Antiproliferative Activity of Non-Calcemic Vitamin D Analogs on Human Melanoma Lines in Relation to VDR and PDIA3 Receptors

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Abstract: Vitamin D is a precursor for secoestroidal hormones, which demonstrate pleiotropic biological activities, including the regulation of growth and the differentiation of normal and malignant cells. Our previous studies have indicated that the inhibition of melanoma proliferation by a short side-chain, low calcemic analog of vitamin D—21(OH)pD is not fully dependent on the expression of vitamin D receptor (VDR). We have examined the effects of classic vitamin D metabolites, 1,25(OH)2D3 and 25(OH)D3, and two low calcemic vitamin D analogs, (21(OH)pD and calcipotriol), on proliferation, mRNA expression and vitamin D receptor (VDR) translocation in three human melanoma cell lines: WM98, A375 and SK-MEL-188b (subline b of SK-MEL-188, which lost responsiveness to 1,25(OH)2D3 and became VDR−/−/−CYP27B1−/−/−). All tested compounds efficiently inhibited the proliferation of WM98 and A375 melanoma cells except SK-MEL-188b, in which only the short side-chain vitamin D analog—21(OH)pD was effective. Overall, 21(OH)pD was the most potent compound in all three melanoma cell lines in the study. The lack of responsiveness of SK-MEL-188b to 1,25(OH)2D3, 25(OH)D3 and calcipotriol is explained by a lack of characteristic transcripts for the VDR, its splicing variants as well as for vitamin D-activating enzyme CYP27B1. On the other hand, the expression of VDR and its splicing variants and other vitamin D related genes (RXR, PDIA3, CYP3A4, CYP2R1, CYP27B1, CYP24A1 and CYP11A1) was detected in WM98 and A375 melanomas with the transcript levels being modulated by vitamin D analogs. The expression of VDR isoforms in WM98 cells was stimulated strongly by calcipotriol. The antiproliferative activities of 21(OH)pD appear not to require VDR translocation to the nucleus, which explains the high efficacy of this noncalcemic pregnacalciferol analog in SK-MEL-188b melanoma, that is, VDR−/−. Therefore, we propose that 21(OH)pD is a good candidate for melanoma therapy, although the mechanism of its action remains to be defined.

Keywords: vitamin D; 1,25(OH)2D3; calcipotriol; 21(OH)pD; vitamin D analogs; melanoma; human melanoma cell lines; VDR translocation; anti-melanoma activity

1. Introduction

Vitamin D is a naturally occurring hormone precursor, which after activation serves as a pleiotropic regulator of homeostasis on cellular and whole organism levels [1,2]. It is produced in
the skin which is subjected to the ultraviolet B (UVB) fraction of sunlight or could be acquired from nutritional sources and supplementation. UVB exposure results in the conversion of epidermal 7-dehydrocholesterol (7DHC) into pre-vitamin D$_3$, which undergoes subsequent isomerization to vitamin D$_3$ \[2–5\]. Vitamin D$_3$ has to be hydroxylated in order to implement its biological activities. The first step takes place in the liver, where vitamin D$_3$ is converted to 25(OH)D$_3$ (25-hydroxyvitamin D$_3$, calcifediol) by CYP2R1, CYP27A1 or CYP3A4 (CYP3A4 belongs to cytochrome P450 superfamily and is involved in drug metabolism, but also has a capacity for vitamin D hydroxylation), and 25(OH)D$_3$ is subsequently hydroxylated by 1α-hydroxylase (CYP27B1) in the kidney. In addition, 1,25(OH)$_2$D$_3$ can be generated locally in many cell types (keratinocytes, dendritic cells, melanocytes, lymphocytes and cancer cells) expressing appropriate enzymatic machinery \[6,7\]. The level of 1,25(OH)$_2$D$_3$ and its metabolites in circulation are tightly regulated through a negative feedback loop by 24-hydroxylase (CYP24A1), which metabolizes calcitriol into water-soluble and inactive calcitroic acid \[8\]. The catalytically inactive splicing variant of CYP24A1 (CYP24SV) may serve as dominant negative regulator of vitamin D$_3$ catabolism and possibly contribute to the extracellular accumulation of 1,25(OH)2D$_3$ \[9\]. Most recently, it was discovered that CYP11A1, the rate limiting enzyme of steroidogenesis, also metabolizes 7DHC, vitamin D$_3$ and lumisterol to their corresponding hydroxyderivatives with full and short side-chains \[10–15\].

