Published in final edited form as:

J Steroid Biochem Mol Biol. 2007 March ; 103(3-5): 286–292.

Conservative Mutagenic Perturbations of Amino Acids Connecting Helix 12 in the $1\alpha,25(\text{OH})_2\text{-D}_3$ Receptor (VDR) to the Ligand Cause Significant Transactivational Effects

Craig M. Bula, June. E. Bishop, and Anthony W. Norman

Department of Biochemistry, University of California, Riverside, Riverside, CA, 92521

Abstract

The positioning of helix 12 activation domain of nuclear receptor proteins is critically important for gene regulation. Perturbations of the helix 12 by larger analogs may alter interactions with transcriptional machinery which might give rise to selectivity. To explore the topology of the ligand binding pocket and how the bound ligand conceivably gives rise to altered transcriptional efficiencies, we have targeted 4 hydrophobic residues which contact the 25-carbon of the ligand, $1\alpha,25(\text{OH})_2$ -vitamin D_3 , and made a series of 13 mutants. Substitution of a smaller hydrophobic residue was poorly tolerated compared to a larger one for transactivation. The larger amino acids are likely better tolerated by promoting stronger van der Waals forces with the ligand. Valine-418 mutants demonstrated an extreme example of this observation with mutation to leucine being transactivationally unaffected with alanine being the most affected of all single mutants. V418L resulted in a 1.3-fold increase in EC_{50} for $1,25\text{-D}$ mediated transactivation whereas V418A resulted in a 53-fold increase when compared to wildtype VDR. Importantly, this difference is not explained by ligand binding data but by differential VDR protease sensitivity implying that V418L VDR mutation assumes a better conformational interaction surface for coactivator than V418A. Importantly, the V418 location may accommodate larger sidechains and may even enhance the interaction with specific nuclear coactivators.

Keywords

Vitamin D; VDR; Structure-Function; Transactivation; Mutagenesis

Introduction

The paradigm for ligands which act as selective modulators of nuclear receptor activation has been set by the estrogen receptor (ER) example such as tamoxifen [1] and raloxifene [2]. For this ER model, partial agonists are capable of eliciting exciting new functionalities in collaboration with the nuclear receptor by allowing only a specific subset of interactions performed by the natural ligand [3,4]. Common to most of these estrogen receptor ligands with selective modalities referred to as SERMs (Selective Estrogen Response Modulators) is the presence of a bulky ligand which prevents the proper positioning of helix 12. This can result in receptor activation in only selected tissues. Already ligands with comparable effects are

Corresponding Author: Dr. Anthony W. Norman Department of Biochemistry University of California, Riverside Riverside, CA 92521
Tel: 951-827-4777 Fax: 951-827-4784 Anthony.Norman@ucr.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

beginning to be identified in the vitamin D nuclear receptor system. Ideally, these ligand analogs could have pharmacological benefits by mediating anti-proliferative and protective effects without acting in the classical target organs, such as intestine bone and kidney, responsible for calcium homeostasis which might produce hypercalcemia.

We set out to study the with respect to the effect of modifying ligand sidechain steric size by modifying the VDR with conservative mutations at the ligand binding surface. By substituting the amino acid we can localize the steric bias and predict how increased or decreased electron density at each location might alter the positioning of helix 12. Using the molecular model derived from the x-ray structure of VDR [5], four amino acid residues were targeted for mutagenesis: leucine-404, leucine-414, valine-418 and phenylalanine-422. These non-polar residues along with histidines 305 and 397 are positioned surrounding the end of carbons 25 – 27 of the $1\alpha,25(\text{OH})_2\text{D}_3$ sidechain in the molecular model (see Figure 1A) and are termed the “hydrophobic crown”. Mutation of these 4 residues to the nearest larger and smaller size hydrophobic amino acid was expected to allow the mutant VDR to retain most transactivational activity because of the predominance of Van der Waals stabilization forces in the region (see Figure 1B). These mutations could then cause small modifications to the position of helix 12 relative to the binding site of nuclear coactivator proteins (see Figure 1C) when bound by ligand which might elicit selective receptor modalities. These mutations could be used to probe the structure-function relationships of unique ligands that have bulky sidechains.

