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New vitamin D analogs as potential therapeutics in melanoma

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Abstract

Extensive evidence shows that the active form of vitamin D3 – 1 α ,25-dihydroxyvitamin D3 – plays an important role in cancer prevention, has tumorostatic activity and may potentially be used in therapy for melanoma. Vitamin D3 and its analogs (secosteroids) exert multiple effects on cancer cells, including inhibition of cell growth and induction of differentiation. Activity of secosteroids depends on multiple cellular factors, including expression of the vitamin D receptor. Despite its endogenous origin, the key drawback for the use of pharmacologically effective doses of 1 α ,25-dihydroxyvitamin D3 is its hypercalcemic effect leading to profound toxicity. The solution may lie in properties of vitamin D3 analogs with modified side chains, which demonstrate low calcemic activity but conserve the anti-tumor properties. Noncalcemic vitamin D compounds were found to be potent in multiple studies that mandate further clinical testing. Finally, recent studies revealed alternative metabolic pathways for secosteroids and new targets in the cells, which opens up new therapeutic possibilities.

Keywords

1 α ,25-dihydroxyvitamin D3; melanoma; secosteroids; VDR; vitamin D; vitamin D analogs; vitamin D receptor

Vitamin D

Vitamin D is a crucial element in the regulation of calcium homeostasis. In humans, vitamin D can either be synthesized in the skin or absorbed from the diet [1]. However, the number of dietary sources is limited and approximately 90–100% of a human's vitamin D requirement comes from the exposure of skin to sunlight [2,3]. In the first step of vitamin D3 synthesis, 7-dehydrocholesterol (7-DHC, also termed provitamin D3 or cholesta-5,7-dien-3 β -ol) is converted to previtamin D3 after exposure to UVB radiation (290–320 nm wavelength) [1,2,4,5]. Next, previtamin D is transformed to vitamin D, tachysterol and lumisterol (**Figure 1**). However, in order to carry out its systemic physiologic functions, cholecalciferol is subsequently translocated to the circulation by vitamin D-binding protein (DBP) [4] and activated through sequential hydroxylations in the liver and kidney (**Figure 2**) [1,2,4]. In the liver, cholecalciferol is metabolized to calcidiol (25-hydroxyvitamin D3)

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by enzyme CYP27A1 (25-hydroxylase). In the kidney, calcidiol is further hydroxylated at position 1 to calcitriol (1 α ,25-dihydroxy vitamin D3) by CYP27B1 (1 α -hydroxylase) [1,6–8]. Vitamin D is also activated in the skin as well in many other peripheral organs [8,9].

1 α ,25-dihydroxyvitamin D3 is the biologically active form of vitamin D3. This hormone acts locally in the kidney and is also transported by DBP to other target tissues expressing vitamin D receptors (VDRs) [4]. VDRs display the typical domain structure of a nuclear receptor with a highly conserved DNA-binding region and ligand-binding domain. Binding between the ligand-binding domain and 1 α ,25-dihydroxy vitamin D3 (or its analogs) induces heterodimerization of VDR and the retinoid X receptor. This event starts the cascade that leads to changes in the expression of genes containing vitamin D response elements in their promoter [3,4,6,7,10]. 1 α ,25-dihydroxyvitamin D3 directly or indirectly controls over 200 genes in a cell- and tissue-specific manner. The most well-known action is its effect on metabolism of vitamin D and calcium; however, other functions are also modified, such as: regulation of the cell cycle and apoptosis; stimulation of differentiation; angiogenesis; chemopreventive and genoprotective effects; xenobiotic metabolism; immunomodulation; and antimicrobial activity [7,8]. The level of vitamin D is tightly regulated by CYP24A1 (24-hydroxylase), the enzyme responsible for its inactivation [1,3].

Epidemiological data indicate that people living at higher latitudes demonstrate lowered levels of 25-hydroxyvitamin D and have an increased risk for lymphoma, colon, pancreatic, prostate, ovarian, breast and other cancers, along with a higher mortality rate from cancer [8,11]. People living in these areas also demonstrate an increased risk of Type 1 and 2 diabetes, multiple sclerosis, Crohn's disease, rheumatoid arthritis, osteoarthritis, abdominal obesity, hypertension and cardio vascular disease [8,12,13]. However, vitamin D overdose has negative side effects, including hypercalcemia and hypercalciuria with accompanying pathology, which restricts the pharmacological use of vitamin D [8,14,15].

Melanoma

Solar radiation has been implicated as a major etiologic factor in skin cancers. Cancers of the skin are usually divided into two general groups: nonmelanoma and melanoma. The first category comprises the most common types of cancers: basal cell carcinoma and squamous cell carcinoma. The third most prevalent form is cutaneous malignant melanoma, representing less than 5% of all skin cancer cases, but being the most lethal one. Such a high mortality rate is related to the poor efficacy of established chemotherapeutic medications [16–18,201].

Cutaneous melanoma originates from malignant transformation of epidermal melanocytes (melanin-producing cells) [19,20,201]. The major risk factor for melanoma is sun exposure [19,21–23]. However, the relationship is complex and less direct than for other nonmelanoma skin cancers. For example, current data indicate that basal cell carcinoma and squamous cell carcinoma arise in skin chronically exposed to sunlight, while many melanomas arise after intermittent sun exposure [19,22,23], and they can develop on acral skin, genital and oral mucosa and in internal organs including the esophagus, tracheobronchial tree, colon, urinary tract and CNS [23,24].

It is well understood that there is a strong association between melanoma incidence and a history of sunburns, UV exposure at an early age and sharp, short bursts of acute exposure in childhood, and sun-sensitive phenotype [13,19,21–23,25]. Nevertheless, some authors have proposed that moderate or even cumulative lifetime occupational exposure to sunlight may have some beneficial effects, including a reduced risk of several cancers [13,25]. It is worth mentioning that exposure to solar UVB (290–320 nm) was correlated with a reduced risk of approximately 14 types of cancer [12]. The surprising effect of UV exposure most probably

arises from sunlight-induced vitamin D production in the skin [26,201]. It has to be underlined that observed anticancer effects may be related to the compensation of vitamin D insufficiency or deficiency alone.

