Functional conformations of the nuclear 1 α ,25-dihydroxyvitamin D₃ receptor

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The nuclear hormone 1 α ,25-dihydroxyvitamin D₃ (VD) has important cell regulatory functions. Various synthetic VD analogues are under investigation to identify candidates with an improved therapeutic profile against hyperproliferative diseases. VD directly activates the transcription factor VD receptor (VDR), which in turn stimulates the expression of a cascade of primary and secondary VD-responsive genes. The activation of the VDR through binding of its natural and synthetic ligands is linked to a conformational change presenting the interface with co-activator proteins, referred to as the (trans)activation function 2 (AF-2) domain. Multiple conformations of the VDR might be the key to understanding a selective action of VD analogues. The method of limited protease digestion was used here to characterize up to three different functional VDR conformations stabilized

individually by VD and its analogues. The relative potency of VDR ligands can be quantified in the interaction with these VDR conformations by determination of a functional dissociation constant, where a two-concentration-point comparison has already provided important information. In this way seven amino acid residues in the AF-2 domain have been analysed as potential ligand contact points. Interestingly, residues Phe-422 and Val-418 seem to interact with all tested VDR ligands, whereas VD analogues such as the anti-psoriatic drug MC903 displayed additional contact points within the AF-2 domain. Taken together, limited protease digestion is a powerful method for studying functional VDR conformations and seems to be very appropriate for screening VD analogues.

INTRODUCTION

The biologically active metabolite of vitamin D₃, 1 α ,25-dihydroxyvitamin D₃ (VD), is a nuclear hormone that is involved in the regulation of a variety of important biological functions such as calcium homeostasis as well as cellular growth, differentiation and apoptosis [1]. The cell-regulatory properties of VD provide it with an interesting potential for the therapy of hyperproliferative diseases like cancer and psoriasis. However, the calcaemic function of VD can cause side effects such as hypercalcaemia, hypercalciuria and soft-tissue calcification [2]. Thus there is a need for the development of low-calcaemic VD analogues with an increased cell regulatory function, being most effective if supported with a simple and informative screening assay [3–5]. The efficacy of a VD analogue is influenced by its characteristic resorption, stability, metabolism and receptor binding rates respectively. Selectivity of VD analogues could be studied on each of these levels, but an investigation of their functional effects seems to be most promising.

VD and its analogues can easily pass through cellular membranes and enter the nucleus to bind their cognate transcription factor, the VD receptor (VDR) [6,7], which is a member of a superfamily of structurally related nuclear receptors [8]. Binding of ligand activates a nuclear receptor, as demonstrated recently by crystal structures of the retinoid X receptor α [9] and the retinoid acid receptor γ [10], by changing the position of an amphipathic α -helix referred to as the (trans)activation function 2 (AF-2) domain [11,12]. This AF-2 domain provides an interface for interaction with cell-specific co-activators that mediate contacts to the basal transcriptional machinery. In this way the activation of the nuclear receptor is transferred into the stimu-

lated activity of RNA polymerase II, a process called trans-activation [13–15]. A crystal structure has not yet been resolved for the VDR, nor have VDR-specific co-activators been characterized, but it can be assumed that the main principles discovered for retinoid receptors are also valid for the VDR. However, functional VDR has been shown to participate in a variety of different protein–DNA complexes [7,16]. It can be assumed that in each of these complexes the VDR takes a different conformation so that the selectivity of VD analogues might be based on the differential recognition of these conformations [17]. This suggests that the conformations of the VDR and their interaction with ligand are of central importance in nuclear VD signalling.

Ligand binding is largely studied by traditional competition assays with radiolabelled ligand; however, this method does not permit the detection of any conformational changes in the receptor. An attractive alternative is the limited protease digestion assay, which is based on the principle that the binding of ligand to the ligand-binding domain (LBD) masks a protease cutting site and creates, under limited reaction conditions, a protease-resistant receptor fragment. Biologically potent VD analogues have been shown with this method to have a higher functional affinity for VDR than the natural hormone [18]. In contrast, a comparable competition study provided misleading results [19]. In addition, limited protease digestion has recently been used to monitor the kinetics of stabilization of the VDR by ligand [20].

In this report, limited protease digestion was used to characterize functional conformations of the VDR in its interaction with VD and three selected VD analogues. Moreover, seven residues of the AF-2 domain of the VDR were investigated as potential ligand contact points. This presents the method of

Abbreviations used: AF-2, (trans)activation function 2; K_{d} , functional dissociation constant; LBD, ligand-binding domain; VD, 1 α ,25-dihydroxyvitamin D₃; VDR, 1 α ,25-dihydroxyvitamin D₃ receptor.

