

CYP11A1-derived vitamin D hydroxyderivatives as candidates for therapy of basal and squamous cell carcinomas

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Abstract. Hydroxyderivatives of vitamin D₃, including classical 1,25(OH)₂D₃ and novel CYP11A1-derived hydroxyderivatives, exert their biological activity by acting as agonists on the vitamin D receptor (VDR) and inverse agonists on retinoid-related orphan receptors (ROR) α and γ . The anti-cancer activities of CYP11A1-derived hydroxyderivatives were tested using cell biology, tumor biology and molecular biology methods in human A431 and SCC13 squamous (SCC)- and murine ASZ001 basal (BCC)-cell carcinomas, in comparison with classical 1,25(OH)₂D₃. Vitamin D₃-hydroxyderivatives with or without a C1 α (OH) inhibited cell proliferation in a dose-dependent manner. While all the compounds tested had similar effects on spheroid formation by A431 and SCC13 cells, those with a C1 α (OH) group were more potent in inhibiting colony and spheroid formation in the BCC line. Potent anti-tumorigenic activity against the BCC line was exerted by 1,25(OH)₂D₃, 1,20(OH)₂D₃, 1,20,23(OH)₃D₃,

1,20,24(OH)₃D₃, 1,20,25(OH)₃D₃ and 1,20,26(OH)₃D₃, with smaller effects seen for 25(OH)D₃, 20(OH)D₃ and 20,23(OH)₂D₃. 1,25(OH)₂D₃, 1,20(OH)₂D₃ and 20(OH)D₃ inhibited the expression of GLI1 and β -catenin in ASZ001 cells. In A431 cells, these compounds also decreased the expression of GLI1 and stimulated involucrin expression. VDR, ROR γ , ROR α and CYP27B1 were detected in A431, SCC13 and ASZ001 lines, however, with different expression patterns. Immunohistochemistry performed on human skin with SCC and BCC showed nuclear expression of all three of these receptors, as well as megalin (transmembrane receptor for vitamin D-binding protein), the level of which was dependent on the type of cancer and antigen tested in comparison with normal epidermis. Classical and CYP11A1-derived vitamin D₃-derivatives exhibited anticancer-activities on skin cancer cell lines and inhibited GLI1 and β -catenin signaling in a manner that was dependent on the position of hydroxyl groups. The observed expression of VDR, ROR γ , ROR α and megalin in human SCC and BCC suggested that they might provide targets for endogenously produced or exogenously applied vitamin D hydroxyderivatives and provide excellent candidates for anti-cancer therapy.

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Introduction

The ultraviolet spectrum of solar radiation (UVR) that reaches surface of the earth includes both UVA and UVB corresponding to the wavelengths of 320-400 and 290-320 nm, respectively. Both induce various biological effects that are wavelength dependent (1-4). UVR exposure can lead to a range of skin pathologies including skin aging, solar keratosis and melanoma or non-melanoma skin cancers (NMSC). Due to its pleiotropic effects UVR is defined as a full cutaneous carcinogen with UVB being an important etiological factor responsible for basal (BCC) and squamous (SCC) cell carcinomas (4-7). The two chromophore dependent (predominantly UVB) and

chromophore independent mechanisms, such as generation of reactive oxygen species (ROS), are involved in epidermal cancerogenesis (2,3,5,8,9), a process further amplified by modification of local homeostasis, including immunosuppression (10). While the mortality for BCC and SCC is low, they are the commonest human cancers with a significant economic burden on the health care system in the USA and worldwide (11). Their incidence increases in immunocompromised patients, including those with organ transplantation, where tumors behave more aggressively. In addition, military personnel are unavoidably exposed in an unprotected manner to high doses of UVR during training or when deployed to locations with high solar radiation. Therefore, occupational overexposure to UVB will be experienced by those who work outdoors or who are exposed intermittently, potentially leading to the development and progression of BCC and SCC. While a number of therapeutic approaches are being tested against these cancers, they are costly and not fully effective, which in part could be secondary to the phenomenon of UVB-induced field of cancerization (12). Therefore, there is an urgent need to establish economical and non-toxic chemopreventive and therapeutic measures against UVB-induced cancer.

UVB also has a beneficial role in biology where transformation of 7-dehydrocholesterol (7DHC) to vitamin D3 (D3) in the skin supplies much of this hormonal precursor for systemic use (13,14). Higher doses of UVB lead to photoisomerization of the previtamin D3 intermediate to lumisterol (L3) and tachysterol (T3) (13). In the classical pathway, D3 is activated by hydroxylation at C25 producing 25(OH)D3 in the liver and subsequently at C1 producing 1,25(OH)₂D3 (kidney) with local (skin) production also occurring (13,15,16). 1,25(OH)₂D3, in addition to regulating calcium homeostasis, has pleiotropic activities that include anti-cancer effects (13,15-18). In the skin, it regulates formation of the epidermal barrier, hair cycling and attenuates cancerogenesis and inflammatory processes (13,16,19-22). 1,25(OH)₂D3 can also protect against UVB-induced cell death and DNA damage (23-26) with topically applied D3 precursor being able to inhibit UVB-induced cancerogenesis in mice (27).

We previously reported an alternative metabolic pathway of D3 activation initiated by the action of CYP11A which converted D3 to 20(OH)D3 and 22(OH)D3 with some further side-chain hydroxylations producing di-hydroxy (such as 20,23(OH)₂D3) and tri-hydroxy species. The major product was 20(OH)D3 and this can be further modified by other CYP enzymes including CYP2R1, CYP27A1 and CYP27B1 (28-34). Many of these pathway products and intermediates have been detected in both humans and animals (28,29,31,33,34). 20(OH)D3 has been shown to be present in serum and to accumulate together with its downstream derivatives in human epidermis (28,29,31). Thus, 20(OH)D3 and related metabolites are physiologically relevant endogenous bioregulators that can be defined as natural products (35). They show antiproliferative, prodifferentiation, anti-inflammatory and anti-cancer effects that are comparable to those of 1,25(OH)₂D3 (30,36-41). They also protect human keratinocytes against UVB damage (42-45) and topically applied 20(OH)D3 attenuates UVB-induced skin damage in *Skh:hr1* mice (46).

While the phenotypic effects of canonical 1,25(OH)₂D3 are predominantly mediated through interaction with the

vitamin D receptor (VDR) through genomic and non-genomic mechanisms (13,47-50), CYP11A1-derived vitamin D hydroxyderivatives, in addition to acting on VDR (51-53), can also act on retinoid-related orphan receptors (RORs) α and γ (54-56) and the aryl hydrocarbon receptor (AhR) (57). Furthermore, clinico-pathological analyses of human melanoma samples have shown that expression of VDR and ROR α and γ changes during tumor progression and correlates with disease outcomes (58-62).

Notably, 20(OH)D3 and 20,23(OH)₂D3 are noncalcemic and non-toxic at extremely high doses in rats and mice (37,39,63,64) unlike the majority of low-calcemic derivatives of D3 that have been chemically synthesized (18,65), identifying the former as potential therapeutics.

Therefore, using a wide range of CYP11A1-derived vitamin D hydroxyderivatives, some modified by 1 α -hydroxylation by CYP27B1, the present study examined their anticancer potential in human SCC and murine BCC lines. In addition, it analyzed the expression of the VDR, ROR α and γ , and megalin/LRP2 which is a transmembrane receptor for vitamin D-binding protein for cellular import of 25(OH)D3 (66-68), in a panel of benign and malignant epidermal lesions.

Materials and methods

Vitamin D derivatives. Vitamin D3 and D2, 1,25(OH)₂D3 and 25(OH)D3 were purchased from MilliporeSigma. Hydroxyderivatives of D3 and D2 were synthesized enzymatically or non-enzymatically as described in Fig. S1 and purified as previously described (30,32,63,69-76). The purity of the compounds was >99%. The compounds were dissolved in ethanol and applied to cell cultures as previously described (44,51,57). All vitamin D-derived compounds were dissolved in ethanol. The active compounds were prepared as stock solutions at 10⁻⁴ M and then diluted with medium to final concentrations ranging from 10⁻⁷ to 10⁻¹² M (as indicated in the figures) and added to cell cultures. In each experiment, the control with vehicle (ethanol) was prepared.

Cell culture. The murine basal cell carcinoma (BCC) cell line ASZ001 and human skin squamous cell carcinoma (SCC) cell lines were used for the experiments. ASZ001 was a gift from Dr Ervin Epstein (University of California, San Francisco, CA) (77,78), A431 was purchased from ATCC (cat. no. CRL-1555) and SCC13 was a gift from Dr. S. K. Katiyar (University of Alabama at Birmingham, Birmingham, AL) (79). The BCC cell line was cultured in 154CF medium (Thermo Fisher Scientific, Inc.) (77,78) and SCC cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Corning Inc.), supplemented with 10% fetal bovine serum (FBS; MilliporeSigma), antibiotic-antimycotic solution (Corning Inc.) and maintained in 5% CO₂ at 37°C (79). For experimental treatment, charcoal-treated FBS (ctFBS) was used as previously described (44).

Proliferation assays. The MTS, sulforhodamine B (SRB) tests were used to measure proliferation of BCC and SCC cells as described previously (80,81). BCC and SCC cells were seeded on 96-well plates in complete culture medium for 24 h in 5% CO₂ at 37°C. The next day the medium was replaced with

serum-free medium and the cells starved for the subsequent 24 h. Cells were treated with secosteroids dissolved in ethanol and added to culture medium with 10% charcoal-treated FBS, for 24, 48 and 72 h. To assess the effects of secosteroids on BCC and SCC cells, the metabolic activity was tested using a tetrazolium compound and an electron coupling reagent (MTS; CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega Corporation), as previously described (80,81): 20 μ l of MTS/PMS solution was added to the cells and incubated for 2 h at 37°C then read the absorbance at 490 nm using Cytation 5 (BioTek Instruments, Inc.). SRB was also used to determine cell number and cell viability by colorimetric evaluation of total protein content as the dye binds to cellular proteins. The BCC and SCC cells were assayed as described previously (82). Briefly, after the treatment with secosteroids, BCC and SCC cells were fixed for 1 h with 10% trichloroacetic acid at 4°C and incubated with 0.4% sulforhodamine B in 1% acetic acid for 30 min at room temperature. Next, the protein-bound dye was dissolved in Tris base solution and the absorbance was measured at 565 nm using Cytation 5 (BioTek Instruments, Inc.).

Spheroids and colony formation. To assess the ability of the secosteroids being tested to inhibit anchorage-independent growth of BCC and SCC cells, the spheroid formation test was performed as previously described (81). Briefly, cells were seeded into ultra-low attachment 96-well plates in culture medium supplemented with epidermal growth factor (MilliporeSigma), basic fibroblast growth factor (MilliporeSigma), insulin (Thermo Fisher Scientific, Inc.), bovine serum albumin (MilliporeSigma) and B27 supplement (Thermo Fisher Scientific, Inc.). After seven days the plate was scanned using a Cytation 5 microplate reader and Gene5 software (BioTek Instruments, Inc.).

For colony formation assay, cells were plated in 6-well plates (1,000 cells/well) and incubated with graded concentrations of the secosteroids for 6 days. After methanol (70%) fixation at 4°C and staining with 0.1% crystal violet, the colonies were counted using Cytation 5 (BioTek Instruments, Inc.) and analyzed by Gene 5 software. The large (>0.5 mm), medium (>0.25 mm) and small colonies (>0.1 mm) colonies were counted.

