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Correlation between secosteroid induced vitamin D receptor activity in melanoma cells and computer-modeled receptor binding strength

Tae-Kang Kim^{a,†}, Jin Wang^{b,†}, Zorica Janjetovic^a, Jianjun Chen^b, Robert C. Tuckey^c, Minh N. Nguyen^c, Edith K. Y. Tang^c, Duane Miller^b, Wei Li^{b,*}, and Andrzej T. Slominski^{a,d,*}

^aDepartments of Pathology and Laboratory Medicine, Center for Cancer Research, University of Tennessee Health Science Center, Memphis, TN, USA

^bPharmaceutical Sciences, University of Tennessee Health Science Center, Memphis, TN, USA

^cSchool of Biomolecular, Biomedical and Chemical Sciences, The University of Western Australia, Crawley, WA, Australia

^dDivision of Dermatology, Department of Medicine, University of Tennessee Health Science Center, Memphis, TN, USA

Abstract

To define the interaction of novel secosteroids produced by the action of cytochrome P450_{scc} with vitamin D receptor (VDR), we used a human melanoma line overexpressing VDR fused with enhanced green fluorescent protein (EGFP) and tested the ligand induced translocation of VDR from the cytoplasm to the nucleus. Hydroxyderivatives of vitamin D₃ with a full length (D₃) side chain and hydroxy-secosteroids with a shortened side chain (pD) stimulated VDR translocation and inhibited proliferation, however, with different potencies. In general the D₃ were more potent than pD analogues. Molecular modeling of the binding of the secosteroids to the VDR genomic binding pocket (G-pocket) correlated well with the experimental data for VDR translocation. In contrast, docking scores for the non-genomic binding site of the VDR were poor. In conclusion, both the length of the side chain and the number and position of hydroxyl groups affect the activation of VDR by novel secosteroids.

Keywords

secosteroid; melanoma; vitamin D receptor; computer modeling

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*Corresponding author and address for reprints: Andrzej Slominski, MD/PhD, Department of Pathology, 930 Madison Avenue, Memphis, TN 38163; Tel: 901-4483741; Fax: 901-4486979; aslominski@uthsc.edu; or Wei Li PhD, Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, 847 Monroe Avenue, room 327, Memphis, TN 38163; Tel: 901-448-7532; Fax: 901-448-6828; wli@uthsc.edu..

[†]Equal first authors

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1. Introduction

Cytochrome P450scc is well known for catalyzing the side chain cleavage reaction where cholesterol is converted to pregnenolone in the first enzymatic step of steroid biosynthesis (Tuckey, 2005). Recently, it has been demonstrated that cytochrome P450scc hydroxylates vitamin D₃ to produce 20(OH)D₃ with subsequent hydroxylations producing 20,23(OH)₂D₃, 20,22(OH)₂D₃, 17,20(OH)₂D₃ and 17,20,23(OH)₃D₃ (Guryev et al., 2003, Slominski et al., 2005, Tuckey et al., 2008b). 1(OH)D₃ can also be converted to 1,20(OH)₂D₃ by this enzyme and subsequently to 1,20,23(OH)₃D₃ (Tuckey et al., 2008a). 20(OH)D₃, 20(OH)D₂ and 20,23(OH)₂D₃ display potent anti-proliferative activity comparable to that for 1,25(OH)₂D₃, the hormonally active form of vitamin D₃ (Janjetovic et al., 2011, Janjetovic et al., 2010, Janjetovic et al., 2009, Slominski et al., 2011a, Zbytek et al., 2008). Importantly, these compounds have markedly lower calcemic activity than 1,25(OH)₂D₃ indicating that they have potential to serve as therapeutic agents (Slominski et al., 2010, Slominski et al., 2011a). We have previously shown that P450scc-derived 20(OH)D₃, 1,20(OH)₂D₃ and 20,23(OH)₂D₃, as well as pD and 20(OH)pD which are UVB-induced transformation products from 7DHP and 20(OH)-7DHP, respectively, have antileukemia activity, while displaying lack or low calcemic activity (Slominski et al., 2010). In addition, the pD compounds also showed antiproliferative activity against melanoma and keratinocytes (Slominski et al., 2009, Zmijewski et al., 2011, Zmijewski et al., 2009) and showed anti-fibrotic activity (Slominski et al., 2011b).

The vitamin D receptor (VDR) is a transcriptional factor with binding of 1,25(OH)₂D₃ to the G-pocket causing the regulation of target genes. Once 1,25(OH)₂D₃ binds to the G-pocket in the VDR, the complex dimerizes with the RXR receptor followed by its translocation to the nucleus (Carlberg, 2003, Nagpal et al., 2005, Reichrath, 2007, Vaisanen et al., 2005, Wang et al., 2005). We have shown that the anti-proliferative and anti-inflammatory effects of 20(OH)D₃, 20(OH)D₂ and 20,23(OH)₂D₃ in human melanoma and keratinocytes are mediated through the VDR (Janjetovic et al., 2010, Slominski et al., 2011a, Zbytek et al., 2008). In order to study the relative potency of hydroxyvitamin D₃ derivatives towards the VDR, we transduced SKMEL-188 melanoma cells with the pLenti-CMV-VDR-EGFP-pgk-puro plasmid, which then stably produced VDREGFP fusion protein (Slominski et al., 2011a). Using this cell line we have evaluated the effect of hydroxyvitamin D₃ and hydroxy-pD derivatives on the VDR translocation from the cytoplasm to the nucleus. We correlated this translocation efficiency with the ability of the secosteroids to inhibit proliferation, and with their estimated binding affinities to the G-pocket of the VDR determined by molecular modeling studies. Since 1,25(OH)₂D₃ can also bind to the membrane VDR in the alternative A-pocket to produce rapid, non-genomic responses, we also included in our analysis the ability of the secosteroids to bind to this site (Boland, 2011, Haussler et al., 2011, Mizwicki et al., 2004) to gain insights into the mechanism of action for these novel compounds.

