DNA interaction partners of the VDR. This provides them with some selectivity for DNA-dependent and -independent signaling pathways and VDRE structures. J. Cell. Biochem. 88: 274–281, 2003.

Key words: vitamin D; vitamin D analogues; nuclear receptor conformations; coactivator proteins; protein-DNA interaction; corepressor proteins

The nuclear receptor (NR) superfamily contains a series of transcription factors (48 human members) that are of high impact, because they can be specifically regulated in their activity by small lipophilic compounds of natural or synthetic origin [Chawla et al., 2001]. 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 [Carlberg and Polly, 1998]. VDR is the only nuclear protein that binds the biologically most active vitamin D metabolite, 1α,25$(OH)_2D_3$, with high affinity ($K_d = 0.1$ nM). This classifies the VDR into the classical endocrine receptor subgroup of the NR superfamily, which also contains the receptors for the nuclear hormones retinoic acid, thyroid hormone, estradiol, progesterone, testosterone, cortisol, and aldosterol [Carlberg, 1995]. Moreover, this indicates that all genomic effects of physiological concentrations of 1α,25$(OH)_2D_3$ and its analogues are mediated by the VDR. Most analogues of 1α,25$(OH)_2D_3$ carry modifications in their side chain (see Fig. 1 for some examples). They have been developed with the goal to improve the biological profile of the natural hormone for a therapeutic application in hyperproliferative diseases, such as psoriasis and different type of cancer, and bone disorders, such as osteoporosis [Bouillon et al., 1995]. In addition, several of these analogues represent interesting model ligands that are useful for studying the molecular mechanisms of vitamin D signaling.

A very helpful and desired prerequisite to the rational design of 1α,25$(OH)_2D_3$ analogues

Abbreviations used: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; AF-2,(trans)activation function-2; CoA, coactivator protein; CoR, corepressor protein; DR3, direct repeat spaced by three nucleotides; DR4, direct repeat spaced by four nucleotides; ER, estrogen receptor; IP9, inverted palindrome spaced by nine nucleotides; NR, nuclear receptor; RE, response element; RXR, retinoid X receptor; LBD, ligand-binding domain; VDR, vitamin D_3 receptor; VDRE, 1α,25$(OH)_2D_3$ response element.

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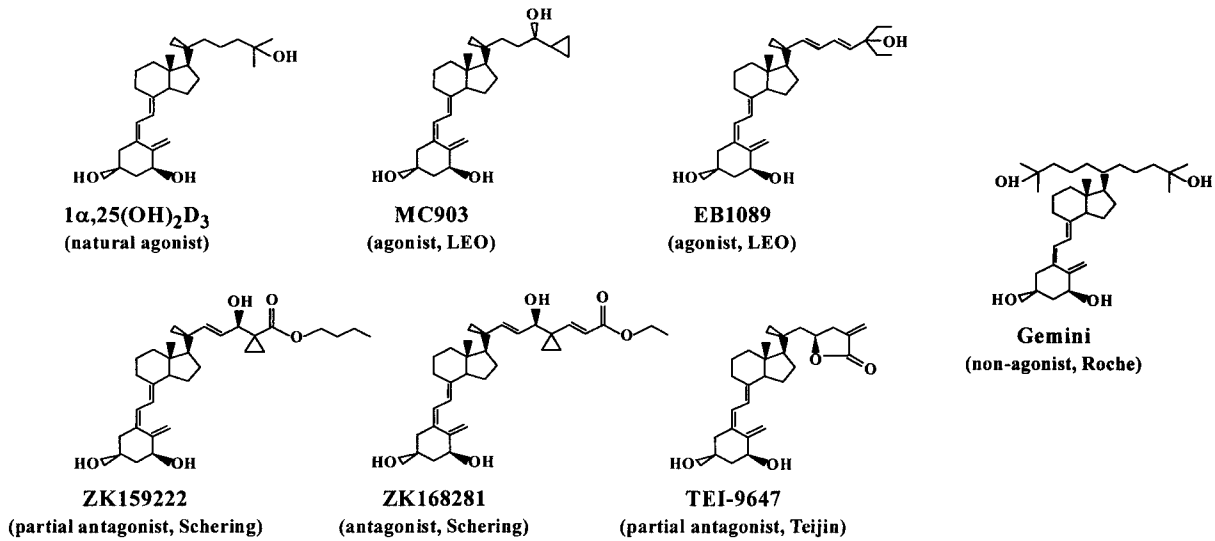