Western blot analysis. RIPA Lysis and Extraction Buffer (Thermo Scientific; cat. no. 89901) was used for whole cell extract and Nuclear Extract kit (Active Motif, Inc.; cat. no. 54001) for nuclear and cytoplasmic fractions as described previously (43,44). Protein concentration was measured with Pierce BCA Protein Assay kit (Thermo Scientific; cat. no. 23225). For protein separation 4-15% Mini-PROTEAN TGX Stain-Free Protein Gels, 10 well, 30 μ l (Bio-Rad Laboratories, Inc.; cat. no. 4568083) were used with 20 μ g of proteins loaded per lane. The proteins were transferred to Immobilon-P PVDF membranes (MilliporeSigma; cat. no. IPVH00010). Prior incubation with primary antibodies the membranes were incubated with 5% skimmed milk in TBS-T buffer (Tris-buffer saline containing 0.1% (v/v) Tween-20 and 5% (w/v) skim milk) for 1 h at room temperature.

Immunodetection of VDR protein expression was performed using VDR antibody (D-6; cat. no. sc-13133; Santa

Cruz Biotechnology, Inc.) at a 1:200 dilution (1 h incubation at room temperature) followed by treatment of secondary antibody, m-IgG κ BP-HRP (cat. no. sc-516102; Santa Cruz Biotechnology, Inc.; 1 h incubation at room temperature at 1:2,000 dilution). ROR α Polyclonal Antibody (cat. no. PA1-812; Invitrogen; Thermo Fisher Scientific, Inc.; 1:1,000) and ROR γ (t) monoclonal Antibody (cat. no. 14-6988-82; Invitrogen; Thermo Fisher Scientific, Inc.; 1:500) were used for ROR α and ROR γ protein expression, respectively at 1 h at room temperature. Goat Anti-Rabbit IgG (cat. no. ab6721; Abcam; 1:5,000) and Anti-rat IgG, HRP-linked Antibody (cat. no. 7077; Cell Signaling Technology, Inc.; 1:2,000) were used as secondary antibodies for ROR α and ROR γ , respectively. The membranes were reprobed with following antibodies to measure the protein loading. Lamin C: lamin A/C antibody (N-18) (Santa Cruz; cat. no. sc-6215) for primary antibody (1 h incubation at room temperature at 1:200 dilution) with donkey anti-goat IgG-HRP (Santa Cruz; cat. no. sc-2020) for secondary antibody (1 h at room temperature at 1:5,000 dilution). α -Tubulin: alpha Tubulin Antibody (Thermo Fisher Scientific, Inc.; cat. no. 62204) for primary antibody (1 h incubation at room temperature at 1:5,000 dilution) and goat anti-mouse IgG-HRP (Santa Cruz; cat. no. sc-2005) for secondary antibody (1 h incubation at room temperature at 1:5,000 dilution). β -Actin: anti- β -Actin-Peroxidase antibody, mouse monoclonal (MilliporeSigma; cat. no. A3854) with 1hr incubation at room temperature at 1:10,000 dilution. After each incubation with primary antibody membranes were washed three times with TBS-T (Tris-buffer saline containing 0.1% (v/v) Tween 20) for 10 min each prior incubation with secondary antibody. Immuno-reactivity was detected using the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Inc., cat. no. 34580) or Immobilon Crescendo Western HRP substrate (MilliporeSigma; cat. no. WBLUF0500). The integrated optical density of the bands was analyzed by ImageJ software, version 1.53a (National Institutes of Health).

Flow cytometry. A431 cells were treated with 100 nM 1,20(OH) $_2$ D $_3$, 1,25(OH) $_2$ D $_3$, 20(OH)D $_3$, or ethanol (vehicle) for 24 h. To test the stimulation of differentiation by vitamin D $_3$ derivatives, after treatment the cells were fixed in 2% paraformaldehyde (Thermo Fisher Scientific, Inc.) in PBS for 1 h at room temperature. Following washing with PBS, pellets were resuspended in 100 μ l of permeabilizing solution containing 0.1% BSA and 0.1% NaN $_3$ in PBS and 0.2 μ g primary antibody against involucrin (Novocastra Laboratories Ltd.). Cells stained with isotype control antibody (IgG $_1$; Caltag Medsystems) were used as controls. After overnight incubation at 4°C, cells were washed with PBS and resuspended in 100 μ l of permeabilizing solution containing sheep anti-mouse FITC-conjugated secondary antibody (1:50; cat. no. NCL-SAM-FITC; Novocastra Laboratories Ltd.). After 3 h of incubation at 4°C, cells were washed with PBS, centrifuged and resuspended in 500 μ l of PBS. Samples were read and analyzed with an Accuri C6 (BD Bioscience) flow cytometer and BD Accuri C6 Plus software 8 (BD Bioscience). The FL-1 signal (collected from 10,000 events inside scatter/forward scatter window after debris exclusion) was recorded. Forward (relative to cell size) and side (relative to cell granularity) scatter were recorded.

The number of events (positive cells)-FL-1 signal values are presented as difference between mean fluorescence intensity of sample stained with specific and isotype control antibody (dMFI). Scatter signal values were presented as MSI (mean signal intensity).

Immunofluorescence studies. VDR, ROR α and ROR γ in BCC and SCC cells were immunostained as previously described (81) (for details see Fig. S2: Scheme for immunohistochemistry and characteristic of reagents used). Briefly, the cells were seeded in a 96-well plate and grown until reaching ~70% confluence then fixed with 4% buffered formalin for 20 min at room temperature. Following permeabilization, the cells were blocked with 0.5% Triton X-100 and 2% bovine serum albumin (BSA) for 1 h at room temperature, followed by overnight incubation with primary antibodies (VDR; clone 9A7; Thermo Fisher Scientific, Inc., cat. no. MA1-710, 1:50) and ROR α (1:400) and ROR γ (1:400) [both antibodies developed at NIEHS and characterized as reported in (54)] overnight at 4°C and incubation with the secondary antibody (goat anti-rat IgG (H+L) Cross-Adsorbed Secondary Antibody, cat. no. A-11006A, Alexa Fluor 488 goat anti-rabbit immunoglobulin G (IgG; H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, cat. no. A-11008; both Thermo Fisher Scientific, Inc.), diluted 1:1,000 for 1 h at room temperature. After washing the cell nuclei were stained with propidium iodide (PI; MilliporeSigma) and examined with a Cytation 5 microplate reader (BioTek Instruments, Inc.). For changes in GLI1 and β -catenin expression, ASZ001 and A431 lines were treated with 20(OH)D₃, 1,20(OH)₂D₃, 25(OH)D₃, 1,25(OH)₂D₃ or vehicle (ethanol) at 10⁻⁷ M at 37°C for 24 h. After washing the cell nuclei were stained with propidium iodide (PI; MilliporeSigma) and examined with a Cytation 5 microplate reader (BioTek Instruments, Inc.).

Immunohistochemistry. For immunohistochemistry, the standard formalin-fixed paraffin-embedded skin samples of normal skin BCC, SCC *in situ* and invasive SCC were included in this study (Table SI). The authors declare that this investigation was carried out following the rules of the Declaration of Helsinki of 1975 (revised in 2008) and this study was approved by the institutional review board (IRB) of the University of Alabama at Birmingham under IRB-940831016 (OCCC Tissue Procurement CORE Facility) and IRB-00000726 (Title E150427002, Dr. A. Slominski PI). The IRB of the University of Alabama at Birmingham, which gave its permission for conducting the present study, waived the requirement to obtain patients' informed consent for this research. VDR, ROR α , ROR γ and megalin in tissues samples of normal skin, BCC and SCC were labelled as previously described (83,84) (for details see Fig. S2). Briefly, heat-induced antigen retrieval (96°C, 20 min) in high pH unmasking solution (Vector Laboratories, Inc.) was performed on formalin-fixed paraffin embedded sections, followed by blocking of endogenous peroxidase for 10 min. The sections were incubated 30 min at room temperature with anti-Lrp2/Megalin antibody (cat. no. ab76969; Abcam) or overnight with other antibodies (anti-VDR; clone 9A7; cat. no. MA1-710) or anti-ROR γ and anti-ROR α [generated as previously described (54)] with subsequent incubation at room temperature with secondary antibodies conjugated

with HRP (ready-to-use anti-rabbit (cat. no. MP-7451) and anti-mouse (cat. no. MP-7452) ImmPRESS antibodies, Vector Laboratories, Inc. for RORs and megalin; anti-rat antibody, (1:200; cat. no. ab97057; Abcam) for VDR. The immunolabelling was visualized with peroxidase substrate ImmPACT NovaRED (Vector Laboratories Inc.) and the sections were mounted with permanent mounting (Thermo Fisher Scientific, Inc.) under coverslips. Sections were analyzed under a light microscope (magnification, x200). The mean staining intensity was assessed by two observers (ATS and AAB).

The analysis of the expression of *RORA* and *RORC* mRNA level followed public genomics data repository Gene Expression Omnibus, accession number GSE7553 <http://www.ncbi.nlm.nih.gov/geo>. For details on data collection, methods and patient's characteristic performed by Riker *et al* (85) please refer to their original paper.

Reverse transcription-quantitative (RT-q) PCR. Cultures at 70% confluency were used for treatment. After treatment for 24 h with secosteroids, cells were detached using trypsin and centrifuged at 271 x g for 5 min at 4°C. Cell pellets were then collected, and total RNA was isolated using an Absolutely RNA Miniprep kit (1.2 ml per 4x10⁶ cells) (Agilent Technologies, Inc.). The RNA purity and quantification were performed with Cytation 5 (BioTek Instruments, Inc.) and the absorbance 260/280 ratio ~2 checked for the purity. Total RNA (0.5 μ g) was used for cDNA synthesis with a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA) following the manufacturer's protocol. The cDNA (750 ng) was used for qPCR. β -actin was used as an internal control. qPCR was performed using Kapa SYBR Fast qPCR Master Mix (Kapa Biosystems; Roche Diagnostics) with QuantStudio 6 Flex Real-Time PCR Systems (Applied Biosystems; Thermo Fisher Scientific, Inc.) in quadruplicate, following the manufacturer's protocol and as previously described (43,44). Primer sequences are listed in Table SII. Data were collected with QuantStudio Real-Time PCR Software v1.2 (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the amount of mRNA was normalized by the comparative 2^{- $\Delta\Delta C_q$} method followed by calculating the fold change, using β -actin as a housekeeping gene as described previously (43,44). The experiments were repeated three times.

Statistical analysis. Data exported from Microsoft Excel were analyzed using GraphPad Prism 7 (GraphPad Software, Inc.) statistical software and are presented as means \pm SD or SEM as indicated in the figures legend. The data were analyzed by one way ANOVA with Dunnett's multi comparison post-hoc test or the Student's t-test as detailed in figure legends.

Results

The present study investigated the *in vitro* antitumor effects of CYP11A1-derived D3-hydroxyderivatives using established lines of human squamous (A431 and SCC-13) and mouse basal cell (ASZ0001) carcinoma. The structures of the secosteroids that were tested are presented in Table I, with stereochemistry shown where known. The MTS tests showed that a range of representative vitamin D3-hydroxyderivatives inhibited

Table I. Chemical structures of vitamin D hydroxyderivatives analyzed.

