Novel Vitamin D Hydroxyderivatives Inhibit Melanoma Growth and Show Differential Effects on Normal Melanocytes

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Abstract. *Background/Aims: To test the activity of novel hydroxyvitamin D_3 analogs (20(OH)D_3, 20,23(OH)$_2$D and 1,20(OH)$_2$$D_3$) on normal and malignant melanocytes in comparison to 1,25(OH)$_2$$D_3$. Materials and Methods: Human epidermal melanocytes and human and hamster melanoma cells were used to measure effects on proliferation and colony formation in monolayer and soft agar. Cell morphology and melanogenesis were also analyzed. QPCR was used to measure gene expression. Results: Novel secosteroids inhibited proliferation and colony formation by melanoma cells in a similar fashion to 1,25(OH)$_2$$D_3$, having no effect on melanogenesis. These effects were accompanied by ligand-induced translocation of VDR to the nucleus. In normal melanocytes 1α-hydroxyderivatives (1,25(OH)$_2$$D_3$ and 1,20(OH)$_2$$D_3$) had stronger anti-proliferative effects than 20(OH)D_3 and 20,23(OH)$_2$$D_3$, and inhibited dendrite formation. The cells tested expressed genes encoding VDR and enzymes that activate or inactivate vitamin D_3. Conclusion: Novel secosteroids show potent anti-melanoma activity in vitro with 20(OH)D_3 and 20,23(OH)$_2$$D_3$ being excellent candidates for pre-clinical testing.*

There is a significant public interest in vitamin D_3 due to its wide beneficial effects in both prevention and therapy for various diseases including cancer (1, 4, 13, 23). These pleiotropic (not fully explained) effects are believed to be secondary to the action of 1,25-dihydroxyvitamin D_3 (calcitriol; 1,25(OH)$_2$$D_3$), which is generated through sequential hydroxylation of vitamin D_3 at positions C25 by CYP27A1 or CYP2R1, and C1 by CYP 27B1 (1, 4, 13, 23).

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Most recently, we defined a previously unrecognized pathway of vitamin D metabolism, initiated by cytochrome P450scc (CYP11A1), that generates *in vitro* novel vitamin D hydroxyderivatives, different from the classical 1,25(OH)$_2$D (27, 38, 41) (Figure 1). This pathway can also operate *in vivo* (31). The main product of CYP11A1-initiated metabolism of vitamin D_3 is 20-hydroxyvitamin D_3 (20(OH)D_3) (11, 27). It can further be hydroxylated by CYP11A1 to 20,23-dihydroxyvitamin D_3 (20,23(OH)$_2$$D_3$) and a number of other hydroxy-products (27, 40, 41). Both 20(OH)D_3 and 20,23(OH)$_2$$D_3$ are biologically active, with anti-leukemic properties (30) and exhibit anti-proliferative and pro-differentiation activities in human epidermal keratinocytes (15, 16, 43), inhibiting NF-κB activity (14, 16). Importantly, 20(OH)D_3 is non-toxic (non-calcemic) in rats (30) and mice (42), at doses as high as 3 μg/kg and 30 μg/kg, respectively. 20(OH)D_3 can be hydroxylated to 1,20(OH)$_2$$D_3$ by CYP27B1 (32, 37) and 1,20(OH)D_3 can also be produced from the 1(OH)D_3 prodrug by hydroxylation at C20, mediated by P450scc (39) (Figure 1). Although 1,20(OH)D_3 is biologically active, addition of the hydroxygroup in position 1α causes a partial calcemic activity (30).

Despite significant progress in understanding mechanisms defining malignant behavior of melanoma cells, there is still no therapy for metastic melanoma [reviewed in (9, 10)]. Although the use of B-RAF inhibitors leads to attenutation of the disease, it has undesirable side-effects and there is a high recurrence rate due to development of resistance to B-RAF inhibitors (9, 34). Other types of therapy are predominantly ineffective for metastatic melanoma (8, 9, 24). Therefore, there is a need to develop new strategies to manage this devastiting disease that has a high mortality rate.

The anti-melanoma activity of 1,25(OH)$_2$$D_3$ *in vitro* was established more than 30 years ago (5). Subsequent studies have also shown inhibitory effects of 1,25(OH)$_2$$D_3$ on some human melanoma lines cultured *in vitro* [reviewed in (6, 21, 36)]. A potential beneficial involvement of vitamin D is also indicated by the reverse correlation between serum levels of