Apart from UV exposure, family and personal history of skin cancer and the presence of nevi may increase individual risk of melanoma. Nevi, especially numerous and atypical, may be potential precursors of malignancy [23,24,27,28].

Some authors have suggested that vitamin D deficiency could be a cofactor in the development of melanoma in body parts not usually exposed to solar radiation [19,27–29]. Moreover, some authors reported that vitamin D can upregulate melanin synthesis in the skin through the positive effect of tyrosinase activity [16,20,30–32]. However, other authors have questioned a direct interaction between vitamin D3 and melanogenesis [20,33,34]. Furthermore, there is no sufficient evidence that skin photo-type influences vitamin D production or that serum levels of 25-hydroxyvitamin D3 affect pigmentation. Therefore, it is possible that the observed effects may be limited to specific conditions (see comments in [33]). Nevertheless, it has been demonstrated that vitamin D has direct or indirect effects on protective mechanisms against UV radiation-induced damage, implicating its role in protection against UV radiation-induced cancers [35,36]. These findings are in agreement with attenuation of oxidative DNA damage in internal organs, making vitamin D an excellent chemopreventive agent against carcinogenesis [37]. Furthermore, vitamin D can protect against damage induced by ionizing radiation [38,39].

So far, the most effective method to treat melanoma has been the proper surgical excision of early stages localized to the skin, which requires precise and early detection [18,24,202]. Accordingly, when diagnosed early, the probability of curing melanoma (*in situ* or radial growth phase) is very high [22,201]. The significant mortality of this disease is due to the ineffectiveness of standard methods of melanoma therapy when the disease enters the metastatic stage [17,18,24,202]. However, there has been remarkable progress towards targeted melanoma therapy by targeting mutant *BRAF*, using KIT inhibitors or using antibody targeting cytotoxic T-lymphocyte-associated antigen 4 [40–42]. Nevertheless, the search for new drugs is mandatory, since melanomas develop resistance to such targeted therapy (of which the best example is the development of resistance to PLX4032; reviewed in [43]). As melanocytes are VDR-expressing cells, they are also potential targets for vitamin D. However, caution is needed in overestimating the importance of 1 α ,25-dihydroxyvitamin D3-based therapy, owing to recent findings that VDR expression decreases with the progression of melanoma [44].

The link between vitamin D & melanoma

Evidence is accumulating that melanoma development may be facilitated by vitamin D insufficiency and deficiency, defects in vitamin D signaling and in VDR function due to genetic factors and decreased expression or protein levels (**Table 1**) [16,25,44–46]

The *VDR* gene contains over 1000 polymorphic sites, and several of them were confirmed by different studies to have an impact on melanoma risk, aggressiveness or prognosis [47–50]. The main restriction fragment length polymorphisms under investigation were rs10735810 (T>A nucleotide change at FokI restriction site), rs1544410 (G>A at BsmI) and rs731236 (T>C at TaqI). For example, the rs10735810 A allele (FokI f allele) [49,51–54] and rs1544410 A allele (BsmI b allele) [49,52–54] seem to act as the risk alleles, while the rs731236 C allele (TaqI t allele) has been identified as the protective allele [53–55]. In addition, the rs4516035 A allele (A>G change at EcoRV restriction site) has been reported to be a melanoma risk allele [56–58]. On the other hand, no association was observed

between restriction fragment length polymorphism rs7975232 (G>T change at ApaI restriction site) as well as rs11568820 (G>A change at Cdx2-binding site) and melanoma risk [49,51].

The linkage disequilibrium between some of the *VDR* polymorphisms and melanoma was also investigated. For example, TaqI, FokI and BsmI have been reported to be in linkage disequilibrium, exerting a potential joint effect on melanoma risk [53]. Furthermore, the TaqI restriction site is in strong linkage disequilibrium with the poly(A) polymorphism [52]. Halsall *et al.* have identified the combined effect on melanoma risk of the A allele of the EcoRV (A-1012G) polymorphism with the f allele of the FokI polymorphism (**Table 1**) [57]. Similarly, evidence was presented that melanoma progression may be facilitated by defects in vitamin D signaling, including decreased expression of *VDR* [16,44]. It must be stressed that analysis of *VDR* expression and polymorphism could serve as important prognostic factors before treatment of melanoma with vitamin D and its analogs.

Vitamin D & its analogs in melanoma treatment

Vitamin D anti-tumor activity

It is well established that the active form of vitamin D, as well as its derivatives, has inhibitory effects on the growth of multiple cancer cell lines including melanoma.

Research has revealed that 1 α ,25-dihydroxyvitamin D3 and its analogs exert multiple effects on melanoma cells (summarized in [59]). Generally, the *VDR*-expressing cells tend to demonstrate inhibition of tumor growth in response to 1 α ,25-dihydroxyvitamin D3 and a number of its analogs (**Table 2**) [5,52,60–65].

Biological effects of 1 α ,25-dihydroxyvitamin D3 in melanoma

In 1981, the Feldman group opened the door for the investigation of vitamin D in melanoma research [66]. Since then, the antiproliferative effect of 1 α ,25-dihydroxyvitamin D3 has been tested in both human and mouse melanoma cell lines. It is well established that vitamin D inhibits the growth of several melanoma cell lines: MeWo [46,63,67], ME18 [68], SM [63], SK Mel 28 [46,63,69], RPMI 7951 [70], and mouse B16 [31]. The dosage of 1 α ,25-dihydroxyvitamin D3 varied in the experiments from 10⁻¹¹ to 10⁻⁵ M, with the effective dose of 10⁻⁸ M. However, not all melanoma cells responded to vitamin D and its analogs. Lack of response was observed in human melanomas such as Mel-Juso [63], SK Mel 5 [46,63,67] and SK Mel 25 [63], as well as in murine melanomas S91 [34], IGR [46,63] and B16 [71,72]. The diverse effects of 1 α ,25-dihydroxyvitamin D3 on mouse melanoma B16 may be due to different experimental setups or, more likely, due to different subclones of B16 used in the presented studies.