2. Materials and Methods

2.1. Source of Secosteroids

1,25(OH)₂D₃ was from Sigma (St. Louis, MO). 20(OH)D₃, 22(OH)D₃, 20,22(OH)₂D₃, 20,23(OH)₂D₃ and 17,20,23(OH)₃D₃ were made by the action of P450scc on vitamin D₃ (Tuckey et al., 2011). 1,20(OH)₂D₃ was made by the action of P450scc on 1(OH)D₃ (Tuckey et al., 2008a), while 1,20,23(OH)₃D₃ was made by the action of CYP27B1 on 20,23(OH)₂D₃ (Tang et al., 2010). pD derivatives were synthesized as previously described (Zmijewski et al., 2011, Zmijewski et al., 2008, Zmijewski et al., 2009). The chemical structure of these secosteroids is shown in figure 1.

2.2. VDR translocation

The effects of hydroxy-secosteroids on VDR translocation were tested using SKMEL-188 cells stably transduced with pLenti-CMV-VDR-EGFP-pgk-puro (VDR and EGFP expressed as fusion protein) (Slominski et al., 2011a). Cells were treated with 10^{-10} to 10^{-7} M secosteroids overnight for 16 hours followed by fixing with 4% PFA (paraformaldehyde). Fixed cells were mounted with fluorescent mounting media (Dako, Denmark) and analyzed with a fluorescent microscope. Translocation to the nucleus was determined by counting cells with a fluorescent nucleus and results are presented as the percentage of the total cells that display nuclear staining as described previously (Slominski et al., 2011a). The data are from at least from 2 separate experiments with pictures were taken randomly from at least 6 different fields and counted as described (Slominski et al., 2011a).

2.3. Anti-proliferative activity

MTS/PMS solution (Promega, Madison, WI) was used to test the effect of secosteroids on cell proliferation (Janjetovic et al., 2010, Li et al., 2010). Briefly, normal human epidermal keratinocytes (5×10^3 cells) were plated into 96 well plates with 100 μ l KGM medium plus KGF per well. After overnight incubation at 37°C with 5% CO₂, media were replaced with fresh media containing the secosteroid (10^{-6} M) and further incubated for 24 h. Media were removed and cells treated with MTS reagent for 4 h then absorbance, indicative of viable cells, was measured at 490 nm.

The proliferation of immortalized human HaCaT keratinocytes was measured from ³H-thymidine incorporation into DNA or by performing MTS assays as described previously, with some modifications (Janjetovic et al., 2011, Janjetovic et al., 2010). Briefly, cells were inoculated into 24-well plates at 5,000 cells/well. After overnight incubation at 37°C, the cells were placed in serum free media to synchronize them at the G0/G1 phase of the cell cycle. After 24 h vitamin D3 derivatives were in fresh media containing growth supplements and incubated for an additional 24 or 48 h. [³H]-Thymidine (specific activity 88.0 Ci/mmol; Amersham Biosciences, Piscataway, NY, USA) was added to a final concentration of 0.5 μ Ci/mL in medium. After 4 h at 37°C, media were discarded, cells precipitated in 10% TCA in PBS (phosphate-buffered saline) for 30 min, washed twice with 1 mL PBS and then incubated with 1 N NaOH/ 1% SDS (250 μ L/well) for 30 min at 37°C. The extracts were collected in scintillation vials and 5 mL of scintillation cocktail was added to each. ³H-radioactivity incorporated into DNA was measured with a beta counter (Direct Beta-Counter Matrix 9600; Packard). For MTS assays, HaCaT keratinocytes were plated in 96-well plates, 5,000 cells/ well. After overnight incubation of cells, 17,20(OH)₂D₃ initially dissolved in ethanol and then diluted in DMEM media supplemented with charcoal treated FBS, was added to the medium, while an equivalent concentration of ethanol was added to the control. After incubation for 44 h, 20 ml of MTT solution (Promega, Madison, WI) was added to the cells. Four hours later, absorbance was recorded at 570 nm using an ELISA plate reader. The number of viable cells was measured in six replicates.