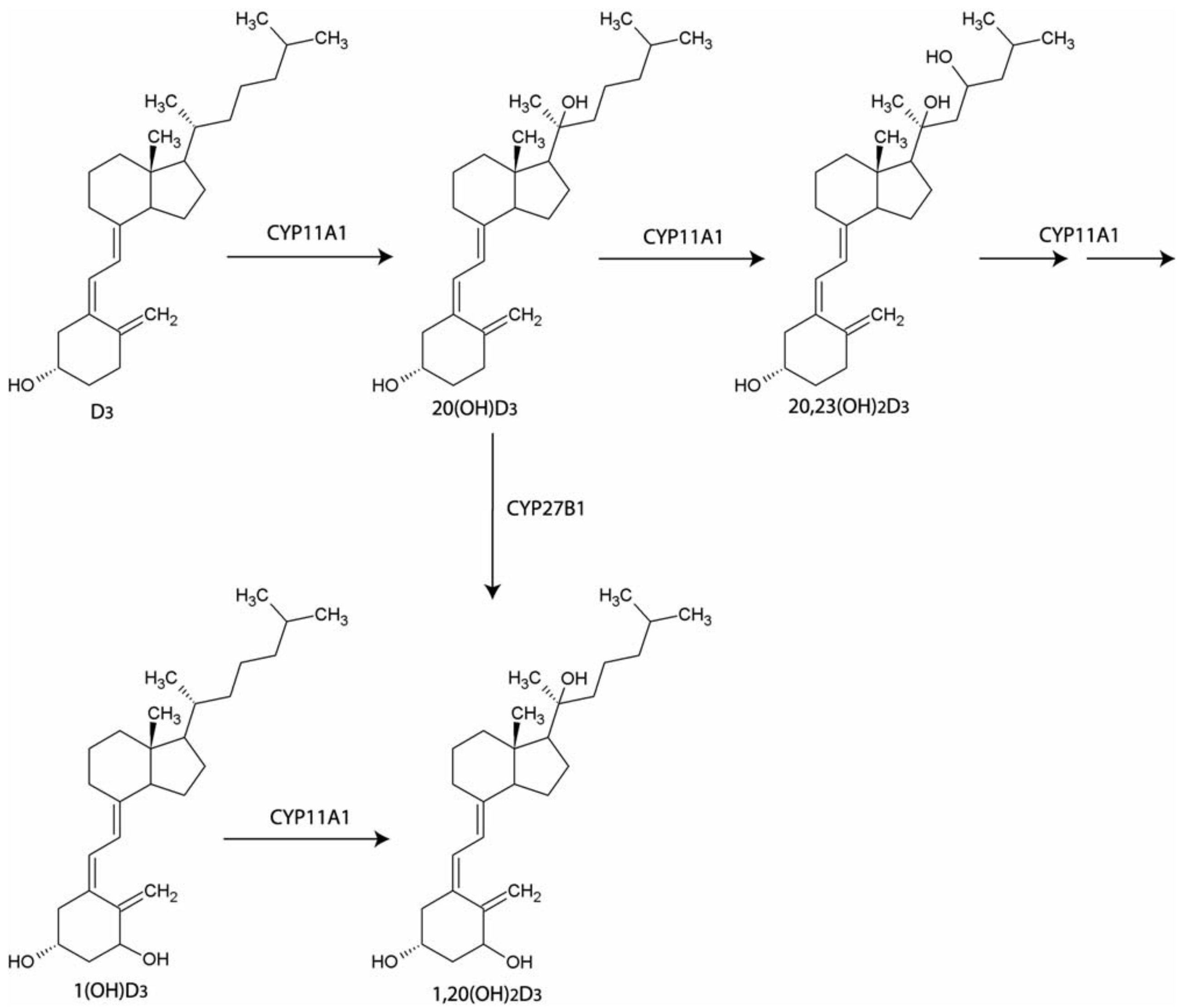

Figure 1. *Structures and enzymatic methods of production of novel vitamin D_3 hydroxy-derivatives.*

1,25(OH)D_3 or local cutaneous production of vitamin D and melanoma progression, and the markedly increased incidence of melanoma in patients having mutations in the vitamin D receptor (VDR) (reviewed in (6, 7, 21, 36)). Furthermore, recent clinicopathological studies demonstrate a decrease or loss of VDR or CYP27B1 expression during melanoma progression, with loss of either of these markers connected with an increased mortality rate (2, 3). These observations indicate that targetting VDR signaling may represent a promising strategy for malignant melanoma treatment. Therefore, we tested several melanoma lines for anti-melanoma activities of novel non-calcemic vitamin D_3 derivatives derived from the action of CYP11A1. The biological activity of all these cell lines was also tested on normal human epidermal melanocytes.

Materials and Methods

Materials. 1,25$(OH)_2D_3$ was from Fluka Chemicals (Sigma-Aldrich, St. Louis, MO). 20(OH)D_3 and 20,23$(OH)_2D_3$ were produced by the enzymatic hydroxylation of vitamin D_3 catalyzed by CYP11A1, while 1,20$(OH)_2D_3$ was produced by CYP11A1-catalysed hydroxylation of 1(OH)D_3, as described previously (39, 41). Products, extracted with dichloromethane, were first purified by preparative thin-layer chromatography, then further purified by reverse phase HPLC as detailed in (39, 41). The hydroxyderivatives of vitamin D_3 were divided (5 μg/vial), dried and stored at –80˚C until use. Stock solutions were prepared in ethanol at a concentration of 100 μM.

Cell culture. Human SKMEL-188 melanoma cells (gift from Dr Ashok Chokraborty, Yale University), established from a human metastatic melanoma, were maintained in Ham's F10 medium supplemented with glucose, L-glutamine, pyridoxine hydrochloride (Cellgrow, Manassas, VA, USA), 5% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA) and 1% penicillin/ streptomycin/ amphotericin antibiotic solution (Sigma, St. Louis, MO), as described previously (29). YUROB, YUKSI and YULAC human melanoma cells (gift of Dr. Ruth Halaban, Yale University) were cultured in Opti-MEM media supplemented with 10% serum (12). Human WM35, WM1341, WM164, WM98D and SBCE2 melanoma cells (gift of Dr Meenhard Herlyn from Wistar Intitute) and the hamster AbC1 melanoma line were cultured as described previously (22, 25). Normal human epidermal melanocytes were established from foreskin of African-American donors following protocols described previously (32). They were grown in melanocyte MBM media supplemented with MGF (Lonza, Walkersville, MD)

Cell proliferation assays. To measure cell growth, human melanocytes (HEMn) and melanoma cells (SK Mel 188) were seeded in 25 cm^2 flasks and grown until 80% confluent. Ham's F10 plus 5% charcoal-stripped FBS media was used for melanoma cells or MBM + MGF for melanocytes. The media were changed every third day and 100 nM of $1,25(OH)_2D_3$, $20,23(OH)_2D_3$, $1,20(OH)_2D_3$, $20(OH)D_3$ or ethanol (solvent control) were added every day. After 7 days the cells were trypsinized, stained with Trypan blue, and viable cells were counted under the microscope.

Testing of DNA synthesis was carried out as described previously (17, 26). Cells were inoculated into 24-well plates at 5,000 cells/well. After overnight incubation at 37˚C, the cultures were placed in serum-free media to synchronize cells at the G_0/G_1 phase of the cell cycle. After 24 h, vitamin D_3 derivatives (100 nM) were added along with fresh media containing growth supplements and incubated for an additional 48 h. After a defined period of time, [^{3}H]-thymidine (specific activity 88.0 Ci/mmol; Amersham Biosciences, Piscataway, NY, USA) was added to a final concentration of 0.5 μCi/mL in the medium. After 4 h of incubation at 37˚C, media were discarded, cells precipitated in 10% TCA for 30 min, washed twice with 1 mL PBS and then incubated with 1 N NaOH/ 1% SDS (250 μL/well) for 30 min at 37˚C. The extracts were collected in scintillation vials and 5 mL of scintillation cocktail was added. ^{3}H-radioactivity incorporated into DNA was measured with a beta counter (Direct Beta-Counter Matrix 9600; Packard).

