20-Hydroxyvitamin D2 is a noncalcemic analog of vitamin D with potent antiproliferative and prodifferentiation activities in normal and malignant cells


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Slominski AT, Kim T, Janjetovic Z, Tuckey RC, Bieniek R, Yu e J, Li W, Chen J, Nguyen MN, Tang FK, Miller D, Chen TC, Holick M. 20-hydroxyvitamin D2 is a noncalcemic analog of vitamin D with potent antiproliferative and prodifferentiation activities in normal and malignant cells. Am J Physiol Cell Physiol 300: C526–C541, 2011. First published December 15, 2010; doi:10.1152/ajpcell.00203.2010.—20-hydroxyvitamin D2 [20(OH)D2] inhibits DNA synthesis in epidermal keratinocytes, melanocytes, and melanoma cells in a dose- and time-dependent manner. This inhibition is dependent on cell type, with keratinocytes and melanoma cells being more sensitive than normal melanocytes. The antiproliferative activity of 20(OH)D2 is similar to that of 1,25(OH)2D3 and of newly synthesized 1,20(OH)2D2 but significantly higher than that of 25(OH)D3. 20(OH)D2 also displays melanocyte. The antiproliferative activity of 20(OH)D2 is similar to that of 1,25(OH)2D3 and of newly synthesized 1,20(OH)2D2 but significantly higher than that of 25(OH)D3. 20(OH)D2 also displays tumorostatic effects. In keratinocytes 20(OH)D2 inhibits expression of cyclins and stimulates involucrin expression. It also stimulates CYP24 expression, however, to a significantly lower degree than that by 1,25(OH)2D3 or 25(OH)D3. 20(OH)D2 is a poor substrate for CYP27B1 with overall catalytic efficiency being 24- and 41-fold lower than for 25(OH)D3 with the mouse and human enzymes, respectively. No conversion of 20(OH)D2 to 1,20(OH)2D2 was detected in intact HaCaT keratinocytes. 20(OH)D2 also demonstrates anti-keratinocyte activity but with lower potency than 1,25(OH)2D3. The phenotypic effects of 20(OH)D2 are mediated through interaction with the vitamin D receptor (VDR) as documented by attenuation of cell proliferation after silencing of VDR, by enhancement of the inhibitory effect through stable overexpression of VDR and by the demonstration that 20(OH)D2 induces time-dependent translocation of VDR from the cytoplasm to the nucleus at a comparable rate to that for 1,25(OH)2D3. In vivo tests show that while 1,25(OH)2D3 as doses as low as 0.8 μg/kg induces calcium deposits in the kidney and heart, 20(OH)D2 is devoid of such activity even at doses as high as 4 μg/kg. Silencing of CY27B1 in human keratinocytes showed that 20(OH)D2 does not require its transformation to 1,20(OH)2D2 for its biological activity. Thus 20(OH)D2 shows cell-type dependent antiproliferative and prodifferentiation activities through activation of VDR, while having no detectable toxic calcemic activity, and is a poor substrate for CYP27B1.

melanocytes; melanoma cells; keratinocytes; leukemia

THE PHOTOCHROMIC ISOMERIZATION of 7-dehydrocholesterol (7DHC) after absorption of UVB photons to the pre-vitamin D3 intermediate, followed by its slow isomerization to three main products including D3, tachysterol, and lumisterol, represent the most fundamental reactions in the photobiology of the skin (6, 22). Similar photochemical process occur in the case of plant-derived ergosterol leading to generation of vitamin D2 (supplementary Fig. S1). After entering the circulation, vitamins D3 and D2 are successively hydroxylated in the liver and the kidney to 1,25(OH)2D3 and 1,25(OH)2D2, respectively, which in addition to regulation of body calcium metabolism, mediate several systemic and local pleiotropic effects (reviewed in Refs. 6 and 21). Some experts (2, 11), although not all (23), express the opinion that active forms of vitamin D3 are more potent than those of vitamin D2, 1,25(OH)2D3 is also produced in the epidermis from D2 and has significant local actions on formation of the skin barrier, functional differentiation of adnexal structures, and modulation of the skin immune system (reviewed in Refs. 6, 21, and 33).

The above active forms of vitamin D3 and D2 bind to the vitamin D receptor (VDR) and induce conformational changes in the receptor, which then heterodimerizes with the retinoic acid receptor (RXR). The complex is subsequently translocated to the nucleus where it regulates transcription of genes containing the VDRE in their promoter region (13, 43, 71, 73). The final phenotypic effects include tumorostatic and anticarcinogenic activities where proliferation, differentiation, and apoptosis of cells of different lineages are affected and protection of DNA against oxidative damage (15, 22, 28, 43, 54). In addition, 1,25(OH)2D3 and its derivatives also display potent anti-keratinocyte activities (25, 40, 42, 44, 55, 65, 74). The above activities of 1,25(OH)2D3 and structural analogs of this compound make them of interest for the treatment of cancer and other hyperproliferative disorders (38). Unfortunately, the toxic effect of hypercalcemia caused by pharmacological doses of 1,25(OH)2D3 or 1,25(OH)2D2 impairs its use in pharmacological therapy. This has induced an extensive effort in chemical synthesis of vitamin D analogs that display reduced calcemic activity but retain powerful antiproliferative activity (38, 63, 64).

Cytochrome P450scs (P450scc) is a mitochondrial enzyme that catalyzes the first step of steroidogenesis where the side chain of cholesterol is cleaved producing pregnenolone (68). We have documented that recombinant P450scc, or isolated adrenal mitochondria containing P450scc, can hydroxylate vitamins D2 (46, 56), D3 (19, 58, 70) and their corresponding precursors, ergosterol (57), and 7-DHC (19, 60, 62). The latter reaction was also demonstrated in adrenal glands incubated ex vivo, which produced 7-dehydropregnenolone, its hydroxyderivatives and 7-dehydroprogesterone (62). P450scc converts vitamin D3 to 20-hydroxyvitamin D3 [20(OH)D3] (19, 58), 20,23-dihy-
hydroxvitamin D$_1$ [20,23(OH)$_2$D$_3$], and 17,20,23-trihydroxvitamin D$_3$ (70) and converts vitamin D$_2$ to 20-hydroxysteroid product of vitamin D$_2$ [20(OH)D$_2$], 17,20-dihydroxysteroid D$_2$ [17,20(OH)$_2$D$_2$], and 17,20,24-trihydroxysteroid D$_2$ [17,20,24(OH)$_3$D$_2$] (46, 56). 20(OH)D$_3$ (58, 70) and 20(OH)D$_2$ (56) are the major products of these reactions indicating that they can readily dissociate from the active side of the enzyme, which indicates their potential to enter the extracellular environment.