2.4. Ligand and VDR docking modeling

We selected the crystal structure of 1,25(OH)₂D₃ in complex with human VDR (PDB code: 1DB1), which has the highest resolution of 1.8 Å (Rochel et al., 2000) among available VDR complexes, for molecular modeling studies. 1,25(OH)₂D₃ binds to the G-pocket in this crystal structure. We also performed similar calculations using a crystal structure of Zebrafish VDR (PDB code: 2HCD, resolution 2.6 Å) (Ciesielski et al., 2007) to investigate whether there are significant differences between species. There are no crystal structures for 1,25(OH)₂D₃ interacting with the A-pocket in VDR; therefore, we followed the literature procedure in building the complex using non-genomic agonist 1,25(OH)₂-lumisterol₃ (JN) based on 1DB1 crystal structure (Mizwicki et al., 2004). Briefly, using the structure of

1DB1, JN was overlaid with 1,25(OH)₂D₃ in the G-pocket by superimposing the C1, C3, and C5 atoms of JN on the C3, C1, and C5 atoms of 1,25(OH)₂D₃, respectively. Subsequently 1,25(OH)₂D₃ was deleted and JN was merged with the rest of the atoms to form the starting complex. The energy of this complex was minimized to produce the model in which the ligand occupies the A-pocket in VDR (Mizwicki et al., 2004). We used Schrodinger Molecular Modeling Suite 2011 (Schrodinger LLC, New York, NY) for the docking practice. All secosteroids were built and prepared using the Ligprep module, and they were docked into the VDR ligand binding site using the Glide module in the software. The best docking complexes were subject to restricted molecular dynamics calculations to release any strains by using MacroModel module with OPLS-2005 force field. The ligand and its surrounding residues within 15 were allowed to move freely, while residues outside the 15-radius were kept rigid. The docking score (Glide score) was recorded and contributions from different interactions for each ligand-VDR complex were analyzed.

Statistical analysis

Data are presented as means ± SD or SEM, when indicated, and analyzed with Student's t-test (for 2 groups) and appropriate post-hoc test (for more than 2 groups) using Prism 4.00 (GraphPad Software, San Diego). Statistically significant differences are considered when $P < 0.05$.

3. Results

3.1. Anti-proliferative effects of secosteroids on human epidermal keratinocytes

Previously we have shown that P450scc-derived secosteroids inhibit cell proliferation and induce cell differentiation through activation of the VDR (Janjetovic et al., 2010, Slominski et al., 2011a, Zbytek et al., 2008). To investigate the relationship between the ability of various secosteroids to inhibit proliferation and their ability to stimulate VDR translocation to the nucleus, we determined the anti-proliferative effects of 20(OH)D₃, 20,23(OH)₂D₃, 1,20(OH)₂D₃, 17,20,23(OH)₃D₃, pD and 20(OH)pD on human epidermal keratinocytes. Figure 2 shows that all these secosteroids display anti-proliferative activity. While there was no significant difference between the activities of 20(OH)D₃, 20,23(OH)₂D₃, 1,20(OH)₂D₃ and 17,20,23(OH)₃D₃ to that of 1,25(OH)₂D₃, (analogues with a fulllength side chain), pD analogues (pD and 20(OH)pD that have a short side chain) caused significantly lower inhibition than 1,25(OH)₂D₃.

These data are consistent with our previous studies documenting dose dependent effects of vitamin D hydroxyderivatives with a full side-chain including 20(OH)D and 20,23(OH)₃D₃, that showed a similar potency to 1,25(OH)₂D₃ (Janjetovic et al., 2011, Janjetovic et al., 2010, Slominski et al., 2010, Slominski et al., 2011a). This comparable potency for inhibition of cell proliferation is also shown in Figure 3, where 20,23(OH)₂D₃, 1,20(OH)₂D₃ and 17,20,23(OH)₃D₃ show anti-proliferative activity towards HaCaT keratinocytes similar to, or better than 1,25(OH)₂D₃. Dose-dependent antiproliferative activity was also seen for 17,20(OH)₂D₃ measured separately using the MTS assay.

3.2. The effect of secosteroids on VDR traslocation

In order to study the effect of hydroxy-secosteroids on VDR translocation, SKMEL-188 cells transduced with pLenti-CMV-VDR-EGFP-pgk-puro were treated with 10⁻¹⁰ to 10⁻⁷ M secosteroid, and the percentage of cells with a fluorescent nucleus was determined. Figure 4 shows that of the P450sccderived secosteroids tested that have a full length side-chain containing at least one hydroxyl group, 1,20(OH)₂D₃, 20,23(OH)₂D₃, 1,20,23(OH)₃D₃ and 20(OH)D₃ are the most potent with EC₅₀ values similar to that for 1,25(OH)₂D₃. 17,20,23(OH)₃D₃, 20,22(OH)₂D₃ and 22(OH)D₃ displayed higher EC₅₀ values than that for

1,25(OH)₂D₃ and could not be accurately determined because the translocation process was not saturated at the highest concentration of secosteroid used. For the mono-, and di-hydroxy vitamin D₃ analogues where saturation was achieved, the maximum translocation percentages were comparable with that for 1,25(OH)₂D₃ (not statistically different by student t-test) with most of the variation arising from differences in the percentage of fluorescent nuclei seen under basal conditions (no secosteroid added) (Figure 4 and 5).