Colony forming assay. The assay followed standard methodology as described previously (19, 43). Briefly, cells were plated in 24-well plates at a density of 192 cells/well in medium containing 5% charcoal-treated FBS, 1% antibiotic solution and vitamin D_3 hydroxyderivatives, at graded concentrations or vehicle control. After 10 days of culture with media changed every 3 days, the colonies were fixed with 4% paraformaldehyde and stained with 5% crystal violet. The number and size of the colonies were measured using an ARTEK counter 880 (Dynex Technologies Inc., Chantilly, VA, USA). Colony forming units were calculated by dividing the number of colonies by the number of cells plated and then multiplying by 100.

Growth in soft agar. The tumorogenicity of human SKMEL-188 and hamster AbC1 melanoma cells was determined by their ability to form colonies in soft agar, as previously described (33). Briefly, cells were detached from the flasks by trypsinization and re-suspended (~1,000 cells/well) in 250 μL of medium containing 0.4% agarose and 5% charcoal-stripped serum (HyClone). Cell suspensions were placed on a 0.8% agar layer in 4×24 well plates. Compounds were added from ethanol stocks (100 μM) to final concentrations of 0.1 nM or 10 nM, in 100 μL media. Each condition was tested in quadruplicate. An ethanol solvent control (amount of ethanol equivalent to test) as well as a media-only control was included in the assay. Cells were allowed to grow at 37˚C with 5% CO_2 over two weeks with secosteroids in fresh media (100 μL) being added after every 72 h. Soft agar colonies were scored and stained with 0.5 mg/ml MTT reagent (Promega), at 500 μL/well after two weeks. Colonies were then counted under the microscope.

Melanogenesis. Cell pigmentation was evaluated macroscopically, while tyrosinase activity (DOPA oxidase) was assayed in cell extracts as described previously (29).

VDR translocation. In order to determine VDR translocation from the cytoplasm to the nucleus, induced by hydroxyvitamin D_3 compounds, SKMEL-188 cells were transduced with pLenti-CMV-VDR-EGFP-pgk-puro, resulting in stable expression of the VDR-EGFP fusion protein (32). The cells were incubated with hydroxyvitamin D_3 derivatives for 2 h, followed by fixing with 4% paraformaldehyde and analyzed under a fluorescent microscope. The cells containing fluorescent nuclei were counted from the pictures taken from at least 6 different fields. Data are presented as a percentage of cells with fluorescent nuclei relative to the total cell number.

Quantitative PCR analysis. RNA from skin cells and tissue was isolated using an Absolutely RNA Miniprep Kit (Stratagen, USA). Reverse transcription was performed using a Transcriptor First Strand cDNA Synthesis Kit (Roche, USA). Real-time PCR was performed using cDNA and a Cyber Green Master Mix (n=3). Reactions were performed at 95˚C for 5 min and next 50 cycles (95˚C for 15 s, 60˚C for 30 s and 72˚C for 30 s). Data were collected on a Roche Light Cycler 480. The amounts were compared to a reference gene (Cyclophilin B) using a comparative C_T method. Relative gene expression data were calculated using the ΔΔCt method. Changes in gene expression are presented as relative quantities using mean ΔCt (normalized target) as a difference between target gene and reference gene in the cycle of appearance in time (C). A list of primers is presented in Table I.

Table I. *Sequences of the primers used for qPCR.*

Oligo	Sequence
Cyclophilin B	L TGTGGTGTTTGGCAAAGTTC
	R GTTTATCCCGGCTGTCTGTC
CYP2R1	L AGCCTCATCCGAGCTTCC
	R CCACAGTTGATATGCCTCCA
CYP11A1	L CCAGACCTGTTCCGTCTGTT
	R AAAATCACGTCCCATGCAG
CYP27A1	L CAGTACGGAACGACATGGAG
	R GGTACCAGTGGTGTCCTTCC
CYP27B1	L CTTGCGGACTGCTCACTG
	R CGCAGACTACGTTGTTCAGG
CYP24	L CATCATGGCCATCAAAACAAT
	R GCAGCTCGACTGGAGTGAC
VDR	L CTTACCTGCCCCCTGCTC
	R AGGGTCAGGCAGGGAAGT