Other experiments demonstrated that 1 α ,25-dihydroxyvitamin D3 inhibited melanoma colony formation at the dose of 5 $\times$ 10⁻⁹ M in human SK Mel-188 [5] and at 10⁻¹⁰ M in mouse B16 [31]. Another study investigated the ability of 1 α ,25-dihydroxy vitamin D3 to induce apoptosis of melanoma cells [73]. 1 α ,25-dihydroxy vitamin D3 was effective at the dose of 1.1 nM in WM1341, but not in the MeWo cell line [73]. It seems that induction of apoptosis by 1 α ,25-dihydroxyvitamin D3 depends on cell-specific factors such as stimulation of PTEN, which results in downregulation of the AKT antiapoptotic pathway, as demonstrated for the gastric cancer cell line HGC-27 (see recent review [74]), but this mechanism has not been well studied in melanoma so far. 1 α ,25-dihydroxyvitamin D3 was also found to inhibit DNA replication at the dose of 10⁻⁸ M in the human MM96 cell line [75].

Yudoh *et al.* reported that 1 α ,25-dihydroxyvitamin D3 had an inhibitory effect on invasiveness, cell adhesion to the extracellular matrix and type IV collagenolytic activity of B16 cells, but did not affect cell migration [71].

Several groups have demonstrated that 1 α ,25-dihydroxyvitamin D3 does not stimulate melanogenesis in melanocytes – the hallmark of melanocyte differentiation (reviewed in [20], while others reported the opposite effect, e.g., stimulation of the tyrosinase activity [16,20,30–32,52,76]). This phenomenon may also be considered as a potential effect of 1 α ,25-dihydroxyvitamin D3 on melanoma cells, although different laboratories reported its adverse effects on pigmentation [16,33,34].

1 α ,25-dihydroxyvitamin D3 also affects the expression of genes involved in vitamin D activation and metabolism. 1 α ,25-dihydroxyvitamin D3 treatment of cells of different lineages results in rapid overexpression of the 24-hydroxylase gene, thus stimulating the degradation of 1 α ,25-dihydroxyvitamin D3. The other targets are 25-hydroxylase (stimulation) and 1-hydroxylase (inhibition) genes [63,67]. The majority of researchers also demonstrated increased expression of the gene encoding VDR after vitamin D stimulation [63,67,77]. On the other hand, lack of effect was observed by other investigators [73,77]. Sensitivity of melanoma to 1 α ,25-dihydroxyvitamin D3 seems to correlate with stimulation of gene expression. For example, MeWo and SK Mel-28 melanomas were found to be sensitive to 1 α ,25-dihydroxyvitamin D3, which also resulted in altered expression of vitamin D-related genes. In other melanomas, such as SK Mel 5 and SK Mel 25, 1 α ,25-dihydroxyvitamin D3 treatment failed to induce expression of the genes and inhibition of cell growth.

Effects of vitamin D precursors & other 5,7-dienes

Vitamin D precursors such as 7-DHC and other 5,7-dienes were tested for their effects on melanoma cells *in vitro*. 7-DHC failed to influence cell growth, melanin content or tyrosinase activity in both human (S91) and mouse (C2M) melanoma cell lines [34,76]. Similarly, ergosterol – the precursor of vitamin D2 – had no effect on either melanin content or tyrosinase activity in this cell line [76]. However, 7-dehydropregnenolone (7-DHP) inhibited cell growth in soft agar as shown in SKMEL-188 (human) and AbC1 (hamster) melanoma cells [64].

It must be noted that a new steroidogenic pathway started by cytochrome P450_{scc} (CYP11A1) acting on 7-DHC was described. It involves the sequential hydroxylation of 7-DHC at positions C22 and C20 followed by cleavage of side chains to produce 7-DHP [64,78,79]. 7-DHP can be further modified by classical enzymes of steroidogenesis, leading to the production of new steroidal 5,7-dienes with modified side chains [64,79]. Those compounds may serve as vitamin D precursors after exposure to UVB [79,80] and are being extensively tested for their potential use in melanoma treatment.

As demonstrated recently, not only are vitamin D analogs derived from those 5,7-dienes active, but 5,7-dienal precursors such as 7-DHP, 20-S and 20-R pregna-5,7-diene-3,17,20-triols or 21-hydroxy 7-DHP, were also found to inhibit melanoma growth [5,65]. Two epimers – 4R (pregna-5,7-diene-3 β ,17 α ,20R-triol) and 4S (pregna-5,7-diene-3 β ,17 α ,20S-triol) – were reported to inhibit colony formation of the SKMEL-188 human melanoma cell line, demonstrating equal or even higher potency than 1 α ,25-dihydroxyvitamin D3. The 4S compound proved to be the most potent, even when compared with its vitamin D-like derivative (4S-pD, as detailed below) [5].

One more steroidal derivative of 5,7-diene is 17-COOH-7DA (3 β -hydroxyandrosta-5,7-diene-17 β -carboxylic acid). It was reported as much more potent than 1 α ,25-

dihydroxyvitamin D3 in inhibiting proliferation, colony formation and DNA replication in human SKMEL-188, WM35, WM1341 and hamster AbC1 melanoma cell lines [81].

Effects of vitamin D analogs

Besides classical vitamin D3 and its natural derivatives 25-hydroxyvitamin D3 and 1 α ,25-dihydroxyvitamin D3, at least 2000 other vitamin D analogs (secosteroids) have been synthesized and extensively tested on several cell lines including melanomas. In addition, other vitamin D isomers, including lumisterol-like compounds, have been shown to be active against melanoma. It should be noted that lumisterol-like compounds, as well as tachysterol-like and vitamin D-like compounds, are the classical products of UV irradiation of 5,7-dienes (see **Figure 1**, where photolysis of 7-DHC was given as an example).