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limited protease digestion as a very informative tool for a functional characterization of the VDR and its ligands.

MATERIALS AND METHODS

Compounds

VD and the three VD analogues MC1288, EB1324 and MC903 (for structures see Figure 1) were kindly provided by L. Binderup and I. Mathiasen (LEO Pharmaceutical Products, Ballerup, Denmark). These ligands were dissolved in propan-2-ol at 4 mM; dilutions were performed in ethanol.

DNA constructs

The cDNA for human VDR was subcloned into the expression vector pSG5 (Stratagene) [21] and was used as template for a linear PCR reaction with *Pfu* DNA polymerase (Stratagene) with a profile of 0.5 min at 94 °C, 1 min at 55 °C and 11 min at 68 °C for 16 cycles. For the seven point mutations V418A, L419A, E420A, V421A, F422A, G423A and N424A (one-letter amino acid codes) the following primer pairs were used: V418A+, 5'-GCTAACGCCCTTGCGCTCGAAGTGTGTTG-3'; V418A-, 5'-CAAACACTTCGAGCGCAAGGGGCGTTAGC-3'; L419A+, 5'-AACGCCCTTGTTGGCCGAAGTGTGTTGGCA-3'; L419A-, 5'-TGCCAAACACTTCGGCCACAAGGGGCGTT-3'; E420A+, 5'-GCCCCTTGCTCGCAAGTGTGTTGGCAATG-3'; E420A-, 5'-CATTGCCAAACACTGTCGAGCACAAGGGGC-3'; V421A+, 5'-CCTTGTGCTCGAAGCGTGTGGAATGAGA-3'; V421A-, 5'-TCTCATTGCCAAACGCTTCGAGCACAAGG-3'; F422A+, 5'-TTGTGCTCGAAGTGGCTGGCAATGAGAT; F422A-, 5'-ATCTCATTGCCAGCCACTTCGAGCACA-3'; G423A+, 5'-GCTCGAAGTGTGTTGCCAATGAGATCTCCT-3'; G423A-, 5'-AGGAGATCTCATTGGCAAACACTTCGAGC-3'; N424A+, 5'-GAAGTGTGTTGGCGCTGAGATCTCCTGA-3'; N424A-, 5'-TCAGGAGATCTCCAGGCCAAACACTTC-3'.

Methylated template DNA was then digested selectively with *DpnI*, and supercompetent Epicurian Coli XL-1 (Stratagene) were transformed with non-digested, PCR-generated plasmid DNA. The respective point mutations were confirmed by sequencing.

Limited protease digestion assay

Linearized cDNA of the wild-type VDR and of the seven point mutations were used for transcription *in vitro* as recommended (Promega). VDR RNA (10 µg) transcribed *in vitro* was mixed with 175 µl of rabbit reticulocyte lysate (Promega), 100 units of RNasin, 20 µl of [³⁵S]methionine (1000 Ci/mmol) and 20 µM amino acid mixture (lacking methionine) in a total volume of 250 µl and incubated at 30 °C for approx. 75 min (total protein concentration 60 µg/µl). For work with VDR that is most functional, the material should be used immediately after translation; storage at any temperature should be omitted. Protein translated *in vitro* (1 µl), 5.5 µl of 50 mM Tris/HCl, pH 7.9, and 1 µl of ligand (or 1 µl of ethanol as a control) were preincubated for 15–30 min at room temperature. Then 2.5 µl of protease [trypsin (Promega), final concentration 27 µg/ml, chymotrypsin (Boehringer Mannheim), final concentration 50 µg/ml or proteinase K (Life Technologies), final concentration 1 µg/ml] was added and the mixtures were incubated for 1–60 min at room temperature, as indicated. The digestion reactions were stopped by adding 10 µl of protein gel loading buffer [0.25 M Tris/HCl (pH 6.8)/20% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 0.025% Bromophenol Blue]. The samples were

denatured at 95 °C for 5 min and subjected to SDS/PAGE [15% (w/v) gel; acrylamide/*N,N'*-methylene-bisacrylamide 33:1 (w/w)] with the Mini-PROTEAN electrophoresis system (Bio-Rad, migration time 90–120 min at 220 V). The gels were electrotransferred to a nitrocellulose filter, dried in air and autoradiographed overnight. The protease-sensitive VDR fragments were located and excised from the filter; radioactivity was measured by scintillation counting. The protease-resistant fragments contain 10 of the 17 methionine residues of the full-length VDR, so that 58.8% of the radioactivity of the full-length VDR equals 100% of the VDR fragments. Calculation of ligand-stabilized VDR was based on the amount of VDR that is resistant to protease digestion in the absence of ligand, subtracted from the value obtained with the respective ligand concentration.