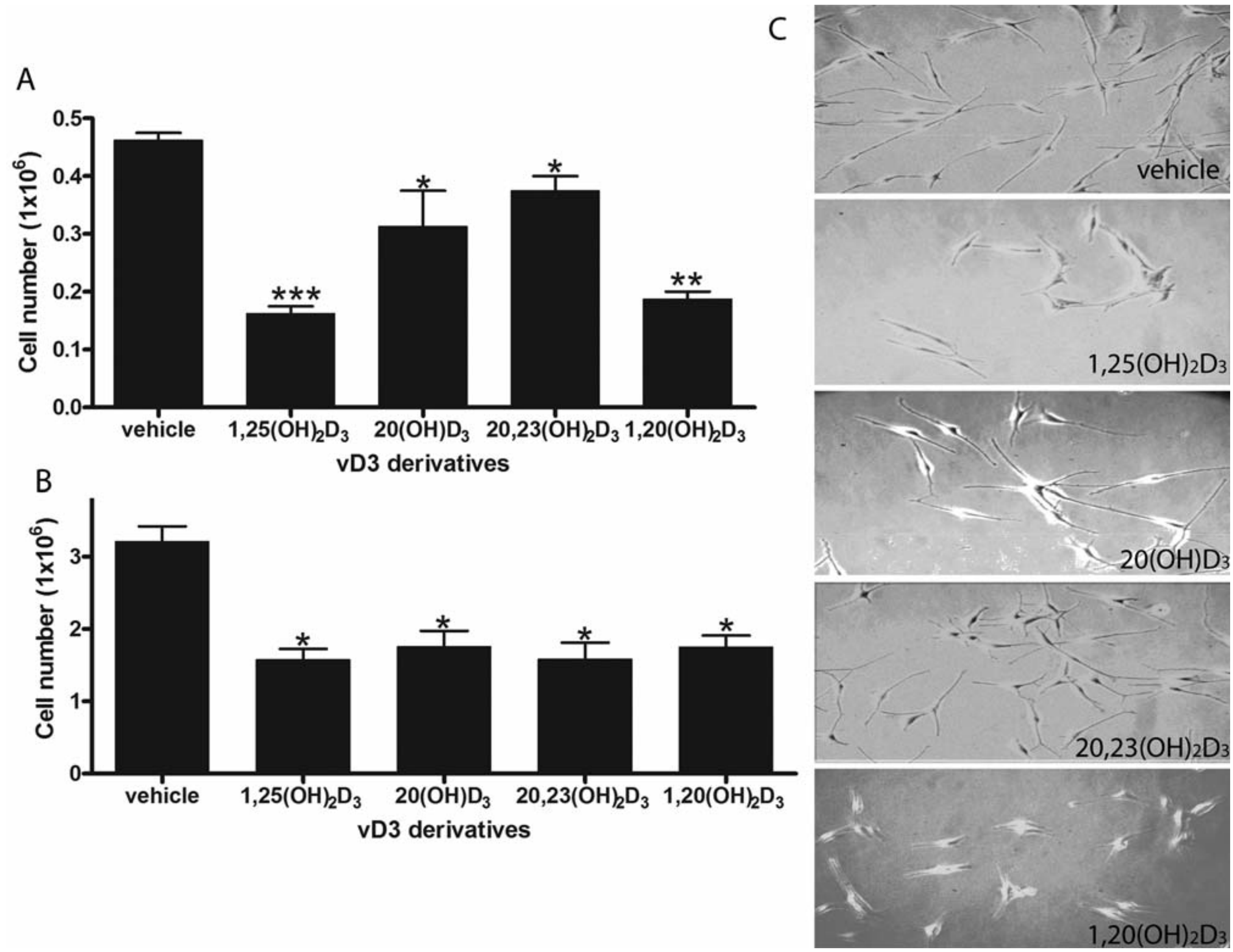

Figure 2. *Comparison of the anti-proliferative activity of the vitamin D derivatives $1,25(OH)_2D_3$, $20(OH)D_3$, $20,23(OH)_2D_3$ and $1,20(OH)_2D_3$ between cultured normal human epidermal melanocytes (A) and human SKMel-188 melanoma cells (B). The cells were treated with the secosteroids (10^{-7} M) for 7 days and their numbers were counted. Data are shown as mean±SD (n=3); *$p<0.05$; **$p<0.01$; ***$p<0.001$. $1,25(OH)_2D_3$ and $1,20(OH)_2D_3$, but not $20(OH)D_3$ or $20,23(OH)_2D_3$ inhibited dendrite formation (C). The differences between ethanol-treated control and treatments were analyzed by the Students t-test.*

Statistical analysis. Data are presented as mean±SD, and they were analyzed with Student's *t*-test (for 2 groups) and appropriate *post-hoc* test (for more than 2 groups) using Prism 4.00 (GraphPad Software, San Diego). Statistically significant differences were considered when $p<0.05$.

Results

$1,25(OH)_2D_3$ and the novel vitamin D_3 hydroxy-derivatives inhibited proliferation of normal and malignant melanocytes, with a differential effect noted for normal melanocytes (Figure 2). Specifically, $1,25(OH)_2D_3$ and $1,20(OH)_2D_3$ showed stronger inhibitory effects on melanocytes than $20,23(OH)_2D_3$ and $20(OH)D_3$ (Figure 2A). In contrast, all compounds caused comparable inhibition of humam melanoma (SKMel-188) growth *in vitro* (Figure 2B). Furthermore, only $1,25(OH)_2D_3$ and $1,20(OH)_2D_3$, but not $20(OH)D_3$ and $20,23(OH)_2D_3$, inhibited dendrite formation by normal melanocytes (Figure 2C). None of the compounds, including $1,25(OH)_2D_3$, had a significant effect on pigmentation and tyrosinase activity in normal and malignant melanocytes (data not shown). A similar inhibitory effect of the secosteroids on DNA sysnthesis was observed in another human melanoma line, YUROB (Figure 3). Interestingly, $20(OH)D_3$, $20,23(OH)_2D_3$ and $1,20(OH)_2D_3$ caused greater inhibition than $1,25(OH)_2D_3$ in this cell line. We also screened other human melanoma cell lines (YUKSI, YUTICA, YULAC, WM35, WM1341, WM164, WM98D and SBCE2), using the MTT assay to estimate the effects of $1,25(OH)_2D_3$, $1,20(OH)_2D_3$, $20(OH)D_3$ and $20,23(OH)_2D_3$ on