According to the genomic pathway, active forms of vitamin D$_3$ exert their biological activities through interactions with the VDR, which is expressed by a majority of cells in the body \[16,17\]. Activated VDR forms a heterodimer with retinoid X receptor (RXR), and this complex is translocated to the nucleus where it binds to the VDREs (vitamin D response elements) and activates the expression of hundreds of human genes \[17,18\]. The activity of this complex is also regulated by the recruitment of co-activators or co-repressors to modulate the expression of selected genes, including those participating in the inhibition of cell cycle progression and the stimulation of differentiation and apoptosis \[19–22\].

In addition to the well-characterized vitamin D$_3$ genomic actions, its analogs may also exert a rapid, non-genomic response (RR: rapid response) \[23–25\]. There is growing evidence that the co-localization of VDR \[26,27\] with caveolae of plasma membrane, as well as the binding of vitamin D to PDIA3 (Protein disulfide-isomerase A3), is responsible for the alternative (non-genomic) action of vitamin D$_3$ and its analogs \[23,28\].

According to epidemiological studies, the incidence of human malignant melanoma has been increasing steadily since the 1970s \[29–32\]. Early detection of melanoma results in a good prognosis for patients; however, the survival rate and therapeutic procedures for advanced or metastatic melanomas are very limited. Ultraviolet radiation, in addition to being fully carcinogenic and a main cause of melanoma \[32–34\], is also the most important factor promoting the formation of vitamin D$_3$. The anti-melanoma activity of vitamin D derivatives have been reported previously \[35–40\]. In addition, the presence of specific polymorphisms of VDR \[41–43\] or a decreased expression of VDR, CYP27B1, CYP24A1 and defects in vitamin D signaling are linked to more advanced stages of melanoma or poorer prognosis \[44–48\]. Furthermore, proper supplementation with vitamin D is believed to be an important factor in cancer prevention \[47,49–51\].

Active forms of vitamin D$_3$ show antiproliferative properties against several types of cancer, including colorectal cancer \[52\], breast cancer \[53\], prostate cancer \[54\] and melanoma \[39,40,55–59\]. Furthermore, the use of 1,25(OH)$_2$D$_3$ as an anticancer drug in very high concentrations (above 50,000 units/day) is currently under clinical investigation; however, it has to be acknowledged that it could potentially cause hypercalcemia \[40,50,60\]. Since vitamin D$_3$ analogs with modified or shortened side-chains were shown to have low or no effect on calcium levels \[59,61–63\], they are potentially better alternatives for calcitriol. So far, more than 3000 vitamin D$_3$ analogs have been synthesized, and their biological activity is still intensively investigated as a single agent or in combination with other cytostatics \[40,64–66\]. In this study, we have investigated the response of three human melanoma cell lines against four vitamin D$_3$ analogs. We have analyzed the antiproliferative potential of analogs
(Figure 1) and found that 21(OH)pD inhibits melanoma growth through a mechanism independent from VDR or PDIA3.

Figure 1. Chemical structure of vitamin D₃ analogs: 1,25(OH)₂D₃ (A), 25(OH)D₃ (B), calcipotriol (C) and 21(OH)pD (D).