Materials and Methods

Methods for plasmid construction, site-directed mutagenesis, culture of cell lines, transient transfection for protein expression, equilibrium binding assays, and transcriptional activation assays are as previously described in Bula et al., 2005 [6]. Briefly, mutant plasmids were made from the pcDNA3.1(-)VDRwt plasmid using the QuikChange[®] site directed mutagenesis kit (Stratagene, Palo Alto, CA) and then sequenced.

The plasmids were transfected into CV-1 cells used for transactivation assays using a 10 minute pre-treatment with 0.2 mg/ml DEAE-dextran in PBS followed by PBS wash and a 30 min incubation with PBS containing 0.1 $\mu\text{g}/\text{well}$ VDR mutant plasmid and 0.5 $\mu\text{g}/\text{well}$ reporter OCpSEAP2 [7]. After addition of 5% charcoal stripped fetal bovine serum for 4 hours, medium was replaced. At 28 hours, cells were dosed in a final ethanol concentration of 0.1% and secreted alkaline phosphatase was measured at 52 hours using the Phospha-Light[™] SEAP assay (Tropix, Bedford, MA). Experiments were carried out in quadruplicate with the data expressed as the mean $\pm$ SEM and fit by non-linear regression analysis to the sigmoidal dose-response equation using GraphPad Prism[®] (GraphPad Software, San Diego, CA).

For overexpression of VDR plasmid for binding assays, Cos-1 cells were transfected as for transactivation with 1mg/ml DEAE-dextran into 150mm culture plates for a duration of 72 hours. Cell lysate was harvested by scraping in hypotonic buffer, TED (10 mM Tris-Cl, 1 mM EDTA, 1 mM Dithiothreitol; pH 7.4) with Complete EDTA-Free Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). Samples were assayed in triplicate with additional duplicate samples containing more than a 100-fold excess nonradioactive $1\alpha,25(\text{OH})_2\text{D}_3$ to determine non-specific binding. Five sets of triplicate samples containing [³H]- $1\alpha,25(\text{OH})_2\text{D}_3$ in 10 μl ethanol were incubated with 0.185 ml cell lysate from Cos-1 cells overexpressing transfected VDR or VDR mutant proteins for 4 hours at 0°C. After washing, bound $1\alpha,25(\text{OH})_2\text{D}_3$ was eluted with 1 ml ethanol and measured for radioactivity. The dissociation constant was determined by non-linear regression with a one-site binding curve equation (GraphPad Prism Software, San Diego, CA).

The sensitivity of ligand-bound VDR receptor or mutants to limited proteolysis was determined using our protease sensitivity assay [7]. [^{35}S]-labeled VDR or mutants were incubated for 20 min at room temperature with ligand (10 μM), followed by treatment with 15 μg trypsin/ml (Sigma, St Louis MO) for 20 min at room temperature. Samples were run on 12% SDS-PAGE. The autoradiography 34 kDa band density was plotted versus log ligand concentration to determine the EC_{50} . This value is a semi-quantitative measure of binding, stability and conformation of ligand in the VDR or VDR mutants that remains relatively constant between experiments.

Results

Using site-directed mutagenesis, leucine-404 and 414 were mutated to both valine and phenylalanine, valine-418 was mutated to alanine or leucine, and phenylalanine-422 was mutated to valine or tryptophan. Some of these mutants were later combined to produce double mutants thus creating a family of “hydrophobic crown” VDR mutants.

The predominant method for analysis of the mutants was a transient transfection reporter assay system for secreted alkaline phosphatase driven by an osteocalcin vitamin D receptor element (ocVDRE-SEAP) which is located 233 basepairs upstream of the transcription start site. Data are presented as dose response curves with relative light units (RLU's) measured from the reporter over the interval of 10^{-11} to 10^{-6} molar $1\alpha,25(\text{OH})_2\text{D}_3$ (see Figure 2). Data points are the average of quadruplicate experiments $\pm$ SEM. Transcriptional effectiveness is measured by inflection point of the sigmoidal dose response curve which is termed the EC_{50} or point at which the dose is 50% effective. This value provides a most reproducible measure of the dose response curve combining both ligand binding and ligand efficacy.