RESULTS

Limited protease digestion was performed with [³⁵S]methionine-labelled human VDR translated *in vitro*, together with the proteases trypsin, chymotrypsin or proteinase K in the presence of a high concentration (10 µM) of VD, its analogues MC903, EB1324 or MC1288, or solvent (ethanol). EB1324 and MC1288 are simple 1β and 20-epi modifications of VD, whereas MC903, an analogue in clinical use against psoriasis [22], has multiple alterations in the side chain (Figure 1). In the presence of the VDR ligands it was found that the protection against digestion with the different proteases was clearly enhanced compared with that of solvent controls (Figure 2). Digestion with chymotrypsin or proteinase K provided VDR fragments with sizes less than 29 kDa, whereas digestion with trypsin generated two VDR fragments with VD and EB1324 and three VD fragments with MC903 and MC1288 (labelled 1, 2 and 3, all in the molecular mass range 33–29 kDa). Therefore limited protease digestion with trypsin was considered to be suitable for analysing VDR–ligand interactions; all experiments that followed were performed with this endoprotease.

The interaction of VDR with a high concentration (10 µM) of VD, MC903 and EB1324 in comparison with solvent was plotted as a function of time (Figure 3A). The protease-resistant VDR fragments 1 to 3 were quantified by scintillation counting and compared with the amount of full-length VDR used in each reaction (Figure 3B). At this ligand concentration, VD and MC903 protected nearly all VDR molecules against digestion with trypsin (for a period of 60 min), whereas the same concentration of EB1324 or the ethanol solvent resulted in a rapid decrease in protease-resistant VDR fragments. At low concentrations of ligand (1 nM; Figure 3C) with all ligands a decrease in trypsin-resistant VDR fragments was observed over time. VD proved to be more effective than MC903, which in turn was more effective than EB1324. However, results shown in Figures 3(B) and 3(C) suggested that an incubation time of 30 min was most effective for both a qualitative and a quantitative comparison of VDR ligands.

Limited protease digestion was performed with graded ligand concentrations, allowing a more detailed quantitative comparison of VDR ligands. As shown for MC903 (Figure 4A) at a fixed incubation time (30 min), decreasing the ligand concentration resulted in a decreased quantity of protease-resistant VDR fragments. Interestingly, the three protease-resistant VDR fragments decreased differently in their quantities, similarly to what was observed with MC1288 [23]. The high-molecular-mass VDR fragment proved to be more resistant to protease digestion than the two low-molecular-mass fragments. The graphical presentation of the relative amount of ligand-stabilized VDR resulted in ligand binding curves that seemed to be characteristic of each

Figure 1 Structure of VD and of its analogues EB1324, MC1288 and MC903

In comparison with VD, EB1324 and MC1288 have each only one conformational change (1 β and 20-epi respectively). MC903 is in clinical use against psoriasis.

Figure 2 Digestion of the VDR with different proteases

[35 S]Methionine-labelled full-length VDR (FL; 1 μ l) synthesized *in vitro* was preincubated with solvent [ethanol (EtOH)] or with 10 μ M VD, MC903, EB1324 or MC1288. The proteases trypsin (T), chymotrypsin (C) or proteinase K (K) in a final concentration of 27, 50 or 1 μ g/ml respectively were added and the mixtures were incubated for 30 min at room temperature. Samples were subjected to SDS/PAGE [15% (w/v) gel], electrotransferred to a nitrocellulose filter, dried in air and autoradiographed. Three protease-resistant VDR fragments (bands 1 to 3) of approximate size 33, 31 and 29 kDa were resolved by trypsin digestion, whereas with chymotrypsin and proteinase K only one protease-resistant fragment of approximate size 28 kDa was observed. This suggested that trypsin was the candidate protease for the subsequent experiments.

VDR-specific ligand (Figure 4B). In contrast with binding curves obtained from traditional ligand binding assays, these curves represented the functional consequence of ligand binding. VD was shown to be more effective than MC903 (by a factor of approx. 100), whereas EB1324 displayed almost no functional effects. The difference between VD and MC903 was quantified by performing Scatchard plots (Figure 4C), from which functional dissociation constants (K_{af}) of 1.0 and 68 nM for VD and MC903 respectively were determined. For VD this confirms results published previously [17,23]. Taken together, the Scatchard analysis suggests that VD has approximately 70-fold higher

sensitivity in functional effects than MC903 and confirms the estimation based on Figure 4(B).

