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The role of CYP11A1 in the production of vitamin D metabolites and their role in the regulation of epidermal functions

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Abstract

Research over the last decade has revealed that CYP11A1 can hydroxylate the side chain of vitamin D3 at carbons 17, 20, 22 and 23 to produce at least 10 metabolites, with 20(OH)D3, 20,23$(OH)_2$D3, 20,22$(OH)_2$D3, 17,20$(OH)_2$D3 and 17,20,23$(OH)_3$D3 being the main products. However, CYP11A1 does not act on 25(OH)D3. The placenta, adrenal glands and epidermal keratinocytes have been shown to metabolize vitamin D3 via this CYP11A1-mediated pathway that is modified by the activity of CYP27B1, with 20(OH)D3 (the major metabolite), 20,23$(OH)_2$D3, 1,20$(OH)_2$D3, 1,20,23$(OH)_3$D3 and 17,20,23$(OH)_3$D3 being detected, defining these secosteroids as endogenous regulators/natural products. This is supported by the detection of a mono-hydroxyvitamin D3 with the retention time of 20(OH)D3 in human serum. In new work presented here we demonstrate that the CYP11A1-initiated pathways also occurs in Caco-2 colon cells. Our previous studies show that 20(OH)D3 and 20,23$(OH)_2$D3 are non-calcemic at pharmacological doses, dependent in part on their lack of a C1α hydroxyl group. In epidermal keratinocytes, 20(OH)D3, 20(OH)D2 and 20,23$(OH)_2$D3 inhibited cell proliferation, stimulated differentiation and inhibited NF-κB activity with potencies comparable to 1,25$(OH)_2$D3, acting as partial agonists on the VDR. 22(OH)D3 and 20,22$(OH)_2$D3, as well as secosteroids with a short or no side chain, showed antiproliferative and prodifferentiation effects, however, with lower

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Conflict of interest
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potency than 20(OH)D3 and 20,23$(OH)_2$D3. The CYP11A1-derived secosteroids also inhibited melanocyte proliferation while having no effect on melano-genesis, and showed anti-melanoma activities in terms of inhibiting proliferation and the ability to grow in soft agar. Furthermore, 20(OH)D3 and 20,23$(OH)_2$D3 showed anti-fibrosing effects in vitro, and also in vivo for the former. New data presented here shows that 20(OH)D3 inhibits LPS-induced production of TNFα in the J774 line, TNFα and IL-6 in peritoneal macrophages and suppresses the production of proinflammatory Th1/Th17-related cytokines, while promoting the production of the anti-inflammatory cytokine IL-10 in vivo. In summary, CYP11A1 initiates new pathways of vitamin D metabolism in a range of tissues and products could have important physiological roles at the local or systemic level. In the skin, CYP11A1-derived secosteroids could serve both as endogenous regulators of skin functions and as excellent candidates for treatment of hyperproliferative and inflammatory skin disorders, and skin cancer.

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Keywords

Epidermis; CYP11A1; P450scc; Keratinocytes; Vitamin D; Immune system

1. Introduction

1.1. Epidermis: an overview

The epidermis represents the most outer layer of the skin and its primary function is to protect body homeostasis against external environmental stressors [1,2]. To fulfill this function, the proliferating basal keratinocytes enter a precise differentiation program and generate several layers of the epidermis, with ultimate formation of a permeability barrier made of cross-linked proteins (cornified cell envelope) and lipids (cornified lipid envelope) [2–4]. This barrier isolates the body from the external environment and protects against microbes and dehydration [2,5]. Lipids and cholesterol derivatives, which are synthesized locally, play an important role in this barrier function [6]. The protective role of the epidermis is complemented by local immune [7,8], pigmentary [9–11], neuroendocrine [12] and activating/inactivating enzymatic [13] systems, which interact in a hierarchical manner [1].

Importantly, the epidermis is intermittently exposed to different wavelengths of ultraviolet radiation (UVR) including UVB (280–320 nm). While the mutagenic, carcinogenic and skin aging effects of UVR are properly recognized, the role of UVB in the photochemical transformation of 7-dehydrocholesterol (7DHC) to vitamin D3 cannot be overestimated since more than 90% of systemic vitamin D3 derives from the skin [14–16].

1.2. Cytochrome P450scc (CYP11A1): an overview

CYP11A1 catalyses the rate-limiting reaction of steroid synthesis, the conversion of cholesterol to pregnenolone, known as the cholesterol side-chain cleavage reaction. This involves three oxidative reactions on the cholesterol side chain producing 22*R*-hydroxycholesterol and 20*R*,22*R*-dihydroxycholesterol as intermediates, with subsequent

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cleavage of the side chain between C20 and C22 [17,18]. These intra-mitochondrial reactions require electrons provided by NADPH via the electron transport proteins, adrenodoxin reductase and adrenodoxin [17,19]. The product of CYP11A1 action, pregnenolone, serves as the precursor for all steroids, which are produced through tissue- and cell-specific pathways involving various cytochromes P450 and/or steroid dehydrogenases [18].