The effects of vitamin D analogs are varied – some of them retain or demonstrate enhanced potency, while some do not exert any significant effect. The form of vitamin D that is hydroxylated only at the 1 α position failed to influence the tyrosinase activity in B16 mouse melanoma cells [31]. The lack of effect may be explained by decreased VDR-binding affinity of 1 α -OH D3 when compared with 1 α ,25-dihydroxyvitamin D3 [31]. The analog with an additional hydroxylation position at 24R – tacalcitol or PRI-2191 (1,24R-dihydroxyvitamin D3) – also had no effect on the growth of B16 mouse melanoma cells [72]. The trihydroxylated forms of vitamin D demonstrate different activities depending on the hydroxylation position. 1,25,26-trihydroxyvitamin D3 inhibited DNA replication, while 1,24,25-trihydroxyvitamin D3 had no effect [75]. 24R,25-dihydroxyvitamin D3 had no significant effect on DNA replication [75] and tyrosinase activity [31]. Previtamin D3 also failed to influence cell growth, melanin content or tyrosinase activity in both human (S91) melanoma cell lines [34]. Vitamin D3 proved to increase melanin content and tyrosinase activity in mouse (C2M) melanoma cells [76], but failed to influence cell growth and melanin content in mouse (S91) melanoma cells [34].

25-hydroxyvitamin D3 is the vitamin D derivative that has been investigated most actively. However, only one study has reported the inhibition of growth of ME18 human melanoma cells [68]. Other studies have demonstrated that 25-hydroxyvitamin D3 exerts no effect on cell growth in MeWo and S91 melanomas [34,63]. Moreover, 25-hydroxyvitamin D3 did not influence DNA replication in MM96 [75] or melanin content in S91 [34] melanoma cells, nor did it affect tyrosinase activity in the mouse B16 melanoma cell line [31]. It seems that the biological activity of 25-hydroxyvitamin D3 in melanomas strongly depends on its conversion to 1 α ,25-dihydroxyvitamin D3; therefore, the expression of 25-hydroxy vitamin D3 1- α -hydroxylase (CYP27B1) is crucial for the antimelanoma properties of 25-hydroxyvitamin D3. It should be mentioned here that 25-hydroxyvitamin D3 is much more stable in solution or plasma compared with 1 α ,25-dihydroxyvitamin D3, thus giving 25-hydroxyvitamin D3 an advantage over 1 α ,25-dihydroxyvitamin D3 in the circulation, since 25-hydroxyvitamin D3 will be activated by CYP27B1 in targeted cells.

1 α ,25-dihydroxyvitamin D2 is a product of sequential hydroxylation of plant-derived vitamin D2 that has been reported to stimulate melanin synthesis and increase tyrosine activity in C2M mouse melanoma cells [76]. However, a vitamin D2 analog (PRI-1906; 24E-24a-Homo-[1S]-1,25-dihydroxyergo-calciferol) had no effect on the growth of B16 melanoma [72]. Recently, new vitamin D2 derivatives – 20-hydroxy D2 and 1,20-dihydroxyvitamin D2 – demonstrated antiproliferative activity against melanoma cells [62]. These derivatives are the products of CYP11A1 action on vitamin D2 [82,83] with subsequent modification by CYP27B1 [62].

In addition, vitamin D analogs with short side chains also possess antiproliferative activities. Those compounds with pregenolone or androsterone-type side chains and the presence or

absence of an additional hydroxyl group at carbon C17, C20 or C21 were shown to inhibit melanoma cell growth and most probably possess low or no calcemic properties. For example, 20-oxopregnacalciferol inhibited cell growth in soft agar in both human SKMEL-188 and hamster AbC1 cell lines [64]. Moreover, two vitamin D-like compounds (4R-pD and 4S-pD) derived from pregna-5,7-diene-3 β ,17 α ,20R-triol and pregna-5,7-diene-3 β ,17 α ,20S-triol (4R and 4S) as well as lumisterol-like derivatives (4R-pL and 4S-pL) were reported to inhibit colony formation of the SKMEL-188 human melanoma cell line, demonstrating equal or even higher potency than 1 α ,25-dihydroxyvitamin D3. Surprisingly, the parental compound (4S) was the most potent, while 4R-pL demonstrated the lowest potency. 4R-pD, 4S-pD and 4S-pL demonstrated similar activity to 1 α ,25-dihydroxyvitamin D3 [5].

Lumisterol (for structure see **Figure 1** – the inert photoproduct of isomerization of previtamin D3 in the skin – was also tested. Interestingly, it showed no effect on cell growth or melanin content in S91 cells [34].

EB1089 or secocalcitol (22,24-diene-24a,26a,27a-trihomo-1 α ,25-dihydroxyvitamin D3) has been demonstrated in two studies to inhibit proliferation of MeWo human melanoma cells [63,67]. Cell lines SK Mel 5, SK Mel 25, IGR and MelJuso proved to be nonresponsive to the antiproliferative activity of EB1089 [63,67]. The effect of EB1089 on the induction of cell apoptosis has also been investigated. Danielsson *et al.* reported that EB1089 induced apoptosis in WM1341 cells but not in MeWo cells [73]. The same study demonstrated that another vitamin D analog – CB1093 (20-epi-22(S)-ethoxy-23yne-24a,26a,27a-trihomo-1 α ,25-dihydroxyvitamin D3) – also stimulates apoptosis in WM1341 cells but not in MeWo cells [73]. Notably, 1 α ,25-dihydroxyvitamin D3 also did not trigger apoptosis in the MeWo cell line. Moreover, CB1093 was capable of inducing apoptosis in several cancer-derived cell lines including mammary epithelial tumor cell lines [84]. This suggests that the vitamin D-induced apoptotic pathway in MeWo melanoma cells is defective.

Mechanisms of vitamin D analog selectivity

Generally, the activity of vitamin D compounds is based on their interactions with a relatively limited spectrum of proteins: nuclear and cell membrane VDR, 1,25D3-MARRS receptor, serum DBP and intracellular vitamin D-binding proteins, vitamin D 24-hydroxylase and perhaps other metabolizing enzymes [10,11,14,26,85].

VDR binding

The majority of vitamin D activity is mediated via nuclear VDR. The key structure in vitamin D responsible for the binding affinity for VDR is presumably the A-ring containing a 1 α -hydroxyl group. It has also been suggested that some other groups may be involved in substitution. On the other hand, the side-chain modifications probably have little effect on VDR binding affinity, and it is the selectivity of a compound that they may affect [26].