2. Results

2.1. New Vitamin D₃ Analogs Effectively Inhibit A375 Cell Proliferation

Our previous studies on human melanomas SK-MEL-188 \[10,39,57,59,62,67–69\], A375 \[55\], and WM98 \[39,68\] demonstrated the sensitivity of melanoma cells to vitamin D₃ analogs with short and full side-chains. The efficacy of compounds was attenuated by melanogenesis, which was associated with the downregulation of VDR expression \[56,68\]. However, it was also shown that the antiproliferative activity of short side-chain analogs of vitamin D₃, such as 21(OH)pD, were not fully dependent on VDR expression in rodent melanomas \[56\], consistent with the poor docking score on the ligand binding domain of the VDR and the poor translocation of VDR to the nucleus \[69\].

To better understand the effect of VDR expression on the differential action of vitamin D₃ analogs, we used three human melanoma lines, A375 and WM98 and a subline b of SKMEL-188 which lost responsiveness to vitamin D during in vitro passaging \[55\], and detected the VDR expression and sensitivity to selected vitamin D analogs (1,25(OH)₂D₃, 25(OH)D₃, 21(OH)pD and calcipotriol). As expected, these compounds \[40,65\] effectively inhibited the proliferation of A375 and WM98 melanomas expressing VDR receptor. IC₅₀ values ranged from pM to µM, and the effects on cell proliferation were strongly dependent on the melanoma cell line and the nature of the compound. Interestingly, non-pigmented SK-MEL-188b was found to be resistant to vitamin D₃ analogs with a full-length side-chain (1,25(OH)₂D₃, 25(OH)D₃ and calcipotriol) and sensitive to short side-chained 21(OH)pD (Figure 2). Overall, 21(OH)pD was found to be the most potent inhibitor of growth in tested melanoma cell lines.
Figure 2. Effects of 1,25(OH)2D3, 25(OH)D3, calcipotriol and 21(OH)pD on growth of human SK-MEL-188b, A375 and WM98 melanoma cells. Cells were seeded into 96-well plates and incubated in a medium supplemented with serial dilution of vitamin D analogs from 0.01 nM to 1000 nM concentration (as described in Material and Methods). The statistical significance of results has been analyzed using one-way ANOVA (GraphPad Software, San Diego, CA, USA) and data are presented as means ± SEM for at least three independent measurements. The cutoff point of significance was defined as \( p < 0.05 \) (* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \), **** \( p < 0.0001 \)).

2.2. The Effect of Vitamin D3 Compounds on the Expression of Genes Related to Vitamin D3 Action or Metabolism

First, we investigated the expression of vitamin D3-related genes in untreated melanoma cells. As shown in Figure 3, PCR fragments characteristic for VDR, CYP27A1 and CYP27B1 mRNAs were below the level of detectability in SK-MEL-188b melanoma, which did not respond to vitamin D. Only the mRNA of PDIA3 was detected in this line. On the other hand, in A375 and WM98, cell lines corresponding to transcripts for all tested genes were detected.

Next, we tested the effects of vitamin D3 analogues on the relative mRNA levels of vitamin D3-related genes in responsive melanoma cell lines (A375 and WM98). The treatment of WM98 melanoma with full length side-chain vitamin D3 analogs (1,25(OH)2D3, 25(OH)D3 and calcipotriol) is presented in Figure 4. Only 1,25(OH)2D3 stimulated the expression of VDR Co-receptor RXR.
and of CYP27A1 (Figure 4B). All three analogs, however, stimulated the expression of CYP3A4, CYP2RI, CYP24A1 and its splicing variant CYP24A1sv. Interestingly, the strongest effect (approximately a 6-fold induction) on CYP2RI mRNA level was observed in cells treated with calcipotriol (Figure 4C). Furthermore, the level of CYP27B1 mRNA was not affected by tested secosteroids (Figure 4G), while the expression of PDIA3 was stimulated only by 25(OH)D3 (Figure 4E).