Data points in the Scatchard plots (Figure 4C) calculated for MC903 apparently lie on two separate lines (broken), suggesting that there are at least two ligand-binding sites in the VDR. Thus the VDR fragment with the highest molecular mass (band 1) was quantified separately from one (for VD) or two (for MC903) lower-molecular-mass fragments (Figure 5). A comparison of the amount of ligand-stabilized VDR found in band 1 (which is designated VDR conformation 1) with the total amount of ligand-stabilized VDR showed that with increasing ligand concentration an increasing quantity of lower-molecular-mass VDR fragments (i.e. the difference between the two curves) were binding ligand. A Scatchard plot analysis of the VDR in conformation 1 provided K_{af} values of 0.59 and 6.1 nM for VD and MC903 respectively. The value for VD stayed in the range of that found with the total amount of ligand-stabilized VDR (Figure 4C) [17], but for MC903 a greater than 10-fold higher functional ligand binding affinity was obtained. This suggested that VDR conformation 1 was a high-affinity ligand binding conformation and confirmed both the visual impression from Figure 4(A) and previous results obtained with MC1288 [23].

To test this idea and earlier indications that the AF-2 domain was involved directly in ligand binding [23], residues Val-418 to Asp-424 were individually mutated into alanine residues. Wild-type VDR and these seven point mutations were incubated with high (10 μ M) and physiological (1 nM) concentrations of VD, MC903 and MC1288 before limited protease digestion was performed (Figure 6). Wild-type VDR at high ligand concentrations provided the typical two-band pattern with VD and the three-band patterns with MC903 and MC1288. Concentrations

Figure 3 Protection of the VDR against protease digestion is time- and ligand-dependent

[35 S]Methionine-labelled VDR (1 μ l) synthesized *in vitro* was preincubated with solvent [ethanol (EtOH)], 10 μ M VD, MC903 or EB1324 at a concentration of 10 μ M (**A**, **B**) or 1 nM (**C**). Trypsin was added to a final concentration of 27 μ g/ml and the mixtures were incubated for 1, 5, 10, 15, 30 or 60 min at room temperature. Samples were subjected to SDS/PAGE [15% (w/v) gel], electrotransferred to a nitrocellulose filter, dried in air and autoradiographed [a representative experiment is shown in (**A**)]. FL indicates full-length receptor. The protease-resistant VDR fragments (marked 1 to 3) from three independent series of experiments were cut out from the filter and their radioactivity was measured in a scintillation counter. The mean of the percentage of protease-resistant VDR was plotted over time for each ligand and for the ethanol control. Bars indicate S.D.

of VD and MC1288 that were 1/10000 of this still resulted in a prominent quantity of VDR fragment 1, but showed no reasonable amounts of VDR fragments 2 and 3. Interestingly, physiological concentrations of MC903 did not stabilize any of the three VDR conformations. A qualitatively similar result was obtained with the VDR point mutation E420A, which suggested that this residue does not have an important role in the interaction with ligand. In contrast with wild-type VDR, the point mutations N424A, G423A and L419A did not provide VDR fragment 1 when preincubated with 10 μ M MC903. This indicated that these point mutations specifically affect the high-affinity interaction of the VDR with MC903. High-affinity binding of the VDR to VD was affected through point mutation V421A, whereas high-affinity binding to MC1288 was affected only to a smaller extent. Finally, with the two point mutations F422A and V418A high-affinity binding of the VDR to all three ligands was drastically

decreased. In contrast, the ligand interactions of the lower-affinity ligand binding conformations were apparently not affected by any of the seven point mutations.