However, many vitamin D analogs retain therapeutic activity and exert much lower hypercalcemic properties [62,86]. One possible explanation is that VDR occurs in a few isoforms (splicing variants) with different specificities to vitamin D compounds, or that vitamin D analogs act through alternative mechanisms (see next section titled ‘Mechanisms of anti-tumor vitamin D analog activity’ for discussion). Recently, the presence of an alternative binding site for vitamin D analogs has been suggested [87]. For example, 25-hydroxyvitamin D3 can preferentially bind to an alternative side chains and trigger a voltage-gated, outwardly rectifying chloride channel, but is less potent than 1 α ,25-dihydroxyvitamin D3 in the stimulation of gene expression [88]. The presence of an alternative binding socket of VDR may explain the different activity of vitamin D analogs. It

has also been confirmed by previous studies that there is a form of VDR that preferentially binds noncalcemic analogs [26]. Interaction with an alternative binding site or an alternative isoform of VDR may also change the affinity of the receptor to coactivators or corepressors [26,89]. For example, 20-epi analogs may form tighter VDR–retinoid X receptor complexes [26]. Selectivity was also demonstrated for 22-oxacalcitriol (OCT), which was found to accelerate binding of VDR to TIF-2; however, it interacted poorly with other coactivators SRC-1 and AIB-1 [89].

Vitamin D analogs can also act as VDR antagonists, while some switch from agonist to antagonist. The switch mechanism is attributed to the balance between coactivators and corepressors in the cell. For example, the Gemini analog acts as an antagonist when corepressors are in excess [26].

Activation of the VDR complex leads to its ubiquitination and starts proteasomic degradation of VDR. Some of the most potent analogs (e.g., KH 1060 and EB 1089) were reported to retard proteasomic degradation of VDR, resulting in enhancement of their potency [89].

The presence of alternative splicing variants of VDR and alternative vitamin D binding sites does not fully explain the complexity of activities of vitamin D, especially those observed after the inactivation of VDR (for review, see [90]). Fortunately, the existence of other vitamin D-interacting proteins, such as ‘rapid-response receptor’, may provide some additional explanations.

Mechanisms of anti-tumor vitamin D analog activity

Classical VDR pathway

The mechanisms of antiproliferative vitamin D analog activity are multiple and complex (**Figure 3**). They vary between cell types; however, most commonly vitamin D influences the cyclin/CDK system. $1\alpha,25$ -dihydroxyvitamin D₃–VDR activates the transcription of CDK inhibitors p19 (*CDKN2D* gene), p21 (*CDKN1A* gene) and p27 (*CDKN1B* gene), and decreases the expression of cyclins D1, D3, E1 and A1 [26]. This leads to the inhibition of CDK activity and hypophosphorylation of the pocket proteins Rb, p107 and p130. Hypophosphorylated pocket proteins (especially p107 and p130) bind the E2F transcription factors, preventing the activation of a wide variety of genes (including DNA replication, repair and other E2F-dependent genes involved in cell cycle progression). These events impair the entry of the cell into the S phase, and thus lead to cell arrest at the G₀/G₁ stage of the cell cycle. In addition, the upstream pathways of the cyclin/CDK system are also affected by vitamin D: the EGF receptor and IGF pathways are downregulated, while the TGF- β pathway is upregulated [14,26,91].

VDR activation by $1\alpha,25$ -dihydroxyvitamin D₃ may also inhibit proliferation by inducing cell differentiation. The mechanism of this event differs depending on the cell type and may depend on: activation of VDR and PI3K complexes; suppression of IL-12 secretion and downregulation of other costimulatory molecules (CD40, CD80 and CD86); induction of the expression of genes that are associated with the differentiated cell of origin; induction of *CDH1* (encoding E cadherin); or inhibition of AKT/mTOR signaling (for details, see recent reviews [14,26,74]).

Apoptosis induction & angiogenesis inhibition

Additional mechanisms for the anti-tumor activity of vitamin D involve stimulation of apoptosis and blocking of angiogenesis. $1\alpha,25$ -dihydroxyvitamin D₃ may repress antiapoptotic, prosurvival proteins (BCL-2 and BCL-X) and induce proapoptotic proteins

(BAX, BAK and BAD) [14,74]. Interestingly, 1 α ,25-dihydroxy vitamin D3 was found to protect cells from apoptosis in primary human melanocytes, but had adverse effects on melanomas [92]. Vitamin D can also cause destabilization of telomerase reverse transcriptase mRNA, inducing apoptosis through telomere attrition [93]. The putative mechanisms may also involve suppression of the expression and activity of COX-2 (mitochondrially encoded cytochrome c oxidase II) [14,26]. Another pro-apoptotic mechanism exerted by 1 α ,25-dihydroxyvitamin D3 involves downregulation of the antiapoptotic factors clusterin and survivin in the melanoma cells [16].

Angiogenesis is a crucial step in the development of melanoma metastases [59]. Vitamin D was demonstrated to inhibit angiogenic factors such as VEGF and IL-8, and upregulate the expression of antiangiogenic factors such as thrombospondin 1 [14]. Therefore, vitamin D and its analogs may potentially prevent or attenuate metastatic disease. Nevertheless, the precise molecular basis of the overall inhibition of cell cycle progression mediated by vitamin D in tumor cells, including melanoma, is very complex and therefore needs further investigation.

