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Design, Synthesis and Biological Activities of Novel Gemini 20*S*-Hydroxyvitamin D_3 Analogs

ZONGTAO LIN[1], **SRINIVASA R. MAREPALLY**[1], **TAE-KANG KIM**[2], **ZORICA JANJETOVIC**[2], **ALLEN SW. OAK**[2], **ARNOLD E. POSTLETHWAITE**[3,4], **LINDA K. MYERS**[3], **ROBERT C. TUCKEY**[5], **ANDRZEJ T. SLOMINSKI**[2,6], **DUANE D. MILLER**[1], and **WEI LI**[1]

[1]Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, Memphis, TN, U.S.A

[2]Department of Dermatology, University of Alabama at Birmingham, Birmingham, AL, U.S.A

[3]Department of Medicine, University of Tennessee Health Science Center, Memphis, TN, U.S.A

[4]Veterans Affairs Medical Center at Memphis, Memphis, TN, U.S.A

[5]School of Chemistry and Biochemistry, University of Western Australia, Crawley, WA, Australia

[6]Veterans Affairs Medical Center at Birmingham, Birmingham, AL, U.S.A

Abstract

Vitamin D_3 (D_3) can be metabolized by cytochrome P450scc (CYP11A1) into 20S-hydroxyvitamin D_3 ($20D_3$) as a major metabolite. This bioactive metabolite has shown strong antiproliferative, antifibrotic, pro-differentiation and anti-inflammatory effects while being non-toxic (non-calcemic) at high concentrations. Since D_3 analogs with two symmetric side chains (Gemini analogs) result in potent activation of the vitamin D receptor (VDR), we hypothesized that the chain length and composition of these types of analogs also containing a 20-hydroxyl group would affect their biological activities. In this study, we designed and synthesized a series of Gemini $20D_3$ analogs. Biological tests showed that some of these analogs are partial VDR activators and can significantly stimulate the expression of mRNA for VDR and VDR-regulated genes including CYP24A1 and transient receptor potential cation channel V6 (TRPV6). These analogs inhibited the proliferation of melanoma cells with potency comparable to that of 1α,25-dihydroxyvitamin D_3. Moreover, these analogs reduced the level of interferon γ and up-regulated the expression of leukocyte associated immunoglobulin-like receptor 1 in splenocytes, indicating that they have potent anti-inflammatory activities. There are no clear correlations between the Gemini chain length and their VDR activation or biological activities, consistent with the high flexibility of the ligand-binding pocket of the VDR.

Keywords

20-Hydroxyvitamin D_3; anti-inflammation; anti-proliferation; Gemini analogs; CYP24A1; vitamin D receptor

Correspondence to: Wei Li, Ph.D., 881 Madison Avenue, Room 561, Memphis, TN 38163, U.S.A. Tel: +1 9014487532, Fax: +1 9014486828, wli@uthsc.edu.

The production, activation and metabolism of vitamin D_3 (D_3) involves the participation of a variety of tissues and organs (1). In the epidermis of the skin, D_3 is produced from ultraviolet (UV) irradiation causing photoconvesion of 7-dehydrocholesterol (7DHC) to pre-D_3, which undergoes thermal isomerization to D_3. On the systemic level, activation of D_3 involves initial hydroxylation in the liver by a 25-hydroxylase [cytochrome *P450 2R1* (CYP2R1) or cytochrome *P450* (CYP27A1)] followed by 1α-hydroxylation by CYP27B1 in the kidney to form its biological active form, 1α,25-dihydroxyvitamin D_3 ($1,25D_3$, Figure 1). $1,25D_3$ acts through the vitamin D receptor (VDR) and regulates the expression of a variety of genes involved in immunomodulation, anti-inflammation, anti-proliferation, pro-differentiation, anti-angiogenesis, pro-apoptosis, mineral homeostasis and vitamin D catabolism (2, 3). One of these genes, *CYP24A1*, encodes D_3 24-hydroxylase which sequentially oxidizes the side chain of $1,25D_3$ producing 1α,24R,25-trihydroxyvitamin D_3 ($1,24,25D_3$), 24-oxo-$1,25D_3$, 24-oxo-1α,23,25-trihydroxyvitamin D_3, 23-oxo-24,25,26,27-tetranor-1α,-hydroxyvitamin D_3, and finally calcitroic acid for excretion (4, 5).

We previously reported the identification and characterization of a new metabolic pathway, in addition to the classical pathway described above, for activation of D_3 (6–9). This pathway is initiated by mammalian CYP11A1 (also known as cytochrome *P450scc*) acting on D_3 to produce 20S-hydroxyvitamin D_3 ($20D_3$) as the major product (7, 10), which can be further metabolized into di- and tri-hydroxy products by CYP11A1 and other vitamin D-metabolizing enzymes such as CYP24A1, CYP27A1 and CYP27B1 (4, 7, 11). Importantly, $20D_3$ and its di- and tri-hydroxymetabolites accumulate *in vivo* in the human epidermis and serum (12). $20D_3$ produces a number of biological effects similar to those of $1,25D_3$, acting as a biased agonist on the VDR (7, 13), but lacks the calcemic effect. High doses of $20D_3$ (up to 30 μg/kg) do not cause hypercalcemia in rats or mice, while $1,25D_3$ has substantial hypercalcemic effects (toxicity) at a dose as low as only 2 μg/kg (14, 15). Thus $20D_3$ has the potential to be used at therapeutic doses without toxicity. $20D_3$ displays antiproliferative, pro-differentiation and anti-inflammatory properties in many cell lines (7, 16). In addition, $20D_3$ is able to inhibit the growth of solid tumors (17) and leukemia (14), indicating its tumorostatic activities, and it has shown antifibrotic activity *in vitro* and *in vivo* (15, 18, 19).

Gemini analogs of D_3 are characterized by having two symmetric side chains at C20. Many Gemini D_3 analogs have been synthesized to investigate the contribution of the extra side chain to their drug-like properties (20, 21). To determine the effects of chain length and composition in the Gemini analogs also possessing a 20-hydroxyl group, we designed and synthesized a series of $20D_3$ Gemini analogs (Figure 2) based on our established synthetic route (16). Biological activities including VDR activation, expression of VDR-regulated genes, and inhibition of proliferation and inflammation were investigated for these Gemini analogs by comparison of their properties with those of positive controls.

Materials and Methods

Chemicals

The starting material pregnenolone acetate was purchased from Bosche Scientific LLC (New Brunswick, NJ, USA) with a purity above 98% as determined by high-performance liquid chromatography (HPLC). HPLC-grade acetonitrile was purchased from Fisher Scientific

(Hampton, NH, USA). De-ionized water was prepared by a Milli-Q purification system for the HPLC mobile phases. Vitamin D_3 (Sigma-Aldrich, St. Louis, MO, USA) was used as standard reference to generate HPLC standard curves to quantify small aliquots of vitamin D_3 analogs.

General methods for chemistry

All reagents and solvents for the synthesis and separation were purchased from commercial sources and were used as received. Reactions of 5,7-diene compounds were carried out in the dark by wrapping the flasks with aluminum foils. Moisture- or air-sensitive reactions were performed under an argon atmosphere. All reactions were routinely monitored by thin layer chromatography (TLC) on silica gel using ethyl acetate and hexane as mobile phases, and visualized by 5% phosphomolybdic acid in ethanol or UV lights. Mass spectra of all compounds were obtained by a Bruker ESQUIRE-LC/MS system (Bruker Corporation, Billerica, MA, USA) equipped with an electrospray ionization (ESI) source. Nuclear magnetic resonance (NMR) spectra were recorded by either a Bruker Avance III 400 MHz or an Agilent Unity Inova 500 MHz spectrometer. High-resolution mass spectrometry (HRMS) was carried out based on our previous methods (22, 23) by a Waters Acquity™ ultra-performance liquid chromatography system (Milford, MA, USA) equipped with a Waters Xevo™ G2-S quadrupole time-of-flight mass spectrometer and an ESI source in positive mode. Ethyl acetate was used for extraction of reaction mixtures and then dried over anhydrous Na_2SO_4, filtered and removed using a rotary evaporator under reduced pressure.