For a long time it was considered that CYP11A1 was expressed predominantly, if not solely, in the classical steroidogenic tissues such as adrenal cortex, ovary, testis and placenta [18]. However, evidence has accumulated that CYP11A1 is expressed in additional organs or tissues including brain, skin, lungs, gastrointestinal (GI) tract, bone, breast, prostate, thymus, immune cells and several cancer cells of different origin [reviewed in [20–24]]. The expression of CYP11A1 in these non-classical steroidogenic tissues/cells, or their malignant counterparts, is relatively low and consequently the products are unlikely to have major systemic effects. However, a range of local effects is highly probable with some already being documented [reviewed in [20,21]].

2. CYP11A1 expression in the skin

Testing for the cutaneous expression of the homologue of the hypothalamic-pituitary-adrenal axis (HPA) [25], identified, for the first time, expression of CYP11A1 and related genes in human skin [26]. Subsequently, expression of CYP11A1 protein and its corresponding enzymatic activity, as well as adrenodoxin, adrenodoxin reductase, the Steroidogenic Acute Regulatory protein (StAR or STARD1) and another member of the START domain family, STARD3 (also known as MLN64), were detected in whole skin and skin cells [13,21,27–29]. CYP11A1 was found to be expressed in the epidermis, adnexal structures and dermis, as well as in cultured keratinocytes, melanocytes, sebocytes, dermal fibroblasts and melanoma cells. Interestingly, CYP11A1 mRNA was found to undergo alternative splicing in skin cells of epidermal origin [13], a phenomenon also observed in bone tissue [23].

Importantly, UVB and UVC, but not the UVA wavelengths of ultraviolet radiation, stimulated CYP11A1 expression when skin was incubated ex vivo or when keratinocytes were co-cultured with melanocytes [30,31]. This stimulation was accompanied by upregulation of the production of corticotropin releasing factor (CRF), urocortin (Unc) and POMC derived peptides, consistent with previously described endogenous expression of those elements of the HPA axis and their stimulation by UVB [12,32–35]. These findings suggest that highly energetic UVR can regulate cutaneous expression of CYP11A1 directly or indirectly, perhaps through a HPA-like organization operating at the local level, with locally produced ACTH and CRF serving as its messengers [25,36–39].

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3. Novel role of CYP11A1 in the metabolism of 7-dehydrocholesterol, ergosterol and vitamin D

3.1. CYP11A1 metabolizes 7DHC and ergosterol

It has been established over the last ten years that CYP11A1 catalyzes the cleavage of the side chain of 7DHC to produce 7-dehydropregnenolone (7DHP). The reaction sequence is similar to that for the conversion of cholesterol to pregnenolone, proceeding through the intermediates 22-hydroxy-7DHC [22(OH)7DHC] and 20,22-dihydroxy-7DHC [20,22$(OH)_2$7DHC] [13,40–42]. Interestingly, purified CYP11A1 shows slightly higher catalytic efficiency toward cleaving the side chain of 7-DHC than that of cholesterol [13].

Ex vivo and ex-utero studies using fragments of rat, dog, pig and rabbit adrenal glands, human placentas and human and pig epidermal keratinocytes, have clearly shown their ability to convert 7DHC to 7DHP. There was also detectable production of the intermediates 22(OH)7DHC and 20,22$(OH)_2$7DHC, as well as other monohydroxy-derivatives of 7-DHC [40,41]. 7DHP undergoes further metabolism by other steroidogenic enzymes in skin with 17-hydroxy-7DHP, 7-dehydroprogesterone and 20-hydroxy-7DHP having being identified.

Ergosterol, another 7 sterol that is the major membrane sterol in fungi, can also be metabolized by CYP11A1 but unlike 7DHC, no cleavage of the side chain occurs [43,44]. This is likely to be due to the double bond in the side chain of ergosterol between carbons 22 and 23 since neither the addition of a C7-C8 double bond (as in 7DHC) nor a 24 methyl group (as in campesterol) [45] prevent scission of the side chain from occurring. Human CYP11A1 was found to catalyze epoxidation of the C22 C23 double bond as well as hydroxylation of the ergosterol side chain at C20, generating 20-hydroxy-22,23-epoxy-22,23-dihydroergosterol as a major metabolite [44]. Experiments with CYP11A1 isolated from bovine adrenals revealed that C24 is the favored site of hydroxylation with 24-hydroxyergosterol and 17,24-dihydroxyergosterol being major products [43]. The major epoxidation products seen with human CYP11A1 are also made by the bovine enzyme [44]. There is evidence that some of the products of CYP11A1 action on ergosterol are biologically active with 24-hydroxyergosterol and 17,24-dihydroxyergosterol displaying antiproliferative activity on skin cells [43].