Antiproliferative activity of vitamin D & its derivatives: beyond VDR

1,25D3-MARRS receptor

1,25-dihydroxyvitamin D3 may also exert its activity via a rapid, pregenomic pathway, through binding with 1,25D3-MARRS receptor. This endoplasmic membrane-associated protein is also known as thiol-disulfide oxidoreductase, ERp57 or PDIA3 [85,94,95]. Binding depends on the conformation of the vitamin D analog. For example, 1,25-dihydroxy-lumisterol, locked in the 6-*cis* configuration, activates the rapid-response pathways and competes with 1 α ,25-dihydroxyvitamin D3 for MARRS receptor binding, but not for VDR binding [86]. However, recent studies have demonstrated the presence of a binding pocket for 'rapid-response' ligands on the VDR [87,88]. Therefore, it is difficult to apply classic binding studies in this case. On the other hand, the low calcemic activity of some vitamin D analogs (e.g., 1 α ,25-dihydroxy-16-ene-23-yne-D3, OCT and calcipotriol) may be the result of their lack of ability to stimulate the nongenomic rapid-response pathway [26]. The 1,25D3-MARRS/ ERp57/PDIA3 protein participates in the folding and quality control of newly synthesized glycoproteins and in the assembly of MHC class I. Interestingly, 1 α ,25-dihydroxyvitamin D3, in addition to its classical activity, was found to induce rapid stimulation of phospholipases C and A2, PKC and ERK [96]. 1 α ,25-dihydroxyvitamin D3 stimulates translocation of 1,25D3-MARRS/ERp57/PDIA3 to the nucleus. Moreover, interaction of 1,25D3-MARRS/ERp57/PDIA3 with nuclear factor NF- κ B and STAT3 has also been demonstrated [97]. Therefore, it was postulated that some activities of 1 α ,25-dihydroxy vitamin D3, previously attributed solely to VDR, may also be mediated by 1,25D3-MARRS (for a recent review, see [95]). It has to be noted that the existence of a rapid-response pathway and the role of PDIA3 in 1 α ,25-dihydroxyvitamin D3 activity is still under debate, but it may, at least in part, explain the activity of vitamin D analogs with low affinity to VDR (e.g., short side-chain analogs).

DBP binding

DBP enhances the circulating half-life of vitamin D compounds and decreases their tissue accessibility. DBP acts as a vitamin D reservoir and guards against vitamin D intoxication (reviewed in [98]). All natural vitamin D metabolites present in the serum demonstrate high binding affinity to DBP, while most analogs bind this protein with low affinity, most probably due to modified side chains [98,99]. An example could be OCT, which demonstrates a DBP-binding affinity 500-times lower than that of 1 α ,25-dihydroxyvitamin D3. Usually, the interaction of such analogs with serum DBP is reduced, which leads to their

rapid clearance and poor absorption from the circulation [99]. Pharmacologically, vitamin D analog administration exerts an early effect, with high peak levels, compared to the slow rise and fall of natural 1 α ,25-dihydroxyvitamin D₃. Consequently, the target cells are exposed to higher levels of the analog. Thanks to rapid clearance of such vitamin D compounds from the tissues, the increase in calcium transport is elicited only transiently, which explains the decreased calcemic effects when compared with 1 α ,25-dihydroxyvitamin D₃ [26,89]. Moreover, levels of DBP decrease with the progression of melanoma [100]. Finally, vitamin D analogs may be used topically, which could enable rapid, targeted and DBP-independent treatment of melanoma.

Target cell metabolism

Target cell metabolism plays an important role in regulating the activity of vitamin D analogs. Binding of vitamin D compounds with VDR activates the transcription of 24-hydroxylase (CYP24A1), the enzyme that oxidates and cleaves the side chains of 1 α ,25-dihydroxyvitamin D₃. Such transformation leads to the lowered VDR affinity and low activity of vitamin D metabolites. Owing to the side-chain modifications, the pattern of vitamin D analog catabolism may be different. Structural changes can decrease the rate of CYP24A1-mediated catabolism of the analog, which would be expected to survive much longer in target cells and activate the VDR for a longer time [26,89].

Therefore, the higher biological activity of the analogs that demonstrate VDR affinity similar to 1 α ,25-dihydroxyvitamin D₃ may be explained by the slower rate of their intracellular catabolism. Another possible mechanism is the conversion of vitamin D analogs to active metabolites through 24-hydroxylase activity. Examples include 1,25-dihydroxy-16-ene-D₃ and 20-epi-1,25-dihydroxy D₃, metabolized by 24-hydroxylase to stable 24-oxo intermediates that retain significant biological activity [26].

Putative mechanisms of unresponsiveness to vitamin D analogs

The results of experiments on the effect of vitamin D and its analogs on melanoma cells, summarized here, are in some cases contradictory, demonstrating that the same analog is effective in some cell lines and not effective in others. The question that arises is why there are differences between the cell lines of the same tumor type. What are the mechanisms that cause the differentiated responses of melanoma cells to treatment with vitamin D and its analogs? Other tumors, such as breast cancer [101] or leukemia [97,98], also behave in a similar way, with some cell lines responsive and others resistant.

As stated above, the majority of vitamin D activity is most likely mediated through VDR signaling. Consequently, the unresponsiveness or resistance of cells to vitamin D and its analogs may be explained by defects in VDR-mediated transcription. Recent studies emphasize the potency of two binding pockets of VDR in substrate recognition and downstream signaling through both genomic and nongenomic pathways, which may in part explain the partition of procalcemic and antiproliferative activities of vitamin D analogs [88,102]. The genomic pathway modulates expression of a variety of genes that contain vitamin D response elements, including those involved in cellular growth, differentiation, apoptosis, invasion and metastasis of tumor cells [63]. The signaling pathways that lead to the activation or suppression of transcription were summarized above. Defects in targets for specific vitamin D compounds may result in an altered response to the treatment. Cell lines with a defective signaling pathway will be nonresponsive to some compounds, but may respond to others that act through different signaling pathways [7,10,102].

Another mechanism that may be involved in vitamin D unresponsiveness is the regulation of VDR levels through alterations in transcription rate and mRNA stability. For example, 1 α ,

25-dihydroxyvitamin D3 induces transcription of the 24-hydroxylase gene in a more pronounced way in responsive cells compared with resistant ones [63]. This may suggest that the machinery essential for correct VDR signaling is defective in the cells that demonstrate resistance to vitamin D treatment.

For the leukemia THP-1 cell line, it was reported that resistance towards vitamin D-induced differentiation correlated with impaired nuclear localization of VDR, but not with its total expression in the cells [103]. Therefore, the presence of VDR in nuclei is essential for the induction of differentiation, while the activation of intracellular signaling pathways is of secondary importance and probably plays a role in amplifying the response. This hypothesis is supported by reports demonstrating that vitamin D analogs with higher differentiation-inducing potential are more potent inducers of MAPK activation [103].