Chemical name	Chemical structure	Chemical name	Chemical structure
1,25(OH) ₂ D ₃		20,23(OH) ₂ D ₃	
1α,25-dihydroxyvitamin D ₃ (1 <i>R</i> ,3 <i>S</i> , <i>Z</i>)-5-(2-((1 <i>R</i> ,7 <i>aR</i> , <i>E</i>)-1-((<i>R</i>)-6-hydroxy-6-methylheptan-2-yl)-7 <i>a</i> -methyloctahydro-4 <i>H</i> -inden-4-ylidene)ethylidene)-4-methylenecyclohexane-1,3-diol		20 <i>S</i> ,23 <i>S</i> -dihydroxyvitamin D ₃ (2 <i>S</i> ,4 <i>S</i>)-2-((1 <i>S</i> ,7 <i>aS</i> , <i>E</i>)-4-((<i>Z</i>)-2-((<i>S</i>)-5-hydroxy-2-methylenecyclohexylidene)ethylidene)-7 <i>a</i> -methyloctahydro-1 <i>H</i> -inden-1-yl)-6-methylheptane-2,4-diol	
1,20(OH) ₂ D ₃		20,24(OH) ₂ D ₃	
1α,20 <i>S</i> -dihydroxyvitamin D ₃ (1 <i>R</i> ,3 <i>S</i> , <i>Z</i>)-5-(2-((1 <i>S</i> ,7 <i>aS</i> , <i>E</i>)-1-(2-hydroxy-6-methylheptan-2-yl)-7 <i>a</i> -methyloctahydro-4 <i>H</i> -inden-4-ylidene)ethylidene)-4-methylenecyclohexane-1,3-diol		20 <i>S</i> ,24 <i>R</i> -dihydroxyvitamin D ₃ (2 <i>S</i> ,5 <i>R</i>)-2-((1 <i>S</i> ,7 <i>aS</i> , <i>E</i>)-4-((<i>Z</i>)-2-((<i>S</i>)-5-hydroxy-2-methylenecyclohexylidene)ethylidene)-7 <i>a</i> -methyloctahydro-1 <i>H</i> -inden-1-yl)-6-methylheptane-2,5-diol	
1,20,23(OH) ₃ D ₃		20,25(OH) ₂ D ₃	
1α,20 <i>S</i> ,23 <i>S</i> -trihydroxyvitamin D ₃ (1 <i>R</i> ,3 <i>S</i> , <i>Z</i>)-5-(2-((1 <i>S</i> ,7 <i>aS</i> , <i>E</i>)-1-((4 <i>S</i>)-2,4-dihydroxy-6-methylheptan-2-yl)-7 <i>a</i> -methyloctahydro-4 <i>H</i> -inden-4-ylidene)ethylidene)-4-methylenecyclohexane-1,3-diol		20 <i>S</i> ,25-dihydroxyvitamin D ₃ 2-((1 <i>S</i> ,7 <i>aS</i> , <i>E</i>)-4-((<i>Z</i>)-2-((<i>S</i>)-5-hydroxy-2-methylenecyclohexylidene)ethylidene)-7 <i>a</i> -methyloctahydro-1 <i>H</i> -inden-1-yl)-6-methylheptane-2,6-diol	
1,20,24(OH) ₃ D ₃		17,20(OH) ₂ D ₂	
1α,20 <i>S</i> ,24 <i>R</i> -trihydroxyvitamin D ₃ (1 <i>R</i> ,3 <i>S</i> , <i>Z</i>)-5-(2-((1 <i>S</i> ,7 <i>aS</i> , <i>E</i>)-1-((5 <i>R</i>)-2,5-dihydroxy-6-methylheptan-2-yl)-7 <i>a</i> -methyloctahydro-4 <i>H</i> -inden-4-ylidene)ethylidene)-4-methylenecyclohexane-1,3-diol		17 <i>R</i> ,20 <i>S</i> -dihydroxyvitamin D ₂ (1 <i>R</i> ,7 <i>aS</i> , <i>E</i>)-4-((<i>Z</i>)-2-((<i>S</i>)-5-hydroxy-2-methylenecyclohexylidene)ethylidene)-1-((2 <i>S</i> ,5 <i>R</i> , <i>E</i>)-2-hydroxy-5,6-dimethylhept-3-en-2-yl)-7 <i>a</i> -methyloctahydro-1 <i>H</i> -inden-1-ol	
1,20,25(OH) ₃ D ₃		20(OH)D ₂	
1α,20 <i>S</i> ,25-trihydroxyvitamin D ₃ (1 <i>R</i> ,3 <i>S</i> , <i>Z</i>)-5-(2-((1 <i>S</i> ,7 <i>aS</i> , <i>E</i>)-1-(2,6-dihydroxy-6-methylheptan-2-yl)-7 <i>a</i> -methyloctahydro-4 <i>H</i> -inden-4-ylidene)ethylidene)-4-methylenecyclohexane-1,3-diol		20-hydroxyvitamin D ₂ (1 <i>S</i> , <i>Z</i>)-3-(2-((1 <i>S</i> ,7 <i>aS</i> , <i>E</i>)-1-((2 <i>S</i> ,5 <i>R</i> , <i>E</i>)-2-hydroxy-5,6-dimethylhept-3-en-2-yl)-7 <i>a</i> -methyloctahydro-4 <i>H</i> -inden-4-ylidene)ethylidene)-4-methylenecyclohexan-1-ol	
1,20,26(OH) ₃ D ₃		22(OH)D ₃	
1α,20 <i>S</i> ,26-trihydroxyvitamin D ₃ (1 <i>R</i> ,3 <i>S</i> , <i>Z</i>)-5-(2-((1 <i>S</i> ,7 <i>aS</i> , <i>E</i>)-1-((2 <i>S</i>)-2,7-dihydroxy-6-methylheptan-2-yl)-7 <i>a</i> -methyloctahydro-4 <i>H</i> -inden-4-ylidene)ethylidene)-4-methylenecyclohexane-1,3-diol		22-hydroxyvitamin D ₃ (1 <i>S</i> , <i>Z</i>)-3-(2-((1 <i>R</i> ,7 <i>aR</i> , <i>E</i>)-1-((2 <i>S</i>)-3-hydroxy-6-methylheptan-2-yl)-7 <i>a</i> -methyloctahydro-4 <i>H</i> -inden-4-ylidene)ethylidene)-4-methylenecyclohexan-1-ol	
20(OH)D ₃		25(OH)D ₃	
20 <i>S</i> -hydroxyvitamin D ₃ (1 <i>S</i> , <i>Z</i>)-3-(2-((1 <i>S</i> ,7 <i>aS</i> , <i>E</i>)-1-((<i>S</i>)-2-hydroxy-6-methylheptan-2-yl)-7 <i>a</i> -methyloctahydro-4 <i>H</i> -inden-4-ylidene)ethylidene)-4-methylenecyclohexan-1-ol		25-dihydroxyvitamin D ₃ (1 <i>S</i> , <i>Z</i>)-3-(2-((1 <i>R</i> ,7 <i>aR</i> , <i>E</i>)-1-((<i>R</i>)-6-hydroxy-6-methylheptan-2-yl)-7 <i>a</i> -methyloctahydro-4 <i>H</i> -inden-4-ylidene)ethylidene)-4-methylenecyclohexan-1-ol	

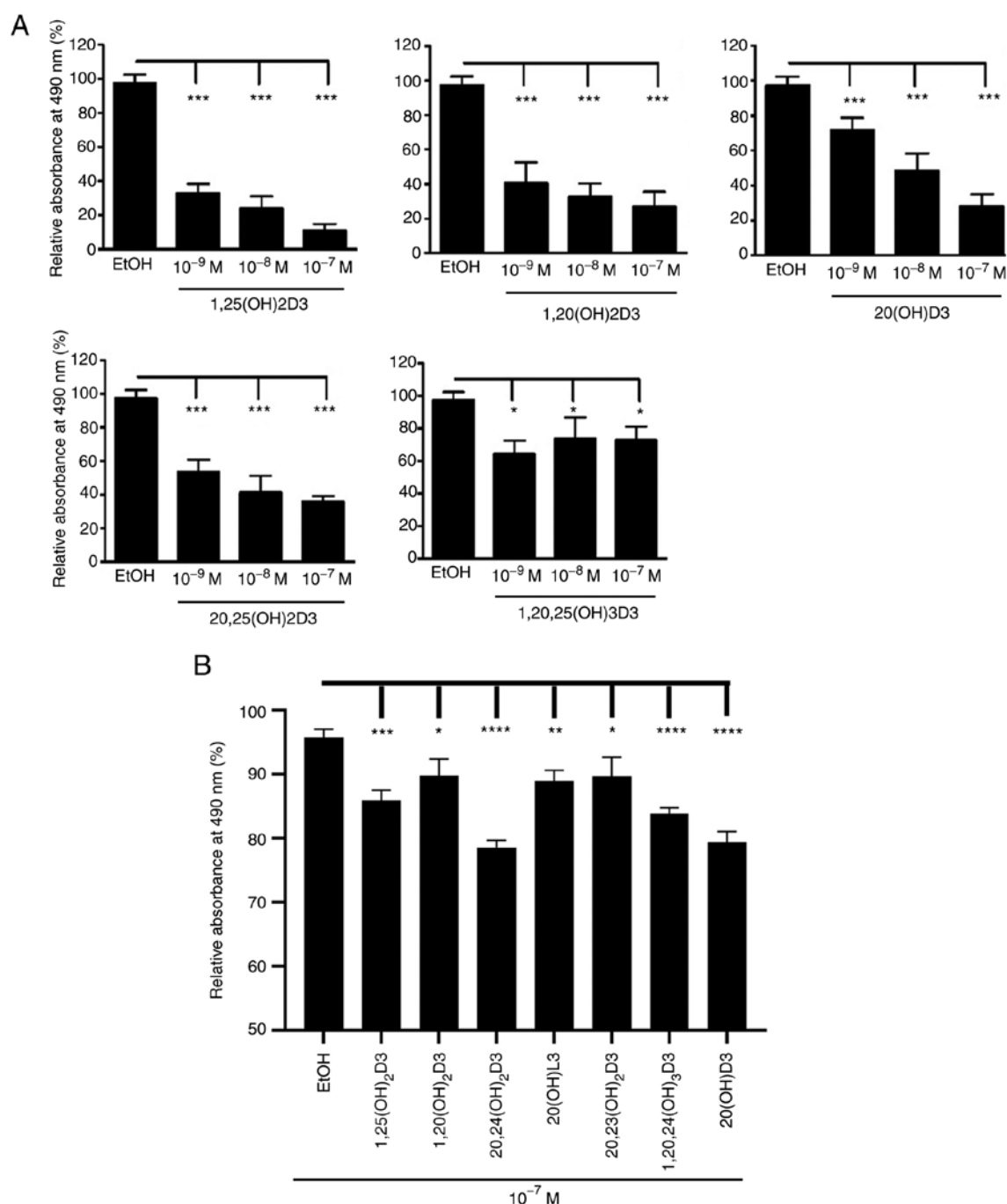


Figure 1. Inhibition of cell proliferation of (A) human A431 cells and (B) murine ASZ001 cells by hydroxy-derivatives of vitamin D3 in comparison with 1,25(OH)₂D₃ (positive) and ethanol (negative) controls. Cells were seeded in 96-well plates and treated with the listed secosteroids for 24 h and cell proliferation assessed using the MTS assay. Data are shown as means ± SEM (n=6) with ****P<0.0001; ***P<0.001; **P<0.01; *P<0.05 by (A) one way ANOVA with Dunnett's multi comparison post-hoc test or (B) Student's t-test. The concentrations of the secosteroids used are shown on the x axis.

proliferation of basal and squamous cell carcinoma cells in a dose-dependent manner (Fig. 1). Similar inhibitory effects were observed using the RBS assay (data not shown).

1,20,24(OH)₃D₃ and 1,25(OH)₂D₃ were selected to test the effect of secosteroids on colony formation by the murine basal cell carcinoma line, ASZ001 (Fig. 2). The two secosteroids caused a dose-dependent inhibition of the formation of large (>0.5 mm), medium (>0.25 mm) and small colonies (>0.1 mm). Using spheroid formation assays the antitumor activities of all the CYP11A1-derived hydroxyderivatives of vitamin D3 under study (Table I) were tested against the ASZ001 cell line (Fig. 3).