In brief, starting material **1** underwent haloform reaction with high yield (96%) to give the 3-hydroxyl acid **2** following our previously published procedure (24). To generate the 20-hydroxyl group, the acid of **2** was firstly esterified, with quantitative yield, by methanol in the presence of sulfuric acid, to produce methyl ester **3**. The ester was then ready for the introduction of the same two aliphatic side chains in one Grignard reaction. The 3-hydroxyl group on **3** was then protected to give ester **4**, following our established acetylation procedure (3), for the introduction of C7 double bond. The 5,7-diene structure was then introduced into **4** by free radical reaction under dibromatin/azo*bis*isobutyronitrile/tetra-*n*-butylammonium bromide/tetra-*n*-butylammonium fluoride conditions to provide the protected methyl ester **5** with acceptable yield (45%). Under dark conditions, the Grignard reaction was carefully carried out using different Grignard reagents in THF to introduce the two side chains to the C20 position where the 20-hydroxyl was formed as a result. Irradiation (15 min) of the 7DHC analogs in ethyl ether by UVB, followed by 3 hours reflux in ethanol gave D_3 structures (10) together with their parent 7DHC, pre-vitamin D_3 (7), lumisterol (8) and tachysterol (9) structures as related impurities. The separation of compounds in the reaction mixture after irradiation was achieved by preparative HPLC using a gradient of acetonitrile in water to afford the final D_3 compounds (10) with high purity (>98%) for biological testing.

HPLC conditions

An Agilent HPLC 1100 series system consisting of a binary pump, a column oven, a degasser, a diode array detector and an autosampler was used for chromatographic analysis. The purity check of 20D_3 analogs was carried out on a Phenomenex Luna-PFP C18 column

(5 μm, 250 mm × 4.6 mm; Phenomenex) maintained at 25°C. The flow rate was 1.0 ml/min. The UV absorption at 263 nm was set for displaying the chromatograms. Isocratic elution using water (A) and acetonitrile (B) was as follows: 60% B for compound **10a**, 70% B for compound **10b**, 70% B for compound **10c**, 80% B for compound **10d** and 90% B for compound **10e**.

Cell culture

Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose, L-glutamine, pyridoxine hydrochloride, 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin antibiotic solution (Sigma-Aldrich, St. Louis, MO, USA) was used to culture immortalized human keratinocytes (HaCaT). Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotic solution. Eagle's minimal essential medium (EMEM) containing 9% charcoal-stripped FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, non-essential amino acids, 2.5 mM 2-mercaptoethanol and 2.5 mM L-glutamine was used to culture splenocytes from DBA/1 mice. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

VDRE-luciferase reporter assay

Jurkat cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were transduced with lentiviral VDRE luciferase using a Cignal Lenti VDRE Reporter (luc) Kit according to the manufacturer's protocol (QIAGEN, Valencia, CA, USA). The cells then went through 1-week selection under puromycin (1.0 μg/ml) treatment. During the selection, the media were changed every other day. For the biological test, transduced Jurkat cells were washed with PBS (1×) and then seeded in a 96-well plate (10,000 cells/well, 100 μl/well) diluted by FBS-free media. All cells were then synchronized by a 24 h incubation. DMSO solutions (10%; 1.0 μl) of each secosteroid were added to each well of cells with final concentrations of 1000, 300, 100, 30, 10, 3 and 1 nM, which were then incubated for another 24 h. The luciferase signal of the cells was measured by the ONE-Glo™ Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's suggested procedure. DMSO (10%) in water was used as the vehicle control and the final concentration of DMSO in culture media was 0.1% during the activity test. Each concentration of analog was tested in triplicate (n=3).

Real-time polymerase chain reaction (RT-PCR)-based expression analysis

HaCaT cells were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and were cultured as described above. The RNA from HaCaT keratinocytes treated with *10a–e*, $1,25D_3$, 22-oxa-$1,25D_3$ or DMSO was isolated using the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA, USA). Reverse transcription (100 ng RNA/reaction) was performed with the Transcriptor First Strand cDNA Synthesis Kit (Roche Inc., Mannheim, Germany). Real-time PCR was performed using cDNA diluted 10-fold in sterile water and a SYBR Green PCR Master Mix. The primers for both forward and reverse lines for *VDR, CYP24A1* and *TRPV6* genes were designed based on the mouse and rat sequences using Primer Quest software (Integrated Device Technology, San Jose, CA, USA). Reactions (in triplicate) were performed at 50°C for 2 min, 95°C for 10 min and then 40 cycles of 95°C

for 15 s, 60°C for 30 s and 72°C for 30 s. Data were collected and analyzed on a Roche Light Cycler 480. The amount of the final amplified product for each gene was compared and normalized to the amount of β-actin as a housekeeping gene using a comparative Ct method (25).

Antiproliferative assay

Antiproliferative assay was performed using the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)/phenazine methosulfate (MTS/PMS) solution (Promega, Madison, WI, USA) as per the manufacturer's instructions (26). Briefly, SKMEL-188 cells were plated in on 96-well plates with Ham's F10 media containing 5% charcoal treated FBS (Atlanta Biologicals, Inc. Flowery Branch, GA, USA). After overnight culture, the medium was changed to serum-free medium to synchronize the cells for 24 h. Subsequently, the cells were incubated with compounds **10a–e** for 48 h. Finally, 20 μl of MTS/PMS solution was added to the cells and they were incubated for another 4 h at 37°C then the absorbance was recorded at 490 nm using Cytation 5 Cell Imaging Multi-Mode Reader (Winooski, VT, USA).

Interferon-γ (IFNγ)-inhibition assay

Compounds **10a–e** and $1,25D_3$ were solubilized in absolute EtOH at 10^{-4} M and diluted to 10–6 M by adding EMEM as described above (27). Splenocytes from DBA/1 mice were isolated, erythrocytes lysed by hypotonic shock, then cells were washed twice with EMEM, and suspended at 2×106/ml in supplemented EMEM described above. To each well of a 48-well tissue culture plate, 450 μl of the splenocytes were added. Analogs (50 μl of the 10^{-6} M stock), or EtOH diluted 1:100 with the above culture medium as negative control, were added to triplicate wells and then incubated at 37°C in 5% CO_2 in a humidified tissue culture incubator for 2 h, after which 1.0 μg/well of rat monoclonal antibody to mouse cluster of differentiation 3 (CD_3) was added. After a 72 h incubation, supernatants from each well were harvested and analyzed by the enzyme-linked immunosorbent assay (ELISA) to determine the levels of D-murine IFNγ (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The concentration of IFNγ in supernatants from cultures containing analogs were compared with that of EtOH-treated control cultures, by ANOVA. Results are expressed as the mean of triplicate determinations±SEM.

Assay of leukocyte-associated immunoglobulin-like receptor 1 (LAIR1) by flow cytometry

Splenocytes from DBA/1 mice were isolated (3) and the level of expression of LAIR1 was determined following overnight culture with each analog at a concentration of 10^{-7} M (or ethanol as vehicle control) by multi-parameter flow cytometry using an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). Cells were labeled with fluorochrome antibodies specific for CD4 (BD Biosciences) and for LAIR1 (eBioscience, San Diego, CA, USA). Gating was performed on CD4+ cells and the data were displayed as mean fluorescence±standard deviation. A minimum of 10,000 cells were analyzed from each treated sample and the final analysis was performed using Flow software (Tree Star, Ashland, OR, USA). Results are expressed as the mean of duplicate values±SEM.