Our recent studies documented that 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$ (26, 27, 61, 76), as well as other novel secosteroidal products of P450scc action (62, 77), are biologically active and regulate the behavior of a number of cell types including the inhibition of proliferation and stimulation of differentiation of human keratinocytes and leukemia cells (26, 27, 61, 62, 76, 77). 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$ act through the VDR as partial receptor agonists (26, 27, 76) having antiproliferative activity as potent as 1,25(OH)$_2$D$_3$, but unlike 1,25(OH)$_2$D$_3$, only weakly stimulate the expression of the CYP24 gene (26, 27, 76). Furthermore, 20(OH)D$_2$ shows a lack of calcemic activity at concentrations as high as 3 μg/kg, a dose that is calcemic for 1,25(OH)$_2$D$_3$, 25(OH)D$_3$, and 1,20(OH)$_2$D$_2$ (61).

In the current study we have examined the biological activity of 20(OH)D$_2$, the major product of vitamin D$_2$ hydroxylation by P450scc (56) (Supplemental Fig. S1) and compared it with 1,25(OH)$_2$D$_3$, the classical form of vitamin D$_3$. In our experiments we have used normal and malignant skin epidermal cells as well as K562 and HL60 leukemia cell lines that are well-defined targets of active forms of vitamin D (3, 5, 7, 32, 51). We also compared the calcemic effects of 20(OH)D$_2$ with those of 1,25(OH)$_2$D$_3$ by histologically analyzing tissues for calcium deposition, indicative of calcemic toxicity.

**MATERIALS AND METHODS**

**Preparation and Purification of 20(OH)D$_2$**

20(OH)D$_2$ was enzymatically generated through hydroxylation of vitamin D$_2$ (Sigma-Aldrich, St. Louis, MO) using an in vitro reconstituted P450scc system as described previously (46, 56). The product was purified by preparative TLC followed by isocratic RP-HPLC on a C18 column (Brownlee Aquapore, 25 cm × 1.0 cm, particle size 20 μm) using 90% methanol in water as mobile phase at a flow rate of 1.5 ml/min, and the identity was confirmed based on mass and UV spectra, as well as on retention times compared with standards previously characterized by NMR (56). The concentration of 20(OH)D$_2$ was determined using an extinction coefficient of 18,000 M$^{-1}$ cm$^{-1}$ at 263 nm (20). The compounds were aliquoted, dried, and stored at −80°C until use.

**Preparation of 1,20(OH)$_2$D$_2$ and Testing of CYP27B1 Activity on 20(OH)D$_2$**

Mouse CYP27B1 was expressed in *Escherichia coli* and purified as described previously (67). Human CYP27B1 was expressed similarly with a COOH-terminal 6-histidine tag. A bacterial membrane fraction containing the human enzyme was prepared and used for catalytic studies since the human enzyme is unstable when extracted from membranes (67). 1α-Hydroxylase activity of purified mouse CYP27B1 was measured in a reconstituted system containing 25(OH)D$_3$, 20(OH)D$_2$ substrate incorporated into phospholipid vesicles (67). For human CYP27B1, the reconstituted system was similar except that bacterial membranes were used and substrates were added from an ethanol stock. After incubation, secosteroids were extracted with dichloromethane and analyzed by reverse-phase HPLC on a C18 column as before (66). Kinetic parameters were determined by fitting the Michaelis-Menten equation to the experimental data using Kaleidagraph 3.6. The concentration of 25(OH)D$_3$ used was kept below 6 × $K_m$ to avoid the substrate inhibition reported to occur at higher concentrations (67).

To produce sufficient product from the action of CYP27B1 on 20(OH)D$_2$ for structural determination by NMR, a 70-ml incubation of 20(OH)D$_2$ in phospholipid vesicles containing 0.025 mol substrate/mmol phospholipid was carried out for 1 h with 0.8 μM mouse CYP27B1. The product was extracted with dichloromethane and purified by reverse-phase HPLC (63), which yielded 80 μg.

To test ability of cultured cells to 1α-hydroxylate 20(OH)D$_2$ compared with 25(OH)D$_3$, $10^9$ HaCaT keratinocytes were incubated in DMEM without phenol red (Sigma-Aldrich) containing 5 μM 25(OH)D$_3$ or 20(OH)D$_2$ for 24 h. After incubation, the mixtures were extracted twice with 2.5 volumes of dichloromethane. The extract was redisolved in methanol and analyzed using an API-3000 LC-MS/MS (Applied Biosystems, Toronto, Canada) mass spectrometer equipped with an ESI source. HPLC separation was performed with a gradient of methanol in water (65%-100%) at a flow rate of 0.075 ml/min for 20 min using a Zebra Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm) (Agilent Technology, Santa Clara, CA).

**Defining the Chemical Structure of 1,20(OH)$_2$D$_2$**

**NMR spectroscopy.** All NMR measurements were performed on a Varian Unity Inova 500 MHz spectrometer equipped with a 3-mm inverse probe (Agilent, Santa Clara, CA). Samples were dissolved in CD$_3$OD and transferred to 3-mm Shigemi NMR tubes (Shigemi, Allison Park, PA). Temperature was regulated at 22°C and was controlled with an accuracy of ±0.1°C. Chemical shifts were referenced to residual solvent peaks for CD$_3$OD (3.31 ppm for proton and 49.15 ppm for carbon). Standard two-dimensional NMR experiments [1H-1H correlation spectroscopy (COSY), 1H-1H total correlation spectroscopy (TOCSY), 1H-13C heteronuclear single correlation spectroscopy (HSCQC), and 1H-13C heteronuclear multiple bond correlation spectroscopy (HMBCC)] were acquired to fully elucidate the structures of the metabolite. All data were processed using ACD NMR processor (Advanced Chemistry Development, Toronto, ON, Canada), with zero-filling in the direct dimension and linear prediction in the indirect dimension.

**Mass spectrometry.** The mass spectrum of 1,20(OH)$_2$D$_2$ was acquired in a Bruker Esquire-LC system (Bruker Daltonics, Billerica, MA) equipped with an electrospray ionization (ESI) source. Data were collected and processed by ACD mass processor (Advanced Chemistry Development).

**Cell Culture**

Normal human keratinocytes (HEKn) and melanocytes were isolated from neonatal foreskin of African American patients following protocols described previously (26, 27). The protocols were approved by the local IRB. Keratinocytes were grown in serum-free keratinocyte basal medium (KBM) supplemented with keratinocyte growth factors (KGF) (Lonza, Walkersville, MD) on collagen-coated plates (27), whereas normal melanocytes were cultured in MBM-4 medium (Lonza) containing MGM-4 (Lonza). Immortalized human epidermal keratinocytes (HaCaT) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% charcoal-treated fetal bovine serum (CT-FBS) (Hyclone, Logan, UT) and 1% antibiotics [penicillin-streptomycin-amphotericin (Sigma-Aldrich)]. Human SK-MEL-188 and hamster A67C melanoma cells were grown in Ham’s F10 supplemented with 5% CT-FBS and 1% antibiotics (59). All cultures were performed at 37°C in 5% CO$_2$.