The inhibition of spheroid formation by different secosteroids, with or without hydroxyl group at C1α and in comparison with canonical 1,25(OH)₂D₃, is shown in Fig. 3A. Only 22(OH)D₃ and 17,20(OH)₂D₂ failed to significantly inhibit spheroid formation at the dose (10⁻⁷ M) tested. Secosteroids having a C1α hydroxyl group generally showed greater inhibition than those lacking this functional group. Fig. 3B and C show the effect of secosteroids on spheroid formation depending on their size. Similar inhibitory effects on spheroid formation by representative secosteroids containing a 1α-hydroxyl group, in comparison with 1,25(OH)₂D₃ (control) were observed in

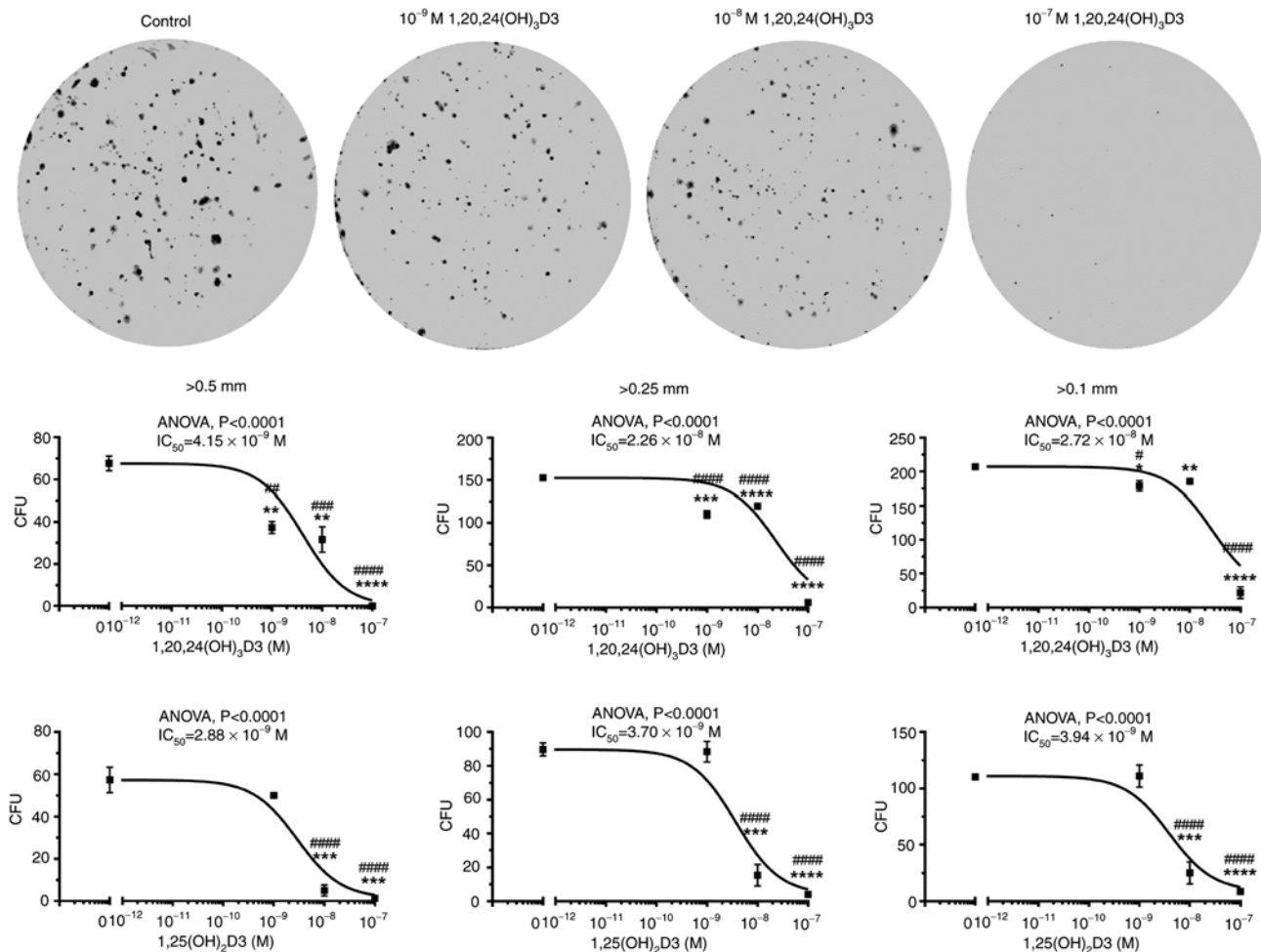


Figure 2. 1,20,24(OH)₃D₃ and 1,25(OH)₂D₃ inhibit colony formation by murine ASZ001 cells. Cells were plated in 6 well-plates (1,000 cells/well) and incubated with graded concentrations of the secosteroids for six days. After methanol fixation and staining with 0.1% crystal violet, the colonies sized >0.5, >0.25, >0.1 mm were counted using Cytation 5 and analyzed by Gene 5 software (BioTek Instruments, Inc.). Representative images for 1,20,24(OH)₃D₃ treatment are shown at the top of the Figure. Data points for the dose responses (lower panels) are shown as means \pm SEM (n=3) *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by Student's t-test; #P<0.05, ##P<0.01, ###P<0.001, ####P<0.0001 at one-way ANOVA with Dunnett's multi comparison post-hoc test. IC₅₀ values were calculated from the non-fitted dose-response curves.

A431 cell carcinoma cells (Fig. 4A). The dose dependency of selected secosteroids on spheroid formation by SCC13 squamous cells is shown in Fig. 4B with significant inhibition being observed already at 10⁻⁹ M.

As the authors have previously shown that the antitumor effects against oral squamous cell carcinoma are associated with inhibition of β -catenin and glioma-associated oncogene 1 (Gli1) (80), the effect of vitamin D-hydroxyderivatives on the expression of β -catenin and Gli1 were tested in the murine ASZ001 carcinoma line. The selected secosteroids, including 25(OH)D₃ and 1,25(OH)₂D₃ as well as CYP11A1-derived 20(OH)D₃ and 1,20(OH)₂D₃, significantly inhibited intracellular expression of these proteins (Fig. 5A-D). These effects were further substantiated by inhibition of the expression of *Gli1* and *Cttnb1* genes, by a panel of vitamin D derivatives (Fig. 5A-D). A similar inhibitory effect on Gli1 expression by 20(OH)D₃, 1,20(OH)₂D₃ and 1,25(OH)₂D₃, but not by 25(OH)D₃ was observed in the A431 line (Fig. 6A and B). The expression of β -catenin was not evaluated as it was below the level of detection by the procedure employed. The effects of 20(OH)D₃ and 1,20(OH)₂D₃ on involucrin expression was

also tested in comparison with 1,25(OH)₂D₃ in the A431 cells. Stimulation by all three secosteroids was observed (Fig. 6C-D).

SCC and BCC lines expressed VDR and ROR α and γ , which are receptors for vitamin D₃ hydroxyderivatives, as well as is the C1 α hydroxylase CYP27B1 (Fig. 7A and B). The relative expression of VDR and ROR γ in cell extracts was the highest in the ASZ001 line with ROR α being similar in the three lines. CYP27B1 expression was highest in the A431 cells and lowest in the ASZ001 cell extracts (Fig. 7A). The nuclear receptors showed both a cytoplasmic and nuclear staining pattern, while western blotting showed predominant location of the VDR in the cytoplasm of the ASZ001 line (Fig. 7A). The effects of representative vitamin D₃ hydroxyderivatives on the gene expression of vitamin D inactivating (*Cyp24A1*) and activating enzymes (*Cyp11a1* and *CYP27b1*) and nuclear receptors (*Vdr*, *Rora* and *Rorc*) were also tested (Fig. 7B). As expected, the secosteroids with a C1 α (OH) showed the strongest stimulation of the expression of *Cyp24a1*. A smaller stimulation of *Cyp27b1* expression (up to 1.5-fold) was seen with all the secosteroids tested except 1,20,24(OH)₃D₃ which showed no effect. Only a marginal effect was observed for

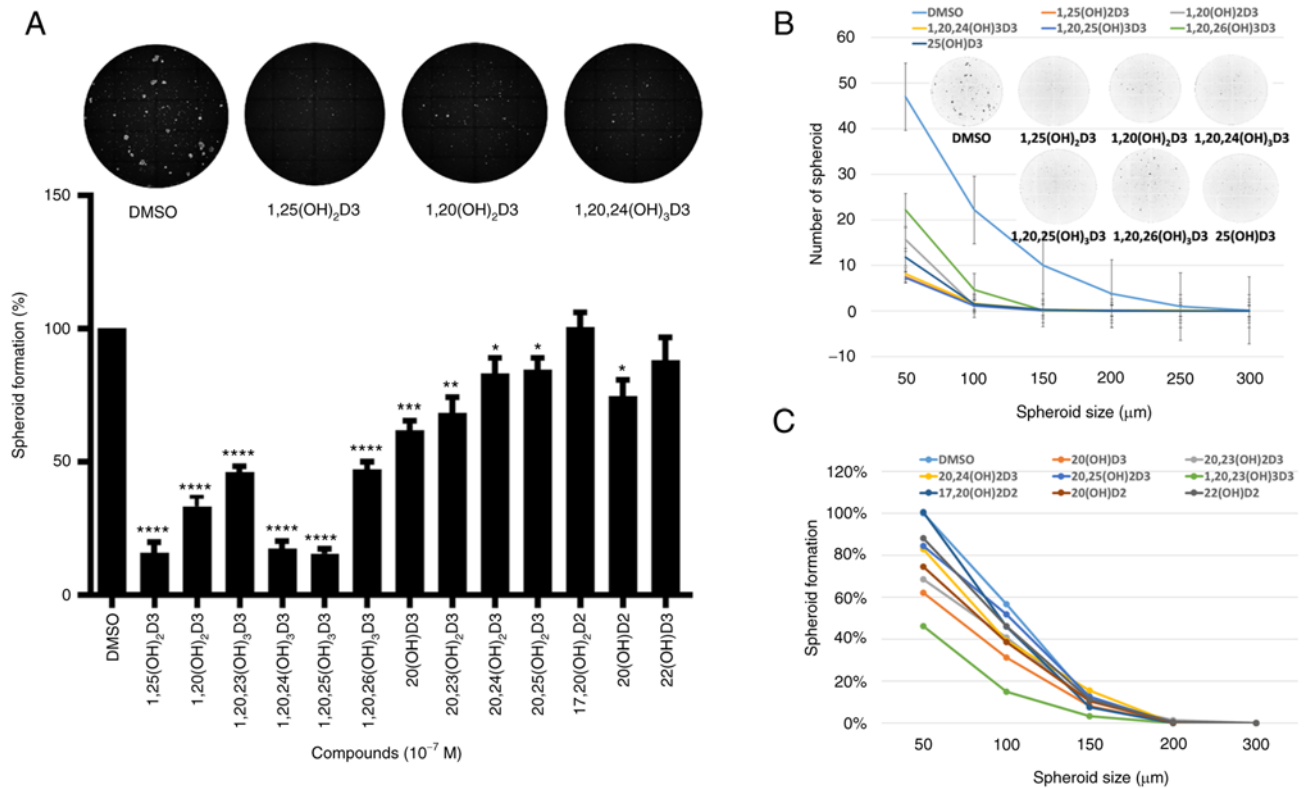


Figure 3. Inhibition of spheroid formation of murine ASZ001 cells by secosteroids. Cells suspended in tumerosphere medium (154CF phenol red free, 0.4% BSA, 20 ng/ml EGF, 10 ng/ml FGF, 5 μg/ml insulin) were treated with the listed secosteroids and seeded into low adhesion 24 well plates (500 cells/well) and incubated for 7 days at 37°C. (A) Spheroids ≥50 μm in size were counted using Cytation 5 (BioTek Instruments, Inc.). Representative images were taken by Cytation 5 at microscopic magnification 4x. Data are shown as means ± SEM (n=6) with *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by Student's t-test. Inhibition of spheroids formation in relation to (B) their size (means ± SD; n=6) or (C) means where SD ≤15% of the mean; n=6.