Fig. 1. Structure of $1\alpha,25(OH)_2D_3$ and selected analogues. $1\alpha,25(OH)_2D_3$ and its analogues are lipophilic molecules, that easily pass cellular membranes and enter the nucleus, where they binds with high affinity to the VDR.

is the detailed understanding of their molecular action via the VDR. 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 and a carboxy-terminal LBD of approximately 300 amino acids (Fig. 2A), which is formed by 12 α-helices. 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 [Moras and Gronemeyer, 1998]. Moreover, the LBD is involved in a variety of interactions with nuclear proteins, such as other NR, corepressor (CoR) and coactivator (CoA) proteins [Glass and Rosenfeld, 2000]. These ligand-triggered protein-protein interactions are the central molecular event of nuclear $1\alpha,25(OH)_2D_3$ signaling.

An essential prerequisite for a direct modulation of transcription via $1\alpha,25(OH)_2D_3$-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 VDRE in the regulatory region of a primary $1\alpha,25(OH)_2D_3$ responding gene [Carlberg and Polly, 1998]. 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 formation 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. In most cases, the heterodimeric partner of VDR is RXR and simple VDREs are often formed by a directly repeat of two hexameric core binding motifs spaced by three nucleotides (DR3). However, strongest DNA binding of VDR–RXR heterodimers is observed to RGTTCA motifs spaced by four nucleotides (DR4) [Toell et al., 2000].

VDR–RXR heterodimers are rather dominant on DR3-type REs, whereas on DR4-type REs they have to compete with a broad variety of other NR complexes. The heterodimerization of the DBDs of VDR and RXR is very similar on DR3- and DR4-type REs suggesting that the VDR is on both types of REs in the same conformation. VDR was also found to bind to some unconventional REs, such as DR-type REs with a larger spacer (e.g., DR6-type VDREs [Carlberg et al., 1993]) or core binding motifs in an inverted palindromic arrangement (e.g., IP9-type VDREs [Schräder et al., 1995]). The wide distance of the DBDs of VDR and RXR on these REs suggests that alternative dimerization interfaces between the two receptors are used and that the VDR–RXR heterodimer takes a different conformation than on a DR3- or DR4-type RE. However, clusters of three or more core binding motifs form the most potent VDREs. Such complex VDREs are found in the promoters of the rat and human genes of the

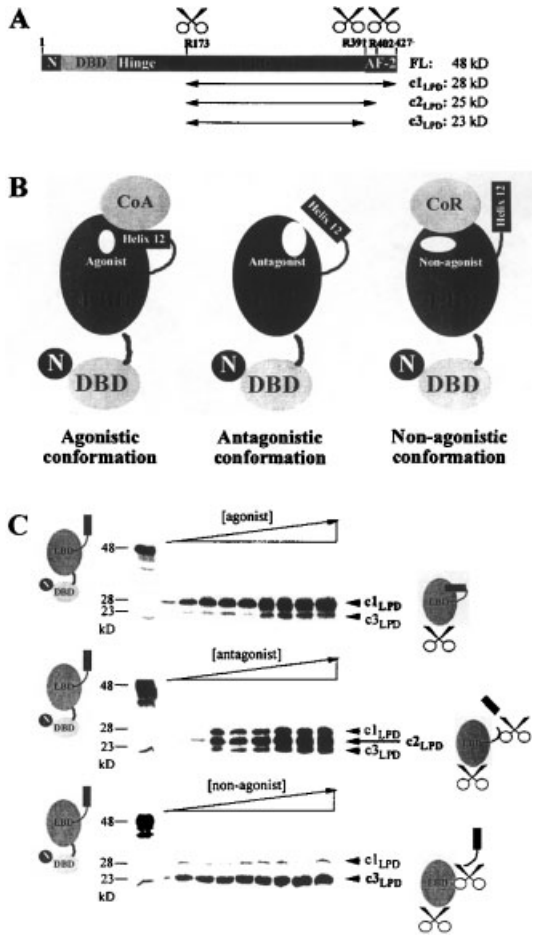

Fig. 2. Relation of VDR structure, VDR conformations, and limited protease digestion patterns. Schematic overview on the primary structure of the VDR containing an amino-terminal region (N), a DBD, a hinge region, and a LBD that includes the AF-2 domain (**A**). The trypsin restriction sites (after arginines at position 173, 391, and 402), that are critical for the generation of VDR fragments 1, 2, and 3 in the limited protease digestion assay, are indicated. The VDR fragments are representatives of different ligand-induced VDR conformations (**B**). Representative limited protease digestion patterns are shown for an agonist, an antagonists, and a non-agonists (**C**).

cytochrome P450 (CYP) 24 and osteocalcin [Schräder et al., 1995; Toell et al., 2000]. Interestingly, both genes represent the apparent most important physiological roles of $1\alpha,25(OH)_2D_3$, which are the regulation of small lipophilic compound metabolism and bone formation [Haussler et al., 1998].