**DNA Synthesis**

Incorporation of [3H]thymidine into DNA was used as a measure of cell proliferation following protocols described previously (26, 76).
Briefly, cells were synchronized at G0/G1 phase of the cell cycle by incubation in serum-free media (26, 29, 76), and then 20(OH)D2 was added with fresh media containing growth supplements (as indicated in the figure legends) and incubated for an additional 24–72 h. After a defined period of time, [3H]thymidine (specific activity 88.0 Ci/mmol; Amersham Biosciences, Picataway, NY) was added to a final concentration of 0.5 μCi/ml in medium, and after 4 h of incubation cells were precipitated with 10% TCA, processed as described previously, and subjected to liquid scintillation counting using a beta counter (Direct Beta-Counter Matrix 9600; Packard).

Cell cycle analysis was performed as described previously (26, 29). After synchronization of 40% confluent cultures of HaCaT keratino-...
cytes or melanoma cells at G1/G0 phase, the cultures were maintained in DMEM media containing 5% CT-FBS with or without 10−7 M 20(OH)D2 for 72 h. The cells were then processed for flow cytometry with staining in propidium iodide (Sigma-Aldrich) as described previously. The cell cycle analysis was performed with a FACS Calibur flow cytometer (UTHSC Flow Cytometry and Cell Sorting Laboratory) with 10,000 cells scored.

**Cell Viability Assay (MTT Assay)**

For determining the number of viable cells in proliferation, we used 5-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Promega, Madison, WI). Human melanoma cells (SKMel-188) and neonatal human epidermal keratinocytes (HEKn), isolated from the foreskin from African American patients and in their third passage, were seeded in a 96-well plate and grown until 80% confluent. Cells were then treated with 0.1–100 nM 1,25(OH)2D3, 20(OH)D2, or 25(OH)D3 (or ethanol vehicle), diluted in KGM supplemented with KGF (Lonza) medium and 0.5% BSA for keratinocytes and in F-10 media for melanoma cells, 100 μl/well. After 44 h of incubation, 20 μl of MTT reagent (5 mg/ml) was added to the cells, which were further incubated for 4 h at 37°C. Media were discarded and cells lysed in isopropanol-0.1 N HCl solution for 30 min with shaking at room temperature. The quantity of formazan product is directly proportional to the number of living cells in culture as measured by the amount of 570 nm absorbance. Data were analyzed using GraphPad Prizm and one-way ANOVA.

**Colony-Forming Assay**

The assay followed standard methodology used in our laboratory as described previously (18, 29, 76). Briefly, cells were plated at a density of 20 cells/cm² in medium containing 5% CT-FBS. Graded concentrations of 20(OH)D2 or 1,25(OH)2D3 (or ethanol vehicle as a

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**Fig. 3. Vitamin D derivatives inhibit DNA synthesis in normal and malignant skin cells in a time- and dose-dependent manner. HaCaT keratinocytes were an incubated for 48 h (A) or 72 h (B) in the presence of graded concentrations of 20(OH)D2 or 1,25(OH)2D3. Dose-dependent inhibition of the proliferation of immortalized normal epidermal melanocytes (PIG1 line) (C), neonatal epidermal melanocytes (D), SKMEL-188 human (E), and AbC1 hamster (F) melanoma cells by 20(OH)D2 or 1,25(OH)2D3 was measured after 72 h of exposure, with the exception of D, where it was measured after 48 h. Data are shown as means ± SD (n ≤ 3); *P < 0.05; **P < 0.01.**
control) were added with media being changed every 3 days. After 7 days of incubation, the colonies were fixed and then stained with 2% crystal violet, and the number and size of the colonies were measured using an ARTEK counter 880 (Dynex Technologies, Chantilly, VA). Colony-forming units were calculated by dividing the number of colonies by the number of cells plated and then multiplying by 100.

**Cell Differentiation and Proliferation of Leukemia Cell Lines**

HL-60 human promyelocytic and K562 human erythroleukemia cells were cultured in RPMI 1640 medium containing 10% CT-FBS and 1% penicillin-streptomycin-amphotericin antibiotic solution (61). The test compounds were added daily at concentrations of $10^{-7}$M with media being changed every 72 h. After 5 days the cells were stained in 0.4% trypan blue (Sigma) and the viable cells counted with a hemocytometer.

Erythroid differentiation of K562 cells was evaluated after 7 days by benzidine staining. Benzidine-positive cells were scored under light microscopy examination ($\times$20) with a minimum of 200 cells scored, and the induction of differentiation was expressed as the number of benzidine-positive cells per 200 cells. For spectrophotometric analysis, the cells were washed and the insoluble formazan deposits in the resulting pellets were solubilized in 1 ml of 90% DMSO, 0.1% SDS, and 0.01 mM NaOH, the samples were centrifuged 5 min at 1,500 g to remove the cellular debris, and then the absorbance of supernatants was measured at 715 nm. Data are expressed as change in $A_{715}/10^{6}$ cells (16).

**Immunofluorescent Staining**

For involucrin immunostaining, normal human epidermal keratinocytes (HeKa) were seeded onto cover slides in six-well plates and treated with $10^{-7}$ M 20(OH)D$_2$ or 1,25(OH)$_2$D$_3$ for 24 h. After treatment, cells were washed, fixed in 4% paraformaldehyde, and processed for immunofluorescent analysis as described previously (76, 78). Primary mouse anti-human-involucrin antibody was used at dilution 1:200 in 1% BSA (Sigma-Aldrich), and incubations were for 3 h at room temperature with shaking. After being washed in PBS, cells were incubated in secondary antibody solution of anti-mouse-FITC conjugate (NCL-SAM-FITC) (1:200 in 1% BSA) for 1 h. Nuclei were stained with propidium iodine (Vectashield), and cells were viewed with a fluorescent microscope and photographed under $\times$40 magnification. Ten to twenty independent images were generated for each condition tested. The percentage of cells expressing involucrin was counted while relative fluorescent intensity and relative fluores-

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**Fig. 4.** The comparison of inhibitory effects of 20(OH)D$_2$ (A) and 1,25(OH)$_2$D$_3$ (B) on the ability of human SKMEL-188 melanoma cells to form colonies. After 7 days, colonies were stained with crystal violet, and the numbers over 0.2 mm and over 0.5 mm were counted. Data are shown as means ± SD (n ≥ 3). *P < 0.05; **P < 0.01. ***P < 0.001. Bottom: representative plates of melanoma cells treated with vehicle (control) or $10^{-7}$ M 20(OH)D$_2$ or 1,25(OH)$_2$D$_3$. 