The treatment of A375 melanoma with four vitamin D3 analogs (1,25(OH)2D3, calcipotriol and 21(OH)pD) decreased the expression of VDR, RXR, PDIA3, CYP2RI genes (Figure 5). Interestingly, the strongest decrease in the mRNA levels of CYP3A4 and CYP2RI was observed in A375 melanoma cells treated with 21(OH)pD. The induction of CYP24A1 gene was hundreds of times folds higher in cells treated with 1,25(OH)2D3 and calcipotriol when compared to 21(OH)pD, showing minimal effect. Finally, the mRNA level of CYP27B1 was not affected by vitamin D analogues and CYP11A1 was elevated in A375 melanoma cells only after treatment with calcipotriol.

![Figure 3](image-url)

**Figure 3.** Differences in mRNA level of VDR, PDIA3, CYP27A1 and CYP27B1 genes in SK-MEL-188b cells in comparison with A375 and WM98 cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reporter gene to normalize all samples. See Table S1 for primer sequences and the predicted length of PCR fragments.
Figure 4. Vitamin D analogs treatment modulate the expression of (A) retinoid X receptor (RXR), (E) disulfide isomerase (PDIA3), 25-hydroxylases ((B) CYP27A1, (C) CYP2R1 or (F) CYP3A4, (G) 1α-hydroxylase (CYP27B1)) and 24-hydroxylases ((D) CYP24A1 or (H) CYP24SV) in WM98 melanoma cell line. Cells were stimulated with 1 µM 1,25(OH)2D3, 25(OH)D3 or calcipotriol for 24 h. Quantitative PCR analyses were performed as described in Materials and Methods. Statistical significance was estimated using t-test and data are presented as means ± SD (n = 3). The cutoff point for significance is defined as p < 0.05 (* p < 0.05, ** p < 0.01).
It is well established that the antiproliferative effects of vitamin D analogs strongly depend on the expression of vitamin D receptor VDR [40,47,65]. As shown in Figure 3, the melanoma cell lines used in the study differ in their basal level of VDR expression, and the transcript is absent in SK-MEL-188b, which is not responsive to vitamin D. Theoretically, an alternative splicing of VDR mRNA (Table S1). Detection of specific variants of VDR was confirmed by sequencing of PCR fragments previously separated on agarose gel (Figure 6B). WM98 and A375 melanoma cell lines express all four PCR fragments (Pr 1, Pr 2, Pr 4 and Pr 5) corresponding to VDR splicing variant c (Figure 6A). In the WM98 melanoma, the cell line presence of an mRNA characteristic for isoforms a (Pr 3) and b (Pr 4, upper band) was also detected. Interestingly, in SK-MEL-188b melanoma cells, only one fragment of mRNA corresponding to the alternative promoter region of VDR (isoform c) was detected. Finally, the effects of two vitamin D3 analogs 25(OH)D3 and calcipotriol on the alternative splicing of VDR pre-mRNA was tested in WM98 cell line (Figure 6C). It was shown that treatment with calcipotriol, but not 25(OH)D3, resulted in at least 7-fold elevation of mRNA levels corresponding to all fragments of the VDR tested (fragments detected by a set of primers: Pr 1 to Pr 5).
Figure 6. (A) Five different PCR primers sets were designed (Pr 1–Pr 5) in order to detect the expression of VDR splicing variants in WM98, A375 and SK-MEL-188b cell lines. The most common isoforms are a, b and c and the rarely identified isoforms are d, e, f and g. The positions of sets of primers designed to differentiate VDR isoforms is also shown. Product 1 (Pr 1) of 132 b.p. is characteristic for isoforms a, b, c and f; Product 2 (Pr 2) of 180 b.p. is universal for all isoforms except f; Product 3 (Pr 3) of 386 b.p. is unique for isoform a; Product 4 (Pr 4), depending on length of the PCR fragment, indicates isoform “b” 532 b.p. or “c” 410 b.p.; Product 5 (Pr 5) of 384 b.p. is characteristic for isoforms a, b, c, d and e, but not g and f (diagram of VDR splicing variants taken from AceView https://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/). (B) Semiquantitative PCR was used to differentiate the VDR isoforms (see Material and Methods for details); molecular weight marker (M) as described in. (C) Effects of vitamin D analogs on the expression of VDR isoforms were analyzed in WM98 melanoma cells. Melanoma cells were treated with 25(OH)D3 or calcipotriol at 1 µM concentration for 24 h. Statistical significance was estimated using t-test and data are presented as means ± SD (n = 3). The cutoff point for significance is defined as p < 0.05 (* p < 0.05, ** p < 0.01, *** p < 0.001).
2.3. Translocation of Vitamin D Receptor by Secosteroids