MOLECULAR SWITCHES OF VITAMIN D SIGNALING

DNA-bound VDR–RXR heterodimers are considered as the molecular switches of nuclear $1\alpha,25(OH)_2D_3$ signaling. Traditional ligand binding assays using radiolabeled ligand 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, 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 [Carlberg et al., 2001]. In the limited protease digestion assay (Fig. 2C), the interaction of the VDR with a ligand protects its LBD in a characteristic way against protease digestion allowing the discrimination and quantification of functional VDR conformations. This assay is traditionally performed in a DNA-independent fashion, but for more accurate results, it better should be done in presence of RXR, DNA, and cofactors (CoAs or CoRs) [Quack and Carlberg, 2000a]. 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 [Quack and Carlberg, 2000b]. The supershift assay simply 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 [Herdick et al., 2000a].

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 [Polly et al., 2000]. 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 an agonistic analogue results in replacing a CoR by a CoA protein of the p160-family, such as SRC-1, TIF2, and RAC3 [Leo and Chen, 2000]. 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 [Rachez et al., 1999]. In this way, ligand-activated VDR–RXR heterodimers

fulfill two tasks, opening chromatin and activating transcription.

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 of the more than 2,000 presently 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 few of these superagonists have been chosen for clinical trials. The clinically most successful $1\alpha,25(OH)_2D_3$ analogue, MC903 (calcipotriol, Fig. 1), is topically applied against keratinocyte dysfunction in psoriasis. A systemically applied $1\alpha,25(OH)_2D_3$ analogue is EB1089 (seocalcitol, Fig. 1). EB1089 is less calcemic than the natural agonist, but up to 100-times more potent in inhibiting the proliferation of malignant cells. Therefore, EB1089 should be used in the therapy of several types of cancer. There are plenty other interesting superagonists, e.g., OCT, ED-71, MC1288, and KH1060, but the discussion of their physiological action is not the focus of this review.

A comparison of the most prominent superagonists with $1\alpha,25(OH)_2D_3$ in ligand-dependent gel shift assays on DR3-type VDREs showed that they all these ligands have an EC_{50}-value of $\sim$0.1 nM for the complex formation of VDR–RXR heterodimers on DNA [Herdick et al., 2000a; Quack and Carlberg, 1999; Toell et al., 2000]. 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 $\sim$0.1 nM [Toell et al., 2000]. This suggests that on classical DR3-type VDREs, none of superagonists is significantly more potent in activating VDR–RXR heterodimers than the natural hormone. Interestingly, some superagonists, such as EB1089, show a preference in activating VDR–RXR heterodimers on IP9-type VDREs than on DR3-type VDREs [Nayeri et al., 1995]. However, this RE-type selectivity is observed only with a subset of all superagonists. 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 CoA selectivity [Herdick et al., 2000a]. Moreover, in this assay system saturating concentrations, all tested superagonists stabilized the same amount of VDR-CoA complexes than $1\alpha,25(OH)_2D_3$. With the natural hormone as well as with all tested superagonists, supershift assays provided 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. Taken together, the concentration value of 0.1 nM seems to be a lower threshold for VDR activation, which even superagonists cannot pass.