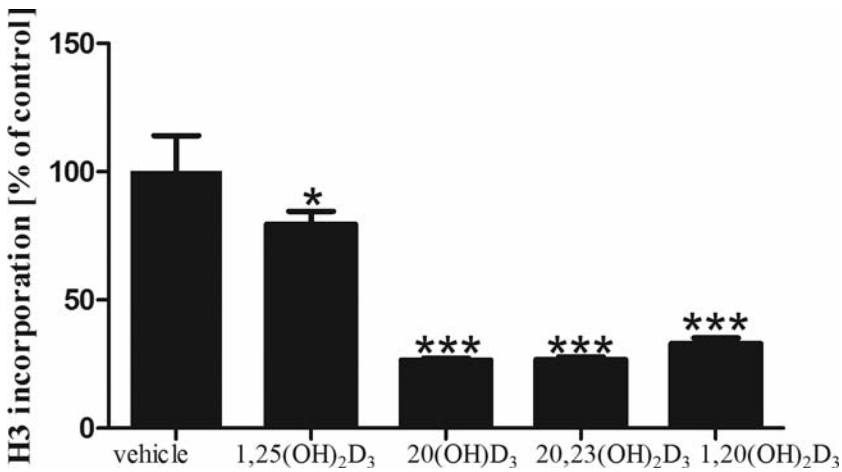

Figure 3. *Novel vitamin hydroxyderivatives inhibit DNA synthesis in human melanoma cells. YUROB cells were treated for 48 h with 1,25(OH)$_2$D$_3$, 20(OH)D$_3$, 20,23(OH)$_2$D$_3$, or 1,20(OH)$_2$D$_3$ (10^{-7} M) and the rate of ^{3}H-thymidine incorporation into DNA served as a measure of proliferative activity. Data are presented as mean±SD (n=4). Statistical significance was estimated using one-way ANOVA. Incorporation into DNA is shown as a percentage (%) of control (ethanol-vehicle treated cells). *p<0.05 and ***p<0.001.*

Table II. *Expression of genes encoding VDR and enzymes metabolising vitamin D_3.*

Cell Line	CYP11A1	CYP27A1	CYP27B1	CYP2R1	CYP24	VDR
WM98D	0.92±0.24	2.95±0.64	3.08±0.23	3.08±0.23	–1.71±0.32	6.55±0.62
YUROB	–3.74±0.24	6.42±0.34	5.46±0.23	5.46±0.23	1.14±0.30	8.15±0.3
YULAC	1.83±0.45	10.68±0.78	4.98±0.29	4.98±0.29	1.03±0.30	5.55±0.3
WM164	9.14±0.11	8.96±0.06	16.28±0.14	19.83±0.1	11.28±0.11	3.56±0.11
WM1341	15.28±0.18	7.90±0.03	15.53±0.23	13.93±0.09	11.56±0.28	4.53±0.19
SK Mel 188	5.24±0.28	–2.51±0.36	5.24±0.28	5.24±0.28	–11±0.35	5.24±0.28
SBCE2	15.46±0.69	8.78±0.32	12.85±0.16	11.04±0.13	5.61±0.47	8.69±0.13

Real-time PCR was performed using cDNA and a Cyber Green Master Mix (n=3). The amounts were compared to a reference gene (Ciclophilin B) using a comparative C_T method. Changes in gene expression are presented as a relative quantity using mean ΔCt (normalized target) as a difference between the target gene and the reference gene in the cycle of appearance in time (C). The lower the number, the higher is the expression level.

cell growth, and found that all of the compounds tested inhibited the growth of these lines *in vitro* (data not shown).

To better define the anti-melanoma activities of the novel secosteroids, we tested their effect on the ability to form colonies by human melanoma liens in monolayer (plating efficiency). We found a dose-dependent inhibitory effect for all compounds, with 1,25(OH)$_2$D$_3$ showing the highest potency (Figure 4). Finally, we tested the ability of the secosteroids to inhibit growth in soft agar (anchorage independent cell growth), and found that 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$ inhibited growth in soft agar of hamster (AbC1) (Figure 5) and human (SKMel-188) melanoma cells (Figure 6). Both of these compounds showed similar effects to those seen for 1,25(OH)$_2$D$_3$.

Using the previously described melanoma line transfected *via* lentivirus with the VDR-GFP construct (18, 32), we found that the novel secosteroids induced translocation of VDR from the cytoplams to the nucleus (Figure 7), consistent with the action of VDR. We also screened human melanoma lines for the expression of genes encoding 25-hydroxylases (CYP27A1 and CYP2R1), 1α-hydroxylase (CYP27B1), 24-hydroxylase (CYP24), cytochrome P450scc (CYP11A1) and VDR; although all of these genes were found to be expressed, there was a considerable variation between the different melanoma lines tested (Table II).

Discussion

In this study we showed, for the first time, that novel vitamin D$_3$ hydroxyderivatives generated by the action of CYP11A1 display differential phenotypic effects against normal epidermal melanocytes and human and hamster melanoma cell lines.