Statistical analysis

The values are reported as the means±SD (or SE). The significance of the differences between different treatments was estimated by unpaired, two-tailed Student's *t*-test with $p<0.05$ considered as being statistically significant. All statistical analyses were performed and some of the figures were produced using GraphPad Prism 6.0 (Graph-Pad Software, San Diego, CA, USA).

Results

The designed $20D_3$ analogs were synthesized starting from commercially available pregnenolone acetate (1). Due to the sensitivity of 5,7-diene structure to light, heat and acidic conditions, we chose to introduce the double bond at the C7 position in later steps (3).

Characterization of $20D_3$ analogs gave the following results

(3S,8S,10R,13S,14S,17S)-3-hydroxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene-17-carboxylic acid (2)—^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.19 (dd, *J*=4.9, 2.2 Hz, 1H), 3.31 (tt, *J*=10.7, 4.8 Hz, 1H), 3.20 (p, *J*=1.6 Hz, 2H), 2.19 (t, *J*=9.3 Hz, 1H), 2.09 (qdd, *J*=13.1, 9.0, 4.0 Hz, 2H), 1.99−1.79 (m, 3H), 1.72 (t, *J*=3.6 Hz, 1H), 1.55 (tdd, *J*=12.2, 6.9, 3.6 Hz, 1H), 1.42 (ddt, *J*=16.5, 12.7, 3.0 Hz, 2H), 1.37−1.23 (m, 3H), 1.12 (tdd, *J*=12.0, 7.8, 5.1 Hz, 2H), 1.03−0.88 (m, 2H), 0.86 (s, 3H), 0.84−0.77 (m, 1H), 0.57 (s, 3H). MS (ESI) *m/z* 317.1 [M-H]–.

(3S,8S,10R,13S,14S,17S)-methyl 3-hydroxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene-17-carboxylate (3)—^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.36 (dt, *J*=5.4, 2.0 Hz, 1H), 3.68 (s, 3H), 3.53 (tt, *J*=11.2, 4.7 Hz, 1H), 2.36 (t, *J*=9.3 Hz, 1H), 2.33−2.17 (m, 2H), 2.17−2.08 (m, 1H), 2.00 (ddt, *J*=7.5, 4.9, 2.7 Hz, 1H), 1.84 (dddd, *J*=19.4, 13.8, 7.1, 3.3 Hz, 3H), 1.77−1.65 (m, 1H), 1.65−1.56 (m, 2H), 1.56−1.45 (m, 2H), 1.45−1.38 (m, 1H), 1.36−1.21 (m, 2H), 1.17−1.04 (m, 2H), 1.02 (s, 3H), 1.00−0.93 (m, 1H), 0.92−0.80 (m, 1H), 0.68 (s, 3H). MS (ESI) *m/z* 355.4 $[M+Na]^+$.

(3S,8S,10R,13S,14S,17S)-methyl 3-acetoxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene-17-carboxylate (4)—^{1}H NMR (400 MHz, Chloroform-d) δ 5.37 (dt, *J*=5.1, 1.6 Hz, 1H), 4.71−4.50 (m, 1H), 3.67 (s, 3H), 2.40−2.32 (m, 2H), 2.32−2.25 (m, 1H), 2.22−2.08 (m, 1H), 2.05 (s, 1H), 2.04 (s, 1H), 2.02−1.95 (m, 1H), 1.92−1.83 (m, 2H), 1.83−1.75 (m, 1H), 1.75−1.67 (m, 1H), 1.67−1.63 (m, 1H), 1.63−1.53 (m, 2H), 1.53−1.37 (m, 2H), 1.35−1.21 (m, 3H), 1.20−1.06 (m, 2H), 1.02 (s, 3H), 1.01−0.94 (m, 1H), 0.92−0.77 (m, 1H), 0.67 (s, 3H). MS (ESI) *m/z* 397.4 $[M+Na]^+$.

(3S,10R,13S,14R,17S)-methyl 3-acetoxy-10,13-dimethyl-2,3,4,9,10,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthrene-17-carboxylate (5)—^{1}H NMR (400 MHz, Methanol-d_4) δ 5.59 (dd, *J*=5.8, 2.6 Hz, 1H), 5.48−5.41 (m, 1H), 4.66 (tdd, *J*=11.4, 4.9, 3.9 Hz, 1H), 3.69

(s, 3H), 2.55−2.50 (m, 1H), 2.49 (q, *J*=2.9 Hz, 1H), 2.38 (ddd, *J*=14.3, 11.9, 2.3 Hz, 1H), 2.24−2.15 (m, 1H), 2.12 (ddd, *J*=12.8, 3.9, 2.5 Hz, 1H), 2.08−2.05 (m, 3H), 2.04 (s, 3H), 1.99−1.80 (m, 3H), 1.71 (ddt, *J*=11.4, 9.8, 5.9 Hz, 2H), 1.65−1.52 (m, 2H), 1.39 (tt, *J*=14.6, 5.4 Hz, 2H), 0.97 (s, 3H), 0.63 (s, 3H). MS (ESI) *m/z* 395.4 $[M+Na]^+$.

(3S,10R,13S,14R,17S)-17-(2-hydroxypropan-2-yl)-10,13-dimethyl-2,3,4,9,10,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol (6a)—^{1}H NMR (400 MHz, Chloroform-d) δ 5.58 (dd, *J*=5.7, 2.6 Hz, 1H), 5.40 (dt, *J*=5.6, 2.8 Hz, 1H), 3.64 (tt, *J*=11.2, 4.2 Hz, 1H), 2.47 (ddd, *J*=14.4, 4.8, 2.3 Hz, 1H), 2.28 (dddd, *J*=18.9, 11.8, 5.9, 3.7 Hz, 1H), 2.18 (ddt, *J*=10.6, 5.7, 2.9 Hz, 1H), 1.97 (ddt, *J*=9.4, 7.1, 2.3 Hz, 1H), 1.93−1.84 (m, 2H), 1.84−1.67 (m, 3H), 1.67−1.53 (m, 2H), 1.53−1.39 (m, 2H), 1.39−1.34 (m, 1H), 1.33 (s, 3H), 1.32−1.22 (m, 2H), 1.22 (s, 3H), 1.2 (m, 1H), 0.95 (s, 3H), 0.78 (s, 3H). MS (ESI) *m/z* 353.4 $[M+Na]^+$. Yield 91%. HPLC purity > 98%. HRMS (ESI) *m/z* 313.2521 $[M+H-H_2O]^+$ (error: −3.2 ppm).

(3S,10R,13S,14R,17S)-17-(3-hydroxypentan-3-yl)-10,13-dimethyl-2,3,4,9,10,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol (6b)—^{1}H NMR (400 MHz, Chloroform-d) δ 5.58 (dd, *J*=5.8, 2.5 Hz, 1H), 5.40 (dt, *J*=5.6, 2.8 Hz, 1H), 3.64 (tt, *J*=11.2, 4.1 Hz, 1H), 2.47 (ddd, *J*=14.4, 4.8, 2.3 Hz, 1H), 2.38−2.22 (m, 1H), 2.22−2.11 (m, 1H), 2.01−1.79 (m, 3H), 1.79−1.60 (m, 4H), 1.56−1.41 (m, 3H), 1.41−1.30 (m, 1H), 1.18−0.99 (m, 1H), 0.97 (d, *J*=6.6 Hz, 2H), 0.95 (s, 3H), 0.90 (m, 2H), 0.88 (s, 2H), 0.88−0.85 (m, 3H), 0.81 (s, 3H), 0.79 (t, *J*=7.6 Hz, 3H). MS (ESI) *m/z* 381.6 $[M+Na]^+$. Yield 85%. HPLC purity >98%. HRMS (ESI) *m/z* 321.2844 $[M+H-H_2O]^+$ (error: 0.0 ppm).