Contrary to expectations, the crystal structures of VDR's LBD with the natural agonist or two superagonists [Tocchini-Valentini et al., 2001], the 20-epi analogues MC1288 and KH1060, as ligand 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 hydrogen bond between the hydroxyl group at carbon 25 of the ligand and H397 in helix 11 of the LBD. The direct ligand contact of H397 enables this amino acid to build an additional contact with F422 of the transactivation function 2 (AF-2) domain of helix 12. Helix 12 makes no direct contact with the ligand, but it forms the "lid" of the ligand-binding pocket and projects its inner hydrophobic surface towards the bound hormone. 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 the AF-2 domain and the positively charged K246 on the surface of helix 3. This charge clamp structure is essential for contacting the NR interaction domains of CoA proteins. It has to be noted that the crystal structure of VDR's LBD was obtained with a truncated version of the receptor that lacks the amino acids between positions 165 and 215 [Rochel et al., 2000]. This so-called insertion domain was deleted, in order to obtain soluble protein for crystallization. Interestingly, the LBD of the only one other member of the NR superfamily

that also carries an insertion domain, the VDR relative pregnane X receptor, could be crystallized without the need of a deletion [Watkins et al., 2001]. In the pregnane X receptor, the insertion domain seems to enlarge the ligand-binding pocket of the receptor, but is not involved in CoA contacts. The same can be assumed for the VDR, so that the above-discussed properties of its agonistic VDR conformation are valid despite the lack of the insertion domain.

Presently, more than 100 different VDR ligands have been analyzed in the limited protease digestion assay. Most of these ligands predominantly stabilize a large fragment of the LBD of the receptor ($c1_{LPD}$, from arginine 173 to the carboxy-terminus at position 427, see Fig. 2) [Quack and Carlberg, 1999]. 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 ~0.1 nM, i.e., with the same threshold concentration that was already observed in gel shift and supershift assays. All 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}-value in the order of 1–20 nM [Quack and Carlberg, 1999]. This suggests that VDR's LBD reaches its full ligand sensitivity only as a component of DNA-bound VDR–RXR heterodimer. Moreover, this observation indicates that superagonists can be differentiated in their ability to activate VDR in solution. This activation may take place in presently not well-characterized, DNA-independent $1\alpha,25(OH)_2D_3$ signaling pathways in the nucleus and the cytoplasma.

Compared to the natural agonist, some superagonists show RE selectivity 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. However, these relative differences in EC_{50}-values are in maximum of a factor of 10, so that promotor and pathway selectivities themselves are not sufficient to explain the improved in vivo profile of superagonists in relation to $1\alpha,25(OH)_2D_3$. 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 [Tocchini-Valentini et al., 2001]. Moreover, KH1060 was shown to stabilize the VDR against endogenous proteolytic degradation over a longer time period than $1\alpha,25(OH)_2D_3$ [van den Bernd et al., 1996]. In addition, in the limited protease digestion assay several superagonists were described to stabilize the agonistic VDR conformation for 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 [Bury et al., 2001b]. 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.

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 block the respective 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, natural 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 [Schapira et al., 2000]. For the VDR, two different types of antagonists have been described. These are the 25-carboxylic esters ZK159222 and ZK168281 [Bury et al., 2000; Herdick et al., 2000c] and the 26,23-lactone TEI-9647 [Ozono et al., 1999] (see Fig. 1 for their structures). 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. One major difference in the action of the two types of antagonists is, that ZK159222 and ZK168281 stabilize the complex formation of VDR–RXR heterodimers on a VDRE with the same potency and nearly the same sensitivity than $1\alpha,25(OH)_2D_3$ [Bury et al., 2000]. In contrast,

TEI-9647 shows both a reduced potency and a more than 10-fold reduced sensitivity [Toell et al., 2001]. The different sensitivity of the antagonists 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 [Toell et al., 2001].

All VDR antagonists stabilize a clearly lower amount of the VDR molecule pool in the agonistic conformation $c1_{LPD}$ than the natural hormone, whereas they specifically stabilize the antagonistic conformation $c2_{LPD}$ (from arginine 173 to arginine 402, see Fig. 2A) [Bury et al., 2000; Herdick et al., 2000c]. Interestingly, the stabilization of the VDR with TEI-9647 compared to with ZK159222 results in a slight migration difference between the VDR fragments that represent conformation $c2_{LPD}$ [Bula et al., 2000; Toell et al., 2001]. This suggests that the two antagonists stabilize different antagonistic conformations. In contrast to the natural hormone and its agonistic analogues, none of the antagonists is able to mediate a significant interaction of the VDR with CoAs [Toell et al., 2001]. However, like $1\alpha,25(OH)_2D_3$, the binding of the both types of antagonists to the VDR induces a dissociation of CoR proteins. This suggests that they stabilize the VDR in a conformation blocking the interaction with coactivators, but this antagonistic conformation appears not to prevent VDR-CoR dissociation [Toell et al., 2001].