DISCUSSION

Limited protease digestion is a powerful method for characterizing various VDR conformations. The interaction of receptor with ligand protects the ligand-interacting region (i.e. basically the LBD) against protease digestion. Use of the endoprotease trypsin displays a characteristic digestion pattern of two or three VDR fragments of 29–33 kDa in size. According to the current model of nuclear hormone signalling, the binding of a ligand induces a conformational change within the nuclear receptor LBD [24,25]. However, in this study it became obvious that two of the VDR fragments (representing conformations 1 and 3) can

Figure 4 Protection of the VDR against protease digestion is dependent on ligand concentration

[35 S]Methionine-labelled wild-type VDR (1 μ l) synthesized *in vitro* was preincubated with the indicated concentrations of MC903. Trypsin was added to a final concentration of 27 μ g/ml and the mixtures were incubated for 30 min at room temperature. Samples were subjected to SDS/PAGE [15% (w/v) gel], electrotransferred to a nitrocellulose filter, dried in air and autoradiographed. FL indicates full-length receptor. A representative experiment is shown in (A). The trypsin-resistant VDR fragments (marked 1 to 3) were cut out from the filter and their radioactivity was measured as before in a scintillation counter. This type of experiment was repeated up to 13 times for VD, MC903 and EB1324; the mean of the percentage of ligand-stabilized VDR was plotted against ligand concentration (B). Ligand-stabilized VDR is calculated as the difference between the protease resistance induced by the ligand and that of the solvent. Scatchard plots of the data for VD and MC903 (C) permitted the calculation of K_{dr} values of 1.0 and 68 nM. However, the data points of both ligands are apparently arranged on two different lines (broken) rather than on one simple line (solid).

be observed even in the absence of ligand. This suggests that before ligand interaction these VDR conformations had already been formed (Figure 7). Therefore the role of a ligand seems more likely to be to stabilize than to induce a conformation. Only those conformations that after interaction with ligand are protected against protease digestion are considered to be functional and can be revealed by limited protease digestion. Depending on the ligand and its concentration, not all VDR molecules are protected against protease digestion; therefore a proportion of the VDR conformations is apparently due to non-functional VDR–ligand interactions (Figure 7). The natural VDR ligand VD, its 1β analogue EB1324 and a wide range of other VD analogues [17,18] interact with only two functional conformations of the VDR, whereas VD analogues such as MC903, MC1288 [23] and ZK157222 [26] interact with an additional third functional VDR conformation.

These three functional VDR conformations differ in their sensitivities to ligand. The highest-molecular-mass VDR fragment of approx. 33 kDa, referred to as functional VDR conformation 1 (Figure 7), shows an interaction with the ligand at low concentrations. At increasing ligand concentrations functional conformations 2 and 3 are also charged with ligand. The extent to which the three functional VDR conformations at a given concentration are charged with ligand is characteristic of each ligand. Potent VDR ligands such as the natural ligand VD show a pronounced quantity of VDR fragment 1 in the limited protease digestion analysis, i.e. only high-affinity VDR conformation 1 is charged with ligand. With ligands such as these and in particular at low, physiological concentrations, the interaction of the VDR with ligand is represented by the ligand interactions of its functional conformation 1. In this case the K_{dr} values calculated on the basis of the total amount of ligand-stabilized VDR were equal to those based only on the ligand-stabilized conformation 1. In contrast, with weak VDR ligands or with VD analogues that interact effectively also with the VDR conformations 2 and 3, the K_{dr} values of each individual VDR conformation should be taken into consideration. The K_{dr} for MC903 that was determined on the basis of the total amount of ligand-stabilized VDR was for this reason more than 10-fold that calculated for its high-affinity conformation.

This raises the question of whether MC903 is a weak or an average VDR ligand. If one considers VDR conformation 1 as the most important and critical conformation, it suggests that the stabilization of VDR conformations 2 and 3 would cause a functional disadvantage for the respective VDR ligand. However, at low nanomolar concentrations of MC903 some of the VDR molecules in conformation 1 seemed to be charged with ligand, whereas only at higher ligand concentrations were those in conformations 2 and 3 also charged. A functional analysis of MC903 on reporter gene constructs of VD response elements performed previously in transiently transfected human breast cancer MCF-7 cells [27] showed that the analogue is active at low nanomolar concentrations. This suggests that charging only a proportion of all VDR molecules (mainly those in conformation 1) with ligand is sufficient to obtain a functional response. Moreover if one assumes that *in vivo* the VDR forms more than the three conformations observed *in vitro*, it is possible that, compared with the natural ligand VD, VD analogues such as MC903 and MC1288 might have an overall greater functional potential.

Comparison of the limited protease digestion patterns of a VDR ligand at high and physiological concentrations provides initial information to characterize its functional binding properties. Further detailed information can be obtained from determination of K_{dr} , which provides a quantification of the relative functional affinity of a VD analogue as presented previously for a series of 20-epi-VD analogues [18]. Ranking of the three VDR ligands MC1288, VD and MC903 used in this study with K_{dr} values based either on all three VDR conformations (0.41 [18], 1 and 68 nM) or only on VDR conformation 1 (0.14 [23], 0.56 and 6.1 nM) correlates with a ranking based on their ability to stimulate gene transcription from DR3-type VD response elements (EC_{50} values of 0.22 [18], 0.3 [27] and 0.6 nM [27]).