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**Table 1.** Differences in the number of benzidine-positive cells between the control and treatment groups.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Number of Benzidine-Positive Cells</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>0.0</td>
</tr>
<tr>
<td>20(OH)D$_2$</td>
<td>100</td>
<td>0.0</td>
</tr>
<tr>
<td>1,25(OH)$_2$D$_3$</td>
<td>100</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Notes:**

- Data are expressed as means ± SD (n ≥ 3).
- *P < 0.05; **P < 0.01. ***P < 0.001.
cent areas were determined by Image J software downloaded from the NIH web site.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA from cells was extracted using an Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA). A reverse transcription reaction was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN). The reaction was performed with a LightCycler 480 Probes Master (Roche Applied Science). The primers and probes were designed with the Universal Probe Library (Roche Applied Science), and the sequences are shown in supplemental Table S1. Real-time PCR was performed using TaqMan PCR Master Mix at 95°C for 5 min followed by 45 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 10 s. The data were collected with a Roche LightCycler 480, and the amount of mRNA was normalized by a comparative Ct method, using cyclophilin B as a housekeeping gene.

Generation of Cell Lines Overexpressing VDR Fused With EGFP

pLenti-CMV-VDR-pgk-puro was constructed by cleavage of the VDR cDNA from the plasmid pRC-CMV-VDR (kind gift from Dr. Daniel Bikle, UCSF) using XhoI and XhoI sites and subcloning it into the same sites of pLenti-CMV-p2a-GFP-pgk-puro (Viral Vector Core lab at UTHSC, Memphis). pLenti-CMV-VDR-pgk-puro plasmid (Viral Vector Core, UTHSC) (75) was amplified using primers 5’-ATACATGGATCCCACTGAGGACATGCGGCCAGC-3’ (forward); 5’-AGCTACTGAGGACATGCGGCCAGC-3’ (reverse), and then digested with BamHI and XhoI and subcloned into the same sites of the pLenti-CMV-p2a-GFP-pgk-puro vector (Viral vector core, UTHSC) (75) to construct pLenti-CMV-VDR-p2a-EGFP-pgk-puro (supplementary Fig. S2). To construct pLenti-CMV-VDR-EGFP-pgk-puro, EGFP cDNA was amplified by PCR from pLenti-CMV-VDR-p2a-EGFP-pgk-puro using primers 5’-GGACCTCTCGAGATGGTGAGCAAGGGCGAGGAG-3’ (forward); 5’-GGCAATTCCTACTTGTGATTACGTCTGCTCCATGCC-3’ (reverse), then digested with XhoI and EcoRI and subcloned into pLenti-CMV-VDR-p2a-EGFP-pgk-puro in which EGFP including p2a gene was removed. The VDR in pLenti-CMV-VDR-p2a-EGFP-pgk-puro was expressed independently of EGFP following 2A peptide self-cleavage, whereas the VDR in pLenti-CMV-VDR-EGFP was expressed with EGFP together as a fusion protein (supplementary Fig. S2). The lentiviral control vector pLenti-CMV-EGFP-pgk-puro was obtained from Viral Vector Core (UTHSC).

Fig. 5. Comparison of the antiproliferative activities of 20(OH)D2, 25(OH)D2, and 1,20(OH)2D2 in human epidermal keratinocytes. A–C: rate of proliferation was determined from the amount of [3H]thymidine incorporated into DNA after 48 h of culture. Data are presented as means ± SD (n = 3). *P < 0.05. D–F: epidermal keratinocytes were treated with graded concentrations of the ligands for 24 h, and the number of viable cells was measured using the MITT assay as described in MATERIALS AND METHODS. Data are shown as means ± SD (n = 3); *P < 0.05. **P < 0.01.
Lentivirus was produced in 293FT cells by UTHSC viral vector core using a method described previously (75). Ten multiplicities of infection of lentivirus (construct pLenti-CMV-VDR-p2a-EGFP-pgk-puro) were used to transduce SKMEL-188 melanoma and HaCaT cells in the presence of 6 μg/ml polybrene in the corresponding culture media (see above). The transduction efficiency was determined using a Nikon Eclipse TE300 microscope (Japan) by green fluorescence.

Silencing of VDR in HaCaT Cells Using VDR shRNA Lentivirus Technology

Scrambled and VDR short hairpin RNA (shRNA) lentiviral particles were purchased from Santa Cruz Biotech (Santa Cruz, CA). The viral particles were transduced following the manufacture’s protocols. Briefly, lentiviral particles were transduced into HaCaT cells cultured in DMEM media containing 5% FBS in the presence of 5 μg/ml polybrene. The transduced cells were selected by further culturing in media containing 5 μg/ml puromycin. DNA synthesis in transduced cells was tested using protocols described above.

Silencing of CYP27B1 in Epidermal Keratinocytes Using siRNA Technology

Keratinocytes were transfected with 10 nM scrambled or CYP27B1 small interfering RNA (siRNA, Santa Cruz) using Lipofectamine plus (Invitrogen, Carlsbad, CA). After transfection, cells were incubated for 24 h, and medium was replaced with fresh containing the compounds to be tested or vehicle (ethanol) at the concentration of 1 μM and incubated for 24 h. Gene expression levels were determined using RT-PCR as described previously.

VDR Translocation Test

With the use of SKMEL-188 transduced by pLenti-CMV-VDR-EGFP-pgk-puro (VDR and EGFP expressed as fusion protein), VDR translocation was determined by counting cells with a fluorescent nucleus. The transduced cells were incubated with drugs for 30, 90, or 240 min and then fixed with 4% paraformaldehyde (PFA). Fixed cells were mounted with fluorescent mounting media (Dako, Denmark) and analyzed with a fluorescent microscope. At least three pictures were taken from different fields per condition, and the cells containing fluorescent nuclei were counted. Data are presented as a percentage of total cell numbers.

Testing of Calcemic Effect and Histological Markers of Toxicity

The calcemic effects of 20(OH)D$_2$ were compared with those of 1,25(OH)$_2$D$_3$ as described previously for 1,20(OH)$_2$D$_3$ and 20(OH)D$_3$, with some modifications (61). Briefly, 2-mo-old rats were obtained from Jackson Laboratory and fed a normal rat chow for 2 wk

![Fig. 6. 20(OH)D$_2$ induces differentiation of normal human epidermal keratinocytes. 20(OH)D$_2$ (10$^{-7}$ M) induced time-dependent involucrin gene expression (A) that was accompanied by increased expression of involucrin protein (B), which was significant in terms of increased number of cells expressing involucrin (C), increase in total relative fluorescence (D) and increased fluorescent area (E). Data are shown as means ± SD (n = 10–20). **P < 0.01. ***P < 0.001.](http://ajpcell.physiology.org/)
before they were divided into seven groups (6 animals per group) and injected with either vehicle (propylene glycol) or three concentrations (0.8, 2.0, and 4.0 μg/kg) of 1,25(OH)2D3 or 20(OH)D2 dissolved in propylene glycol, for 7 consecutive days. A day after the final dosing, selected organs including liver, kidney, heart, skin, and intestine were collected, fixed in 4% formalin and further processed to paraffin blocks. Five-micrometer sections were deparaffinized and processed further for staining with hematoxylin and eosin (H&E). The H&E-stained sections were examined independently by two board-certified anatomic pathologists. Experiments on rats were approved by IRB protocol from Boston University.