Transactivation data from VDR constructs with mutations that include V418 are shown compared to VDRwt in Figure 2. Intriguingly, V418L transactivates very well whereas the transactivational EC_{50} of V418A is increased 53-fold (see Table 1). The addition of the V418L to the VDR-L404F mutation decreases the EC_{50} by nearly half (6.5 nM versus 11 nM [L404F]) improving transactivation but unexpectedly reducing the transactivation of VDR-L404V to less than one-tenth (EC_{50} of 390 nM [L404V-V418L] versus 37 nM [L404V]).

The EC_{50} values for $1\alpha,25(\text{OH})_2\text{D}_3$ in numerous experiments were averaged and the activation was normalized relative to the $1\alpha,25(\text{OH})_2\text{D}_3$ control run in each experiment. The data is graphed as a scatter plot with error bars indicating SEM for each mutant as labeled (see Figure 3). The combination of EC_{50} values (x-axis) and percent wildtype activation values (y-axis) shows clear differentiation of the mutants into 4 groups in the order of decreasing transactivational activity: **(A)** V418L, a group by itself with the ability to transactivate equally well as VDRwt, **(B)** the hydrophobic crown mutations having larger amino acids sidechains added to the ligand binding pocket (L404F, L414F and F422W), **(C)** the hydrophobic crown mutations having smaller amino acid sidechains substituted in the ligand binding pocket (L404V, L414V, V418A and F422V), and **(D)** a group of 2 double mutants with the highest transactivation EC_{50} belonging to a mutant with a combination of the best tolerated mutant combined with the best tolerated mutant containing a smaller sidechain amino acid. Perhaps, adding too much electron density in VDR-L404F/L414F at the same location has occluded entry of the ligand to the LBD. This would explain why VDR-L404F/L414F is not part of the better transactivation group despite the larger amino acid substitutions.

Transcriptional activation measures a combination of both ligand binding to the VDR and efficacy of the ligand-receptor complex to create a productive transcriptional complex. To differentiate which of these two processes is responsible for the resulting transactivational response observed, we have used equilibrium ligand binding assays to ascertain the affinity of

1 α ,25(OH) $_2$ D $_3$ in select mutants. This assay is performed using lysates from Cos-1 cells transiently transfected with the VDR construct of interest (see figure 12). The K $_D$ values of both the V418L and V418A mutants are very high, 4.0 and 6.7 nM, respectively, in relation to the 0.51 nM K $_D$ of VDRwt (see Figure 4 and Table 1). The poor affinity of the 1 α ,25(OH) $_2$ D $_3$ ligand for V418A could be responsible for the reduced transactivation. Surprisingly, the K $_D$ of the V418L mutant is also very poor suggesting the very good transactivation must be due to an improvement in transactivational efficacy rather than ligand binding affinity.

Equilibrium dissociation constant data, K $_D$, is provided for 7 of the 13 hydrophobic crown targeted mutants in Table 1. Despite the dissociation constant showing very poor affinity for some mutants, there is little correlation to transactivational activity. For example, the highest dissociation constant of all of the hydrophobic crown mutants, 9.9 nM for L404F/V418L, is paired with the mutant with transactivational activity second only to V418L. Similarly, the second best dissociation constant is paired with the worst transactivation of all with an EC $_{50}$ of 390 nM for L404V/V418L. The transactivational activity of V418 mutants uncharacteristically do not correlate with the protease sensitivity assay either. Because the V418 mutants are so unusual, it is possible that the subtle change engineered at V418 might significantly modify the protein interaction surface by the repositioning of helix 12.