Secosteroids with a short side chain (pD derivatives) showed a lower and more variable level of VDR translocation to the nucleus than for 1,25(OH)₂D₃ (Figure 6). For this reason it was not possible to determine EC₅₀ values with these compounds. pD and 17(OH)pD stimulated VDR translocation at the lowest concentration tested (10⁻¹⁰ M) while the other derivatives with short side chains caused stimulation at higher concentrations (Figure 6). At the highest concentrations tested (10⁻⁷ M), hydroxy-pD derivatives showed a statistically significant (student t-test) decrease in the percentage of VDR translocated to the nucleus compared with 1,25(OH)₂D₃ (Figure 5). This pattern is consistent with our previous finding showing rather poor anti-leukemic effects of analogues with a short side chain, which contrasted to consistent and strong effects of compounds with a full side chain (Slominski et al., 2010). However, in dermal fibroblasts we found very good anti-fibrotic effect of 17,20(OH)₂pD at subnanomolar concentrations (Slominski et al., 2011b). This suggests cell-type specificity in phenotypic effects perhaps driven by VDR interaction with different co-factors. This challenging problem of differential activity of novel secosteroids with a shortened side-chain is a subject of our future investigations.

3.3. Molecular modeling studies of secosteroidal compounds and VDR

In order to gain an insight into the differential biological activities of the vitamin D₃ analogues used in this study and their molecular interactions with the VDR when binding to the G-pocket, we performed docking studies by computer modeling using the high resolution crystal structure of human VDR (Figure 7) in complex with their native ligand, 1,25(OH)₂D₃. In this analysis, vitamin D₃ analogues having both a 1-hydroxyl group and a hydroxyl group on the side chain showed the best docking score for binding to the VDR. In addition, vitamin D₃ metabolites having a full-length side chain (D₃ analogs) showed theoretically better affinity for the VDR than those having shortened chains (pD analogs).

Figure 8 shows that there is a strong correlation between the EC₅₀ value for nuclear translocation and the score for docking to the G-pocket in the VDR (Spearman $r = 0.89$, $p = 0.0072$). Due to the difficulty in measuring the EC₅₀ values of secosteroids with a short side chain, largely due to their lower potencies and limited availability of these compounds, we tested the correlation of the docking scores of all the secosteroids under study with the extent of translocation observed at a ligand concentration of 10⁻⁷ M. Figure 10 shows that the correlation between docking scores and nuclear translocation activity of all secosteroids is highly significant, with Pearson $r = 0.70$ and $P = 0.0082$. When the analysis was restricted to the vitamin D₃ analogues having a full length side chain, the significance of this correlation increased dramatically (Pearson $r = 0.93$ with $P = 0.0007$). Further analysis of the contributions from hydrogen bonding and hydrophobic interactions shows that the hydrogen bonding contribution to the docking score correlates significantly with nuclear translocation for all analyzed secosteroids (Pearson $r = 0.57$, $P = 0.047$). The significance of this correlation also increased when the analysis was restricted to derivatives with a full length side chain (Pearson $r = 0.94$, $P = 0.0007$). However, hydrophobic interactions were not significantly correlated with nuclear translocation (see lower panels of figure 8, $P > 0.05$). As shown in supplemental figure 1, the docking scores to zebrafish VDR also correlate well with biological activity with highly similar trend as seen in human VDR. This indicates high evolutionary conservation of such interactions among the vertebrates.

In contrast to the data for docking to the G-pocket which is responsible for genomic effects of the VDR, no correlation was found between nuclear translocation and the theoretical ability of the secosteroids to bind to the A-pocket in the VDR which is associated with rapid, non-genomic actions (Mizwicki et al., 2004) (Figure 10). The secosteroids 22(OH)D₃, pD, 20(OH)pD and 17(OH)2pD, showed docking scores for this site as low as that for 1,25(OH)₂D₃, but others with more bulky side chains docked poorly to this site. The docking score for 1,25(OH)₂lumisterol₃ (JN) a potent agonist for this site (Haussler et al., 2011, Mizwicki et al., 2004) was -9.41.

4. Discussion

Previous studies have shown that both P450scc-derived vitamin D derivatives with a full-length side chain and derivatives with a shortened side chain significantly inhibited proliferation of normal and malignant cells (Janjetovic et al., 2011, Janjetovic et al., 2010, Li et al., 2010, Slominski et al., 2010, Slominski et al., 2011a, Slominski et al., 2011b, Slominski et al., 2009, Tuckey et al., 2008a, Tuckey et al., 2011, Zbytek et al., 2008, Zmijewski et al., 2011, Zmijewski et al., 2009). Inhibition of proliferation is likely to be a genomic action of the ligand bound at the G-pocket in the VDR. To test the capacity of these secosteroids to initiate the genomic response, we examined their ability to stimulate the translocation of a fluorescently-labeled VDR construct from the cytoplasm of a stably transduced melanoma cell line, to the nucleus. Examination of the dose dependency showed that P450scc-derived vitamin D₃ derivatives, 1,20(OH)₂D₃, 20,23(OH)₂D₃, 1,20,23(OH)₃D₃ and 20(OH)D₃ displayed comparable potencies to that for 1,25(OH)₂D₃, while 17,20,23(OH)₃D₃, 20,22(OH)₂D₃ and 22(OH)D₃ had lower potencies. All of these secosteroids have a full length, 8-carbon side chain. In contrast, hydroxy-pD derivatives with a short side chain generally showed lower potencies than that for 1,25(OH)₂D₃, and more variable translocation to the nucleus making it difficult to accurately determine their EC₅₀ values.