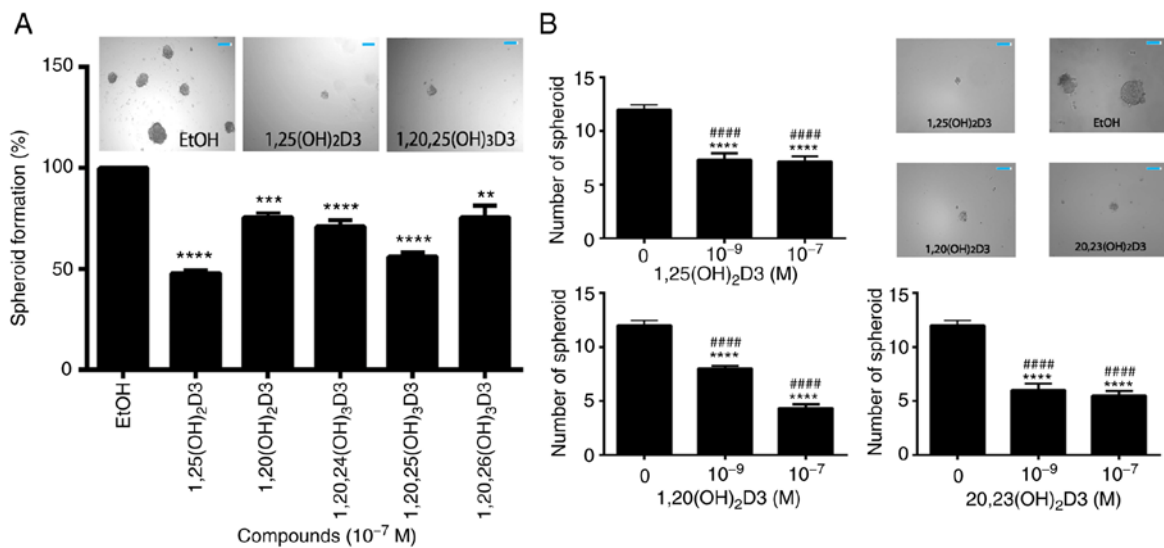


Figure 4. Inhibition of (A) human A431 and (B) human SCC13 cell spheroids formation by secosteroids. Cells suspended in tumerosphere medium (DMEM, 0.4% BSA, 20 ng/ml EGF, 10 ng/ml FGF, 5 μg/ml insulin) were treated with the selected secosteroids and seeded into low adhesion 96 well plates (1,000 cells/well) then incubated for 7 days at 37°C. Spheroids ≥50 μm in size were counted using Cytation 5 (BioTek Instruments, Inc.). Scale bar=200 μm. Data represent means ± SEM (n=6) with **P<0.01, ***P<0.001, ****P<0.0001 by the Student's t-test in A or ****P<0.0001 at one-way ANOVA with Dunnett's multi comparison post-hoc test.

Cyp11a1 expression with 1,20(OH)₂D₃ decreasing it and 1,25(OH)₂D₃ increasing it. The majority of the vitamin D₃ hydroxyderivatives tested stimulated *Vdr* gene expression to some degree, with 1,20,24(OH)₃D₃ showing the strongest

effect (1.8 fold). All the secosteroids had an inhibitory effect on *Rora*. The effects on *Rorc* were small and variable, mainly inhibiting expression but stimulating it in the case of 1,25(OH)₂D₃ (Fig. 7B).

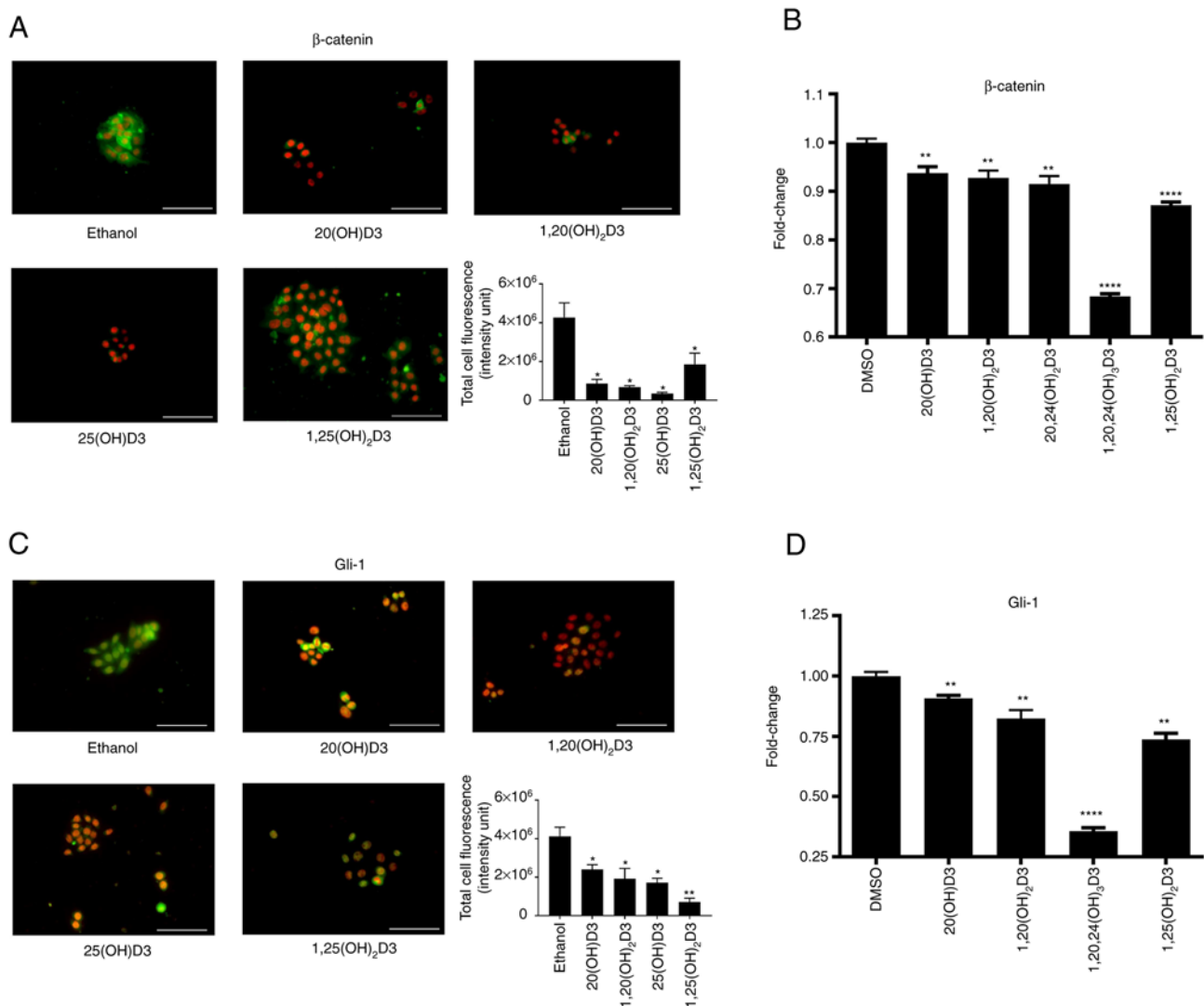


Figure 5. Inhibition of β -catenin and Gli1 signaling in the ASZ001 murine basal cell carcinoma cell line by vitamin D hydroxyderivatives. Cells were treated with 10^{-7} M of representative secosteroids in comparison with vehicle control (ethanol), for 24 h. The cells were stained with (A) murine antibodies against β -catenin (green) or (C) rabbit antibodies against Gli1 (green), or RNA was isolated for qPCR assay of (B) *Cttnb1* (gene coding β -catenin) or (D) *Gli1* (gene coding Gli1) gene expression. Cell nuclei are red (counterstained with propidium iodide). Scale bar=100 μ m. For protein expression the images were analyzed to calculate the corrected total cell fluorescence [CTCF=integrated density-(area of selected cell x mean fluorescence of background readings)] using ImageJ. For gene expression, data were normalized by the comparative $2^{-\Delta\Delta C_q}$ method followed by calculating the fold change, using β -actin as a housekeeping gene. Data are presented as means \pm SEM (n=4) with *P<0.05, **P<0.01, ****P<0.0001 by the Student's t-test.

The relative expression of the above nuclear receptors and megalin/LRP2 in normal skin (n=35), BCC (n=12), SSC *in situ* (n=22) and SCC (n=14) were analyzed by immunohistochemical staining. The characteristics of samples analyzed in this study are presented in Table SI. VDR, ROR α and ROR γ showed mostly nuclear staining, with very faint cytoplasmic staining in normal, peritumoral and cancerous tissues (Fig. 8). Megalin/LRP2 was localized mostly in the cytoplasm with less expression on cell membranes. Megalin/LRP2 was also found in normal, peritumoral and cancerous tissues (Fig. 8).

Semiquantitative analysis of the VDR and megalin/LRP2 expression showed similar levels of immunostaining in SCC *in situ* and invasive and unaffected peritumoral skin; however, their expression was significantly lower in comparison with normal control skin samples (Fig. 9A). Compared to BCC, the VDR expression level was similar in normal and peritumoral skin and BCC samples (Fig. 9). On the other

hand, megalin/LRP2 showed significantly lower expression levels in BCC in comparison with normal and peritumoral skin (Fig. 9A). ROR α and ROR γ expression in peritumoral skin was similar to normal skin, while their expression was significantly reduced in SCC (*in situ* and invasive) and BCC (Fig. 9A). This trend was also found for the mRNA expression of *RORA* and *RORC* (Fig. 9B) using the genomic data from the public repository (Gene Expression Omnibus, accession number GSE7553).

Discussion

The present study demonstrated that active forms of vitamin D3, including classical 1,25(OH)₂D3 and novel CYP11A1-derived hydroxyderivatives, show *in vitro* anticancer activities against human A431- and SCC13- and murine ASZ001-carcinoma cell lines as measured by cell proliferation assays and colony- and