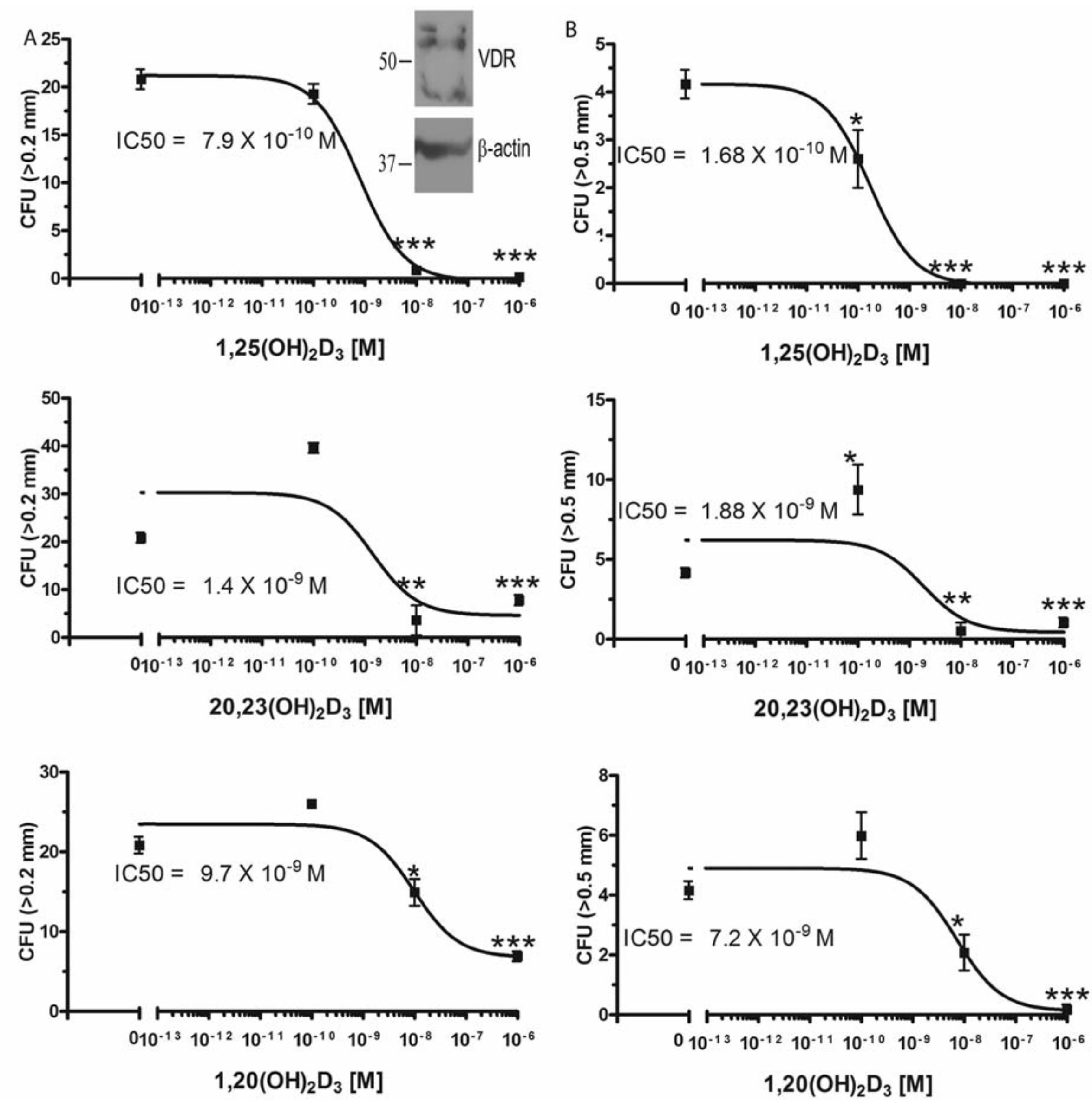

Figure 4. *Novel vitamin D hydroxyderivatives inhibit the ability of human melanoma cells to form colonies in monolayer (platting efficiency). SBCE2 cells were plated at a density of 20 cells/cm^2, grown in the presence or absence of 1,25(OH)$_2$D$_3$, 20,23(OH)$_2$D$_3$ or 1,20(OH)$_2$D$_3$, and after 10 days the formation of colonies larger than 0.2 mm (A) or 0.5 mm (B) in diameter was determined. Data are shown as mean±SD (n = 4); statistical significance was estimated using one-way ANOVA and presented as *p<0.05, **p<0.01 and ***p<0.001. Insert shows western blot detection of VDR in SBCE2 human melanoma cells. The whole extracts from cells were subjected to immunoblotting with anti-VDR, and anti-β-actin (internal control) as described before (3). The numbers on the left in the insert represent molecular weight in kD.*

Thus, the classical hormonally active form of vitamin D$_3$, 1,25(OH)$_2$D$_3$, and novel 1,20(OH)D$_3$ significantly inhibited proliferation of normal epidermal melanocytes and inhibited dendrite formation. 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$ displayed a lower inhibitory effect on proliferation and no effect on cell morphology. This selectivity was absent in human melanoma, where all compounds inhbibited proliferation by a similar degree with no effect on cell morphology.

P450scc hydroxylates vitamin D$_3$ (D$_3$) in a sequential fashion: D3→20(OH)D$_3$→20,23(OH)$_2$D$_3$ (31, 41). In addition, 20(OH)D$_3$ *in vitro* and *in vivo* is hydroxylated by CYP27B1 in position 1α (the same enzyme that generates 1,25(OH)$_2$D$_3$) to produce 1,20(OH)$_2$D$_3$ (32, 37). Our previous studies have shown that addition of a hydroxyl group to C1α modifies the action of the parental 20(OH)D$_3$ by producing some calcemic activity and increasing the ability to stimulate CYP24