The ligand-induced translocation of VDR receptor into the nucleus was studied using A375 cells stably transduced with pLenti-CMV-VDR-EGFP lentiviral construct [70]. 1,25(OH)_{2}D_{3} or calcipotriol induced a dose-dependent and efficient translocation of VDR-GFP fusion protein into the nucleus (Figure 7). A lack of VDR-GFP translocation to the nucleus was observed in cells treated with 21(OH)pD.

![Figure 7](image)

**Figure 7.** Effects of vitamin D analogs: (A) 1,25(OH)_{2}D_{3}, (B) calcipotriol or (C) 21(OH)pD at 0 (C—control), 0.01, 0.1 or 1 µM concentrations on VDR translocation to the nucleus (green fluorescence shown in a gray scale). Melanoma A375 cells were transduced using pLenti-CMV-VDR-GFP-pgkpuro construct: see Materials and Methods. Vitamin D analogs except 21(OH)pD induce VDR translocation to the nucleus after 1 h of incubation in a concentration-dependent manner. Each panel shows four random micrographs for each concentration taken by fluorescent microscope under 100× magnification.

3. Discussion

There is growing evidence that vitamin D_{3} analogs are excellent candidates for melanoma therapy [40,47,65,71,72]. This is supported by the strong correlation between an adequate level of 25(OH)D_{3} (>30 ng/mL in the serum) and a decreased incidence or severity of several cancers,
including melanoma [62,73–77]. However, the use of an active form of vitamin D₃, 1,25(OH)₂D₃ is limited due to its potential hypercalcemic effects; thus, the development and biological evaluation of new low calcemic vitamin D₃ derivatives is highly desirable [40,55,65,72].

Here, we present results showing the different sensitivities of three melanoma cell lines (SK-MEL-188b, A375 and WM98) to vitamin D₃ analogs. Recently, we described vitamin D₃-resistant clone of SK-MEL-188 (SK-MEL-188b) [55]. Contrary to parental SK-MEL-188, this clone was insensitive to the treatment with 1,25(OH)₂D₃, or with several analogs of vitamin D₂, except of PRI-1731 with the inverse orientation of A ring in comparison to parental 1,25(OH)₂D₃ by the introduction of (5Z,7Z) modification [55,78,79]. This clone does not express the coding region of VDR and of CYP27B1. Therefore, we call it SK-MEL-188/VDR⁻⁻–CYP27B1⁻⁻. Interestingly, a short side-chain analog of vitamin D₃ with a pregnacalciferol-type configuration, such as 21(OH)pD, was shown to inhibit the growth of the SK-MEL-188b line. It has to be underlined that this analog, also showed high potency against two other melanoma cell lines in the study (A375 and WM98) and minimal stimulation of CYP24A1. This indicates that the antiproliferative properties of 21-OHpd are independent on its action on the VDR, which is consistent with our previous findings on pregnacalciferol derivatives acting as poor activators of VDR per VDR translocation studies and molecular modeling [69]. One of the possible mechanisms of 21(OH)pD action could include an activation of non-genomic pathways [25,80], including those linked to the PDIA3 protein [81,82]. However, interaction with other nuclear steroid receptors cannot be excluded. In addition, other receptors such as retinoic orphan acid receptors (ROR)α and γ can be considered since vitamin D hydroxyderivatives can act as reverse agonists on these receptors [62,83] and related pregnalumisterol derivatives can also act on RORα and RORγ [15]. These considerations require future in-depth investigations to define specific receptors for pD compounds (secosteroids with shortened side-chain).