Moreover, the limited protease digestion assay seems to be an ideal tool for the analysis of VDR modifications (e.g. point mutations, truncations, covalent modifications such as phosphorylations) and their effects on the interaction with ligand. A relative decrease in, or a disappearance of, a VDR fragment in the digestion pattern indicates, independently of the kind of receptor modification, that the ability of a VDR ligand to stabilize the respective VDR conformation was decreased or

Figure 5 Analysis of individual VDR conformations

Experiments as shown in Figure 4(A) were repeated in triplicate with VD (left panel) and MC903 (right panel). The two or three protease-resistant VDR fragments that were stabilized by VD or MC903 respectively were cut out from the filter and their radioactivity was measured as before in a scintillation counter. The mean of the percentage of ligand-stabilized VDR conformation 1 (band 1) and that of the sum of the two or three conformations (total) were plotted against ligand concentration. The data derived from VDR conformation 1 are presented in Scatchard plots (insets), permitting the calculation of K_{dr} values of 0.59 and 6.1 nM for VD and MC903 respectively.

Figure 6 Ligand-dependent conformations of VDR with AF-2 point mutations

The [³⁵S]methionine-labelled point-mutated VDR proteins N424A, G423A, F422A, V421A, E420A, L419A and V418A were obtained by transcription/translation *in vitro* of respective plasmid templates generated by site-directed mutagenesis of wild-type receptor (wt). They were preincubated with either 10 μM or 1 nM VD, MC903 or MC1288; limited protease digestion was performed as described in the legend to Figure 4(A). A representative gel is shown.

even completely lost. Residue Phe-422 has previously been shown to have a central role in the functionality of the AF-2 domain by apparently contacting both the ligand and co-activators [23,28]. The analysis presented here suggests that Val-418 has a similar

role. Interestingly, both residues (Phe-422 and Val-418) seem to be the contact points not only for VD and MC903 but also for the 20-epi analogue MC1288. This is in contrast with the report of Liu et al. [28] that Phe-422 does not contact MC1288.

Figure 7 Model of the functional VDR conformations analysed by limited protease digestion

The full-length VDR has a size of 48.3 kDa and is composed of a DNA-binding domain (DBD) and an LBD connected by a flexible hinge region. The model is based on the assumption that the unliganded VDR has at least four different conformations, which are named 1 to 4. The VDR contains at least three different contact sites (small grey triangles) for the ligand (bigger black triangle) within its ligand-binding cleft in close vicinity to the transactivation domain AF-2. The four VDR conformations can be differentiated by different uses of these ligand contact sites: in conformation 1 (upper right) the first contact site, in conformation 2 (lower right) the second site, in conformation 3 (lower left) the third site and in conformation 4 (upper left) none of these sites are used. This can be visualized experimentally by incubation with a protease such as trypsin. In conformation 1 the complete LBD is protected and a VDR fragment of approx. 33 kDa can be observed, whereas in conformations 2 and 3 different C-terminal regions (including the AF-2 domain) are digested, which results in VDR fragments of a approximate sizes 31 and 29 kDa. In contrast, in conformation 4 the VDR is completely digested. Compared with their respective unliganded conformations, the binding of ligand stabilizes each of conformations 1, 2 and 3 characteristically. In contrast with conformation 4, conformations 1, 2 and 3 are considered to be functional, as binding of ligand to the ligand-binding cleft is assumed to result in the presentation of the AF-2 domain. The three functional conformations 1, 2 and 3 have been characterized as high-affinity, medium-affinity and low-affinity conformations. Abbreviation: kD, kDa.

However, it has been shown previously [23] that a point mutation of Phe-422 decreased the functional affinity to VD to less than 1/100, but to MC1288 only to 1/10. In addition, the analysis reported here indicated that VDR ligands might have different contact points within the AF-2 domain. MC903 seems to contact not only Phe-422 and Val-418 but also Asn-424, Gly-423 and Val-421. Because VD analogues seem to contact the VDR primarily through their side chain [29], this could be explained by the fact that MC903 has a drastically modified side chain compared with VD and MC1288 (see Figure 1).

Taken together, functional VDR conformations seem to play a central role in nuclear VD signalling and can be characterized easily by limited protease digestion, thus allowing very informative screening assays to be designed and implemented for the identification of clinically effective VD analogues.

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