(3S,10R,13S,14R,17S)-17-(3-hydroxypenta-1,4-dien-3-yl)-10,13-dimethyl-2,3,4,9,10,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol (6c)—^{1}H NMR (400 MHz, Chloroform-d) δ 6.06 (dd, *J*=17.3, 10.7 Hz, 1H), 5.96 (dd, *J*=17.3, 10.7 Hz, 1H), 5.57 (dd, *J*=5.7, 2.5 Hz, 1H), 5.39 (dt, *J*=5.6, 2.7 Hz, 1H), 5.23 (ddd, *J*=37.5, 17.3, 1.3 Hz, 2H), 5.06 (ddd, *J*=27.4, 10.7, 1.3 Hz, 2H), 3.71−3.55 (m, 0H), 2.47 (ddd, *J*=14.3, 4.9, 2.4 Hz, 1H), 2.28 (t, *J*=12.9 Hz, 1H), 2.18 (ddd, *J*=12.6, 4.8, 2.5 Hz, 1H), 1.95 (t, *J*=9.7 Hz, 1H), 1.91−1.80 (m, 2H), 1.79−1.62 (m, 3H), 1.57 (m, 1H), 1.49−1.38 (m, 2H), 1.34−1.23 (m, 3H), 1.18 (td, *J*=13.0, 4.9 Hz, 1H), 0.93 (s, 3H), 0.88−0.82 (m, 1H), 0.73 (s, 3H). MS (ESI) *m/z* 377.4 $[M+Na]^+$. HPLC purity >98%. HRMS (ESI) *m/z* 337.2528 $[M+H-H_2O]^+$ (error: −0.9 ppm).

(3S,10R,13S,14R,17S)-17-(4-hydroxyheptan-4-yl)-10,13-dimethyl-2,3,4,9,10,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol (6d)—^{1}H NMR (400 MHz, Chloroform-d) δ 5.58 (dd, *J*=5.7, 2.5 Hz, 1H), 5.40 (dt, *J*=5.6, 2.7 Hz, 1H), 3.64 (tt, *J*=11.3, 4.1 Hz, 1H), 2.47 (ddd, *J*=14.3, 4.8, 2.3 Hz, 1H), 2.29 (ddq, *J*=13.7, 11.2, 2.1 Hz, 1H), 2.15 (ddd, *J*=12.6, 4.8, 2.6 Hz, 1H), 2.02−1.92 (m, 1H), 1.92−1.79 (m, 3H), 1.79−1.68 (m, 2H), 1.67−1.53 (m, 3H), 1.53−1.47 (m, 1H), 1.47−1.39 (m, 2H), 1.39−1.27 (m, 3H), 1.27−1.17 (m, 2H), 0.95 (s, 3H), 0.94−0.85 (m, 14H), 0.81 (s, 3H). MS (ESI) *m/z* 409.5 $[M+Na]^+$. Yield 63%. HPLC purity >98%. HRMS (ESI) *m/z* 369.3157 $[M+H-H_2O]^+$ (error: 0.0 ppm).

(3S,10R,13S,14R,17S)-17-(5-hydroxynonan-5-yl)-10,13-dimethyl-2,3,4,9,10,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol (6e)—^{1}H NMR (400 MHz, Chloroform-d) δ 5.51 (dd, *J*=5.8, 2.5 Hz, 1H), 5.33 (dt, *J*=5.6, 2.8 Hz, 1H), 3.57 (tt, *J*=11.2, 4.0 Hz, 1H), 2.45−2.34 (m, 1H), 2.22 (t, *J*=12.7 Hz, 1H), 2.12−2.05 (m, 1H), 1.89 (t, *J*=9.5 Hz, 1H), 1.85−1.72 (m, 3H), 1.72−1.61 (m, 2H), 1.61−1.47 (m, 6H), 1.46−1.31 (m, 3H), 1.31−1.16 (m, 6H), 1.16−1.09 (m, 1H), 1.08 (s, 2H), 0.88 (s, 3H), 0.87−0.76 (m, 14H), 0.74 (s, 3H). MS (ESI) *m/z* 437.5 $[M+Na]^+$. Yield 55%. HPLC purity > 98%. HRMS (ESI) *m/z* 397.3468 $[M+H-H_2O]^+$ (error: −0.5 ppm).

(S,Z)-3-((E)-2-((1S,3aS,7aS)-1-(2-hydroxypropan-2-yl)-7a-methylhexahydro-1H-inden-4(2H)-ylidene)ethylidene)-4-methylenecyclohexanol (10a)—^{1}H NMR (400 MHz, Chloroform-d) δ 6.15 (d, *J*=11.3 Hz, 1H), 5.96 (d, *J*=11.2 Hz, 1H), 4.97 (dt, *J*=2.6, 1.4 Hz, 1H), 4.74 (d, *J*=2.5 Hz, 1H), 3.88 (s, 1H), 2.75 (dd, *J*=12.1, 4.3 Hz, 1H), 2.56−2.44 (m, 1H), 2.32 (ddd, *J*=13.1, 7.9, 4.8 Hz, 1H), 2.21 (dd, *J*=13.1, 7.5 Hz, 1H), 2.10 (ddd, *J*=13.6, 8.5, 4.7 Hz, 1H), 2.05−1.97 (m, 1H), 1.96−1.88 (m, 1H), 1.88−1.79 (m, 1H), 1.76−1.52 (m, 4H), 1.45 (m, 2H), 1.25 (s, 1H), 1.23 (s, 3H), 1.17 (s, 2H), 1.13 (m, 1H), 1.12 (s, 3H), 0.63 (s, 3H). MS (ESI) *m/z* 353.4 $[M+Na]^+$. HPLC purity >98%. HRMS (ESI) *m/z* 313.2533 $[M+H-H_2O]^+$ (error: 0.6 ppm).

(S,Z)-3-((E)-2-((1S,3aS,7aS)-1-(3-hydroxypentan-3-yl)-7a-methylhexahydro-1H-inden-4(2H)-ylidene)ethylidene)-4-methylenecyclohexanol (10b)—^{1}H NMR (400 MHz, Methanol-d4) δ 6.24 (d, *J*=11.2 Hz, 1H), 6.04 (d, *J*=11.2 Hz, 1H), 5.09−5.02 (m, 1H), 4.77 (dd, *J*=2.8, 1.2 Hz, 1H), 3.78 (tt, *J*=8.8, 4.0 Hz, 1H), 2.87 (dd, *J*=11.8, 3.9 Hz, 1H), 2.55 (dd, *J*=12.9, 4.1 Hz, 1H), 2.43 (dt, *J*=13.6, 5.0 Hz, 1H), 2.26−2.16 (m, 1H), 2.16−2.07 (m, 2H), 2.07−1.92 (m, 2H), 1.92−1.79 (m, 1H), 1.79−1.61 (m, 6H), 1.61−1.45 (m, 6H), 1.45−1.35 (m, 2H), 0.93−0.79 (m, 1H), 0.89 (t, *J*=7.5 Hz, 1H), 0.83 (t, *J*=7.5 Hz, 3H), 0.74 (s, 3H). MS (ESI) *m/z* 381.6 $[M+Na]^+$. HPLC purity >98%. HRMS (ESI) *m/z* 321.2842 $[M+H-H_2O]^+$ (error: −0.6 ppm).