3.2. Metabolism of vitamin D3 by CYP11A1

Although the side chains of vitamin D3 and cholesterol are identical, CYP11A1 only catalyses hydroxylation of the side chain of vitamin D3 and cannot cleave it, despite 20,22$(OH)_2$D3 being one of the products. The favored site of hydroxylation is at C20 rather than C22, with 20(OH)D3 being the major metabolite [42,46–48]. NMR analysis and chemical synthesis have revealed that this product is the 20S epimer [49]. The K_m for the conversion of vitamin D3 to 20(OH)D3 by CYP11A1 is higher than that for the metabolism of cholesterol [50]. Docking of vitamin D3 into the crystal structure of human CYP11A1 places C20 rather than C22 closest to the heme iron, consistent with the preferred hydroxylation site seen experimentally [51]. Other identified sites of hydroxylation of the vitamin D3 side chain by CYP11A1 are C17, C22 and C23, and at least 10 metabolites are produced. Hydroxylations at these positions occur rarely as the initial hydroxylation event,

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but commonly as the second or third hydroxylation. A summary of the major pathways for metabolism of vitamin D3 by CYP11A1 is shown in the upper section of Fig. 1. Besides 20(OH)D3, major metabolites are 20,23$(OH)_2$D3, 20,22$(OH)_2$D3, 17,20$(OH)_2$D3 and 17,20,23$(OH)_3$D3, with 17(OH)D3 and 22(OH)D3 being among the minor products identified to date [46–48]. It is important to note that CYP11A1 does not hydroxylate 25(OH)D3 [46] so that the CYP11A1 does not compete with CYP27B1 for activation of 25-hydroxyvitamin D3. However, CYP11A1 can hydroxylate the prodrug 1(OH)D3 to the biologically active product, 1,20$(OH)_2$D3, providing an alternative route to 25-hydroxylation for activation of this compound [50].

While initial studies on the metabolism of vitamin D3 by CYP11A1 were carried out using purified bovine enzyme, more recent ex vivo and ex-utero incubations of vitamin D3 with fragments of adrenal glands and human placentas (which express CYP11A1) have clearly demonstrated that the same CYP11A-caltalysed reactions occur. These studies show that 20(OH)D3 is produced as the major metabolite, with 22(OH)D3, 20,23$(OH)_2$D3, 20,22$(OH)_2$D3 and 17,20,23$(OH)_3$D3 also being detected [52]. Both the adrenal and placenta express CYP27A1, CYP2R1 and CYP27B1, and convert vitamin D3 to 25(OH)D3, and 25(OH)D3 to 1,25(OH)2D3 [52]. Our conclusion from these studies is that tissues with high expression of CYP11A1 metabolize vitamin D3 by both the classical pathway [D3 $\rightarrow$ 25(OH)D3 $\rightarrow$ 1,25$(OH)_2$D3] and the novel pathway [D3 $\rightarrow$ 20(OH)D3 + 22(OH)D3 $\rightarrow$ 20,22$(OH)_2$D3 + 20,23$(OH)_2$D3 $\rightarrow$ 17,20,23$(OH)_3$D3]. The several-fold higher production of 20(OH)D3 than 25(OH)D3 suggests that the latter pathway predominates in these tissues. A metabolite corresponding to 20(OH)D3 was also detected in human serum at a level 20 times lower than that of 25(OH)D3, providing further evidence that metabolism of vitamin D3 by CYP11A1 occurs in vivo [52]. Several of the products of the CYP11A1-mediated pathway can be 1α-hydroxylated by purified human CYP27B1, although with lower catalytic efficiency than for 25(OH)D3 [53]. Despite the low efficiency, these CYP27B1-dependent 1α-hydroxylations do appear to occur in vivo with 1,20$(OH)_2$D3 and 1,20,23$(OH)_3$D3 being identified in ex-utero incubations of placentas and adrenals with vitamin D3 [52]. It should be noted that CYP11A1-derived products with a 17α-hydroxyl group such as 17,20$(OH)_2$D3 and 17,20,23$(OH)_3$D3 are not substrates for CYP27B1 [53].

An identical panel of vitamin D3 hydroxy-derivatives was detected in human and pig epidermal keratinocytes incubated with 50 μM vitamin D3, to that detected in incubations of placenta and adrenal tissue [52]. However, in contrast to placentas and adrenals, the levels of 20(OH)D3 and 25(OH)D3 were similar. Skin cells showed slightly higher production of 22(OH)D2 and similar or higher production of 1,25$(OH)_2$D3 in comparison to 20,22$(OH)_2$D3 and 20,23$(OH)_2$D3. Importantly, endogenous production of 20(OH)D3, 25(OH)D3, 22(OH)D3, 20,23$(OH)_2$D3, 20,22$(OH)_2$D3, 1,25$(OH)_2$D3 and 17,20,23$(OH)_3$D3 has been detected in HaCaT keratinocytes cultured in the presence of 5% serum as the only source of vitamin D3 substrate [52]. This demonstrates that high levels of exogenous vitamin D3, often used experimentally to facilitate detection and identification of products, are not required for the CYP11A1-mediated pathway to occur in skin cells.

In new studies presented here, we show CYP11A1-dependent metabolism of vitamin D3 in another epithelial cell type, colonic Caco-2 cells (Fig. 2), which represent a recognized cell

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model for colonic and enteric epithelium [24,54]. LC/MS analyses have demonstrated that Caco-2 cells can metabolize exogenous vitamin D3 to 20(OH)D3 as the predominant metabolite, with lesser production of 22(OH)D3 and 25(OH)D3, and subsequent metabolism to 20,23$(OH)_2$D3, 1,20$(OH)_2$D3 and 1,25$(OH)_2$D3. Thus, epithelial cells of the gastrointestinal (GI) tract can metabolize vitamin D3 through the novel CYP11A1-initiated pathway (Fig. 2) as well as through the classical pathway in a similar manner to the major steroidogenic organs listed above.