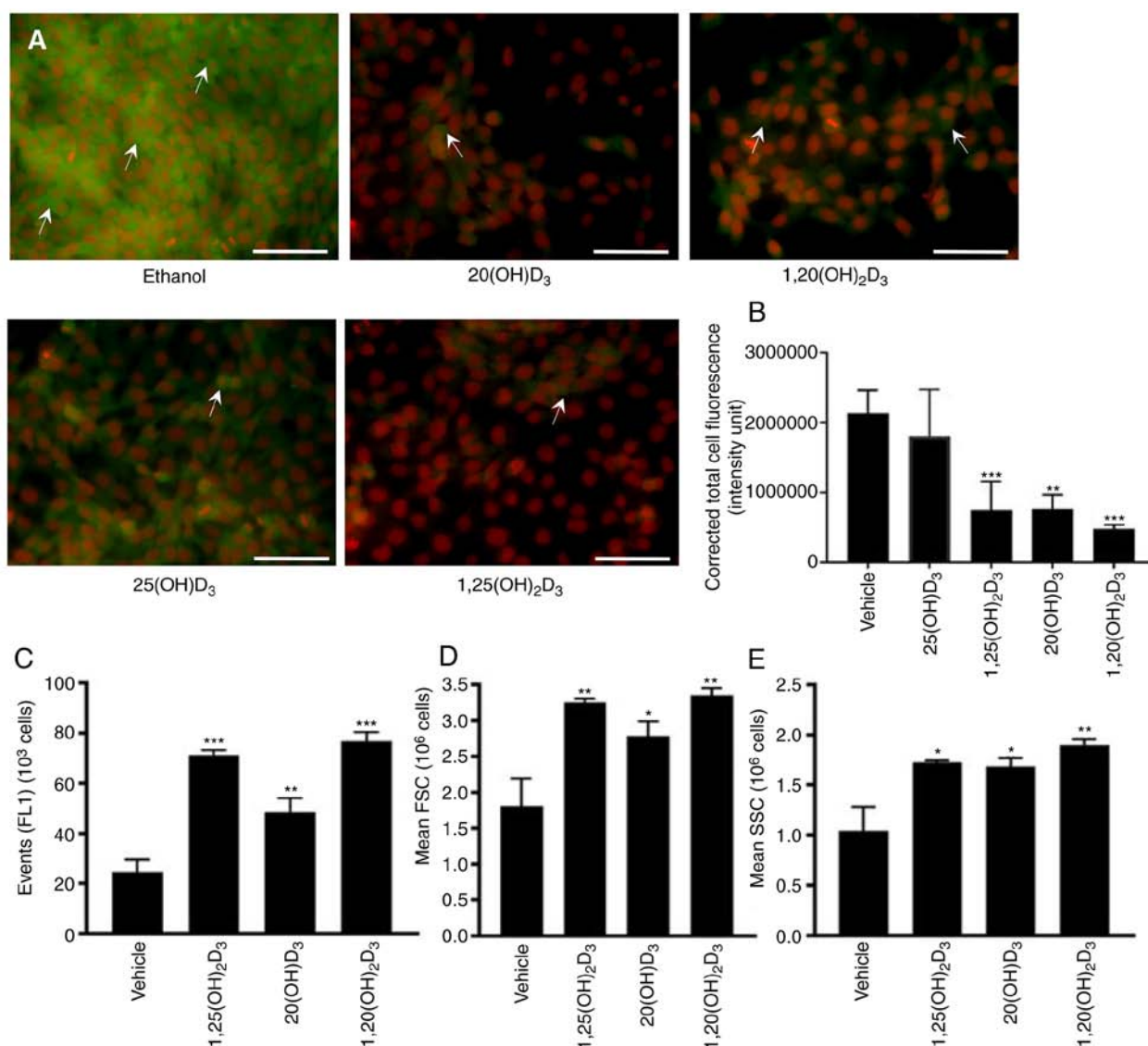


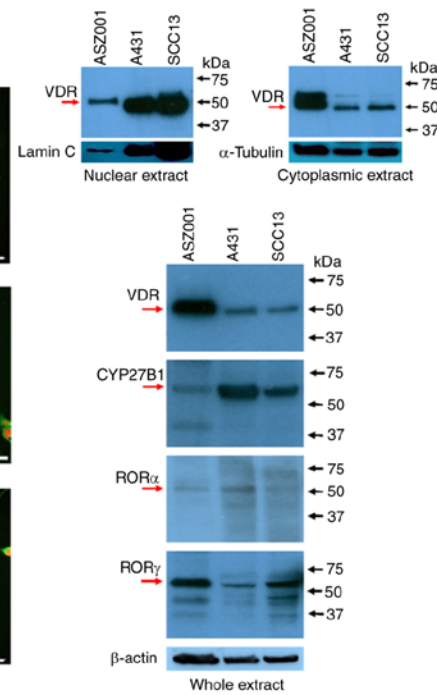
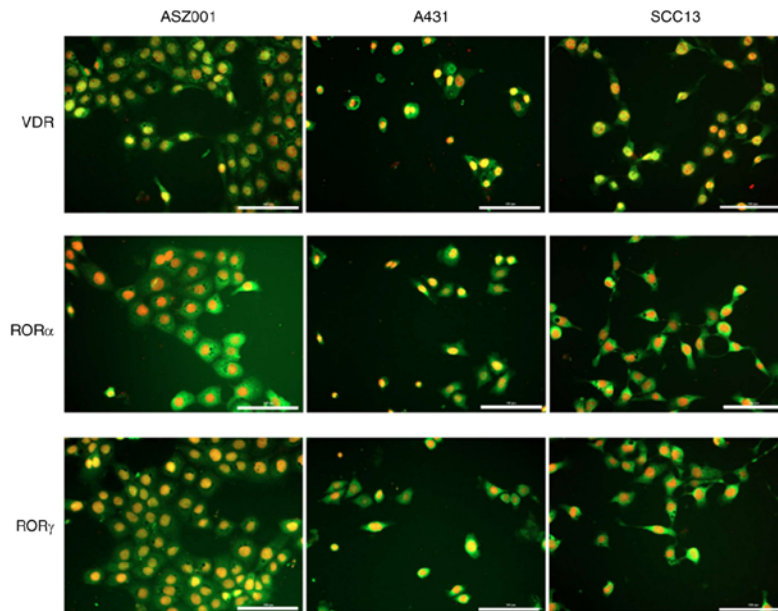
Figure 6. Inhibition of GLI1 and stimulation of involucrin expression by vitamin D3 hydroxyderivatives in the A431 human carcinoma cell line. (A) Immunohistochemistry panel showing inhibition of GLI1 (green, marked by arrows) in cells treated with 10^{-7} M of selected secosteroids in comparison with the vehicle (ethanol) control, for 24 h. Cell nuclei are red (counterstained with propidium iodide). Scale bar=100 μ m. (B) Quantification was performed as described in the legend to Fig. 5 and relative values are presented as means \pm SD (n=5) with * P <0.01, *** P <0.001 by Student's t-test. Stimulation of involucrin expression by 10^{-7} M of the secosteroids in comparison with the vehicle (ethanol) control. After 24 h of incubation cells were harvested, fixed in 2% PFA, stained with anti-involucrin antibody and read with a flow cytometer. Data are presented as (C) the number of positive events (FL-1 or green cells), (D) forward scatter (relative to cell size), or (E) side scatter (relative to cell granularity). Data are presented as means \pm SD (n=3) with * P <0.05, ** P <0.01, *** P <0.001 by the Student's t-test. Histograms (representative flow chart for each treatment) supporting evidence for the reported quantification is presented in Fig. S3.

spheroid-formation assays. While the anti-tumor effects against NMSC of 1,25(OH)₂D₃ were expected (27,77,86-92), the above data are the first reports on the inhibitory effects of CYP11A1-derived D3-hydroxyderivates on cutaneous lines of SCC and BCC. These data are also consistent with the ability of CYP11A1-derived vitamin D hydroxyderivatives to inhibit proliferation of primary and immortalized non-tumorigenic epidermal keratinocytes (51,55,93) and their anti-cancer properties in oral squamous cell carcinoma (80) and melanoma (94-96). The significance of these findings is emphasized by the lack or low calcemic activities of the best characterized of CYP11A1-derived hydroxyderivatives, namely 20(OH)D₃, 20,23(OH)₂D₃ and 1,20(OH)₂D₃, and their lack of measurable toxicity at pharmacological doses (39). Furthermore, they have been identified in the human body (29,31,34,45,97,98) and are

considered as natural products (35). Thus, CYP11A1-derived hydroxyderivatives of vitamin D are excellent candidates for principal or adjuvant therapeutics in the treatment of cutaneous SCC and BCC.

Some differences in the efficacies of the vitamin D3 hydroxyderivatives were observed depending on the cell line and the positions of the hydroxyl groups on the secosteroids, particularly the presence of the C1 α -hydroxyl group. While the anti-proliferative and anti-tumor effects of D3 hydroxyderivatives were similar in both SCC lines, compounds with a C1 α (OH) showed higher efficacy in the BCC line for spheroid formation. These differences can be rationalized by significantly higher expression of the VDR and lower expression of CYP27B1 in the ASZ001 line in comparison with A431 and SCC13 lines. Specifically, the VDR shows high selectivity

A



B

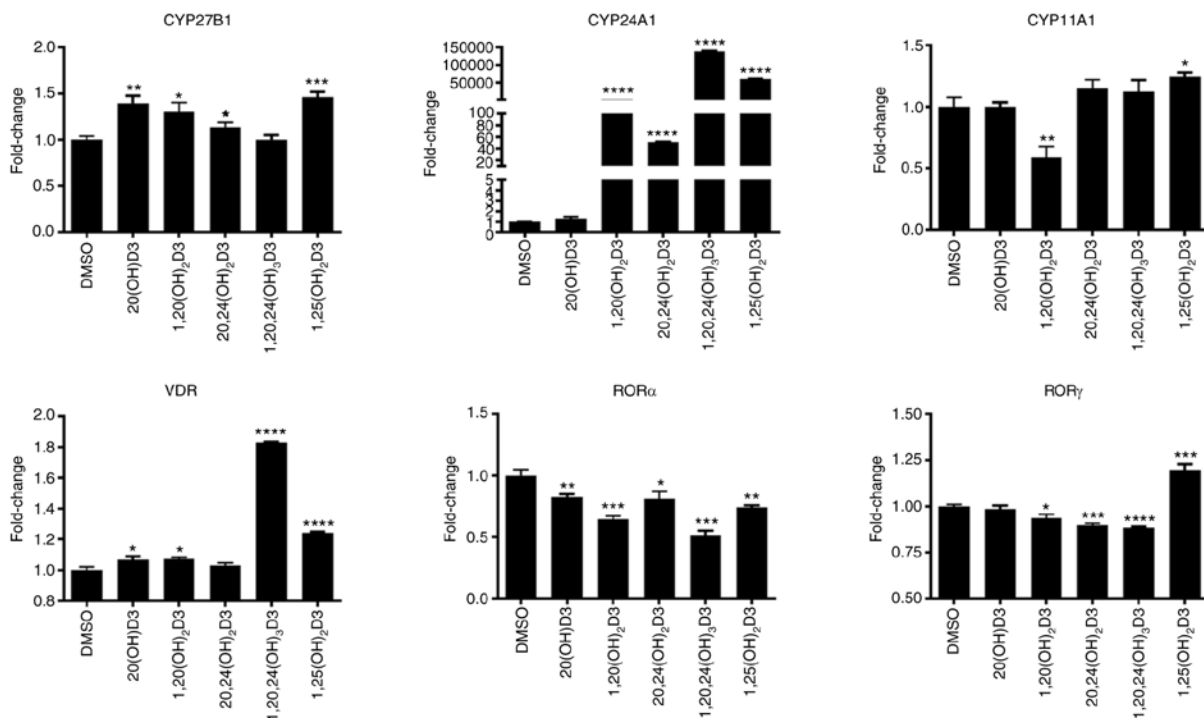


Figure 7. Expression of nuclear receptors for vitamin D3 hydroxyderivatives and CYP enzymes involved in vitamin D metabolism in murine ASZ001, human A431 and human SCC13 carcinoma lines. (A) Immunofluorescence detection (left panel) was performed using antibodies against VDR (MA1-710) and ROR α and ROR γ (54). The antigen is in green, while cell nuclei are red-counterstained with propidium iodide; scale bar=100 μ m. Right panel shows detection of VDR and ROR α and ROR γ by western blotting (arrows) using specific anti-receptor antibodies (see Materials and methods) for isolated cytoplasmic or nuclear fractions (VDR) from the cells or whole extracts (CYP27B1, ROR α and ROR γ). Human hepatoma and human melanoma SKMEL-188 cells were used as positive controls for ROR α and ROR γ . Loading was evaluated using antibodies against lamin C and α -tubulin for nuclear or cytoplasmic fractions, respectively, while with anti- β -actin for whole extracts. (B) shows expression at the mRNA level of *Cyp27b1*, *Cyp24a1*, *Cyp11a1*, *Vdr*, *Rora* and *Rorc*, respectively in ASZ001 murine carcinoma line treated with 10^{-7} M of the secosteroids listed on the x-axis. Data represent fold change, using β -actin as a housekeeping gene and are shown as means \pm SEM (n=4) with *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by Student's t-test. ROR, retinoid-related orphan receptor; VDR, vitamin D receptor.