(S,Z)-3-((E)-2-((1S,3aS,7aS)-1-(3-hydroxypenta-1,4-dien-3-yl)-7a-methylhexahydro-1H-inden-4(2H)-ylidene) ethylidene)-4-methylenecyclohexanol (10c)—^{1}H NMR (400 MHz, Methanol-d4) δ 6.22 (d, *J*=11.2 Hz, 1H), 6.12−5.96 (m, 3H), 5.22 (ddd, *J*=32.5, 17.3, 1.7 Hz, 2H), 5.05 (dd, *J*=2.8, 1.2 Hz, 1H), 5.00 (ddd, *J*=32.5, 17.3, 1.7 Hz, 2H), 4.76 (dd, *J*=2.8, 1.2 Hz, 1H), 3.78 (tt, *J*=9.1, 3.9 Hz, 1H), 2.90−2.79 (m, 1H), 2.60−2.49 (m, 1H), 2.42 (dt, *J*=13.6, 5.0 Hz, 1H), 2.27−2.19 (m, 1H), 2.14 (dddd, *J*=13.7, 10.6, 4.6, 1.6 Hz, 2H), 1.99 (q, *J*=9.2 Hz, 2H), 1.88−1.74 (m, 2H), 1.74−1.60 (m, 2H), 1.60−1.38 (m, 3H), 1.28 (td, *J*=12.8, 3.9 Hz, 2H), 1.03−0.67 (m, 1H), 0.65 (s, 3H). MS (ESI) *m/z* 377.4 $[M+Na]^+$. HPLC purity > 98%. HRMS (ESI) *m/z* 337.2528 $[M+H-H_2O]^+$ (error: −0.9 ppm).

(S,Z)-3-((E)-2-((1S,3aS,7aS)-1-(4-hydroxyheptan-4-yl)-7a-methylhexahydro-1H-inden-4(2H)-ylidene)ethylidene)-4-methylenecyclohexanol (10d)—^{1}H NMR (400 MHz, Chloroform-d) δ 6.23 (d, *J*=11.2 Hz, 1H), 6.03 (d, *J*=11.3 Hz, 1H), 5.05 (dt, *J*=2.6, 1.4 Hz, 1H), 4.82 (d, *J*=2.5 Hz, 1H), 3.95 (s, 1H), 2.82 (dd, *J*=11.7, 4.1 Hz, 1H), 2.57 (dd,

J=13.2, 3.8 Hz, 1H), 2.40 (ddd, *J*=13.1, 7.9, 4.8 Hz, 1H), 2.29 (dd, *J*=13.2, 7.5 Hz, 1H), 2.18 (ddd, *J*=13.6, 8.5, 4.7 Hz, 1H), 2.12−2.02 (m, 1H), 2.02−1.86 (m, 2H), 1.87−1.75 (m, 1H), 1.69 (ddd, *J*=11.6, 9.0, 5.6 Hz, 5H), 1.56 (d, *J*=9.9 Hz, 3H), 1.45−1.27 (m, 5H), 1.27−1.15 (m, 2H), 1.09 (s, 1H), 0.90 (dt, *J*=10.2, 7.1 Hz, 7H), 0.74 (s, 3H). MS (ESI) *m/z* 409.5 $[M+Na]^+$. HPLC purity >98%. HRMS (ESI) *m/z* 369.3152 $[M+H-H_2O]^+$ (error: −1.4 ppm).

(S,Z)-3-((E)-2-((1S,3aS,7aS)-1-(5-hydroxynonan-5-yl)-7a-methylhexahydro-1H-inden-4(2H)-ylidene)ethylidene)-4-methylenecyclohexanol (10e)—^{1}H NMR (500 MHz, Chloroform-d) δ 6.23 (d, *J*=11.2 Hz, 1H), 6.03 (d, *J*=11.3 Hz, 1H), 5.05 (s, 1H), 4.82 (s, 1H), 3.95 (s, 1H), 2.82 (d, *J*=13.1 Hz, 1H), 2.57 (d, *J*=13.1 Hz, 1H), 2.39 (dd, *J*=13.5, 6.6 Hz, 1H), 2.29 (dd, *J*=13.2, 7.5 Hz, 1H), 2.23−2.11 (m, 1H), 2.06 (d, *J*=15.8 Hz, 1H), 1.99 (t, *J*=9.5 Hz, 1H), 1.92 (s, 1H), 1.84−1.75 (m, 1H), 1.74−1.63 (m, 4H), 1.56 (m, 2H), 1.44−1.33 (m, 1H), 1.33−1.22 (m, 8H), 1.19 (q, *J*=7.2 Hz, 1H), 1.08 (s, 1H), 0.91 (q, *J*=6.9 Hz, 10H), 0.74 (s, 3H). MS (ESI) *m/z* 437.5 $[M+Na]^+$. HPLC purity > 98%. HRMS (ESI) *m/z* 397.3469 $[M+H-H_2O]^+$ (error: −0.3 ppm).

To evaluate the capability of these analogs to interact with the VDR, a previously established Jurkat cell line transduced with a VDRE luciferase vector construct was used to carry out the reporter assays. We compared the activity of five analogs with that of two positive controls ($1,25D_3$ and 22-oxa-$1,25D_3$) to activate the VDR *via* binding to the synthetic VDRE in this construct. In preliminary studies, these analogs lacked the ability to activate the VDR at a concentration of 0.1 μM, however, as shown in Figure 3A, all five analogs significantly activated VDR at a concentration of 1.0 μM. The detected luciferase signal increased 31%, 20%, 27%, 20% and 33% as compared with blank controls for compounds **10a**, **10b**, **10c**, **10d** and **10e**, respectively. Compared to $1,25D_3$ and 22-oxa-$1,25D_3$, these analogs caused weak activation of the VDR causing 33- and 30-times less activation respectively, at 0.1 μM.

We compared the activity of the five synthetic analogs on the expression of *CYP24A1* gene in HaCaT cells to that of $1,25D_3$ and 22-oxa-$1,25D_3$. As shown in Figure 3B, after 6 h treatment with 0.1 μM of each analog, relative mRNA levels for *CYP24A1* were 4.7-, 3.2-, 3.4-, 1.9- and 1.6-fold higher than that of the negative control for analogs **10a**, **10b**, **10c**, **10d** and **10e**, respectively. In comparison, cells treated with 0.1 μM $1,25D_3$ or 22-oxa-$1,25D_3$ showed a 366- or 370-fold increase in mRNA for *CYP24A1*, respectively. Following 24 h of treatment (Figure 3C), the positive controls still caused greater stimulation of *CYP24A1* gene expression than did the Gemini analogs, with the degree of stimulation for all compounds being less than at 6 h. Only **10a** and **10c** significantly stimulated expression over that of the control while $1,25D_3$ and 22-oxa-$1,25D_3$ caused 10- and 78-fold stimulation, respectively.

The ability of the new Gemini analogs to regulate the expression of the *VDR* gene was studied using HaCaT cells. As shown in Figure 3D, the mRNA expression level was stimulated by 1.4- to 3-fold by the Gemini analogs following 24 h of treatment with 0.1 μM. In addition, the two positive controls, $1,25D_3$ and 22-oxa-$1,25D_3$, also stimulated VDR expression by 1.3- and 1.7-fold, respectively, in comparison with the negative control. Interestingly, compound **10c** and **10e** caused significantly higher stimulation of the expression of the *VDR* gene than did $1,25D_3$ ($p<0.05$), and analogs **10a**, **10b** and **10d**

displayed much higher stimulation of VDR expression than both $1,25D_3$ and 22-oxa-$1,25D_3$ ($p<0.01$). These results suggest that the new analogs may increase D_3 catabolism, not only by mild stimulation of the expression of the hydroxy-D_3-catabolizing enzyme (CYP24A1), but also through increased expression of its own receptor, the VDR.