Our recently published studies with purified expressed enzymes have shown that both CYP27A1 which acts as a 25-hydroxylase on vitamin D3 and a 26-hydroxylase on cholesterol, and CYP24A1 which inactivates 1,25$(OH)_2$D3, can also metabolize 20(OH)D3 [55,56]. Human CYP27A1 was found to hydroxylate 20(OH)D3 at both C25 and C26 producing 20,25$(OH)_2$D3 and 20,26$(OH)_2$D3 in approximately equal proportions. Rat CYP24A1 was observed to hydroxylate 20(OH)D3 at C24, as it does with 1,25$(OH)_2$D3, producing 20,24$(OH)_2$D3 as the major product. It was also found to hydroxylate 20(OH)D3 at C25 producing 20,25$(OH)_2$D3. Other minor dihydroxy-derivatives were also produced but remain to be identified. We have also reported that 20,24$(OH)_2$D3, 20,25$(OH)_2$D3 and 20,26$(OH)_2$D3 can be 1α-hydroxylated by CYP27B1 with a catalytic efficiency similar to that for 1α-hydroxylation of 25(OH)D3 and much higher than for the parent 20(OH)D3 [53]. These reactions add an additional level of complexity to the CYP11A1-initiated pathway (with the products also being biologically active as described later) and are illustrated in Fig. 1.

3.3. Metabolism of vitamin D2 by CYP11A1

As for vitamin D3, purified CYP11A1 can also hydroxylate the side chain of vitamin D2 without its cleavage, with the preferred site for the initial hydroxylation being at C20 [57,58]. The major products were identified as 20(OH)D2 and 17,20$(OH)_2$D2, with lesser production of 17,20,24$(OH)_3$D2 and another unidentified dihydroxyvitamin D2 metabolite. The reaction sequence is as follows: D2 → 20(OH)D2 → 17,20$(OH)_2$D2 → 17,20,24$(OH)_3$D2. Despite the side chains of vitamin D3 and its ergosterol precursor being identical, unlike ergosterol [44], no epoxidation of the double bond between C22 and C23 occurs for vitamin D2, indicating a different positioning of the side chain in the active site. CYP27B1 was found to 1α-hydroxylate 20(OH)D2 to 1,20$(OH)_2$D2, however, with an efficiency much lower than for the conversion of 25(OH)D2 to 1,25$(OH)_2$D2 [59].

4. Conversion of 7-steroids into vitamin D-like compounds

The skin is becoming recognized as a steroidogenic tissue containing all enzymes necessary for production and metabolism of glucocorticoids, mineralocorticoids, androgens and estrogens [reviewed in [12,21,60]]. Therefore, 7DHP produced locally by the action of CYP11A1 on 7DHC, can further serve as the substrate for the production of 7-pregnenes, -androgens and -estrogens, which have been identified in patients with Smith–Lemli–Opitz Syndrome (SLOS) [61,62]. Thus, in the skin 7DHP may undergo further metabolism to 7-dehydroprogesterone, hydroxy-7DHP and potentially other 7-hydroxysteroids through existing steroidogenic enzymes [13,40,63]. After exposure to UVB, these 5,7-dienes could potentially be converted to androsta-calciferols (aD) and pregna-calciferols (pD), i.e.,

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vitamin D compounds with a short or no side chain [13,63–67]. These interconversions are summarized in Fig. 3. The corresponding intra-epidermal production of androsta- and pregna- lumisterol-like or tachysterol-like compounds (aL, pL, aT and pT, respectively) can also be envisioned [60,63–66], as well as secosteroidal derivatives of 22(OH)7DHC and 20,22$(OH)_2$7DHC since these intermediates in the transformation of 7DHC to 7DHP can be released from the enzyme [40,41].

5. Phenotypic effects of CYP11A1-derived secosteroids on skin cells

The CYP11A1-derived hydroxy-vitamin D derivatives have been found to be biologically active on a range of normal and malignant cells with skin cells being most thoroughly tested to date (Table 1). Importantly, both 20(OH)D3 and 20,23$(OH)_2$D3 lack toxic calcemic activity in rodents, as described later.

5.1. Keratinocytes

The effects of 20(OH)D3 and 20,23$(OH)_2$D3 on keratinocyte proliferation and differentiation have been studied in detail [49,68–73]. These secosteroids inhibit DNA synthesis and colony formation, cause cell cycle arrest and stimulate the differentiation program of keratinocytes with a potency comparable to that of 1,25$(OH)_2$D3. However, unlike 1,25$(OH)_2$D3 they only modestly stimulate the transcription of the CYP24A1 gene and thus do not markedly induce their own metabolism by CYP24A1. Both secosteroids act as partial agonists of the vitamin D receptor (VDR), as demonstrated by gene silencing experiments [68–70]. Similar antiproliferative effects to those of 20(OH)D3 and 20,23$(OH)_2$D3 are seen for 17,20$(OH)_2$D3 and 17,20,23$(OH)_3$D3 [71,73]. The CYP11A1-derived secosteroids with a 22-hydroxyl group, 22$(OH)_2$D3 and 20,22$(OH)_2$D3, also show both antiproliferative and prodifferentiation effects, but are less potent than 20(OH)D3 and 20,23$(OH)_2$D3 [48]. As expected, chemically synthesized 20S(OH)D3 has the same properties as the enzymatically generated secosteroid [49], whereas the 20R(OH)D3 epimer behaves similarly to 20S(OH)D3 at high concentrations, but stimulates rather than inhibits proliferation at low (0.1 nM) concentrations [72].