For vitamin D₃ derivatives with a full-length side chain where EC₅₀ values could be calculated, there was a good correlation between the log of EC₅₀ values and their docking scores to the G-pocket in human VDR. Thus, the theoretical docking score provides a reasonable estimate of the potency of the different secosteroids with a full length side chain analyzed in this study, to stimulate VDR translocation to the nucleus. In the study of relationship of docking scores for G-pocket in human VDR and their experimental VDR activities, a significant correlation was seen between the extent of translocation observed at a ligand concentration of 10⁻⁷ M for the full set of secosteroids analyzed in this study and their calculated docking score. The hydrogen bond component of the docking score, but not the hydrophobic interaction component, also correlated significantly with the extent of nuclear translocation. A stronger correlation between the extent of nuclear translocation and the docking score was observed when the secosteroids with a short side chain were excluded from the analysis. Very similar relationship was observed when the analysis was performed using data obtained from molecular modeling of the Zebra fish VDR showing high correlation between docking score and their VDR activity having Pearson $r = -0.66$ and $P = 0.0141$. The hydrogen bond contribution is also well correlated with VDR activity in full and short side chained secosteroids having Pearson $r = -0.66$ and $P = 0.0147$, even though it is not statistically significant without short side chained (supplemental figure 1). This demonstrate high conservation of secosteroidal ligand and receptor interaction among distant vertebrate organisms. A close examination of the ligand binding domain in the VDR reveals that there is a large hydrophobic sub-pocket in the region where the secosteroid side chain binds (Figure 11A and 11B). While vitamin D₃ analogs with a full length side chain can fill this sub-pocket providing favorable hydrophobic interactions and potential hydrogen bonding to the hydroxyl groups on the side chain, the pD analogs with a shortened side chain leave this region empty (Figure 11B). This is likely to be the major reason why pD

analogs bind less efficiently to the G-pocket in the VDR in comparison to the D₃ analogs. Even though it is well known that the ligand binding domain of the VDR has some flexibility and can tolerate larger side chain D₃ analogs such as the GEMINI compounds (Ciesielski et al., 2007, Takaku et al., 2008), it is unlikely that this sub-pocket dramatically contracts to better interact with the shortened side chain in the pD analogs.

Recent evidence indicates that the VDR can mediate rapid, non-genomic responses, also dependent on ligand binding (Boland, 2011, Haussler et al., 2011, Mizwicki et al., 2004). In this case the ligand binds to a second site (A-pocket) that shows some overlap with the primary ligand binding site occupied by 1,25(OH)₂D₃ in the crystal structure of human VDR (Boland, 2011, Haussler et al., 2011, Mizwicki et al., 2004) (Figure 11C). This second site is also able to bind to 1,25(OH)₂D₃ but shows different specificity for ligands than the primary site. 1,25(OH)₂lumisterol₃ is a potent agonist for this site but shows no genomic activity. Examples of nongenomic physiological responses include the opening of Cl⁻ and Ca²⁺ channels in osteoblasts, regulation of lipid synthesis, modulating the activity of kinases and phosphatases, and the photoprotective effect of 1,25(OH)₂D₃ against UV damage (Dixon et al., 2011, Haussler et al., 2011, Mizwicki and Norman, 2009). No correlation was found between the docking scores to this A-pocket and nuclear translocation, as might be expected. When we examine the possible binding poses identified by molecular modeling to the A-pocket, there is no consistent binding alignment for either the full side chain or the short side chain secosteroids, as exemplified in Figure 11D. The secosteroids tested have much poorer docking scores to this A-pocket compared with their binding to the G-pocket. While we cannot rule out the possibility on an imperfect A-pocket model generated by molecular modeling, the standard nongenomic agonist JN showed a very good docking score in this model (-9.41). Therefore, the results from this modeling exercise are consistent with the P450scc-derived secosteroids producing their main biological effects by interacting with the G-pocket. Interestingly, the secosteroids 22(OH)D₃, pD, 20(OH)pD, 17(OH) pD dock to the A-pocket as well as 1,25(OH)₂D₃ suggesting they may act, at least in part, via the rapid response mechanism. Their docking scores are however notably lower than for the agonist, JN.