The solution to the problem of resistance to vitamin D treatment may be solved by applying a combination of drugs with distinct activity, as the response to vitamin D seems to be cell line- and anti-tumor activity-specific. Perhaps the cell line demonstrating resistance to the antiproliferative effect of one vitamin D compound will respond to another.

Expert commentary & five-year view

Despite great progress at the molecular level and the development of new pharmacological strategies, surgical intervention is the best practice in melanoma treatment.

The active form of vitamin D is a well-known regulator of growth and regeneration of the epidermis [1,2,4], but not much is currently known about its influence on melanocyte physiology. For example, it is still unclear whether vitamin D regulates pigmentation [104]. Although some recent studies demonstrated that vitamin D and its analogs do not influence pigmentation [34], other studies reported that pigmentation can influence 1 α ,25-dihydroxyvitamin D3 activity, with pigmented cells being more resistant towards treatment [44]. In addition, in pigmented melanoma cells, expression of VDR is decreased, which may in part explain the lower efficiency of vitamin D [44,61]. Moreover, high basal NF- κ B activity in nonpigmented melanoma cells was recently shown to increase sensitivity to vitamin D3 derivatives [61]. These findings may indicate limitations for vitamin D use in melanoma treatment. However, the combined use of inhibitors of melanogenesis such as d-penicillamine and *N*-phenylthiourea (which sensitize melanoma cells for radio- and chemotherapy [100]), may improve the final effects of secosteroids.

Recently, studies have also demonstrated that resistance of some melanoma cells to vitamin D treatment may be caused by specific miRNAs or by epigenetic-modulating drugs influencing VDR expression [105]. Therefore, epigenetic regulators such as trichostatin A and 5-Aza 2'-deoxycytidine may possibly sensitize melanoma cells towards vitamin D and its analogs by increasing the expression of VDR.

Recent interest in the pluripotent activity of vitamin D resulted in the synthesis and extensive testing of more than 2000 vitamin D analogs. Potential targets for both prophylactic and therapeutic use of secosteroids range from bone fracture prevention to immune diseases and cancer. According to the NIH [203], approximately 125 ongoing clinical trials related to cancer and vitamin D are enlisted; however, only three records concern melanoma and vitamin D, with only one currently recruiting participants (ClinicalTrials.gov identifier: NCT01264874 [204]). Judging from the growing interest in vitamin D and its derivatives in next 5 years, there will be an outburst of animal, preclinical and clinical studies aimed at using secosteroids as a potential antimelanoma factor. Moreover, new low-calcemic derivatives of vitamin D, such as 20-hydroxyvitamin D3, may be included in those trials [86].

On the other hand, increasing awareness of the problem of vitamin D deficiency in modern societies should result in increased monitoring and supplementation of vitamin D. It is also possible that some low-calcemic vitamin D analogs may be considered as an alternative for classical 1 α ,25-dihydroxyvitamin D₃. Furthermore, the ‘personalized medicine’ concepts may influence studies of vitamin D and its potential use in the treatment of melanoma. It will be possible to predict the potential outcomes of therapy by expression profiling of VDR, DBPs and other proteins involved in its metabolism. Melanoma pigmentation and expression of the genes involved in melanogenesis should also be taken into consideration. In fact, one of the clinical trials scheduled to start this year is going to test the influence of vitamin D on a signaling pathway involved in the development of melanoma, and aims to identify new melanoma biomarkers (ClinicalTrials.gov identifier: NCT01477463 [205]).

There is also growing evidence that vitamin D activity is not strictly dependent on VDR. This is worth exploring in the future in order to fully understand the molecular mechanisms of vitamin D interaction with multiple targets. The existence of an alternative pathway of vitamin D activity (e.g., the so-called ‘rapid-response pathway’), although still questioned, would provide an explanation.

Bearing in mind the relatively high dose of vitamin D or its analogs used in treatment (e.g., 50,000 units for 1 α ,25-dihydroxyvitamin D₃) in a single dose in some inflammatory/autoimmune diseases (see [1] and references within), it is also probable that secosteroids may have a direct influence on cell metabolism, cell membrane stability and levels of reactive oxygen species in other cell types. Similar effects may be expected when vitamin D and its analogs are used topically.

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Key issues

- There are indications that the level of 25-dihydroxyvitamin D3 in blood correlates with the prevalence of multiple types of cancer, including melanoma.
- Vitamin D supplementation decreases the risk of some cancers, including melanoma.
- The active form of vitamin D (1 α ,25-dihydroxyvitamin D3) was found to be effective in anticancer therapy, although its clinical use is limited owing to the high risks of hypercalcemia as a major side effect.
- Low-calcemic vitamin D analogs may be successfully introduced into melanoma therapy.
- Further biological evaluation of the variety of newly synthesized compounds, such as derivatives of androsta- and prena-5,7-dienes, may give promising results and present putative candidates for clinical testing.
- Genetic variations such as vitamin D receptor (*VDR*) polymorphism, epigenetic downregulation of VDR expression or high pigmentation of melanoma cells decrease the effectiveness of vitamin D and its analogs, which may be overcome by the use of specific inhibitors of melanogenesis.
- Vitamin D and its low-calcemic analogs may also be successfully used in combination therapy with other chemotherapeutics such as cisplatin or bevacizumab.
- There is growing evidence that VDR-independent pathways may also play a role in the anticancer activity of vitamin D and its derivatives.

Figure 1. Synthesis of vitamin D
7-DHC: 7-dehydrocholesterol.

Figure 2.
Metabolism of vitamin D.