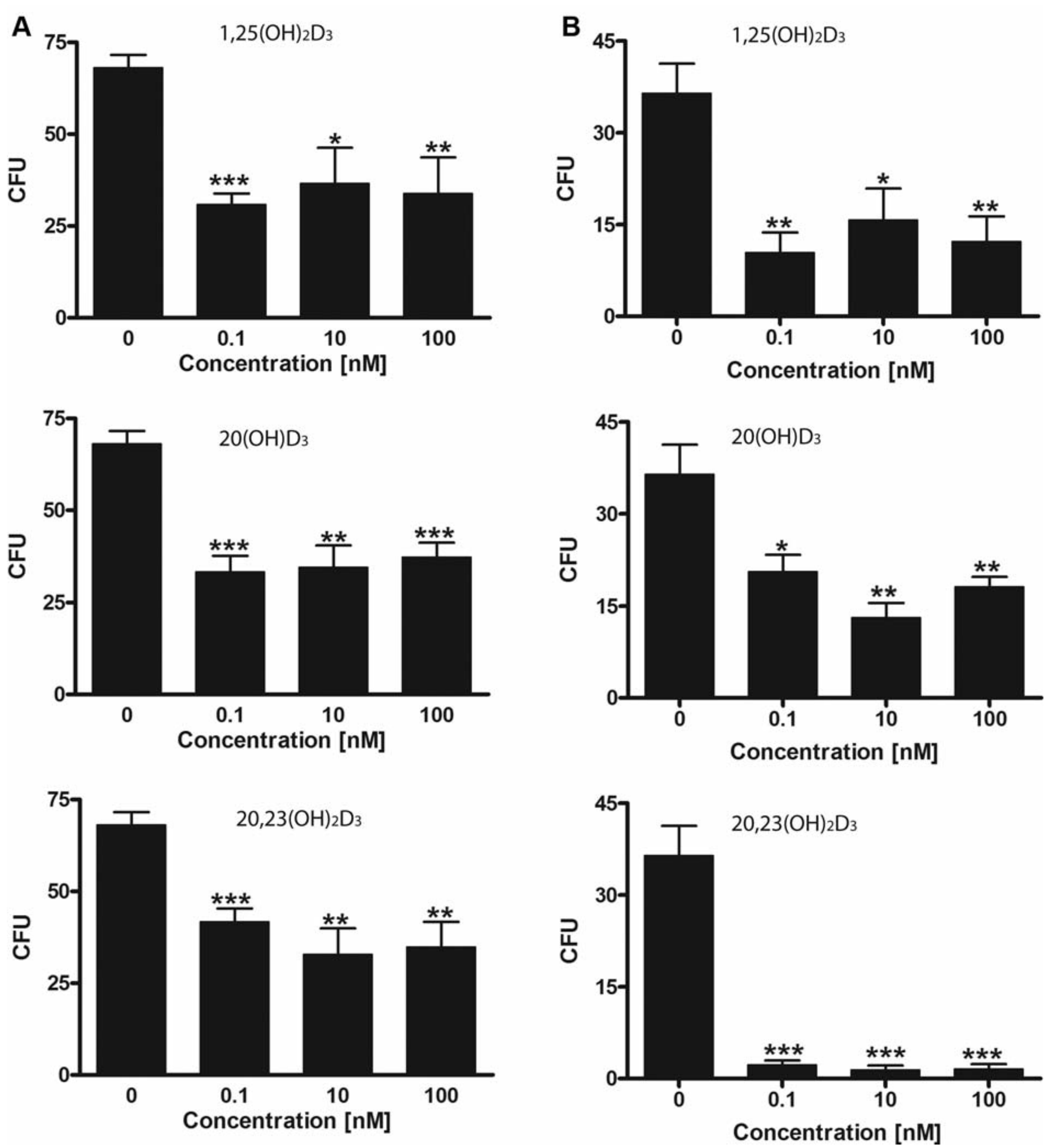

Figure 5. *Novel vitamin hydroxyderivatives inhibit the anchorage-independent growth (ability to form colonies in soft agar) of hamster melanoma cells. AbC1 melanoma cells were plated in soft agar at 1,000 cells/well and grown in the presence or absence of 1,25(OH)$_2$D$_3$, 20(OH)D$_3$ or 20,23(OH)$_2$D$_3$. After two weeks colonies with a diameter larger than 0.2 mm (A) or 0.5 mm (B) were counted. Data are shown as mean±SD (n=4); statistical significance was estimated using one-way ANOVA and presented as *p<0.05, **p<0.01 and ***p<0.001.*

expression (30, 32). In these studies we showed that addition of 1α-hydroxyl group increases the ability of 20(OH)D_3 to modulate the phenotype of normal melanocytes in a similar way to 1,25(OH)$_2D_3$. However, proliferation of melanoma cells is inhibited in a similar manner by compounds without a 1α-hydroxyl group, which is similar to the effects described in leukemias (30) and normal keratinocytes (39).

1,25(OH)$_2D_3$ is a recognized inhibitor of melanoma proliferation acting in a context-dependent fashion, making vitamin D a good candidate to treat skin cancers [reviewed in

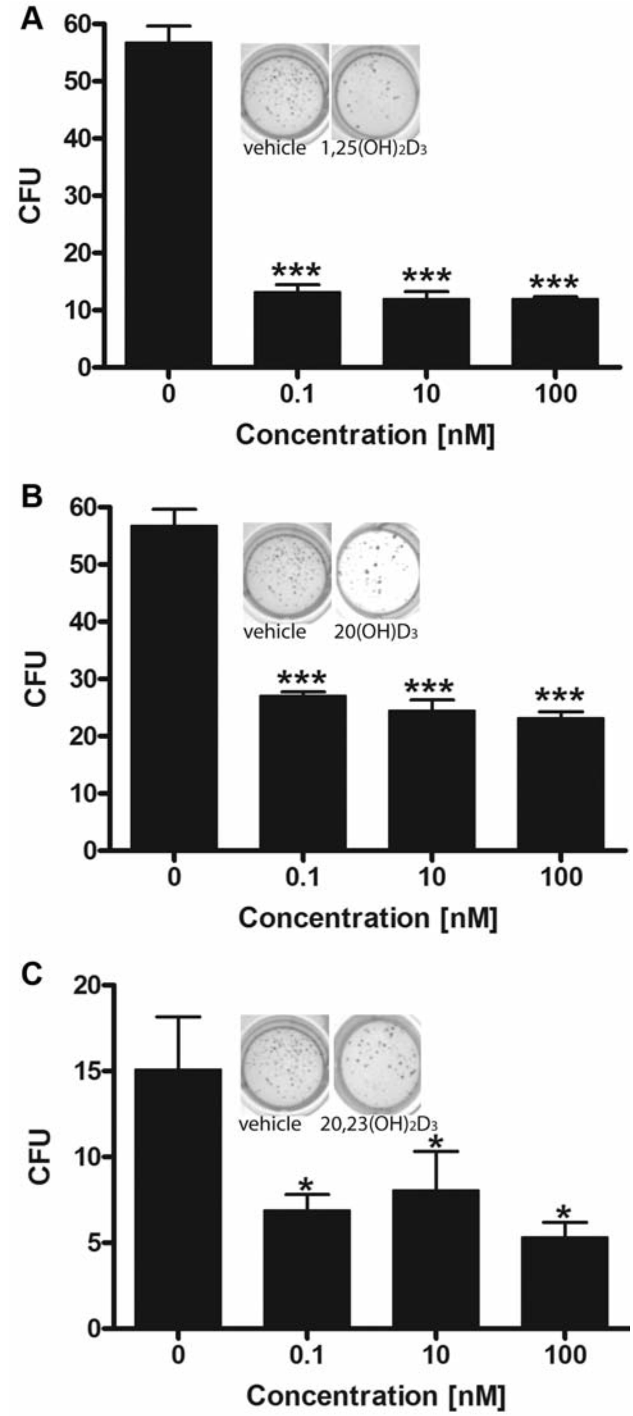

Figure 6. *Novel vitamin hydroxyderivatives inhibit the anchorage-independent growth (ability to form colonies in soft agar) of human melanoma cells. SKMel-188 human melanoma cells were grown in soft agar in the presence or absence of 1,25(OH)$_2$D$_3$ (A), 20(OH)D$_3$ (B) or 20,23(OH)$_2$D$_3$ (C). Panels A and B are from the same, while panel C from a separate experiment. After two weeks colonies with a diameter larger than 0.5 mm were counted. Data are shown as mean±SD (n=4); statistical significance was estimated using one-way ANOVA and presented as *p<0.05 and **p<0.001. Insert: Representative plates incubated with solvent (ethanol) or 10^{-7} M secosteroids.*