for 1,25(OH) $_2$ D3 (48,99) and other C1 α -hydroxyderivatives of D3 (52,53,55). Thus, higher levels of VDR and its greater level in the cytoplasmic fraction of ASZ001 cells indicated

its relatively high availability in this BCC line for binding of C1 α -hydroxyderivatives, necessary for its translocation to the nucleus and induction of transcriptional activity. Furthermore,

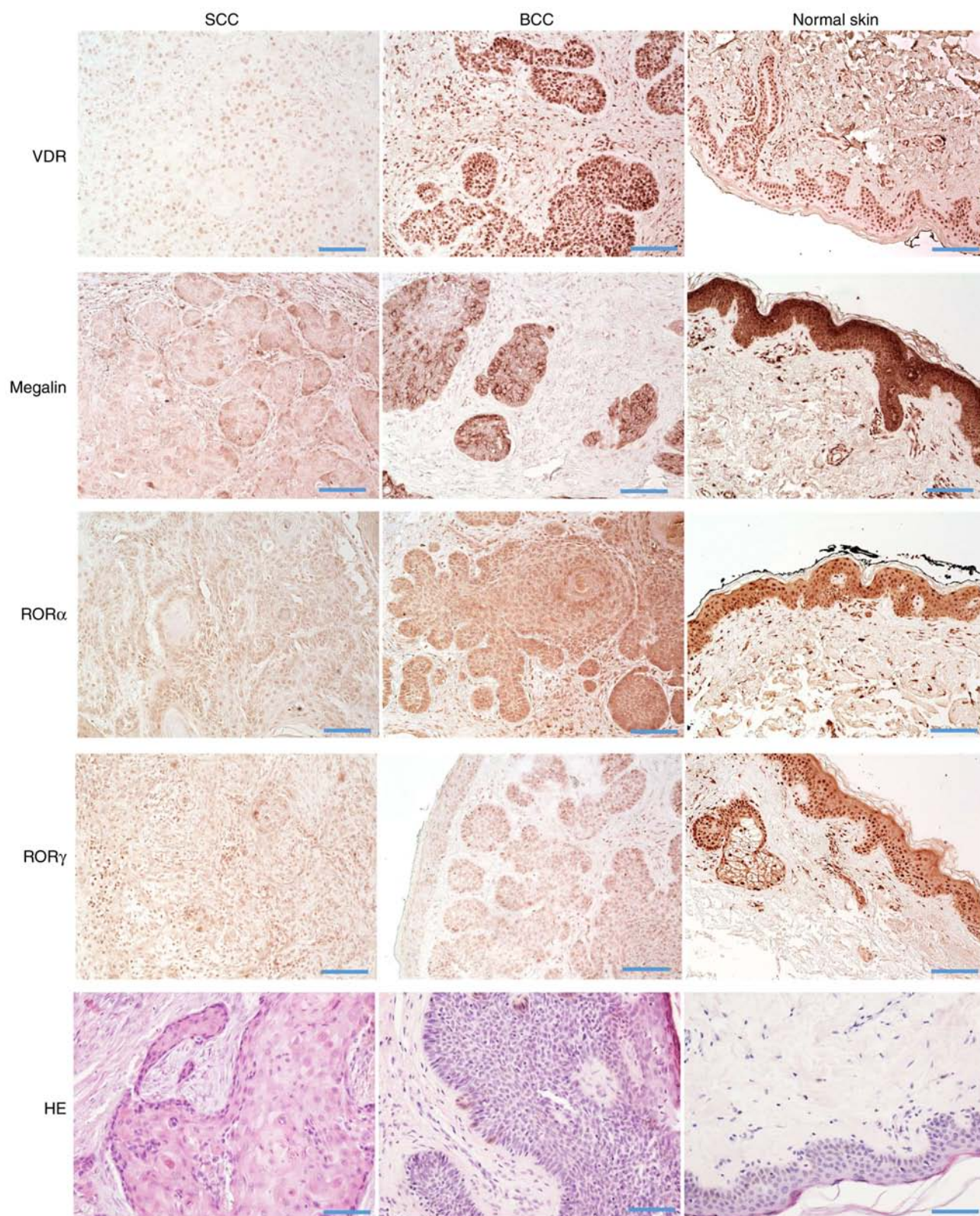


Figure 8. Immunohistochemical detection of VDR, ROR α , ROR γ , megalin and hematoxylin and eosin stained sections of human SCC (left column), BCC (middle column) and normal skin (right column) on archival formalin-fixed paraffin-embedded sections. Scale bar=100 μ m. Immunohistochemistry was performed on the on archival formalin-fixed paraffin-embedded sections. The methodology for IHC is described in the Materials and methods and with flow chart in Fig. S2. SCC, squamous cell carcinomas; BCC, basal cell carcinomas.

the relatively low expression of CYP27B1 in these cells pointed to less efficient hydroxylation of CYP11-derived hydroxyderivatives that lack a C1 α (OH), thus potentiating the difference between secosteroids with and without the C1 α (OH).

D3-hydroxyderivatives can also activate other nuclear and non-nuclear receptors besides the VDR (42,100). Specifically, CYP11A1-derived D hydroxyderivatives act as inverse agonists on ROR α and γ (54,55), which are expressed in

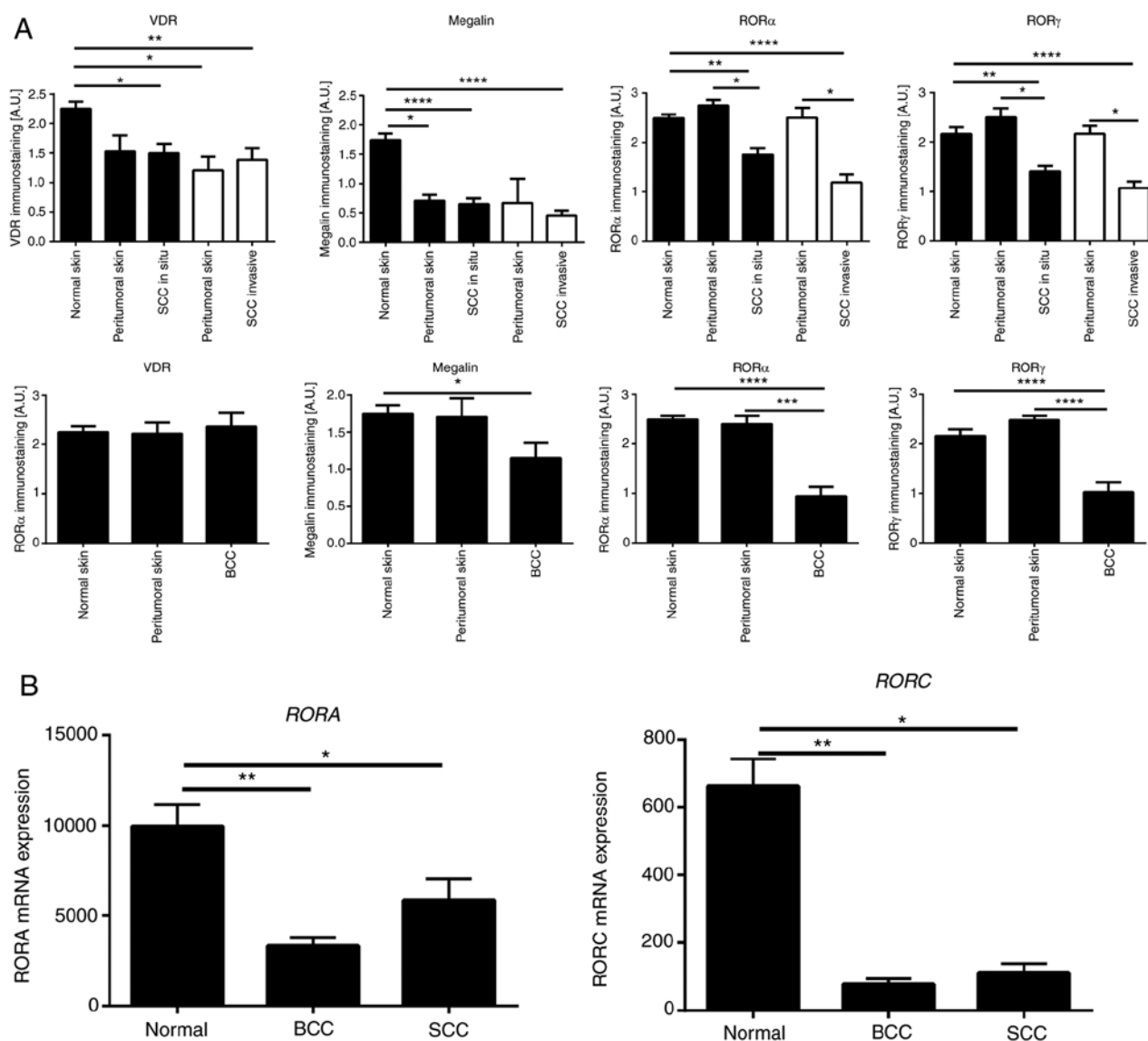


Figure 9. Quantitative analysis of nuclear receptor expression in SCC and BCC. (A) Quantitative comparison of immunostaining of VDR, ROR α , ROR γ and megalin in SCC (n=14), SCC *in situ* (n=22), BCC (n=12), normal (n=35) and peritumoral skin samples. Data are presented as mean values \pm SD. Statistically significant differences were determined with ANOVA followed by Dunn's multiple comparisons test with *P<0.05, **P<0.01, with ***P<0.001, ****P<0.0001. The immunohistochemical stained archival formalin-fixed paraffin-embedded sections were used for quantifications as described in the Materials and methods. (B) Quantitative comparison of mRNA expression of *RORA* (probe 226682_at) and *RORC* (probe 228806_at) in SCC (n=11), BCC (n=15) and normal skin (n=4) was performed using data from the genomic repository (Gene Expression Omnibus, accession number GSE7553 <http://www.ncbi.nlm.nih.gov/geo/>). Data are shown as means \pm SD. Statistically significant differences were determined with the ANOVA followed by Dunn's multiple comparisons test with *P<0.05, **P<0.01. SCC, squamous cell carcinomas; BCC, basal cell carcinomas; ROR, retinoid-related orphan receptor; VDR, vitamin D receptor.

these lines, providing additional regulatory targets. This is further substantiated by our study in which knocking out ROR γ in murine fibroblasts reverses the antiproliferative and anti-fibrotic effects of D3 hydroxyderivatives on these phenotypic traits (56). In other tumor models, the expression of these receptors decrease during melanoma progression (59) and there is a positive correlation between the expression of RORs and the expression of HIF-1 α (hypoxia-inducible factor 1 α) (60). Notably, D3-hydroxyderivatives can affect gene expression of enzymes involved in activation or inactivation of active forms of D3, as well as their receptors (Fig. 7B). Specifically, they caused a small stimulation of *CYP27B1* expression in a manner unrelated to the absence or presence of a C1 α (OH). They significantly stimulated *CYP24A1* [an inactivating enzyme (101,102)] expression with selectivity for compounds

containing a C1 α (OH). This further substantiated the selectivity of the derivatives containing a C1 α (OH) for the VDR, since *CYP24A1* is a well-established downstream target of the VDR (16,103). There was little effect on *CYP11A1* expression and small but variable stimulation of *VDR* expression by the various secosteroids. This is consistent with previous reports showing that *VDR* expression can be stimulated by active forms of vitamin D (30,104). All compounds inhibited *RORA* expression and the majority also inhibited *RORC* expression with the exception of 1,25(OH) $_2$ D3, which stimulated the expression of this gene. In summary, the actions of hydroxyderivatives of D3 are complex involving both VDR-dependent and independent (RORs) mechanisms. Their actions may also be influenced by their *in vivo* metabolism by vitamin D-activating and inactivating enzymes and by their abilities to regulate these