Two other melanoma lines were responsive to vitamin D (WM98 and A375) and showed relatively high expression levels of VDR transcript. Thus, the studies presented above on three melanoma lines are consistent with reports showing that the expression of VDR is the key factor responsible for the antitumor activities of 1,25(OH)₂D₃ [18,47,84,85]. Of note is that its decreased expression correlates with advanced melanoma staging, progression and decreased overall patient survival and disease-free survival time [45,46]. Accordingly, VDR polymorphism [41,42,72] and decreased levels of 25(OH)D₃ in the serum [33,86] positively correlate with melanoma prevalence and poor prognosis.

Here, we show that sensitivity to tested vitamin D₃ hydroxyderivatives with full side-chains depends on VDR expression and its alternative splicing. Interestingly, sensitive melanoma lines (A375, WM98) expressed all three major VDR splicing variants, suggesting that they may play an important but not necessarily identical function in vitamin D₃ signaling. Further studies are required to elucidate the significance of the expression of VDR splicing variants. Furthermore, the expression of vitamin D₃-related genes was altered by vitamin D₃ analogs, and the effects on the expression of VDR, RXR, PDIA3, CYP2R1 or CYP24A1 were stronger in WM98 melanoma cells in comparison to the A375 line. This phenomenon could explain higher sensitivity of WM98 to 1,25(OH)₂D₃ in comparison to A375 line.

In summary, low calcemic vitamin D₃ analogs such as 25(OH)D₃, 21(OH)pD or calcipotriol showed similar antiproliferative activity to 1,25(OH)₂D₃ in melanoma cell lines expressing VDR spliced variants. Furthermore, the short side-chain analog 21(OH)pD was found to be superior among vitamin D analogs and was the only one, which inhibited the growth of the melanoma subline negative for VDR, indicating a mechanism of action that is VDR-independent.

4. Materials and Methods

4.1. Cell Lines and Vitamin D Analogs

In our study, we determined the inhibitory effects of vitamin D₃ analogs against immortalized human melanoma cell lines SK-MEL-188b, WM98 and A375. SK-MEL-188b cells were cultured
in F10 medium supplemented with 10% fetal bovine serum (FBS, Sigma, Poznan, Poland) and 1× antibiotic-antimycotic solution (Anti-Anti, Sigma). A375 and WM98 cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% anti-anti. To eliminate the influence of sterols present in fetal bovine serum in the experiments, we used 5% charcoal-striped FBS (GE Healthcare Life Sciences, Warsaw, Poland). Vitamin D₃ analogs 1,25(OH)₂D₃, 25(OH)D₃ and calcipotriol were acquired from the Pharmaceutical Research Institute (Warsaw, Poland), while the new short side analog 21(OH)pD was synthesized in collaboration with ProChimia Surfaces (Gdynia, Poland) as described previously [49,50]. The chemical structures of these secosteroids are presented in Figure 1.

4.2. SRB Assay

Melanoma cells were seeded on 96-well plates at a density of 8000 per well in an appropriate medium supplemented with 5% charcoal-striped FBS and 1× anti-anti solution. After 24 h, the medium was replaced with the fresh one containing vehicle and serial dilutions of vitamin D₃ analogs at concentrations from 10 pM to 1 µM. Following incubation for 48 h, 100 µL of 20% TCA (trichloroacetic acid) was added and cells were incubated for 1 h in 4 °C. The medium was discarded and cells were washed 5 times with sterile water. Afterwards, cells were dried and 100 µL of SRB (0.4 g sulforhodamin B in 100 mL 1% acetic acid) was added into the plate wells for 15 min incubation at room temperature. Next, cells were washed 5 times with 1% acetic acid, dried and resolved with 150 µL of 10 mM Tris (pH = 10.5) for 10 min in room temperature. The absorbance was measured spectroscopically at 570 nm with 96-well plate reader (BioTek, Winooski, VT, USA).