Figure 3. Mechanisms for the antiproliferative actions of vitamin D analogs in melanoma

As shown for 1 α ,25-dihydroxyvitamin D₃, stimulation of cancer cells may activate several pathways that are relevant to melanoma treatment. 1 α ,25-dihydroxyvitamin D₃ acts through VDR, regulating calcium homeostasis and also activating genes involved in its metabolism. It can also inhibit cyclins and thus affect the expression of E2F-dependent genes involved in DNA replication, repair and cell-cycle regulation. 1 α ,25-dihydroxyvitamin D₃, through VDR, interacts with the transcription factor NF- κ B, thus influencing the immune response, which can be useful in melanoma treatment. The influence of melanogenesis and pigmentation on the activity of secosteroids and vice versa is still under discussion; therefore, the interactions are marked with question marks. The activity of vitamin D analogs in melanoma cells could also be affected by intracellular metabolism by CYP24A1 (inactivation) or CYP27B1 (activation by hydroxylation in position 1). Downregulation of antiapoptotic BCL-2 and BCL-XL and upregulation of proapoptotic BAX, and BCL-X5, as well as BAK and BAD, leads to activation of caspases and cell death (apoptosis). The interaction between vitamin D analogs and 1,25D₃-MARRS/ERp57/PDIA3 protein, especially in melanoma cells, is still unclear but is a very promising topic for research (interactions shown with question marks). See the text for more details.

RXR: Retinoid X receptor; VDR: Vitamin D receptor.

Table 1

VDR polymorphisms and melanoma risk.

Study (year)	Cdx2 (G/A)	EcoRV (A-1012G) (A/G)	FokI (C/T) (F/f)	TaqI (T/C) (T/t)	BsmI (A/G) (B/b)	Apal(G/T) (A/a)	Ref.
Povey <i>et al.</i> (2007)		+ ↑A allele					[58]
Halsall <i>et al.</i> (2009)		+ ↑A allele					[56]
Han <i>et al.</i> (2007)	-		+ ↑f genotype		-		[51]
Hutchinson <i>et al.</i> (2000)			+ ↑f allele	-			[52]
Li <i>et al.</i> (2007); Li <i>et al.</i> (2008)			+ ↑f allele	+ ↑T allele	+ ↑b allele		[53,54]
Randerson-Moor <i>et al.</i> (2009)	-	-	+ ↑f allele	+ ↑T allele	+ ↑b allele	-	[49]
Santonocito <i>et al.</i> (2007)		-	-		+ ↑bb genotype		[55]
Barroso <i>et al.</i> (2008)			-	-			[106]
Gapska <i>et al.</i> (2009)			-	-	-		[50]

↑: Allele is associated with an increased risk or worse prognosis of melanoma; +: Polymorphism is associated with an altered risk or prognosis of melanoma; -: Lack of association was observed.

Table 2

The effects of vitamin D compounds on melanoma cells.

Function affected	Effect	Vitamin D compound	Ref.
Proliferation	Inhibition	1 α ,25-dihydroxy D3	[31,46,61–63,66–70] [†]
		20-hydroxyvitamin D3	[61,62]
		25-hydroxyvitamin D3	[68] [†]
		EB1089	[63,67] [†]
		CB1093	[73]
		17-COOH-7DA	[81]
		No effect	
	No effect	1 α ,25-dihydroxy D3	[34,46,63,67,71,72] [†]
		7-DHC	[34]
		Previtamin D3	[34]
		Vitamin D3	[34]
		25-hydroxyvitamin D3	[34,63] [†]
		Lumisterol	[34]
		EB1089	[63,67] [†]
		PRI-1906	[72]
		PRI-2191	[72]
Apoptosis	Induction	1 α ,25-dihydroxy D3	[73] [†]
		EB1089	[73] [†]
		CB1093	[73] [†]
	No effect	1 α ,25-dihydroxy D3	[73] [†]
		EB1089	[73] [†]
		CB1093	[73] [†]
Colony formation	Inhibition	1 α ,25-dihydroxy D3	[5,31,62]
		7-DHP [‡]	[64]
		20-hydroxyvitamin D3	[62]
		21-hydroxy 7-DHP	[65]
		21(OH)oxy-piT	[65]
		21-(OH)pL	[65]
		21-(OH)pD	[65]
		Pregnenolone	[64]
		20-oxopregnacalciferol	[64]
		4R	[5]
		4S	[5]
		4R-pD [§]	[5]
		4R-pL [¶]	[5]

Function affected	Effect	Vitamin D compound	Ref.
		4S-pD [§]	[5]
		4S-pL [¶]	[5]
		17-COOH-7DA	[81]
DNA synthesis	Inhibition	1 α ,25-dihydroxy D3	[75] [†]
		1,25,26-trihydroxyvitamin D3	[75]
		17-COOH-7DA	[81]
		21-hydroxy 7-DHP	[65]
		21(OH)oxy-piT	[65]
		21(OH)pL	[65]
		21(OH)pD	[65]
	No effect	1 α ,25-dihydroxy D3	[71] [†]
		25-hydroxyvitamin D3	[75]
		1,24,25-trihydroxyvitamin D3	[75]
		24R,25-dihydroxyvitamin D3	[31]
Translocation of VDR to nucleus	Promotion	1 α ,25-dihydroxy D3	[61]
		20-hydroxyvitamin D3	[61]
		21(OH)oxy-piT	[65]
		21(OH)pL	[65]
		21(OH)pD	[65]
Expression of VDR	Increased	1 α ,25-dihydroxy D3	[63,67,77] [†]
	No effect	1 α ,25-dihydroxy D3	[73] [†]
Expression of 1-hydroxylase	Decreased	1 α ,25-dihydroxy D3	[67]
Expression of 24-hydroxylase	Increased	1 α ,25-dihydroxy D3	[63,67,77]
Expression of 25-hydroxylase	Increased	1 α ,25-dihydroxy D3	[63,67,77]
24-hydroxylase activity	Increased	1 α ,25-dihydroxy D3	[39]
Tyrosinase activity	Increased	1 α ,25-dihydroxy D3	[31]
		1 α ,25-dihydroxyvitamin D2	[76]
	No effect	7-DHC	[76]
		25-hydroxyvitamin D3	[31]
		1 α -hydroxyvitamin D3	[31]
		24R,25-dihydroxyvitamin D3	[31]
		Ergosterol	[76]

VDR: Vitamin D receptor.

[†]Contradictory effects occur in different cell lines.

[‡]Product of transformation of 7-dehydrocholesterol (7-DHC) by P450scc.

[§]D-like derivatives of pregna-5,7-diene epimers.

[¶]L-like derivatives of pregna-5,7-diene epimers.