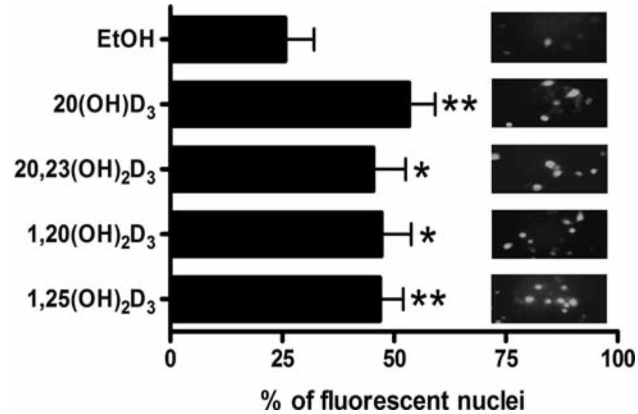

Figure 7. *The effect of vitamin D$_3$ derivatives on the translocation of VDR from the cytoplasm to the nucleus. The panel on the right shows photographs of the cells with fluorescent VDR-EGFP fusion protein in the nucleus. The left pannel shows the percentage of cells with fluorescent nuclei. Data are presented as means±SEM (n≥6). The differences between ethanol-treated controls and treatment samples were analyzed by the student's t-test: p<0.05 (*), p<0.01 (**).*

(6, 7, 36)]. Unfortunately, pharmacological use of vitamin D or its analogs is limited because of hypercalcemic effects causing secondary organ failure and possible death (35). Thus the major obstacle in using 1,25(OH)$_2$D$_3$ for melanoma treatment is its small therapeutic window defined by its calcemic effects. The CYP11A1-derived 20(OH)D is non-calcemic at doses as high as 3-4 μg/kg in rats (30, 32) and 30 μg/kg in mice (42). We have also observed that 20,23(OH)$_2$D$_3$ is non-calcemic in mice (unpublished). Our initial studies also demonstrated that 20(OH)D$_3$ and related 20(OH)D$_2$ show anti-proliferative activity towards the human SKMel-188 melanoma line (14, 32). In this study we extended the spectrum of melanoma lines and parameters tested and found inhibitory effects of 20(OH)D$_3$ as well as the previously untested 20,23(OH)$_2$D$_3$ in 10 human melanoma lines. Using selected human melanoma cell lines we showed that both 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$ inhibit plating efficiency as well as the ability to grow in soft agar, illustrating their anti-tumorogenic activity. We also found that 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$ inhibit the growth of the hamster melanoma line AbC1 in soft agar with a slightly stronger effect for 20(OH)$_2$D$_3$. This identifies this line, as well as human lines SBCE2, SKMel-188 and YUROB, as excellent testing models for planned pre-clinical studies in animals. We also excluded from further testing the mouse S91 Cloudman line that did not respond or responded poorly to vitamin D$_3$ derivatives, which is consistent with other reports on this cell line [reviewed in (36)].

The phenotypic effects of vitamin D$_3$ are mediated trough an interaction with VDR, and the activity of vitamin D$_3$ depends on its sequetial hydroxylations by CYP27A1 or

CYP2R1, and CYP27B1 (classical activating), and CYP24 (classical inactivating) pathways (13, 23). This study showed that the novel secosteroids tested stimulate VDR translocation from the cytoplasm to the nucleus, confirming our previous finding of activation of VDR by CYP11A1-derived vitamin D analogues (18, 32). Expression of genes encoding the enzyme that metabolizes vitamin D was heterogenous without a clear association with a significant modulatory effect. Since all melanoma lines tested express CYP11A1, we believe that exogenously added $20(OH)D_3$ enters metabolic pathways mediated by this enzyme with production of other equi-or potentially more potent compounds, including $20,23(OH)_2D_3$. This is further rationalized by our previous finding that $20(OH)D_3$ is a relatively poor substrate for CYP27B1 (32, 37), and our demonstration that phenotypic activity of $20(OH)D_2$ does not require its activation in position 1α (32). The role of 25- and 24-hydroxylases on the activity of $20(OH)D_3$ remains to be tested.

There are conflicting reports on regulation of melanin pigmentation by $1,25(OH)_2D_3$ [reviewed in (28, 36)]. In the present study we observed a lack of a significant effect (stimulation or inhibition) of $1,25(OH)_2D_3$ and novel vitamin D_3 analogs on melanogenesis in pure cultures of melanocytes or melanoma cells. This is in agreement with studies published by others showing lack of such an effect in cell cultures (20). However, we cannot entirely exclude a role of vitmain D_3 on the regulation of melanin pigmentation *in vivo* because $1,25(OH)_2D_3$ and $1,20(OH)_2D_3$ inhibited the formation of dendrites, which are involved in the transfer of melanosomes to the keratinocytes. Nevertheless, the lack of effect of $20(OH)D_3$ and $20,23(OH)_2D_3$ on these functions indicate that the vitamin D derivatives without a hydroxyl group at C1α are not involved in the regulation of melanin pigmentation.

In conclusion, we have shown that novel non-calcemic $20(OH)D_3$ and $20,23(OH)_2D_3$ demonstrate potent anti-melanoma activity *in vitro* with lesser effects on normal melanocytes. Both $20(OH)D_3$ and $20,23(OH)_2D_3$ are excellent candidates for pre-clinical testing, since they are non-calcemic and non-toxic, and they also show anti-cancer activity on leukemia, breast and liver cancers (30, 42).

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