The protease sensitivity assay (PSA) measures the ability of a given ligand to form the VDR into a rigid molecule tightly formed around the ligand and eliminating availability of positively charged lysine and arginine residues to trypsin digestion. While the presence of the 34 kDa band requires the cohesion of helix 12 to the VDR to prevent scission by trypsin, unlike transactivation however it does not require that the activation helix 2 be presented in a proper conformation to nuclear coregulator proteins. Differences in the EC $_{50}$ values between the PSA and transactivation values may highlight changes in nuclear coregulator recognition for specific mutants which might imply the sensitivity of that location to steric conflict. Comparison of the PSA and transactivation values of select hydrophobic crown mutants in Table 1 shows the largest differences occurs for F404F which has a 5-fold increase in transactivation versus PSA EC $_{50}$ and V418A with a 3-fold increase. L404F is able to coalesce the VDR around the ligand as well as VDRwt but has a modestly affected transactivation ability possible suggesting increased corepressor binding or decreased coactivator binding. V418A does not bind ligand or induce the holo conformation well and is possible further crippled with its ability to recruit coactivator. In contrast, V418L has a better than 2-fold transactivation than PSA EC $_{50}$ value. This might suggest that there is a shift from corepressor toward coactivator binding for this mutant.

Discussion

The residues of the hydrophobic crown are very important in the VDR because they, along with tyrosine-401, form the entire interface of helix 12 with the ligand [5]. With the exception of histidine-305, all of the residues in contact with carbon-25 of the ligand sidechain are part of helix 12. These properties would suggest that the positioning of helix 12 would be most sensitive to differences in structure of the ligand sidechain terminus. With the exception of V418A [8] all of the hydrophobic crown mutants are novel. Since mutation of these residues was meant to emulate the addition of ligand density at a given position, these have the benefit of being more easily modeled since their position is stationary relative to ligand motion. It was our hope that unique properties attributable to any of these mutants could direct the design of novel ligands which might be able to interact with the receptor in a similar manner and manifest the same properties.

Mutants of the hydrophobic crown that add electron density rather than remove it were shown to be better tolerated; thus, V418L, L404F, L414F and F422W transactivated better than

L404V, L414V, V418A and F422V. These mutant amino acid substitutions may be so large that they occlude the ligand binding site, as in VDR-L404F/L414F. Van der Waals forces are very important in recognizing ligand with a much smaller margin for error than for the hydrogen bonding interactions. Van der Waals forces are effective only over a small range between the repulsive force and the attractive exchange force that varies with distance between the atomic radii times an exponent, r^6 . For that reason, a change in distance of up to an angstrom as made by these mutants cannot be tolerated; however, subtle movements in any of the large number of residues in the protein could make up for this distance in either direction. This would of course depend on the rigidity of the position in the amino acid at the site in question.

The rigidity at a particular amino acid can be theoretically approximated by the average atomic B-factor of an amino acid in the crystallographic structure. The B-factor or “Debye-Waller factor” is defined as the degree to which the electron density is “spread out” indicating the true static or dynamic mobility of an atom or group of atoms. The average atomic B-factor for the amino acids that make up the hydrophobic crown are moderate-to-large indicating that they should be fairly capable of moving to accommodate the small changes in electron density inside the hydrophobic pocket. The observation that substitution of larger amino acids results in greater accommodation by the VDR of the ligand indicates that the hydrophobic pocket is more easily enlarged rather than shrunk to fill in empty space. Decreases in electron density following the substitution of a smaller amino acid would result in loss of Van der Waals forces and decreased stabilization. Also, if the ligand binding domain were to compress around this space it may resemble the “compressed pocket” inactive state of the RAR receptor [9] and be less capable of transactivation.

The most obvious example of the paradigm that more electron density is better tolerated can be obtained from evaluating the results for residue valine-418. When valine-418 was changed to the modestly larger leucine (V418L), transactivation was identical to that of the wildtype VDR. When changed to the modestly smaller alanine, however, the transactivation was severely affected by 53-fold increase in EC_{50} . Therefore, a significant function must be assigned to the contact of the ligand to V418. Interestingly, the transactivation of the V418L mutant is unchanged despite a very large 8-fold increase in the dissociation binding constant, K_D .