Studies on vitamin D2-derived 20(OH)D2 have demonstrated that it can also inhibit proliferation and induce the differentiation program of keratinocytes [59,71,73]. This is mediated at least in part through the activation of VDR, as demonstrated by the attenuation of cell proliferation after silencing of the VDR, by enhancement of the inhibitory effect through stable overexpression of VDR and from the 20(OH)D2-induced, time-dependent translocation of VDR from the cytoplasm to the nucleus with a potency comparable to that for 1,25$(OH)_2$D3 [59]. This translocation was measured with newly generated stable cell lines transduced with pLenti-CMV-VDR-EGFP-pgk-puro where VDR and EGFP are expressed as a fusion protein [59,73].

Some of the CYP11A1-derived secosteroids can undergo 1α-hydroxylation by CYP27B1 producing 1,20$(OH)_2$D3, 1,20$(OH)_2$D2 and 1,20,23$(OH)_3$D3, as illustrated in Fig. 1. These products show similar inhibition of keratinocyte proliferation and stimulation of differentiation and VDR expression to those of their precursors, but display greater stimulation of CYP24A1 expression [50,59,71,73,74].

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20(OH)D3 and 20,23$(OH)_2$D3 inhibit NF-κB activity in normal and immortalized keratinocytes and have anti-inflammatory properties [69–71]. Both inhibit the transcriptional activity of NF-κB as demonstrated by electrophoretic mobility shift assays, NF-κB-driven reporter gene activity assays and measurements of translocation of p65 from the cytoplasm to the nucleus (i.e., its inhibition). Thus, stimulation of the expression of IκBα sequesters NF-κB in the cytoplasm and attenuates its transcriptional activity. Gene silencing of the VDR abrogated the 20(OH)D3 effect on IκB [69,70]. Similar inhibition of NF-κB by 1,25$(OH)_2$D3 through increased expression of IκBα and sequestration of the NF-κB complex in the cytoplasm has been reported by other authors [75].

We have compared the effects of secosteroids with a short side chain to those of their 5,7-diene precursors, and found that while they inhibited proliferation of keratinocytes and activation of NF-κB, activity 5,7-dienes were more potent than their 9,10-secosteroidal derivatives [41,63,71]. For example, 7DHP inhibited the proliferation of HaCaT keratinocytes in a dose-dependent manner, with pregnacalciferol (pD) showing little or no antiproliferative activity [40,41]. Interestingly, other 5,7-dienes such as 3β-hydroxyandrosta-5,7-diene-17β-carboxylic acid [76], 20(OH)7DHC [63] and ergosterol hydroxy-derivatives [43] also inhibited keratinocyte proliferation.

5.2. Epidermal melanocytes and melanoma

20(OH)D3, 20(OH)D2, 20,23$(OH)_2$D3, 1,20$(OH)_2$D3 and 1,20,23$(OH)_3$D3 inhibited proliferation and the behavior of normal and malignant melanocytes in a similar manner to 1,25$(OH)_2$D3 [59,63,71,77,78], however, with a notable difference between epidermal melanocytes and melanoma cells [78]. In normal melanocytes addition of a 1α-hydroxyl group to 20(OH)D3 potentiated the inhibitory effect on proliferation and inhibited dendrite formation. Interestingly, none of the above secosteroids had any identifiable effect on melanogenesis.

The above compounds also showed potent antimelanoma activity, as evaluated by inhibition of proliferation, colony formation in monolayer (plating efficiency) and soft agar assays (anchorage independent growth), that was similar to or higher than that seen for 1,25$(OH)_2$D3 [59,63,71,77,78]. In human SKMEL-188 melanoma cells, inhibition of growth by 20(OH)D3 was accompanied by inhibition of NF-κB activity with a more pronounced effect on amelanotic than fully melanized cells [77]. Of note, expression of the VDR and CYP27B1 decreased during melanoma progression, and melanization and melanogenesis negatively affected both overall- and stage III disease-survival [79–81].

Initial tests performed on 20,24$(OH)_2$D3, 20,25$(OH)_2$D3 and 20,26$(OH)_2$D3, products of 20(OH)D3 hydroxylation by CYP24A1 [56] and/or CYP27A1 [55] (Fig. 1), revealed that they display stronger inhibition of colony formation by SKMEL-188 melanoma cells than the parent 20(OH)D3 [53,56]. This is consistent with the prediction of additional hydrogen bonds from the C24-, C25- and C26-hydroxyl groups of these secosteroids to residues in the VDR causing tighter binding (described in Section 7), as seen for the 25-hydroxyl group of 1,25$(OH)_2$D3 [82]. In the case of CYP24A1, the increased potency of the products indicates that the enzyme plays an activating role, rather than the inactivation seen with 1,25$(OH)_2$D3. Addition of a 1α-hydroxyl group to 20,25$(OH)_2$D3 by CYP27B1 further increased this anti-

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melanoma activity with the opposite effect seen for 1α-hydroxylation of 20,26$(OH)_2$D3 [53]. The role of CYP27A1 and CYP24A1 in the in vivo metabolism of 20(OH)D3 and other CYP11A1-derived secosteroids remains to be established, but the in vitro results challenge the dogma that CYP24A1 is always an inactivating enzyme.