In conclusion we have shown that P450scc-derived vitamin D derivatives, and chemically synthesized vitamin D₃ analogues with a short side chain, can inhibit keratinocyte proliferation. Using stably transfected melanoma cells we have demonstrated that the ability of these secosteroids to stimulate translocation of the VDR from the cytoplasm to the nucleus is significantly correlated with their theoretical ability to bind to the G-pocket in VDR, with the strongest correlation being for derivatives with a full-length side chain. Lastly, we have shown that while some of the secosteroids tested can dock into the rapid-response A-pocket on the VDR as well as 1,25(OH)₂D₃, this binding does not correlate with nuclear translocation of the VDR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Boland RL. VDR activation of intracellular signaling pathways in skeletal muscle. *Mol Cell Endocrinol.* 2011; 347:11–6. [PubMed: 21664245]
- Carlberg C. Molecular basis of the selective activity of vitamin D analogues. *J Cell Biochem.* 2003; 88:274–81. [PubMed: 12520526]
- Ciesielski F, Rochel N, Moras D. Adaptability of the Vitamin D nuclear receptor to the synthetic ligand Gemini: remodelling the LBP with one side chain rotation. *J Steroid Biochem Mol Biol.* 2007; 103:235–42. [PubMed: 17218092]
- Dixon KM, Norman AW, Sequeira VB, Mohan R, Rybchyn MS, Reeve VE, Halliday GM, Mason RS. 1 α ,25(OH)-vitamin D and a nongenomic vitamin D analogue inhibit ultraviolet radiation-induced skin carcinogenesis. *Cancer Prev Res (Phila).* 2011; 4:1485–94. [PubMed: 21733837]
- Guryev O, Carvalho RA, Usanov S, Gilep A, Estabrook RW. A pathway for the metabolism of vitamin D3: unique hydroxylated metabolites formed during catalysis with cytochrome P450scc (CYP11A1). *Proc Natl Acad Sci U S A.* 2003; 100:14754–9. [PubMed: 14657394]
- Haussler MR, Jurutka PW, Mizwicki M, Norman AW. Vitamin D receptor (VDR)-mediated actions of 1 α ,25(OH)₂vitamin D3: genomic and non-genomic mechanisms. *Best Pract Res Clin Endocrinol Metab.* 2011; 25:543–59. [PubMed: 21872797]
- Janjetovic Z, Brozyna AA, Tuckey RC, Kim TK, Nguyen MN, Jozwicki W, Pfeffer SR, Pfeffer LM, Slominski AT. High basal NF-kappaB activity in nonpigmented melanoma cells is associated with an enhanced sensitivity to vitamin D3 derivatives. *Br J Cancer.* 2011; 105:1874–84. [PubMed: 22095230]
- Janjetovic Z, Tuckey RC, Nguyen MN, Thorpe EM Jr. Slominski AT. 20,23-dihydroxyvitamin D3, novel P450scc product, stimulates differentiation and inhibits proliferation and NF-kappaB activity in human keratinocytes. *J Cell Physiol.* 2010; 223:36–48. [PubMed: 20020487]
- Janjetovic Z, Zmijewski MA, Tuckey RC, DeLeon DA, Nguyen MN, Pfeffer LM, Slominski AT. 20-Hydroxycholecalciferol, product of vitamin D3 hydroxylation by P450scc, decreases NF-kappaB activity by increasing IkappaB alpha levels in human keratinocytes. *PLoS One.* 2009; 4:e5988. [PubMed: 19543524]
- Li W, Chen J, Janjetovic Z, Kim TK, Sweatman T, Lu Y, Zjawiony J, Tuckey RC, Miller D, Slominski A. Chemical synthesis of 20S-hydroxyvitamin D3, which shows antiproliferative activity. *Steroids.* 2010; 75:926–35. [PubMed: 20542050]
- Mizwicki MT, Keidel D, Bula CM, Bishop JE, Zanello LP, Wurtz JM, Moras D, Norman AW. Identification of an alternative ligand-binding pocket in the nuclear vitamin D receptor and its functional importance in 1 α ,25(OH)₂-vitamin D3 signaling. *Proc Natl Acad Sci U S A.* 2004; 101:12876–81. [PubMed: 15326291]
- Mizwicki MT, Norman AW. The vitamin D sterol-vitamin D receptor ensemble model offers unique insights into both genomic and rapid-response signaling. *Sci Signal.* 2009; 2:re4. [PubMed: 19531804]
- Nagpal S, Na S, Rathnachalam R. Noncalcemic actions of vitamin D receptor ligands. *Endocr Rev.* 2005; 26:662–87. [PubMed: 15798098]
- Reichrath J. Vitamin D and the skin: an ancient friend. revisited, *Exp Dermatol.* 2007; 16:618–25.
- Rochel N, Wurtz JM, Mitschler A, Klaholz B, Moras D. The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. *Mol Cell.* 2000; 5:173–9. [PubMed: 10678179]
- Slominski A, Semak I, Zjawiony J, Wortsman J, Li W, Szczesniowski A, Tuckey RC. The cytochrome P450scc system opens an alternate pathway of vitamin D3 metabolism. *FEBS J.* 2005; 272:4080–90. [PubMed: 16098191]
- Slominski AT, Janjetovic Z, Fuller BE, Zmijewski MA, Tuckey RC, Nguyen MN, Sweatman T, Li W, Zjawiony J, Miller D, Chen TC, Lozanski G, Holick MF. Products of vitamin D3 or 7-dehydrocholesterol metabolism by cytochrome P450scc show anti-leukemia effects, having low or absent calcemic activity. *PLoS One.* 2010; 5:e9907. [PubMed: 20360850]
- Slominski AT, Kim TK, Janjetovic Z, Tuckey RC, Bieniek R, Yue J, Li W, Chen J, Nguyen MN, Tang EK, Miller D, Chen TC, Holick M. 20-Hydroxyvitamin D2 is a noncalcemic analog of vitamin D