Movement of the helix 12 so as to generate the “closed pocket” conformation necessary to allow transactivation should greatly improve the dissociation constant. Still the ligand binding assay is only concerned with the stable association of ligand without regard to knowledge of what is the orientation of helix 12 (open, partially closed or closed) or transactivation complex stability. Ultimately, the position of this residue would indicate an important function in stabilizing the closed conformation as well through contact with carbon-27 of $1\alpha,25(OH)_2D_3$. Without this strong interaction, the increased freedom might allow the sidechain to spin eliminating stability attributable by hydrophobic interactions.

The other hydrophobic crown mutations are not as well tolerated as V418L with modestly larger residues despite each having a lower K_D (see Table 1). Considering the drastic change in size of the phenylalanine-422 to tryptophan and leucine-404 to phenylalanine, these mutations are well tolerated. At an increase of EC_{50} of only 10-fold, an entire pyrrole ring has been added to phenylalanine and 3 more carbons with π -bonds have been added to leucine to form the phenyl group for phenylalanine. Though the phenylalanine to tryptophan mutation is the largest increase of electron density of all the mutants, it still maintains the essential conjugated phenyl group to interact with the other three conjugated amino acids H305, H397 and Y401. The phenyl groups added at positions 404 and 414 might also be better tolerated because they extend the conjugated π -system.

Double mutants of the hydrophobic crown amino acids do not transactivate well with the exception of L404F/V418L. This is an improvement over L404F alone but is less active than V418L (see Figure 4 and Table 1). It is possible that the distance of these two mutations from one another makes them better tolerated or that they are relatively symmetrical with respect to ligand thus preventing it from being skewed to the side. Unexpectedly, this mutant has the highest dissociation constant of all tested (9.9 nM, almost 20-fold higher than VDRwt) despite a less than 5-fold increase in transactivational EC₅₀ (6.5 nM versus 1.4 nM for VDRwt).

Smaller mutations of the hydrophobic crown were the more affected VDR mutants having EC₅₀ values of 20 to 57 fold higher than VDRwt. The receptor would appear then to be better capable of absorbing small changes through the structure than stretching to accommodate missing electron density. Without this strong interaction, the increased freedom of movement in the ligand might allow the sidechain to rotate eliminating the close association of non-polar electron orbitals necessary to benefit from van der Waals interactions over the entire region. This would be very destabilizing and diminish the interaction between ligand and helix 12.

Previous studies have shown that the point mutation of F422A decreased the functional affinity with 1 α ,25(OH)₂D₃ to less than one percent of the wildtype using the protease sensitivity assay. V418A was also shown to have a similar decreased affinity [8,10] and again for F422A in the E421M/F422A double mutant [7]. Protease sensitivity EC₅₀ values generally agree with the transactivation values for the hydrophobic crown mutants except for two of all mutants tested, L404F and V418A. The transactivational EC₅₀ for L404F is 5-fold higher than its protease sensitivity EC₅₀ despite having a protease sensitivity assay EC₅₀ significantly lower than VDRwt. The same is true for V418A that has an 80% reduced EC₅₀ for protease sensitivity relative to transactivational EC₅₀ though, unlike the previous case, this EC₅₀ is 5-fold higher (less potent) than VDRwt. This might suggest that the coactivator binding surface has been affected to change the overall displacement of helix 12. This is possibly the result of slightly skewing the helix 12 to one side or the other due to the position of both of L404 and V418 against either of the two terminal methyl groups on the sidechain.

In summary, we have shown that mutations of the four amino acids comprising the hydrophobic crown can affect ligand binding and transactivation efficiency by altering the interaction of VDR with its natural ligand. A corollary to this observation is that it seems likely that analogs having the same steric increase in size are also capable of the highly effective transactivation shown by mutant V418L. A large enough analog may even protrude into the protein-protein interface and selectively obscure binding of specific nuclear coregulators.

Acknowledgements

The work described in this manuscript was supported by NIH grant Dk-09012-30 (AWN).