Tests performed with 7-steroids and their hydroxy-derivatives, as well as with their pregna or androsta-calciferol (pD and aD), lumisterol-like (pL and aL) and tachysterol-like (pT and aT) photoderivatives, reveal that they have anti-melanoma activity including inhibition of growth in soft agar [63–65,71]. Interestingly, secosteroids with a short side chain showed lower potency for the inhibition of melanocyte proliferation than those with a full (8 carbon) side chain, but had similar inhibitory potency on melanoma cells [63]. The stimulation of melanogenesis appeared to increase the antiproliferative effect of some of the secosteroids with a short side chain [64,65], which is in contrast to 20(OH)D3 where the effect was opposite [77].

5.3. Other solid tumors and leukemia

The CYP11A1-derived secosteroids and some of their lumisterol and tachysterol derivatives showed potent anti-tumor activity against breast cancer, hepatoma and glioma in cell culture and by soft agar assays [71,83]. They also showed anti-leukemic activities, with secosteroids having a full-length side chain displaying significantly stronger prodifferentiation and antiproliferative effects than the pD and pL derivatives [84].

6. Anti-fibrotic and anti-inflammatory effects of CYP11A1-derived secosteroids

Secosteroids with a short side chain and their corresponding 5,7-diene precursors, had anti-fibrotic activities as defined by inhibition of TGF-β1 induced collagen protein production and hyaluronan synthesis, with a similar potency to 1,25$(OH)_2$D3. Inhibition was seen at concentration as low as 10^{-10} M [63,85], which is in contrast to effects on keratinocytes requiring higher concentrations [63]. Both 20(OH)D3 and 20,23$(OH)_2$D3 displayed similar anti-fibrotic activities on human dermal fibroblasts from scleroderma or normal donors [86]. These secosteroids, as well as 20,22$(OH)_2$D3, 17,20,23$(OH)_3$D3 and 20(OH)D2, inhibited proliferation of fibroblasts in culture. Importantly, in our first in vivo studies on the action of CYP11A1-derived secosteroids (other than examining calcemic activity, see below), 20(OH)D3 inhibited bleomycin-induced fibrosis in C57BL/6 mice in vivo at a dose of 3 μg/kg, [86], well within its non-calcemic range [83]. This demonstrates an in vivo anti-fibrosing potential for 20(OH)D3.

Initial evidence for the anti-inflammatory properties of the CYP11A1-derived secosteroids came from their inhibition of NF-κB (a master regulator of pro-inflammatory responses) activity in epidermal keratinocytes [69–71] (see Section 5.1). Studies carried out on normal human peripheral blood mononuclear cell (PBMC) cultures have also shown that 20(OH)D3 reduces production of the proinflammatory cytokine TNFα, induced by LPS (10 ng/ml), by 56% ($p < 0.01$) [63]. In new studies presented here we further show that 20(OH)D3 significantly inhibits (>3 times) LPS-induced TNFα production in the J774 line which

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expresses the VDR, but not in the RAW264.7 cell line where VDR expression is very low (Fig. 4). Furthermore, in peritoneal macrophages isolated from DBA1 mice with collagen-induced arthritis (CIA), 20(OH)D3 significantly inhibited LPS-induced production of proinflammatory cytokines TNFα and IL-6 (Fig. 4C). Lastly, the new data in Table 2 shows that 20(OH)D3 markedly suppresses arthritis in the DBA/1 Lac J CIA model of rheumatoid arthritis (RA) that is associated with down regulation of Th1 and Th17 cytokine production (i.e., inhibition of the proinflammatory phenotype). These data demonstrate that there is a potent suppression of the production of Th1 cytokine, IFN_{Y}, by either specific antigen or anti-CD3 stimulated lymphoid cells, and marked suppression of the Th17 cytokine, IL-17, by anti-CD3 stimulated lymphoid cells. There were different effects on the Th1 cytokine, IL-2, and Th-2 cytokines IL-4 and IL-10, depending on whether the cells were stimulated with specific antigen (i.e. CII 259–273 peptide; for details and definition see Table 2 footnote and [87]) or anti-CD3 antibody. Namely, when lymphoid cells were stimulated with antigen only, 1,25(OH)D3 and 20(OH)D3 suppressed IL-2 production, however, when lymphoid cells were stimulated with anti-CD3 antibody (Experiment 2), all analogs suppressed IL-2 production. The effects of the secosteroids on Th2 cytokines were different depending on whether lymphoid cells were stimulated with specific antigen or with anti-CD3 antibody. Antigen stimulation of lymphoid cells in the presence of 1,25(OH)D3 or 20(OH)D3 increased production of anti-inflammatory cytokines IL-4 and IL-10 (Experiment 1) while all analogs showed a trend to suppress IL-4 and IL-10 production by anti-CD3 stimulated lymphoid cells (Experiment 2), but not reaching statistical significance ($p >$ 0.05). The reasons for this dichotomy of responses to antigens versus T cell receptor activation by anti-CD3 antibody are not known, but similar differences in Th2 cytokine modulation has been previously shown with 1,25(OH)2D3 [88].