A well-known target of $1,25D_3$ is the stimulation of the expression of the *TRPV6* gene (encoding an intestinal calcium channel) (28). Because calcium plays an important role in keratinocytes differentiation, we evaluated the effects of the new Gemini $20D_3$ analogs on the expression of this gene by immortalized human epidermal keratinocytes (HaCaT cells). The mRNA levels of *TRPV6* after a 24-h treatment were increased by 1.4- and 2.6-fold on $1,25D_3$ and 22-oxa-$1,25D_3$ treatment, respectively (Figure 3E). In contrast, the mRNA level for TRPV6 was increased by 5.9-fold for analog **10a** relative to the negative control, but only by 1.5- to 2.7-fold for the other Gemini analogs, similarly to that for $1,25D_3$ and 22-oxa-$1,25D_3$. Interestingly the analog with the shortest side chain (**10a**) caused 4-fold higher expression of the *TRPV6* gene expression than analog **10e** with the longest side chain, indicating that the short aliphatic side chain is most favorable for regulation of the *TRPV6* gene.

One of the $20D_3$ analogs, **10e**, inhibited SKMEL-188 melanoma cell proliferation with comparable potency (1.24×10^{-9} M) to that seen for $1,25D_3$ (1.05×10^{-9} M), while analog **10d** had 15-fold lower potency than $1,25D_3$. Analogs **10a**, **10b** and **10c** displayed no significant antiproliferative activity against the growth of SKMEL-188 melanoma cells. These results suggest that a long side chain is required to inhibit proliferation with a potency similarly to that of $1,25D_3$.

$1,25D_3$ acts as an immunomodulatory agent and displays anti-inflammatory activity (3, 5). Thus many analogs of $1,25D_3$ have been developed with the hope that they can be used as anti-inflammatory agents (5). To test whether the new Gemini-$20D_3$ analogs can exert anti-inflammatory effects, IFNγ concentrations in the medium used to culture mouse splenocytes were measured using our established assay (3). The Gemini analogs were tested at a concentration of 0.1 μM (Figure 4B). $1,25D_3$ reduced the IFNγ concentration by 60% compared to the ethanol control. All the analogs significantly reduced IFNγ levels, with the most active compound being **10b**, which caused a 62% reduction in the IFNγ concentration (62%), comparable to that seen for $1,25D_3$.

Part of the anti-inflammatory activity of $1,25D_3$ is mediated by the up-regulation of *LAIR1*. LAIR1 is a receptor expressed on T-cells and other immune cells believed to down-regulate the immune response (29). The ability of the new Gemini analogs (100 nM) to up-regulate LAIR1 protein in splenocytes was compared with the EtOH negative control and a 22-oxa-$1,25D_3$ (22-Oxa) positive control (Figure 4C). All the new Gemini analogs caused a significant increase in LAIR1 levels, generally comparable to that seen for the 22-Oxa positive control, with the highest stimulation being seen for analog **10e** (59%). The data suggest that all the Gemini-$20D_3$ analogs are strong anti-inflammatory agents.

Discussion

To completely remove the 3-acetyl group on intermediate **5**, at least four equivalences of Grignard reagents were required to form the desired product **6**. Among these 7DHC analogs, **6c** was not obtained through a normal Grignard reaction in our initial trials due to the formation of an α,β-unsaturated ketone after the first attack of the vinyl side chain. Fortunately, the addition of anhydrous $CeCl_3$ following a reported procedure (24) solved this problem and we ended up with an 86% yield. Grignard reagents with longer side chains (C≥5) were used to produce more 7DHC analogs (6), however after UVB irradiation, we were unable to separate the corresponding D_3 structures from the mixture.

The Gemini-$20D_3$ analogs showed similar ability to activate the VDR and bind to a synthetic VDRE reporter construct, to their parent compound ($20D_3$) reported in our previous study (11). Receptor activation was less than for than classical VDR activators ($1,25D_3$ and 22-oxa-$1,25D_3$). These results are consistent with these analogs acting as biased agonists on the VDR, similar to $20D_3$ (7), where the ligand can influence the relative binding to different VDREs.

The *CYP24A1* gene has two VDREs and is highly responsive to $1,25D_3$, but poorly responsive to $20D_3$ (7, 11). CYP24A1 is responsible for the catabolism of $25D_3$ and $1,25D_3$ and can act on numerous vitamin D analogs (4, 5). CYP24A1 initially hydroxylates the vitamin D side chain at C24 converting $1,25D_3$ into $1,24,25(OH)3D_3$ (3, 4). The ability of Gemini-$20D_3$ analogs to stimulate the expression of the *CYP24A1* gene, although only weakly, supports that they act through the VDR. Their inability to cause the massive induction seen with $1,25D_3$ and 22-oxa-$1,25D_3$ likely results in lower CYP24A1 protein levels and thus lower rates of catabolism, therefore promoting prolonged action. However, the ability of CYP24A1 to metabolize these analogs remains to be determined. Our data show that the Gemini $20D_3$ analogs stimulate the expression of the *VDR* gene in HaCaT cells, suggesting they can up-regulate the basal expression level of their own receptor, the VDR. This might be an alternate way for them to exert their biological activities other than by directly modulating target genes, such as *CYP24A1*. Moreover, Gemini $20D_3$ analogs were able to up-regulate the mRNA level of *TRPV6* which is a membrane calcium channel involved in the first step of calcium absorption in the intestine. The expression of *TRPV6* is reported to be vitamin D-dependent in mice and humans, and is greatly decreased in animals that do not express VDR (30). In addition, TRPV6 is a direct target of the VDR and positively controls cell proliferation and apoptosis resistance in prostate cancer (31). Investigating the modulating effects of D_3 analogs on *TRPV6* gene is, thus, very important in order to understand the correlation between vitamin D compounds and their antiproliferative activity.

We have previously reported that $20D_3$ has antiproliferative activity using a colony-forming model. $20D_3$ showed comparable inhibition of colony formation to that of $1,25D_3$ (16, 32), suggesting there is a great potential to use $20D_3$ as a antitumor therapeutic agent, especially given its low calcemic activity. To evaluate the antiproliferative activity of our Gemini analogs, we tested them on the growth of SKMEL-188 cells. Compound **10e**, and to a lesser extent **10d**, had half-maximal inhibitory concentrations (IC_{50}s) (Figure 4) similar to $1,25D_3$.

However, analogs with shorter side chains did not show significant inhibitory effects, indicating that a longer aliphatic side chains is necessary for antiproliferative activity.

IFNγ, or type II interferon, is the only member in the type II class of IFN. It is well known for its immunostimulatory and immunomodulatory effects and is critical for both innate and adaptive immunity (33). For this reason, IFNγ is treated as a common inflammatory marker. Our previous studies have shown that D_3 metabolites down-regulated IFNγ produced by mouse splenocytes (3, 7), inhibited interleukin 17 production by mouse T-lymphocytes (27) and down-regulated nuclear factor kappa-light-chain-enhancer of activated B-cells, which is a master regulator of pro-inflammatory actions (34, 35). The Gemini $20D_3$ analogs caused similar decreases in IFNγ concentrations in cultured splenocytes to that of other D_3 metabolites. To further validate their anti-inflammatory effects, flow cytometric measurements of LAIR1 protein levels in splenocytes were made. LAIR1, also designated as CD305 (cluster of differentiation 305), is encoded by the *LAIR1* gene and is an inhibitory receptor expressed in many peripheral cells in both innate and adaptive immune systems, such as natural killer cells, T-cells and B-cells (36, 37). It is an important anti-inflammatory marker due to its ability to prevent lysis of cells recognized as self during an immune response. Together with the inhibitory effects on IFNγ, the up-regulation of LAIR1 levels confirms a role for these analogs in the regulation of the immune responses and inflammation.