4.3. Classical PCR and Real-Time PCR Analysis

SK-MEL-188b, A375 and WM98 melanoma cells were treated with 1,25(OH)₂D₃, 25(OH)D₃ and calcipotriol (only WM98 line) for 24 h (Figure 5 and Figure S1) or collected without treatment (Figures 3 and 4). RNA was isolated using a Total RNA Kit (A&A Biotechnology, Gdynia, Poland). Reverse transcription (500 ng RNA/reaction) was carried out with a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). Classic PCR and real-time PCR were performed using 5-fold diluted cDNA and 2× PCR Master Mix (A&A Biotechnology) or real time 2× PCR Master Mix SYBR Set A, B (A&A Biotechnology). The primers to amplify fragments of ACTB, VDR, RXR, PDIA3, CYP27A1, CYP2R1, CYP3A4, CYP27B1, CYP24A1, CYP24SV genes were designed with Primer Quest software (Integrated Device Technology, San Jose, CA, USA) (Table S1). The data was collected on a MJ Mini BioRad cycler (BioRad, Hercules, CA, USA) or Termocycler StepOne Real-Time PCR Systems (Life Technologies, Carlsbad, CA, USA). PCR products were visualized using the Mupid-One electrophoresis system (BioRad) and ethidium bromide staining.

4.4. VDR Translocation

The melanoma A375 cell line was used to study the VDR receptor translocation to nucleus after vitamin D₃ analog treatment. Cells were transduced by pLenti-CMV-VDR-EGFP-pgkpuro plasmid, where VDR and GFP were expressed as a fusion protein in cytoplasmic compartment. Cells were cultured on 12 wells plate (0.5 × 10⁵ cells/well) in DMEM supplemented with 5% charcoal-striped FBS and 24 h later medium was replaced with DMEM containing 1,25(OH)₂D₃, calcipotriol and 21(OH)pD at 0.01 µM, 0.1 µM or 1 µM concentrations. After 1 h of incubation VDR translocation from cytoplasm to nucleus was determined with fluorescent Eclipse TE300 microscope (Nikon, Tokyo, Japan).

4.5. Statistical Analysis

SRB viability data was presented using one-way Anova test to compare experimental groups: cells were treated with vitamin D₃ analogs with a concentration from 0.01 nM to 1000 nM with control samples (GraphPad Software). The antiproliferative potency of vitamin D₃ analogs were compared by the calculation of EC50 for every compound (half maximal effective concentration).
Real-time PCR data was analyzed with the comparative ΔΔ-Ct method normalized to the reference gene ACTB. As a control, a probe with sterile water was used instead of cDNA. Data are presented as mean ± SD (n = 4–6). Student’s t-test (for two groups) or one-way ANOVA with appropriate post-hoc test (for more than two groups) were used to analyze data using Excel (Microsoft) or Prism 7.00 (GraphPad Software), respectively. Statistically significant differences are denoted with asterisks: p < 0.05 (* p < 0.05; ** p < 0.01; *** p < 0.0001).

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/9/2583/s1.

Author Contributions: T.W. and A.P. and J.W. performed the main experiments, took part in data analysis and preparation of the manuscript; M.A.Z. and A.T.S. designed experiments; M.A.Z., T.W., J.W. and A.T.S. analyzed data and prepared final version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

1α,25(OH)2D3 1α,25-dihydroxyvitamin D₃ (calcitriol)
21(OH)pD 21-hydroxypregnacalciferol
25(OH)D₃ 25-hydroxyvitamin D₃ (calcifediol)
7-DHC 7-dehydrocholesterol (provitamin D₃, cholesta-5,7-dien-3β-ol)
7-DHP 7-dehydropregnenolone
MARRS receptor Membrane-Associated Rapid Response to Steroid binding protein (other names: ERp57, GRp58, Pdia3)
PDIA3 Protein disulfide-isomerase A3
ROS reactive oxygen species
UVA/B ultraviolet radiation A and B
VDR vitamin D receptor
VDRE vitamin D response elements

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