7. CYP11A1-derived secosteroids work via the VDR

20(OH)D3/D2 and 20,23$(OH)_2$D3 act as partial agonists on the VDR as demonstrated by VDR gene silencing and VDR gene overexpression experiments [see above and [59,68–70]]. Also, the phenotypic effects correlate well with ligand-induced translocation of the VDR to the nucleus [48,59,73,78]. Molecular modeling using the VDR crystal structure has predicted that 20S(OH)D3/D2 and 20,23$(OH)_2$D3 overlap well with the native ligand [1,25$(OH)_2$D3], occupying the same binding pocket and showing an excellent fit [59,73,84,86]. These secosteroids posed the side chains toward the surface of VDR and buried the secocholesta head deeply inside the pocket bottom [73].

We have repeated molecular modeling for 20(OH)D3, 20,23(OH)D3 and their 1α-hydroxy-derivatives in comparison to 1,25$(OH)_2$D3, and included the recently characterized secosteroids 20,24(OH)2D3, 20,25(OH)2D3, 20,26(OH)2D3 and their 1α-hydroxy-derivatives (Fig. 5). To calculate the glide score we used the extra precision (XP) glide module of the software [89] as opposed to the standard precision mode (SP) used in previous studies. These calculations, while confirming previous analyses, provided more precise numbers and importantly established a theoretical correlation with activity for the new CYP11A1-derived hydroxy-dervatives that were not analyzed previously (see Section 5). As shown in Fig. 5A, the native ligand of the VDR, 1,25$(OH)_2$D3, has a docking score of −16.99 (more negative scores predict more favorable binding to the VDR). It forms six

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hydrogen bonding interactions to Ser237, Arg274, Ser278, Phe143, His305 and His397. 20(OH)D3 only displays two hydrogen bonding interactions to the VDR which involve the C3 hydroxyl group, giving a docking score of −12.86 (Fig. 5B). No hydrogen bonding interactions were predicted to form from the 20-hydroxy group. The addition of 1α-hydroxy group is predicted to improve its binding to the VDR with a total of four hydrogen bonds (Fig. 5C, docking score 14.75). The addition of a hydroxyl group to 20(OH)D3 at C24, C25,– or C26 permits additional hydrogen bonds between the side chain and the VDR (Fig. 5D-F), with 26-OH forming only one hydrogen bond and having the weakest docking score. When a 1α-hydroxy group was added to these three secosteroids, the predicted binding affinity increased as expected due to the additional hydrogen bonding interactions to Ser237 and Arg274. The docking score for 1,20,24/25/26$(OH)_3$D3 were –17.91, –16.33, and –16.48, respectively.

Using pLenti-CMV-VDR-EGFP-pgk-puro constructs [59], we carried out testing of the ligand-induced translocation of the VDR from the cytoplasm to the nucleus [73]. Vitamin D3 hydroxy-derivatives with a full-length side chain and hydroxy-secosteroids with a shortened side chain (pD) stimulated VDR translocation and inhibited proliferation, however, with the former being more potent than pDs. Molecular modeling of the binding of these secosteroids to the VDR genomic binding pocket (G-pocket) correlated well with the experimental data for VDR translocation. In contrast, docking scores for binding to the non-genomic binding site [A-pocket] of the VDR were poor, suggesting that they do not act via the A-pocket [73].

8. Lack of toxicity of CYP11A1-derived secosteroids

20(OH)D3 at a doses as high as 3.0 μg/kg had no calcemic activity in rats whereas 1,25$(OH)_2$D3 at the same dose raised calcium to 16.0 ± 1.2 mg/dL [84]. Although addition of a hydroxyl group at C1α by CYP27B1 conferred some calcemic activity to 20(OH)D3 [84], the products of the CYP11A1-mediated pathway, including 20(OH)D3 and 20(OH)D2, are poor substrates for CYP27B1 [53,59]. We repeated the calcemic testing of 20(OH)D3, 20,23$(OH)_2$D3 and 20(OH)D2 in mice [59,83,86] and this also showed that these secosteroids lack calcemic effects. Even when we administered 20(OH)D3 at doses as high as 30 μg/kg to C57BL/6 mice daily for 14 days there were no significant differences in sera Ca^{++} levels compared to control mice. There was also a lack of toxicity as determined by serum chemistry and histological analyses of heart, liver, spleen and kidney [83]. Although pD derivatives with a short side chain have been reported to lack calcemic effects [90], we tested 17,20S(OH)2pD and 17,20R(OH)2pD in mice and have found that they are non-calcemic up to the highest dose tested of 3 μg/kg [85]. They also lacked effects on serum inorganic phosphate levels.