Conclusion

To conclude (Figure 5), the new Gemini $20D_3$ analogs were able to activate the VDR. Analysis of gene expression at the mRNA level showed that the analogs regulated *CYP24A1*, *VDR* and *TRPV6* genes, consistent with their effects being mediated through activation of the VDR (7). In addition, these analogs displayed antiproliferative and anti-inflammatory activity, which might also correlate with their VDR activation process. This study suggests that Gemini $20D_3$ analogs have great potential as therapeutic agents on the immune system.

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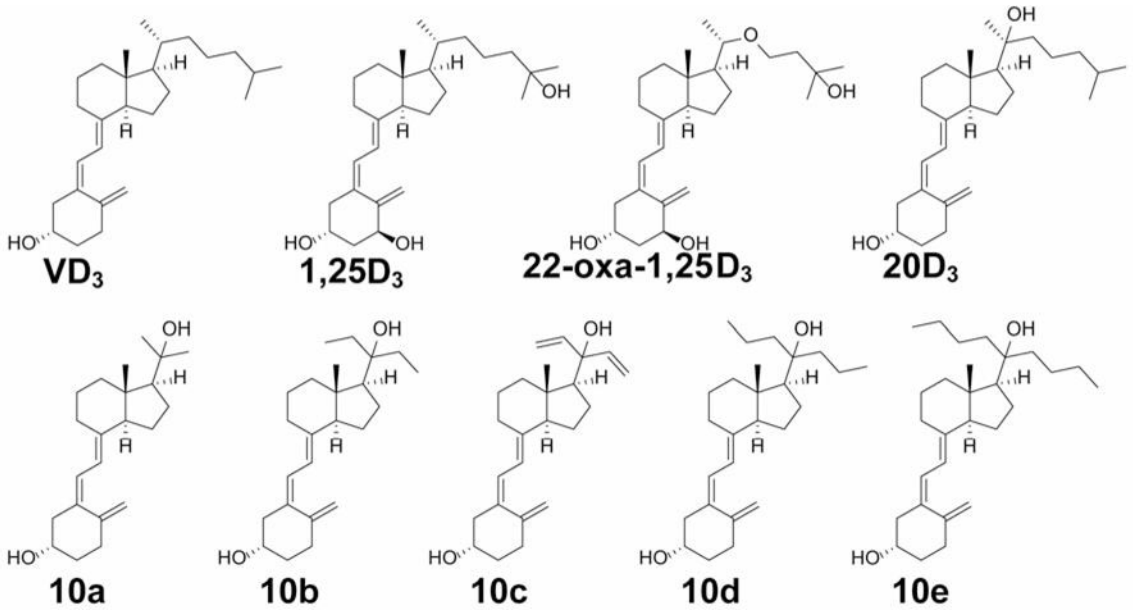

Figure 1.

Chemical structures of vitamin D_3 (VD_3), 1α,25-dihydroxyvitamin D_3 ($1,25D_3$), 22-oxa-1α, 25-dihydroxyvitamin D_3 (22-oxa-$1,25D_3$), 20S-hydroxyvitamin D_3 ($20D_3$) and its five Gemini analogs (**10a–10e**).

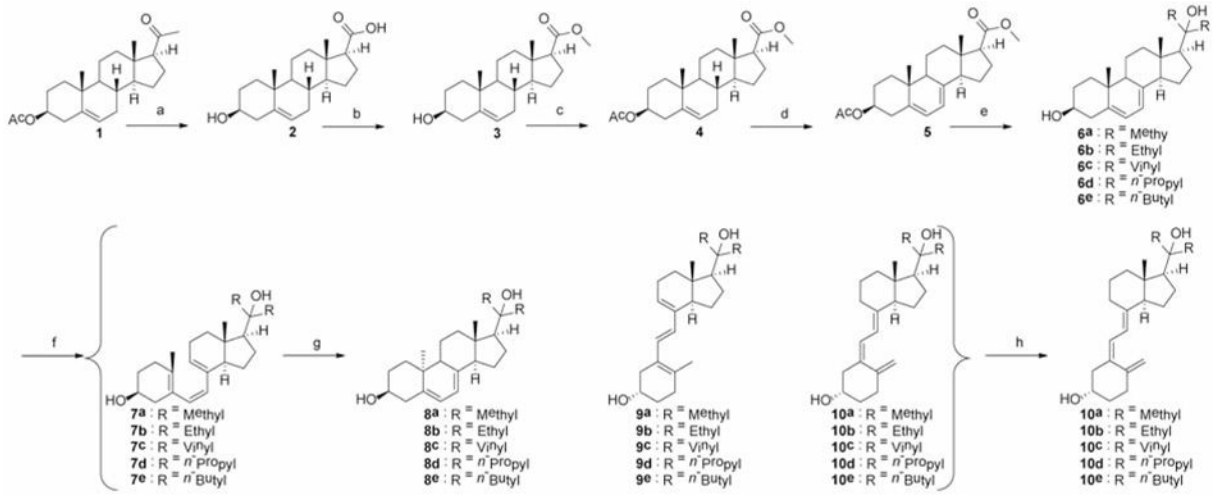

Figure 2.

Synthetic route for producing Gemini analogs of 20-hdroxyvitamin D_3. Reagents and conditions: (a) NaOH, Br_2, 0°C then warmed to r.t., overnight. (b) H_2SO_4, methanol, reflux 2 h. (c) Acetic anhydride, pyridine, 4-dimethylaminopyridine (DMAP), 6 h. (d) Dibromantin, azobisisobutyronitrile (AIBN), benzene: hexane (1:1), reflux 20 min; tetra-n-butylammonium bromide (TBAB), tetrahydrofuran (THF), r.t., 75 min, then tetra-n-butylammonium fluoride (TBAF), r.t., 50 min. (e) Grignard reagent in THF, THF, 0°C then warmed up to r.t., 8 h (**6c**: vinyl magnesium bromide, $CeCl_3$, −78°C then warmed up to r.t., 24 h). (f) Ultraviolet B (UVB), diethyl ether, 15 min. (g) Ethanol, reflux, 3 h. (h) High-performance liquid chromatography, acetonitrile:H_2O.

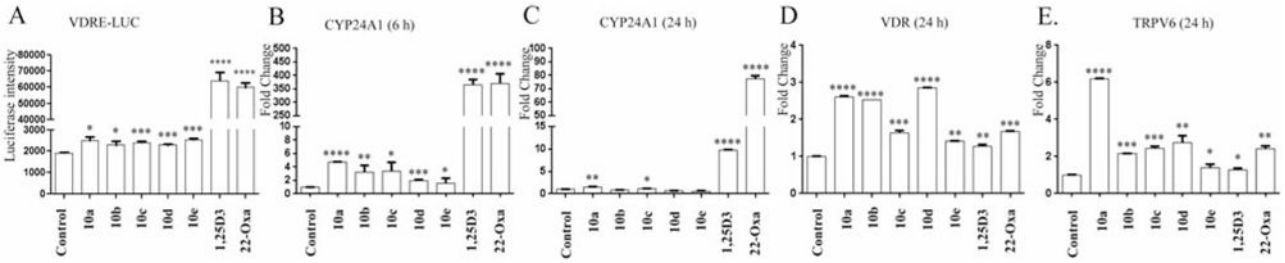

Figure 3.