- with potent antiproliferative and prodifferentiation activities in normal and malignant cells. *Am J Physiol Cell Physiol.* 2011a; 300:C526–41. [PubMed: 21160030]
- Slominski AT, Li W, Bhattacharya SK, Smith RA, Johnson PL, Chen J, Nelson KE, Tuckey RC, Miller D, Jiao Y, Gu W, Postlethwaite AE. Vitamin D analogs 17,20S(OH)2pD and 17,20R(OH)2pD are noncalcemic and exhibit antifibrotic activity. *J Invest Dermatol.* 2011b; 131:1167–9. [PubMed: 21228816]
- Slominski AT, Zmijewski MA, Semak I, Sweatman T, Janjetovic Z, Li W, Zjawiony JK, Tuckey RC. Sequential metabolism of 7-dehydrocholesterol to steroidal 5,7-dienes in adrenal glands and its biological implication in the skin. *PLoS One.* 2009; 4:e4309. [PubMed: 19190754]
- Takaku H, Miyamoto Y, Asami S, Shimazaki M, Yamada S, Yamamoto K, Udagawa N, DeLuca HF, Shimizu M. Synthesis and structure-activity relationships of 16-ene-22-thia-1 α ,25-dihydroxy-26,27-dimethyl-19-norvitamin D3 analogs having side chains of different sizes. *Bioorg Med Chem.* 2008; 16:1796–815. [PubMed: 18032055]
- Tang EK, Li W, Janjetovic Z, Nguyen MN, Wang Z, Slominski A, Tuckey RC. Purified mouse CYP27B1 can hydroxylate 20,23-dihydroxyvitamin D3, producing 1 α ,20,23-trihydroxyvitamin D3, which has altered biological activity. *Drug Metab Dispos.* 2010; 38:1553–9. [PubMed: 20554701]
- Tuckey RC. Progesterone synthesis by the human placenta. *Placenta.* 2005; 26:273–81. [PubMed: 15823613]
- Tuckey RC, Janjetovic Z, Li W, Nguyen MN, Zmijewski MA, Zjawiony J, Slominski A. Metabolism of 1 α -hydroxyvitamin D3 by cytochrome P450_{sc} to biologically active 1 α ,20-dihydroxyvitamin D3. *J Steroid Biochem Mol Biol.* 2008a; 112:213–9. [PubMed: 19000766]
- Tuckey RC, Li W, Shehabi HZ, Janjetovic Z, Nguyen MN, Kim TK, Chen J, Howell DE, Benson HA, Sweatman T, Baldisseri DM, Slominski A. Production of 22-hydroxy metabolites of vitamin D3 by cytochrome p450_{sc} (CYP11A1) and analysis of their biological activities on skin cells. *Drug Metab Dispos.* 2011; 39:1577–88. [PubMed: 21677063]
- Tuckey RC, Li W, Zjawiony JK, Zmijewski MA, Nguyen MN, Sweatman T, Miller D, Slominski A. Pathways and products for the metabolism of vitamin D3 by cytochrome P450_{sc}. *FEBS J.* 2008b; 275:2585–96. [PubMed: 18410379]
- Vaisanen S, Dunlop TW, Sinkkonen L, Frank C, Carlberg C. Spatiotemporal activation of chromatin on the human CYP24 gene promoter in the presence of 1 α ,25-Dihydroxyvitamin D3. *J Mol Biol.* 2005; 350:65–77. [PubMed: 15919092]
- Wang TT, Tavera-Mendoza LE, Laperriere D, Libby E, MacLeod NB, Nagai Y, Bourdeau V, Konstorum A, Lallemand B, Zhang R, Mader S, White JH. Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. *Mol Endocrinol.* 2005; 19:2685–95. [PubMed: 16002434]
- Zbytek B, Janjetovic Z, Tuckey RC, Zmijewski MA, Sweatman TW, Jones E, Nguyen MN, Slominski AT. 20-Hydroxyvitamin D3, a product of vitamin D3 hydroxylation by cytochrome P450_{sc}, stimulates keratinocyte differentiation. *J Invest Dermatol.* 2008; 128:2271–80. [PubMed: 18368131]
- Zmijewski MA, Li W, Chen J, Kim TK, Zjawiony JK, Sweatman TW, Miller DD, Slominski AT. Synthesis and photochemical transformation of 3 β ,21-dihydroxypregna-5,7-dien-20-one to novel secosteroids that show antimelanoma activity. *Steroids.* 2011; 76:193–203. [PubMed: 21070794]
- Zmijewski MA, Li W, Zjawiony JK, Sweatman TW, Chen J, Miller DD, Slominski AT. Synthesis and photo-conversion of androsta- and pregna-5,7-dienes to vitamin D3-like derivatives. *Photochem Photobiol Sci.* 2008; 7:1570–6. [PubMed: 19037511]
- Zmijewski MA, Li W, Zjawiony JK, Sweatman TW, Chen J, Miller DD, Slominski AT. Photo-conversion of two epimers (20R and 20S) of pregna-5,7-diene-3 β , 17 α , 20-triol and their bioactivity in melanoma cells. *Steroids.* 2009; 74:218–28. [PubMed: 19028513]

Highlights

- We tested ligand induced vitamin D receptor translocation by novel secosteroids.
- We modeled secosteroids bindings to the genomic and non-genomic pockets.
- Good correlation between binding to the genomic pocket and translocation exists.
- No correlation exists between binding to the non-genomic pocket and translocation.
- Novel vitamin D hydroxyderivatives inhibit cell proliferation.
- Both chain length and the number or positions of hydroxyl groups are important.