Statistical Analysis

Data were analyzed with GraphPad Prizm Version 4.0 (GraphPad Software, San Diego, CA) using the t-test or one-way ANOVA with appropriate post hoc tests. Differences were considered significant when \( P < 0.05 \). The data are presented as means ± SD.

RESULTS

Absolute Configuration at C20 in The 20(OH)D2 Metabolite

There are two possible isomers for this 20(OH)D2 metabolite: 20\( \text{S}(\text{OH})\text{D2} \) and 20\( \text{R}(\text{OH})\text{D2} \), depending on the absolute configuration at C20. Accumulating evidence strongly suggests that this purified metabolite is 20\( \text{S}(\text{OH})\text{D2} \) for the following reasons. First, this is a pure compound instead of a mixture of diastereomers as indicated by both HPLC and NMR studies (56). Second, extensive literature indicates that protons of 21-Me have very distinctive chemical shifts for 20\( \text{S-} \) and 20\( \text{R-} \)hydroxylation (12, 14, 34, 41, 45). For example, the \(^1\text{H}-\text{NMR} \) chemical shift for 21-Me in 20\( \text{S-} \)-OH-cholesterol is 1.17 to 1.28 ppm (24), and 20\( \text{R-} \)-OH-cholesterol is 1.00 to 1.12 ppm, depending on NMR solvents (24). The value of the proton NMR chemical shift for 21-Me in 20(OH)D2 is 1.31 ppm (56), strongly indicating the 20\( \text{S-} \)-configuration in this metabolite. This value is basically the same as that for leucisterol (1.34 ppm), which has very similar structure in the side chain compared with 20(OH)D2 (17). Whereas unambiguous assignment requires challenging stereospecific synthesis of both isomers and X-ray crystallography, a major study currently underway, collectively current evidence strongly suggests the 20\( \text{S-} \)-configuration in this metabolite. It should be noted that the 20\( \text{S-} \)-configuration is consistent with the known mechanism of P450 hydroxylation. The heme-bound oxygen atom abstracts hydrogen from the substrate at C20 forming a hydroxyl group, which is then transferred to the site of hydrogen abstraction on the substrate, all on the same face of the molecule (39).

Identification of 1,20(OH)2D2

Mass spectrometry (Fig. 1A) and NMR analysis (Figs. 1B, 2, and supplementary Fig. S3) of the metabolite produced by the action of CYP27B1 on 20(OH)D2 confirmed its structure to be 1\( \text{,20-dihydroxyvitamin D2} \). The calculated mass for 1\( \text{,20(OH)2D2} \) is 428.3, and the observed molecular ion is 451 [M + Na]\(^+ \) (Fig. 1A).

The sites of hydroxylation were unambiguously assigned to be at 1 and 20 positions based on the NMR spectra for this metabolite. All five methyl groups (18-, 21-, 26-, 27-, 28-CH\(_3\)) are intact (Figs. 1B and 2A). The doublet of 21-CH\(_3\) in vitamin D2 became a singlet in the metabolite (1H at 1.29 ppm, 13C at 28.8 ppm, Fig. 2A, 1H-13C HSQC, projection), indicating the loss of scalar coupling from 20-CH. Thus the presence of a 20-OH group in the metabolite is unambiguously established. This is consistent with our previously reported results (56). For the other hydroxylation site, 1H-NMR and 1H-13C HSQC revealed a methine peak at 4.35 ppm (13C at 71.2 ppm, Fig. 2B), which is in the same spin system with the methine at C3 (4.13 ppm) based on the
1H-1H total correlation spectroscopy (TOCSY) spectrum (Fig. 2C). Therefore, the hydroxylation must be on the A ring. However, this unknown methine group is not adjacent to the C3 methine because they do not couple with each other as indicated by the 1H-1H COSY NMR spectrum (Fig. 2D). Thus the only possible site for the hydroxylation is the C1 position. The methine proton at the C1 position is a triplet (Fig. 2, B–D), with a coupling constant of 5.0 Hz to the adjacent two protons at C2. This small coupling constant indicates its equatorial (1α-OH) rather than axial (1β-OH) position, which would result in a much larger coupling constant. Thus the above analysis unambiguously confirms the metabolite to be 1α,20(OH)2D2, consistent with the well-established 1α-hydroxylase activity of CYP27B1 on a range of vitamin D analogs (66, 67). In the rest of the text, for simplicity, we refer to this metabolite as 1,20(OH)2D2.

20(OH)D2 Regulates Behavior of Normal and Malignant Skin Cells

We tested the effect of 20(OH)D2 on proliferation of HaCaT keratinocytes by assaying [3H]thymidine incorporation into DNA (Fig. 3, A and B). It inhibited DNA synthesis in time- and dose-dependent manners with more pronounced effects seen at 72 h than at 48 h of culture; the EC50 value was 3.15 × 10⁻¹¹ M, which was lower than for 1,25(OH)2D3 (Fig. 3B).

Next, we tested the response of normal epidermal melanocytes and melanoma cells to 20(OH)D2 at concentrations 10⁻¹¹ to 10⁻⁷ M and compared this to HaCaT keratinocytes. The dose-dependent inhibition curves were constructed and the EC50 values were 2.05 × 10⁻⁹, 1.21 × 10⁻¹⁰, 3.1 × 10⁻¹⁰, and 1.93 × 10⁻¹¹ M for immortalized normal epidermal melanocytes (PIG1 line), neonatal epidermal melanocytes, SKMEL-188, and AbC1 melanoma lines, respectively (Fig. 3). Thus the effect was dependent on cell type with HaCaT keratinocytes and melanoma cells being slightly more sensitive than immortalized normal melanocytes. We also compared the antiproliferative activity of 20(OH)D2 to that of 1,25(OH)2D3 for normal melanocytes and melanoma cells (Fig. 3, D–F). Interestingly, 20(OH)D2 was more potent than 1,25(OH)2D3 (EC50 = 3.6 × 10⁻¹⁰ M) in inhibition of normal melanocytes, however, was equipotent with 1,25(OH)2D3 in inhibiting SKMEL-188 melanoma proliferation (EC50 = 3.1 and 2.2 × 10⁻¹⁰ M). We have also used the MTT assay to show that human melanoma growth is inhibited by 20(OH)D2 (P < 0.01).