References

1. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL. Cell 1998;95(7):927–937. [PubMed: 9875847]
2. Nichols M, Rientjes JM, Stewart AF. EMBO J 1998;17(3):765–773. [PubMed: 9451001]
3. Shang Y, Brown M. Science 2002;295(5564):2465–2468. [PubMed: 11923541]
4. Katzenellenbogen BS, Katzenellenbogen JA. Defining the “S” in SERMs. Science 2002;295:2380–2381. [PubMed: 11923515]
5. Rochel N, Wurtz JM, Mitschler A, Klaholz B, Moras D. Mol. Cell 2000;5(1):173–179. [PubMed: 10678179]
6. Bula CM, Huhtakangas JA, Olivera CJ, Bishop JE, Norman AW, Henry HL. Endocrinology 2005;146(12):581–586.

7. Liu YY, Collins ED, Norman AW, Peleg S. J. Biol. Chem 1997;272(6):3336–3345. [PubMed: 9013574]
8. Nayeri S, Kahlen JP, Carlberg C. Nucleic Acids Res 1996;24(22):4513–4518. [PubMed: 8948643]
9. Renaud JP, Rochel N, Ruff M, Vivat V, Chambon P, Gronemeyer H, Moras D. Nature 1995;378(6558): 681–689. [PubMed: 7501014]
10. Nayeri S, Carlberg C. Biochemical Journal 1997;327(2):561–568. [PubMed: 9359430]

Figure 1.

The “hydrophobic crown” of 1α,25(OH)₂D₃ and helix 12 of the VDR. (A) Six amino acids of the VDR, Leu404, Leu414, Val418, Phe 422, His397 and His305, form the “hydrophobic crown” of VDR. These amino acids are shown rendered in space-filling mode surrounding 4 atoms of 1α,25(OH)₂D₃ indicating the position of carbon-25 (central), carbon-26 (left), carbon-27 (right) and the 25-hydroxyl in red. Coordinates were derived from the x-ray crystal structure protein database identification number 1DB1, *Rochel et al., (2000)*. (B) Amino acids which participate in the many hydrophobic interactions which stabilize helix 12 (violet) are rendered in space-filling mode with colors to represent the helix of origin (helix 3: red-orange, helix 4-5: orange, helix 11: purple; see figure 3). Non-hydrophobic amino acids are indicated in wireframe mode. (C) Amino acids that stabilize the position of helix 12 in 1α,25(OH)₂D₃-bound VDR are colored in Corey-Pauling-Koltun (CPK) color scheme and labeled in white. Broken white lines outline the hydrophobic cleft that is known to recruit co-activator proteins as described in *Darimont et al., 1998*. The largely hydrophobic nature of the region and presence of only 3 intra-helix electrostatic interactions (Glu420-Lys264, Arg154-Leu414, and Arg154-Met412) suggest that conservative mutations of the “hydrophobic crown” amino acids should be non-disruptive.

Figure 2.

Transactivational activity of 1α,25(OH)2D3 in VDR-418 mutants compared to VDRwt.

CV1 cells were co-transfected with the osteocalcin VDRE driven SEAP reporter and plasmid VDR or VDR mutants. A representative graph of data is plotted as relative light units versus log dose of the ligand 1α,25(OH)2D3. Data points are averaged from quadruplicate samples and shown for either VDRwt (▲), V418L (◆), L404F-V418L (●), V418A (□) and L404V-V418L (○). Error bars indicate standard error. Non-linear regression analysis with the sigmoidal dose equation was performed to yield dose response curves shown in the same color as the data points. Table 1 presents a summary of the transactivation EC50 concentration where EC50 is the inflection point of the transactivation sigmoidal dose response.

Figure 3.

For the VDR hydrophobic crown mutants, larger volume amino acid substitutions are generally more transactivationally active than smaller amino acid substitutions. CV1 cells were transfected with the hydrophobic cleft site-directed VDR mutants or VDR wildtype to generate dose response curves with $1\alpha,25(\text{OH})_2\text{D}_3$ from triplicate samples. The data was transformed to show a single point at the computed EC₅₀ on the X-axis with the Y-plane indicating percent activity relative to $1\alpha,25(\text{OH})_2\text{D}_3$ activation of VDRwt. Error bars indicate standard error from 3 replicates. Each point is annotated with the name of the mutant. A light grey circle circumscribed around the data point indicates that the amino acid is larger in volume than the original. A half circle indicates that a double mutant has both a larger and smaller substituted amino acid. EC₅₀ values are tabulated in Table 1. Dotted boxes or arrows differentiate the mutants into four groups based on transactivation values.