Gemini analogs of 20S-hydroxyvitamin D_3 ($20D_3$) activate the VDR in a vitamin D response element-luciferase (VDRE-LUC) reporter assay and regulate cytochrome P450 24A1 (CYP24A1), vitamin D receptor (VDR) and transient receptor potential cation channel V6 (TRPV6) genes. A: Jurkat cells transduced with a VDRE-LUC reporter construct were treated for 24 h with Gemini analogs (**10a–10e**, 1.0 μM), 1α,25-dihydroxyvitamin D_3 ($1,25D_3$) (0.1 μM), 22-oxa-$1,25D_3$ (22-Oxa) (0.1 μM) or 10% dimethyl sulfoxide (DMSO) (final concentration 0.1% DMSO) as a negative control. Gemini $20D_3$ analogs increased mRNA levels for CYP24A1 (B and C), VDR (D) and TRPV6 (E). HaCaT cells were treated with 100 nM of Gemini analogs, $1,25D_3$, 22-Oxa or DMSO only (solvent) as a control. The mRNA was isolated and the real-time polymerase chain reaction was performed using specific primers for CYP24A1, VDR and TRPV6 genes. Data are presented as mean±SE (n=3). *p<0.05, **p<0.01, ***p<0.005 ****p<0.001 compared to the control.

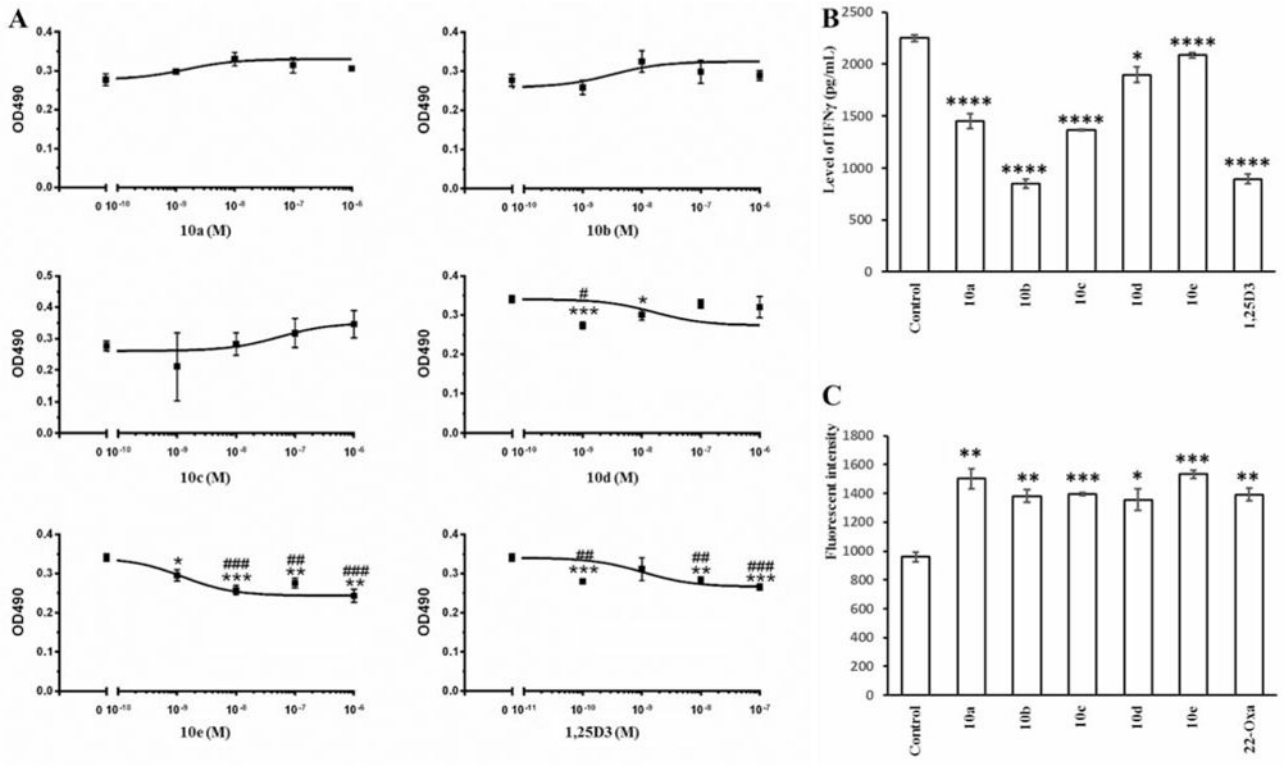

Figure 4.

Antiproliferative effects of Gemini 20S-hydroxyvitamin D_3 ($20D_3$) analogs on human SKEML-188 melanoma cells and anti-inflammatory effects on splenocytes. A: SKEML-188 melanoma cells were treated with analogs, 1α,25-dihydroxyvitamin D_3 ($1,25D_3$) or dimethyl sulfoxide (DMSO) (solvent) as a control to assess their inhibitory effects on cell growth. Gemini $20D_3$ analogs reduced interferon-γ (IFNγ) concentration (B) and up-regulated leukocyte-associated immunoglobulin-like receptor 1 (LAIR1) levels (C) in mouse splenocytes. Splenic cells were treated by 100 nM of Gemini analogs, $1,25D_3$, 22-Oxa or EtOH only (solvent) as a control. Data are presented as mean±SE (n=3). *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 compared to the control. Half-maximal inhibitory concentration (IC_{50}): 1.65×10^{-8} M (**10d**), 1.24×10^{-9} M (**10e**) and 1.05×10^{-9} M ($1,25D_3$).

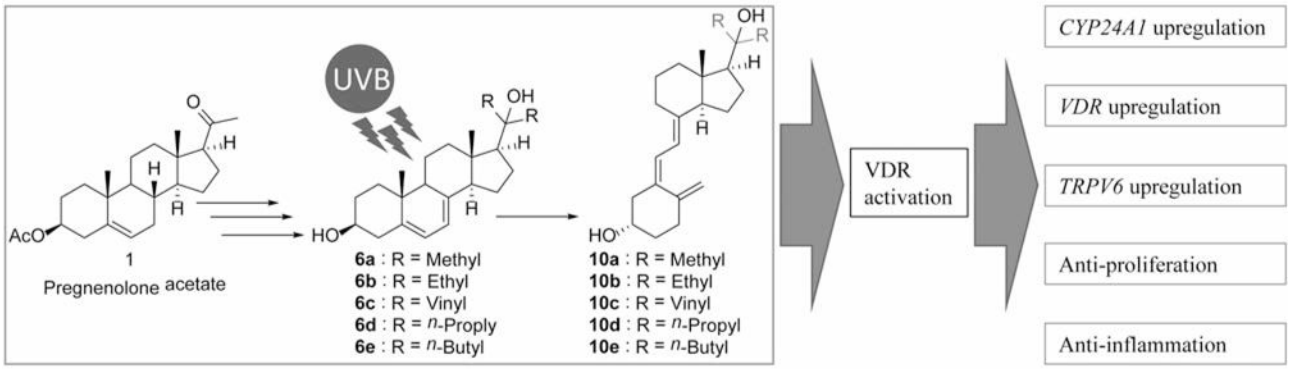

Figure 5.

Summary of synthesis and biological activities of Gemini 20S-hydroxyvitamin D_3 ($20D_3$) analogs used in this study. The synthesis starts from pregnenolone acetate to obtain 7-dehydrocholesterol intermediates which are then irradiated by UVB to produce D_3 structures. These analogs likely exert their activities, including gene up-regulation, antiproliferative and anti-inflammatory effects, through activation of the vitamin D receptor (VDR).