Morphological evaluation of the cells showed a lack of identifiable cytotoxic effects (not shown). The flow cytometry studies have confirmed the above finding showing that 20(OH)D2 slows cell cycling [to a similar degree as 1,25(OH)2D3] of HaCaT and melanoma cells through an increase in G1/G0 and a decrease in S and G2/M phases, with no effect on subG1 (a marker of apoptosis) (supplementary Fig. S4).

To further define the tumorostatic effect of 20(OH)D2, we examined its effect on the ability to form colonies by human SKMEL-188 melanoma cells compared with 1,25(OH)2D3. 20(OH)D2 significantly inhibited colony formation at concentrations as low as 10⁻¹¹ M. Analysis of all colonies formed (size >0.2 mm) showed that 20(OH)D2 was more potent than 1,25(OH)2D3 (Fig. 4).
20(OH)D2 Inhibits Proliferation and Stimulates Differentiation of Normal Human Epidermal Keratinocytes and is More Potent Than 25(OH)D3

20(OH)D2 inhibited growth of normal epidermal keratinocytes in a similar manner to that by 1,25(OH)2D3, and was significantly more potent than 25(OH)D3 (supplementary Fig. S5A). The inhibition of proliferation by 20(OH)D2 was reflected by the attenuation of the expression of cyclin B1, D1, and E1 mRNA expression (supplementary Fig. S5B). Next, we compared the effects of 20(OH)D2 with those of 25(OH)D3 and 1,20(OH)2D2 on human keratinocytes growth (Fig. 5). 20(OH)D2 and 1,20(OH)2D2 inhibited DNA synthesis similarly, whereas 25(OH)D3 had no significant effect (Fig. 5, A–C). We also measured cell proliferation using the MTT assay and, again, while 20(OH)D2 and 1,20(OH)2D2 shown strong inhibition, 25(OH)D3 was without significant effect (Fig. 5, D–F).

20(OH)D2 stimulated involucrin gene expression, which was accompanied by a significant increase in involucrin protein expression (Fig. 6), confirming that 20(OH)D2 induces the keratinocyte differentiation program. In additional experiments we compared the effects of 20(OH)D2 with those of 25(OH)D3, using 1,25(OH)2D3 as a positive control (supplementary Fig. S6A). Again, 20(OH)D2 was markedly more potent than 25(OH)D3 in the induction of involucrin gene expression (11-fold difference). Further testing has also shown that 20(OH)D2 and 1,20(OH)2D2 are significantly more potent (2- and 1.5-fold, respectively) in the induction of VDR gene expression than 25(OH)D3 (supplementary Fig. S6B).

The above experiments clearly show that 20(OH)D2 is more potent than 25(OH)D3 in the inhibition of cell proliferation and induction of differentiation.

20(OH)D2 is a Poor Inducer of CYP24 Gene Expression Compared With 1,25(OH)2D3

Induction of CYP24 expression by 20(OH)D2 required a comparatively high concentration of the ligand (10^-6 M), and induction was low or absent at a concentration of 10^-8 M. This is in contrast to strong stimulation of CYP24 expression by 1,25(OH)2D3 at the same concentrations (Fig. 7). This difference was similar to that described previously for the related 20(OH)D3 versus 1,25(OH)2D3 (34) or 20,23(OH)2D3 versus 2,25(OH)2D3.

Fig. 9. 20(OH)D2 (10^-7 M) induces time-dependent translocation of VDR from the cytoplasm to the nucleus. Data are presented as means ± SD (n ≥ 3). *P < 0.05; **P < 0.01; ***P < 0.001. EtOH: 0.1% ethanol.
1,25(OH)₂D₃ (26). We have also compared the effect of 10⁻⁷ M 20(OH)D₂ with that of 1,20(OH)₂D₃ on normal keratinocytes after a 24 h incubation and found that 20(OH)D₂ is about eight times less potent than 1,20(OH)₂D₃ (P < 0.001) in the stimulation of CYP24 expression (supplementary Fig. S7A). In an additional experiment using siRNA technology, we found that silencing of CYP27B1 completely abrogated the effect of 20(OH)D₂ and only partially the effect of 25(OH)D₃ on CYP24 expression (supplementary Fig. S7B). The latter is consistent with recent reports indicating that 25(OH)D₃ can also act as an agonist vitamin D receptor ligand (35) or through another mechanism (49).

**Phenotypic Effects of 20(OH)D₂ Are Mediated Through Interaction With VDR**

We have performed several experiments documenting that 20(OH)D₂ exerts its phenotypic effects through activation of VDR (Fig. 8). First, stable overexpression of VDR by lentiviral technology significantly increased inhibition of cell proliferation by 20(OH)D₂ in keratinocytes and melanoma cells (Fig. 8, A and B). Second, silencing of the VDR gene attenuated 20(OH)D₂-mediated inhibition of cell proliferation (Fig. 8C). Finally, 20(OH)D₂ induced time-dependent translocation of VDR from the cytoplasm to the nucleus at a comparable rate to that seen for 1,25(OH)₂D₃ (Fig. 9).

**Anti-Leukemic Effects of 20(OH)D₂**

To study the anti-leukemic effect of 20(OH)D₂, we used K562 and HL60 lines to evaluate erythroid and monocytic differentiation, respectively. Thus, after 5 days of treatment, both 10⁻⁸ M and 10⁻⁶ M 20(OH)D₂ or 1,25(OH)₂D₃ induced significant increases in the number of cells synthesizing hemoglobin, with 1,25(OH)₂D₃ being significantly more potent (supplementary Fig. S8A). The same effect was seen when the level of cell differentiation was determined from the relative concentration of hemoglobin as hemin, measured spectrophotometrically in an equal number of cells (supplementary Fig. S8B).

The induction of differentiation of HL-60 cells toward monocyte-like morphology was evaluated by nitro blue tetrazolium (NBT) reduction and cell morphology, after 5 days of treatment. Both compounds tested induced cell differentiation in a dose-dependent manner as measured by the percentage of cells positive for NBT and by the absorbance at 715 nm of the supernatants from lysates (supplementary Fig. S8, C and D). Again 1,25(OH)₂D₃ was more potent than 20(OH)D₂. Similarly, 1,25(OH)₂D₃ was more potent in the reduction of cell number than 20(OH)D₂ (supplemental Table S2).