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 $\sim$20% of that of the natural ligand, whereas ZK168281 displayed an agonistic potential of only less than 5% [Toell et al., 2001; Väisänen et al., 2002]. 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 [McDonnell, 1999]. Therefore, ZK159222 and TEI-9647 also could be referred to as selective VDR modulators.

Molecular dynamics 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 interaction between residues H397 and F422 [Väisänen et al., 2002]. Due to the disturbed H397-F422 interaction, helix 12 is much more flexible. Therefore, the helix will be mostly in a position, in which the distance of the residues K246 and E420 deviates from the optimized value of 19 Å. This decreases the affinity to coactivators or even makes interaction impossible. Although the side chains of both antagonists have the same number of atoms (see Fig. 1), 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 a nearly completely prevents coactivator binding [Väisänen et al., 2002], i.e., 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 coactivator proteins to contact the VDR via the charged clamp formed by K246 and E420. 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. Moreover, TEI-9647 has been described to stabilize a different antagonistic conformation than ZK159222 [Bula et al., 2000; Toell et al., 2001], which fits with molecular dynamic simulations that suggest the existence of various antagonistic conformations of helix 12 [Väisänen et al., 2002].

ZK159222 was shown to display tissue-specific agonism [Herdick et al., 2000d] 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 [Herdick et al., 2000d].

NON-AGONISTIC ACTION OF VDR LIGANDS

The limited protease digestion assay monitors, in which conformation the VDR was at the moment of the protease "snapshot". In the case that this is in majority $c1_{LPD}$, the ligand is considered as an agonist, whereas dominance of $c2_{LPD}$ indicates an antagonist (Fig. 2). However, most ligands stabilize a minor portion of the VDR molecule pool in the so-called non-agonistic conformation $c3_{LPD}$ (from arginine 173 to arginine 391, see Fig. 2A) [Herdick and Carlberg, 2000b]. Interestingly, analogues with two side chains at carbon 20, such as Gemini and its derivatives, were found to stabilize monomeric VDR in solution even preferentially in the conformation $c3_{LPD}$ [Bury et al., 2001a]. The term "non-agonistic" indicates that the ligand binds the receptor in a conformation that does not enable an interaction with CoA proteins, i.e., helix 12 has not moved in comparison to the apo-form of the receptor (Fig. 2C). The main difference between the antagonistic and the non-agonistic conformation is that in the latter case CoR proteins did not dissociated from the receptor, i.e., that interaction with a CoR protein blocks the binding of a CoA protein [Herdick and Carlberg, 2000b]. Due to its bulky side chain that causes steric hindrance to helix 12 an antagonist, such as ZK168281, can never convert to a superagonist. In contrast, the non-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 [Herdick et al., 2000a]. 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. However, even in situations when Gemini acts as an agonist, it showed to be much more affected by higher CoR levels than other agonists, i.e., its potency decreases in the presence of CoRs [Herdick and Carlberg, 2000b].

The molecular mechanisms of the non-agonistic actions of VDR ligands are not fully understood, but it can be assumed that in a non-agonistic conformation the VDR resembles to its apo-form. This would mean that the side chain of the ligand is taking a different position than in the agonistic conformation and is not contacting H397. As a consequence of that, the H397-F422 contact is not formed and helix 12 is not stabilized in the agonistic position. Molecular dynamics simulations of the VDR-Gemini complex suggested that the ligand-binding pocket of the VDR has two extra cavities that may harbor a side chain (Väisänen, Peräkylä, and Carlberg, unpublished results). This means that Gemini has three possibilities to bind to the VDR, which are represented by the three possible combinations of two of these side chain locations. In one of these combinations, none of Gemini's two side chains are using the traditional location, which seems to be the preferred choice in the presence of CoR proteins. In case of an excess of CoA versus CoR proteins, the two other binding possibilities of Gemini seem to get favored, which shifts the $c1_{LPD}/c3_{LPD}$ ratio to values greater than 1 and let Gemini turn from an non-agonist to an agonist.

CONCLUSION

DNA-bound VDR–RXR heterodimers are the molecular switches in $1\alpha,25(OH)_2D_3$ signaling. The agonistic, antagonistic and non-agonistic conformation of VDR's LBD within this molecular switch should explain the functional profile of all VDR ligands. 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 presently appears to be the most informative way for their in vitro evaluation. Finally, the pharmacokinetic profile, such as cellular uptake, transport and in particular metabolic stability, is an important modulary factor of the molecular characteristics of a VDR ligand and should not be neglected.

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