Figure 4.

The equilibrium dissociation binding constants of VDR-V418L and VDR V418A are similar despite extremely different transactivation EC_{50} values for $1\alpha,25(\text{OH})_2\text{D}_3$. Cos-1 cells were transfected with either VDR-V418L or VDR-V418A site-directed mutants. Cells were harvested and aliquots of suspension were bound to the indicated concentrations of $[\text{H}^3]\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ or the same volume of vehicle ethanol for 4 hours at 0°C . Samples were then bound to hydroxyapatite and washed 3 times with TED/Triton-X100 to remove unbound ligand. Specific binding of 98 Ci/mmol $[\text{H}^3]\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ was determined by liquid scintillation spectrometry and disintegrations per minute (DPM) were transformed to concentration bound (nM). (A) Data points for V418L ($\diamond$) and V418A ($\square$), as shown, are averaged for 3 points minus 2 points with excess nonradioactive $1\alpha,25(\text{OH})_2\text{D}_3$. The one-site hyperbola curve was fit to the data by non-linear regression analysis with of VDR-V418L is shown as a solid blue line and V418A as a solid red line. (B) The same data is shown normalized and plotted as percent maximum $1\alpha,25(\text{OH})_2\text{D}_3$ bound relative to concentration added. Maximum specific activity before normalization of bound ligand equaled 33000 and 3500 dpm for V418L and V418A, respectively.

Table 1

Summary of dissociation constants, protease sensitivity EC₅₀ and transactivation EC₅₀ for VDRwt and VDR “hydrophobic crown” mutants. Data obtained from both equilibrium binding assays and protease sensitivity assays for VDRwt and the hydrophobic crown mutants are shown. The table is grouped relative to the number of mutations and the increase or decrease in electron density inside the ligand binding pocket. Dissociation constants (K_D), protease sensitivity assay EC₅₀s, and transactivation EC₅₀ values for 1 α ,25(OH)₂D₃ in these mutants are tabulated with the value $\pm$ SEM and the number of replicates (n). Values in boldface type are significantly different than VDRwt in their category with $p < 0.01$.

VDR Construct	Ligand-Binding K _D (nM)	1 α ,25(OH) ₂ D ₃ Protease Sensitivity EC ₅₀ (nM)	1 α ,25(OH) ₂ D ₃ Transactivation EC ₅₀ (nM)
VDRwt	0.51 $\pm$ 0.08 (n=4)	2.6 $\pm$ 0.4 (n=9)	1.4 $\pm$ 0.2 (n=20)
1-Mutation			
Increase			
L404F	1.6 $\pm$ 1.1 (n=2)	2.2 $\pm$ 0.3 (n=7)	11 $\pm$ 6 (n=3)
L414F	3.6 (n=1)	14 $\pm$ 1 (n=4)	12 $\pm$ 3 (n=3)
V418L	4.0 (n=1)	4.3 $\pm$ 1.6 (n=4)	1.8 $\pm$ 0.8 (n=3)
F422W	2.5 $\pm$ 1.2 (n=2)		16 $\pm$ 3 (n=3)
Decrease			
L404V		25 $\pm$ 12 (n=2)	37 $\pm$ 2 (n=3)
L414V		15 $\pm$ 3 (n=4)	28 $\pm$ 4 (n=3)
V418A	6.7 (n=1)	15 $\pm$ 3 (n=4)	74 $\pm$ 24 (n=3)
F422V			80 $\pm$ 36 (n=3)
2-Mutations			
Decrease			
L404V/L414V			69 $\pm$ 17 (n=2)
Mixed			
L404F/L414V			110 $\pm$ 45 (n=3)
L404V/V418L	2.2 $\pm$ 1.1 (n=2)		390 $\pm$ 150 (n=3)
Increase			
L404F/V418L	9.9 (n=1)		6.5 $\pm$ 1.5 (n=3)
L404F/L414F			93 $\pm$ 55 (n=3)