20(OH)D₂ is Noncalcemic

To test the systemic toxicity due to hypercalcemia, rats were treated ip with 0.8, 2, or 4 μg/kg of either 20(OH)D₂ or 1,25(OH)₂D₃ every day for 7 days. Histological examination of the organs harvested at the end of the experiment showed that 1,25(OH)₂D₃ induced calcium deposits in the heart and kidney at doses as low as 0.8 μg/kg, while this effect was absent for 20(OH)D₂ even at dose as high as 4 μg/kg (Fig. 10). This shows that the cytotoxic effect of 1,25(OH)₂D₃ is associated with renal or cardiovascular insufficiency due to ectopic calcification. Examination of other organs including liver, lungs, small bowel, and skin showed that there was a lack of calcification or morphologically identifiable toxic effects for both 20(OH)D₂ and 1,25(OH)₂D₃.

**20(OH)D₂ is a Relatively Poor Substrate For CYP27B1**

To test the likelihood that 20(OH)D₂ rapidly undergoes 1α-hydroxylation in target cells to 1α,20-dihydroxyvitamin D₂, which could then mediate its biological effects, we tested the ability of vitamin D 1α-hydroxylase (CYP27B1) to metabolize 20(OH)D₂. This was done with mouse CYP27B1, where purified bacterially expressed enzyme was available (66, 67), and with human CYP27B1 in bacterial membranes (Fig. 11A). Mouse CYP27B1 converted only 6% of the 20(OH)D₂ to 1α,20-dihydroxyvitamin D₂, during a 2-min incubation, whereas 88% of 25(OH)D₃ was converted to 1,25(OH)₂D₃ under identical conditions (Fig. 11A, bottom). Detailed kinetic analysis revealed that both sources of enzyme displayed a higher Kₘ with 20(OH)D₂ as substrate than with 25(OH)D₃, and a lower maximum velocity (kₗₘₚₐₓ) than 20(OH)D₂. The overall catalytic efficiency (kₗₘₚₐₓ/Kₘ for mouse CYP27B1, Vₘₚₐₓ/Kₘ for human CYP27B1) was 24-fold lower for 20(OH)D₂ than for 25(OH)D₃ with the mouse enzyme, and 41-fold lower for the human enzyme.

The enzymatic analysis showing that 20(OH)D₂ is a relatively poor substrate for CYP27B1 was confirmed using cultured cells (Fig. 11B). Thus, whereas incubation of HaCaT keratinocytes with 25(OH)D₃ led to production of 1,25(OH)₂D₃, no 1,20(OH)₂D₂ was detected in simultaneous incubations with 20(OH)D₂ (Fig. 11B).

**Biological Activity of 20(OH)D₂ on Keratinocytes Does Not Require its Transformation to 1,20(OH)₂D₂**

To test whether 20(OH)D₂ activity requires its transformation into 1,20(OH)₂D₂, we silenced CYP27B1 gene expression using siRNA technology (Fig. 12A). The silencing of the CYP27B1 gene only slightly (by 8%) decreased the stimulation of involucrin expression by 20(OH)D₂, with the stimulation still being highly significant compared with the control (vehicle treatment) (Fig. 12B). In addition, CYP27B1

![Fig. 10. 1,25(OH)₂D₃ induces calcium deposition in kidneys and heart (arrows), whereas 20(OH)D₂ is noncalcemic, similar to the control (untreated animals). Bar: 100 μm.](http://ajpcell.physiology.org/)
silencing had no effect on 20(OH)D$_2$-induced VDR gene expression (Fig. 12C). Furthermore, silencing of CYP27B1 did not significantly attenuate inhibition of DNA synthesis by 20(OH)D$_2$ (Fig. 12D).

**DISCUSSION**

In this paper we have extensively investigated the biological activities of 20(OH)D$_2$, a novel and major product of the
P450scc-mediated metabolism of vitamin D$_2$ (46, 56). We have also defined the chemical structure of the product generated in vitro by the action of CYP27B1 on 20(OH)D$_2$ as 1,20(OH)$_2$D$_2$ and shown that it is also biologically active. 20(OH)D$_2$ shows potent, cell-type selective, antiproliferative and prodifferentiation effects, having similar (normal and malignant skin cells) or lower potency (leukemia cells) to calcitriol [1,25(OH)$_2$D$_3$] and significantly higher potency than 25(OH)D$_3$. This antiproliferative activity requires the VDR with a ligand-induced receptor translocation to the nucleus. In vivo experiments have shown that 20(OH)D$_2$ at concentrations as high as 4 μg/kg has no identifiable toxic effects, whereas 1,25(OH)$_2$D$_3$ induced calcium deposition in the heart and kidney at the same or even lower (0.8 μg/kg) concentrations. 20(OH)D$_2$ is a poor substrate for CYP27B1, and its antiproliferative and prodifferentiation activities did not require transformation to 1,20(OH)$_2$D$_2$.

Experiments performed on skin cells have demonstrated that 20(OH)D$_2$ inhibits proliferation of epidermal keratinocytes and melanocytes and melanoma cells in dose- (EC$_{50}$ values were between $10^{-11}$ and $10^{-9}$ M) and time-dependent manners, with keratinocytes and melanoma cells being slightly more sensitive than normal melanocytes. The antiproliferative activity of 20(OH)D$_2$ toward skin cells was similar to that of 1,25(OH)$_2$D$_3$ and of enzymatically synthesized 1,20(OH)$_2$D$_2$ (the structure of this new compound was confirmed by NMR) but significantly higher than that of 25(OH)D$_3$. Flow cytometry studies demonstrated that 20(OH)D$_2$ slows cell cycling through an increase in G1/G0 and a decrease in S and G2/M phases rather than having a proapoptotic or toxic effect. In keratinocytes this activity includes stimulation of cell differentiation as documented by induction of involucrin expression by 20(OH)D$_2$ at both mRNA and protein levels. In melanoma cells, it includes inhibition of colony formation suggesting that this antiproliferative activity may reflect its antitumorigenic activity. Thus 20(OH)D$_2$, a product of P450scc action on plant-derived vitamin D$_2$ (46, 56), has very similar antiproliferative/prodifferentiation activity to the related product of vitamin D$_3$ metabolism 20(OH)D$_3$ (27, 76) as well as to the classical active form of vitamin D$_3$ 1,25(OH)$_2$D$_3$ (8, 22), while being significantly more potent than 25(OH)D$_3$.