Figure 1.

Structures of **(A)** secosteroids with full side chains (vitamin D₃-like), and **(B)** secosteroids with shortened side chains (pD-like)

Figure 2.

Effect of secosteroids on proliferation of human epidermal keratinocytes. Data are shown as means $\pm$ SEM (n 6). ##, $P < 0.01$ versus 1,25(OH)₂D₃ and **, $p < 0.01$; ***, $p < 0.001$ versus ethanol (EtOH) control by student t-test.

Figure 3.

Novel di- and trihydroxyvitamin D₃ derivatives inhibit growth of human keratinocytes.

HaCaT cells were treated for 48 h with 1,25(OH)₂D₃ (A), 20,23(OH)₂D₃ (B), 1,20(OH)₂D₃ (C) and ³H-thymidine incorporation into DNA was measured. In separate experiments we tested the effect of 17,20,23(OH)₃D₃ on ³H-thymidine incorporation into DNA after 24 (D) and 48 h (E). The effect of 17,20(OH)₂D₃ on cell proliferation was measured using the MTS assay (F). Data are presented as mean ±SD, n=4. Incorporation into DNA is shown as a percentile (%) of control (ethanol treated cells). Statistical significance is presented as *, p<0.05, **, p<0.01 and ***, p<0.001.

Figure 4.

The effect of P450scc-derived hydroxyvitamin D₃ derivatives on VDR translocation from cytoplasm to nucleus. Data are shown as mean $\pm$ SEM (n = 6). The dose dependent stimulation of VDR translocation was analyzed by one-way ANOVA with #, P < 0.05 and ##, P < 0.01. The differences between control and treatment were analyzed with student's t-test where *, P < 0.05, **, P < 0.01 and ***, P < 0.001.

Figure 5.

Comparison of the effects of different secosteroids on VDR translocation in cells treated with ligands at a concentration of 10^{-7} M. 1, positive control for full length side chained compounds. 2, positive control for short side chained compounds. EtOH, negative ethanol control. Data are shown as mean $\pm$ SEM (n = 6). The differences between positive control and treatment were analyzed with student's t-test where *, $P < 0.05$ and **, $P < 0.01$.

Figure 6.

The effect of hydroxy-pD derivatives with a short side chain on VDR translocation from the cytoplasm to nucleus. Data are shown as mean $\pm$ SEM (n = 6). The dose-dependent stimulation of VDR translocation was analyzed by one-way ANOVA with #, $P < 0.05$ and ##, $P < 0.01$. The differences between control and treatment were analyzed with student's t-test where *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$.

Figure 7.
Docking score for human VDR (G-pocket) with secosteroids

Figure 8.
Correlation of the EC50 with their score for docking to the G-pocket of human VDR. (1) 1,20(OH)₂D₃, (2) 1,25(OH)₂D₃, (3) 1,20,23(OH)₃D₃, (4) 20,23(OH)₂ D₃, (5) 17,20,23(OH)₃D₃, (6) 20(OH)D₃, (7) 20,22(OH)₂D₃, (8) 22(OH)D₃.

Figure 9.

Correlation of the VDR translocation activity at secosteroids (10^{-7} M) with their score for docking to the G-pocket of human VDR. Left panels, correlation analysis for all secosteroids. Right panels, correlation analysis for secosteroids with a full-length side chain (vitamin D3 derivatives). Circled area show short-chained secosteroids. (1) 1,20(OH)₂D₃, (2) 1,25(OH)₂D₃, (3) 1,20,23(OH)₃D₃, (4) 20,23(OH)₂D₃, (5) 17,20,23(OH)₃D₃, (6) 20(OH)D₃, (7) 20,22(OH)₂D₃, (8) 22(OH)D₃, (9) pD, (10) 17(OH)pD, (11) 17,20(OH)₂pD, (12) 20(OH)pD, (13) 21(OH)pD.

Figure 10.

Correlation of VDR translocation activity of different secosteroids (10^{-7} M) and their scores for docking to the alternative, non-genomic ligand binding pocket (A-pocket) on the human VDR. (1) 1,20(OH)₂D₃, (2) 1,25(OH)₂D₃, (3) 1,20,23(OH)₃D₃, (4) 17,20,23(OH)₂D₃, (5) 20(OH)D₃, (6) 20,22(OH)₂D₃, (7) 20,23(OH)₂D₃, (8) 22(OH)D₃, (9) pD, (10) 20(OH)pD, (11) 17(OH)pD, (12) 17,20(OH)₂pD, (13) 21(OH)pD.

Figure 11.

Binding of secosteroids to the VDR. (A) the position of ligand binding pocket for the genomic response (G-pocket) relative to the VDR (PDB code: 1DB1). (B) zoomed G-pocket showing the overlap of a short chain analog (pD) and a full chain analog (1,20(OH)₂D₃) in this pocket. (C) the position of ligand binding domain for the non-genomic response (alternative binding pocket or A-pocket) relative to the VDR (PDB code: 1DB1). (D) zoomed A-pocket showing the overlap of a short chain analog (pD), a full chain analog (1,20(OH)₂D₃), and the original ligand JN in this pocket.