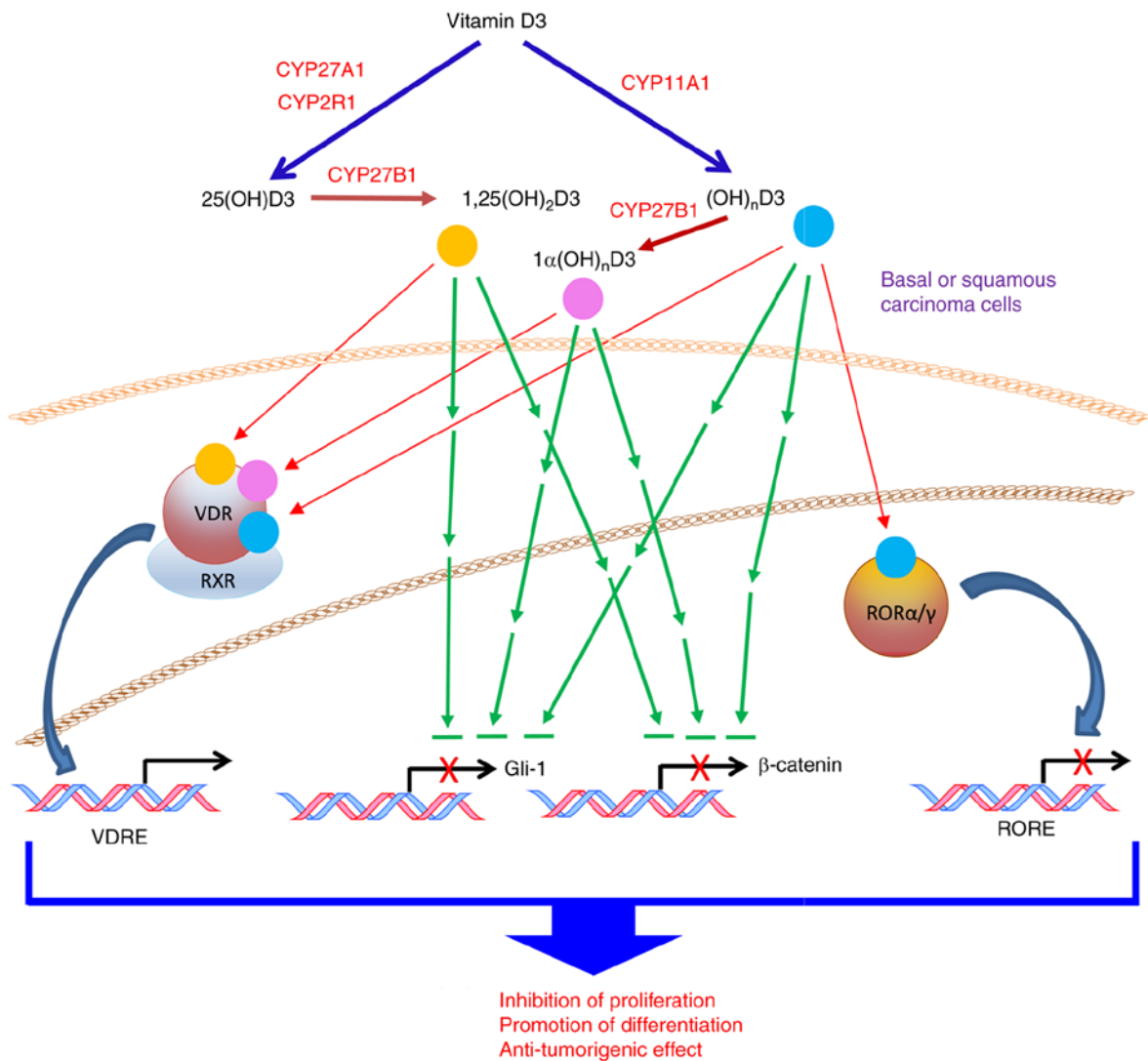


Figure 10. Proposed mechanism of action of novel vitamin D3 hydroxyderivatives. ROR, retinoid-related orphan receptor; VDR, vitamin D receptor.

enzymes, as well as their abilities to regulate expression of their target receptors. This opens exciting possibilities for future studies.

Application of vitamin D and of 1,25(OH)₂D3 can inhibit BCC development and growth through inhibition Hh signaling (27,77,87,88). Similarly, the authors have shown that D3 hydroxyderivatives can inhibit Hh signaling and β-catenin expression in oral SCC (80), while another study has shown that 1,25(OH)₂D3 inhibits β-catenin signaling in epidermal keratinocytes (105). In agreement with these studies the present study showed that classical and CYP11A1-derived D3 hydroxyderivatives can inhibit GLI1 and β-catenin expression in the murine ASZ001 cell line and Gli1 in the human A431 line, identifying these pathways as realistic targets for bioregulation.

Immunocytochemistry performed on human biopsies of SCC *in situ*, invasive SCC and BCC showed nuclear expression of all three receptors. This finding is consistent with previous reports on the VDR expression in NMSCs biopsies (90,106). However, some differences in the level of immunoreactivity require explanation. Studies by Reichrath *et al* (90,106) report an increase in VDR immunoreactivity in SCC and BCC

compared with normal skin. The present study showed a slight but significant decrease in VDR expression in SCC in comparison with normal skin and no difference in VDR expression between BCC and control skin. These quantitative differences could be secondary to differences in the patient population, fixation protocols, tissue processing or different antibodies used. However, in agreement with Reichrath *et al* (90,106), the present study did not observe any marked differences between different types of SCC and BCC and observed an inhibition of SCC growth *in vitro* by D3-hydroxyderivatives. With respect to RORα and γ, these receptors have a similar expression in peritumoral and normal skin and show comparatively lower expression in SCC and BCC. This pattern is consistent with decreased expression of the receptors observed during the progression of melanocytic lesions to a more malignant phenotype (59) or with decreased expression of corresponding genes in SCC and BCC as evaluated from the public genomics data repository (GSE7553). RORs are targets for CYP11A1-derived hydroxyderivatives (54,55). Therefore, a decrease in their expression may reduce the anti-tumor effects of the hydroxyderivatives via their inverse agonist activity on RORs. Finally, the present study observed decreased expression in the SCC and

BCC samples of transmembrane glycoprotein-LRP2/megalin, which is implicated in vitamin D₃ transport into the cell (66-68). The decrease in expression of RORs, VDR and LRP2/megalin was recently reported in hyperproliferative/inflammatory diseases, such as psoriasis (84). Thus, disturbed expression of LRP2/megalin, VDR, ROR α and ROR γ in skin cancerous lesions suggests defects in the vitamin D endocrine system in cancerous tissue that could facilitate tumor growth. Their detection in all SCC and BCC samples indicated that they can serve as realistic targets for therapeutic activity of novel vitamin D hydroxyderivatives. However, it should be noted that not only cancerous keratinocytes express the receptors for vitamin D and are targets for CYP11A1-derived hydroxymetabolites of vitamin D but they also regulate a differentiation program in normal epidermal keratinocytes. Specifically, hydroxymetabolites of vitamin can induce differentiation, inhibit proliferation, act as photoprotectants and as anti-aging and anti-inflammatory factors in normal epidermal keratinocytes (51,53,93,107,108).

In conclusion, the present study identified several CYP11A1-derived vitamin D analogs which are endogenously produced (29,31,33,34,45,98), as potential cutaneous anti-cancer therapeutics. Their anti-cancer activity can be mediated via different mechanisms. It is well known that 1,25(OH)₂D₃ promotes the differentiation of keratinocytes (93,109). The present study demonstrated that 20(OH)D₃, 1,20(OH)₂D₃ and 1,25(OH)₂D₃ also stimulated involucrin expression in skin cancer cells. The other mechanisms relate to effects on Hh/GLI1 and β -catenin signaling pathways uncontrolled activities of which promote cancer development and progression (107,110). The present study showed that GLI1 and β -catenin are downregulated following treatment with CYP11A1-derived D₃ hydroxyderivatives. Expression of nuclear receptors for these hydroxyderivatives were further observed in all samples of SCC and BCC, which potentially can be targeted *in vivo* for anti-tumor activity. This is the first time, to the best of the authors' knowledge, that a range of CYP11A1-derived vitamin D hydroxyderivatives have been tested against cutaneous SCC and BCC. The finding that RORs are expressed in skin cancer cells is also new. It has previously been shown that 1,25(OH)₂D₃ inhibits the growth of BCC in mice through the activation of the VDR and inhibition of Hh signaling, in a VDR-independent manner, with the effects being stronger than those of the cyclopamine (88). It was also reported that D₃ inhibits Hh signaling and growth of murine BCC cells to a similar degree as cyclopamine with an effect independent of the VDR (77). Notably, topical application of the D₃ prohormone can inhibit UVB-induced BCC with orally delivered D₃ having no effect (27). In addition, calcipotriol (analog of 1,25(OH)₂D₃) inhibits cutaneous cancerogenesis in mice (86) and in clinical studies shows a synergistic effect with other therapeutic modalities against actinic keratosis (86,111). While calcemic and other toxic effects place limits on the therapeutic use of 1,25(OH)₂D₃ or its synthetic derivatives at pharmacological doses, major CYP11A1-derived vitamin D derivatives are noncalcemic and non-toxic or show low calcemic effects at supra-pharmacological doses (37,39,63,64). They have also been detected in natural products (35) and show potent protective properties against UVB-induced damage (43,44). Besides the VDR they can act on other nuclear

receptors (42,57,112), including RORs (54), extending the therapeutic possibilities for their use. The present study provided exciting challenges for medicinal chemistry to develop more receptor-specific ligands based on the lead structures of the CYP11A1-derived hydroxyderivatives.

In summary, the present study identified CYP11A1-derived vitamin D hydroxyderivatives as excellent candidates for therapy or prevention of NMSCs, which can act on distinct nuclear receptors and inhibit GLI1 and β -catenin signaling pathways as a part of their anti-cancer activity with a proposed mechanism of action shown in Fig. 10.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ATS conceived and supervised the study, analyzed the data and wrote, revised and edited the manuscript. AAB performed the experiments, analyzed the data and wrote, revised and edited the manuscript. TKK performed the experiments, analyzed the data and drafted the manuscript. MME performed the experiments and analyzed the data. ZJ performed the experiments, analyzed the data and drafted the manuscript. SQ performed the experiments and analyzed the data. RMS performed the experiments, analyzed the data and drafted the manuscript. ASWO performed the experiments, analyzed the data and drafted the manuscript. CL performed the experiments, analyzed the data and drafted the manuscript. EP performed the experiments and analyzed the data. WL synthesized vitamin D derivatives, drafted the manuscript and revised and edited the manuscript. AMJ provided anti-RORs antibodies, analyzed the data and drafted, revised and edited the manuscript. RCT synthesized vitamin D derivatives and drafted, revised and edited the manuscript. EKYT synthesized vitamin D derivatives. CE analyzed the data and drafted, revised and edited the manuscript. MA analyzed data, provided cell lines and drafted, revised and edited the manuscript. ATS and AAB confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The authors declare that this investigation was carried out following the rules of the Declaration of Helsinki of 1975 (revised in 2008) and this study was approved by the Institutional Review Board of the University of Alabama at Birmingham under IRB-940831016 (OCCC Tissue Procurement CORE Facility) and IRB-00000726 (Title E150427002; Dr. A. Slominski PI).

Patient consent for publication

The IRB of the University of Alabama at Birmingham, which gave its permission for conducting this study, waived the requirement to obtain patients' informed consent for this research.

Competing interests

The authors declare that they have no competing interests.

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