The similarity of the effects of 20(OH)D$_2$, 1,25(OH)$_2$D$_3$, and of novel 1,20(OH)$_2$D$_2$ raises the question of whether 20(OH)D$_2$ requires further hydroxylation at position 1α, similar to 25(OH)D$_3$, to reach full biological activity. Therefore, we performed additional experiments to examine this possibility. We have found that 20(OH)D$_2$ is a relatively poor substrate for CYP27B1 with the overall catalytic efficiency for CYP27B1 being 24-fold lower for 20(OH)D$_2$ than for 25(OH)D$_3$ with the mouse enzyme and 41-fold lower for the human enzyme. Furthermore, transformation of 20(OH)D$_2$ to 1,20(OH)$_2$D$_2$ in intact keratinocytes could not be detected, which was in contrast to production of 1,25(OH)$_2$D$_3$ when 25(OH)D$_3$ was added to cells. We have also shown that 20(OH)D$_2$ causes significantly higher inhibition of proliferation of human keratinocytes and induction of involucrin and

![Fig. 12](http://ajpcell.physiology.org/)

**Fig. 12.** Effect of CYP27B1 siRNA on CYP27B1 gene expression (A) and stimulation of involucrin (B) and VDR (C) gene expression and cell proliferation (D). Keratinocytes were transfected with 10 nM scrambled or CYP27B1 small interfering RNA (siRNA) using Lipofectamine plus. After transfection, cells were treated with the compounds to be tested or vehicle (ethanol) at the concentration of 1,000 nM and incubated for 24 h. Gene expression levels were determined using RT-PCR. Total RNA was extracted and subjected to real-time RT-PCR analysis with cyclophilin B used as a housekeeping gene. Data are presented as means ± SD (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001. Cells were also treated with the compounds and incubated with thymidine. DNA incorporation is measured using B-counter.
VDR gene expression compared with 25(OH)D3. This is in agreement with our additional work with the analogous vitamin D3 metabolite [20(OH)D3], which is significantly more active than 25(OH)D3 in the inhibition of keratinocyte proliferation and differentiation (76). In addition, silencing of the CYP27B1 gene using siRNA technology neither prevented stimulation of VDR and involucrin gene expression by 20(OH)D2 nor inhibition of keratinocyte proliferation. Therefore, we can safely conclude that the antiproliferative and prodifferentiation activities of 20(OH)D2 do not require its transformation to 1,20(OH)2D2.

We have enzymatically synthesized 1,20(OH)2D2 from 20(OH)D2, and after confirming its structure by NMR, we have tested its biological activity. It had similar or more potent (in some assays) antiproliferative activity against human keratinocytes compared with the 20(OH)D2 precursor. This pattern is very similar to the previously described antiproliferative and prodifferentiation effects of the analogous 1,20(OH)2D3 and 20(OH)D2 in leukemia cells (61) and in human keratinocytes (69). However, the addition of the 1α-hydroxyl group can explain remarkable differences in the stimulation of the expression of CYP24 [the enzyme that inactivates the active forms of vitamin D (50, 52, 53)] between 20(OH)D2 and 25(OH)D3 or 1,25(OH)2D3, with 20(OH)D2 being a rather poor inducer. Importantly, silencing of the CYP27B1 gene completely abrogated the 20(OH)D2-mediated induction of CYP24 expression, indicating that metabolic hydroxylation at the 1α-position is required for this activity. In the related model for vitamin D3, 1,20(OH)2D3 is also more potent than 20(OH)D3 in induction of CYP24 expression, and it does exhibit some calcemic activity, although not that of 1,25(OH)2D3, whereas 20(OH)D3 is noncalcemic (61). Again, the 20(OH)D2, investigated in this study is nontoxic and noncalcemic at comparatively high concentrations. Therefore, in agreement with the previous data, the current study demonstrates a requirement for the 1α-hydroxyl group for stimulation of CYP24 expression by 20(OH)D2 and 20(OH)D3, and perhaps its calcemic activity (61), which is in contrast to the regulation of other cellular functions in human keratinocytes such as proliferation and differentiation.

Since the prodifferentiation and antiproliferative activities of 20(OH)D2 toward epidermal cells are very similar to the endogenously produced 1,25(OH)2D3 (5, 9, 33), we have performed detailed experimental studies to define the involvement of the VDR in this process. The evidence for involvement of VDR was provided using genetically modified keratinocytes and melanoma cells. Specifically, silencing of the VDR in skin cells attenuated the inhibitory effect of 20(OH)D2 on cell proliferation, whereas overexpression of VDR enhanced the antiproliferative effect. These data, in conjunction with the 20(OH)D2-induced time-dependent translocation of VDR from the cytoplasm to the nucleus comparable to that for 1,25(OH)2D3, clearly demonstrate the involvement of the VDR in mediation of the above phenotypic actions, which is again similar to what has been observed for 20(OH)D3 (27, 76) and 20,23(OH)2D3 (26). The ability of 20(OH)D2 to bind to the VDR is illustrated by molecular modeling based on the crystal structure of the VDR (supplementary Fig. S9), which shows that both 20(OH)D2 and 1,20(OH)2D3 metabolites overlap with the native ligand well and bind to the VDR with high affinity. The presence of the 20-OH moiety contributes to the binding with increased van der Waals interaction and provides a unique ability to reduce the hypercalcemic effects for this type of analog, which is consistent with literature reports that certain modifications at C20 are very beneficial for reducing hypercalcemia (4, 30, 31, 36, 64), as well as with our present (Fig. 10) and previous studies (61). In addition, the absence of hydrogen bonding interactions from the 1α-OH to the VDR is likely to contribute to the reduced calcemic activity, since addition of 1α-OH to 20(OH)D3 partially restores the calcemic effect (61).

In contrast to normal and malignant skin cells, 20(OH)D2 is less potent than 1,25(OH)2D3 in its anti-leukemic effects, suggesting that the local cellular environment and/or cell lineage affect the receptor activity. Such VDR activity could depend on RXR availability and/or on the presence and activity of coactivators and corepressors (8, 37, 48). The involvement of these factors in 20(OH)D2 and 20(OH)D3 (and their derivatives) mediated-VDR-dependent signaling represents an exciting new challenge in this field, which could also explain the remarkably different activities on the induction of CYP24 expression.

The above properties make 20(OH)D2 an excellent candidate for treatment of cutaneous hyperproliferative disorders or for use as an adjuvant in the therapy of malignant melanoma. Specifically, it has strong antiproliferative and tumorostatic activities similar to 1,25(OH)2D3 without being toxic. 20(OH)D2 shows no calcemic activity at concentrations (0.8–4 μg/kg) that for 1,25(OH)2D3 induce calcium deposits in the kidney and heart. Furthermore, 20(OH)D2 is produced by enzymatic action of P450scc either in a reconstituted system or in isolated mitochondria (56). This makes it a good candidate for a natural product, although confirmation of this awaits its direct detection in animals or plants. Finally, efficient methods of its production via enzymatic or chemical routes are available (supplementary Fig. S1).

In conclusion, evidence is presented that the newly discovered vitamin D2 derivative 20(OH)D2 shows potent and cell-type-dependent antiproliferative and prodifferentiation activity toward normal and malignant cells through activation of VDR, while having low or no toxic/calcemic activity.