9. Conclusions

The unexpected discoveries of new secosteroidogenic pathways initiated by CYP11A1 and modified by the activities of CYP27B1, CYP27A1, CYP24A1, or modified by UVB radiation in the case of hydroxyl-7DHC derivatives, 7DHP and its metabolites, represent a fundamental and ground breaking advance in vitamin D-related studies. These discoveries

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open up new paradigms for medicine and biology potentially of extreme importance with many biological applications. This potential is supported by the diverse phenotypic activities of the products or intermediates of these pathways, their very low toxicity, and the detection of endogenous production of some of these secosteroids including 20(OH)D3. In the skin, these novel secosteroids could serve both as endogenous regulators of skin functions and as excellent candidates for treatment of hyper-proliferative, fibrosing and inflammatory skin disorders including psoriasis and skin cancer. Some of the CYP11A1-derived secosteroids may serve as endogenous ligands for the VDR providing a potential explanation for why $VDR^{-/-}$ mice develop alopecia while $CYP27B1^{-/-}$ do not, i.e., an endogenous ligand other than 1,25(OH)2D3 that does not require 1α-hydroxylation may activate the VDR locally. Furthermore, the CYP11A1-derived secosteroids can serve as excellent candidates for treatment of systemic automimmune diseases such as rheumatoid arthritis (RA), lupus erythomatousus (LE), inflammatory bowel diseases (IBD), sclerosis multiplex (SM) and other autoimmune disorders affecting large segments of the population. Finally, they are excellent candidates for further preclinical testing as therapeutics or adjuvants for solid tumors or tumors of hematopoietic origin, or deadly melanoma. In the case of melanoma, there is already sufficient experimental data to justify further preclinical testing of these novel secosteroids [53,56,59,63–65,71,73,77,78,80,81,91,92].

In summary, CYP11A1 initiates new pathways of vitamin D metabolism in a range of tissues and products could have important physiological roles at local and systemic levels. The metabolites display high biological potencies with specific effects dependent on cell lineage, and show a lack of calcemic or other toxic effects at pharmacological doses far above the toxicity levels of 25(OH)D3 or 1,25$(OH)_2$D3.

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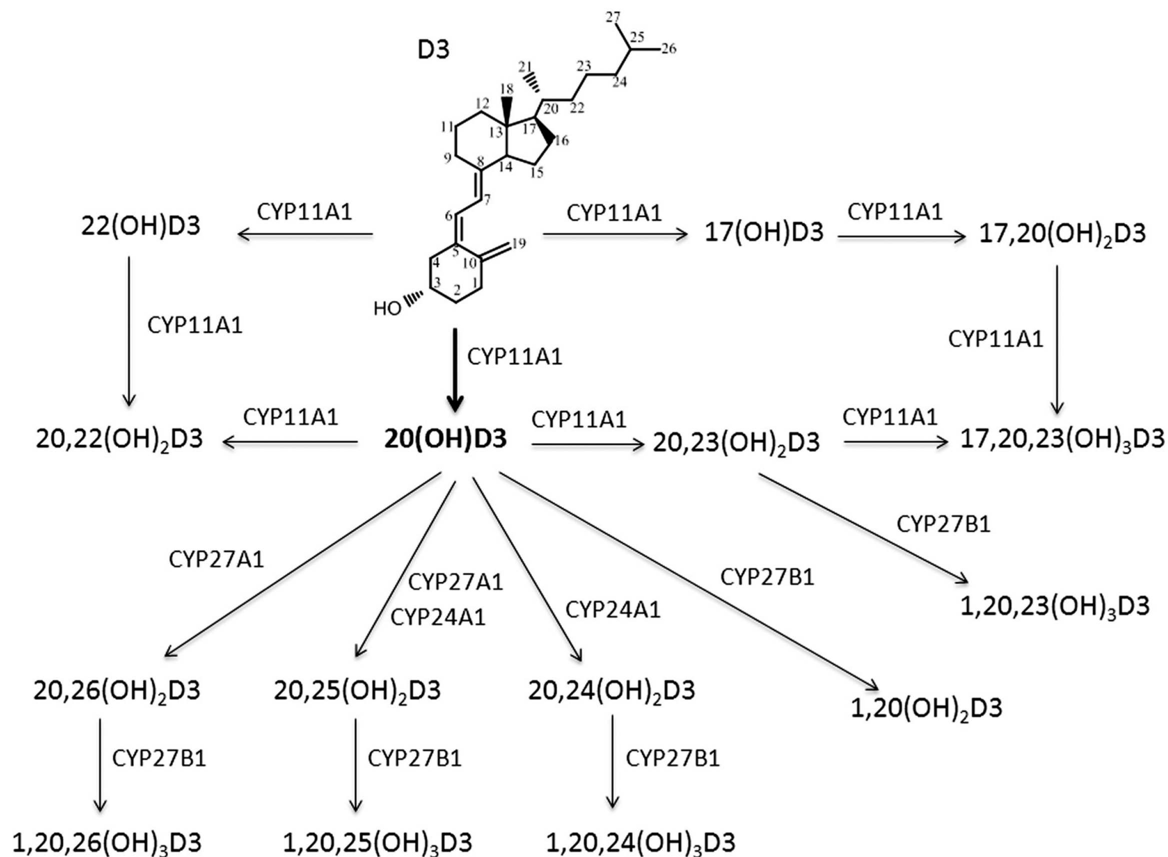

Fig. 1.

Summary of the major pathways for metabolism of vitamin D3 by CYP11A1 and the role of CYP27A1, CYP27B1 and CYP24A1 in the further hydroxylation of products. See the text for published references to the reactions listed.

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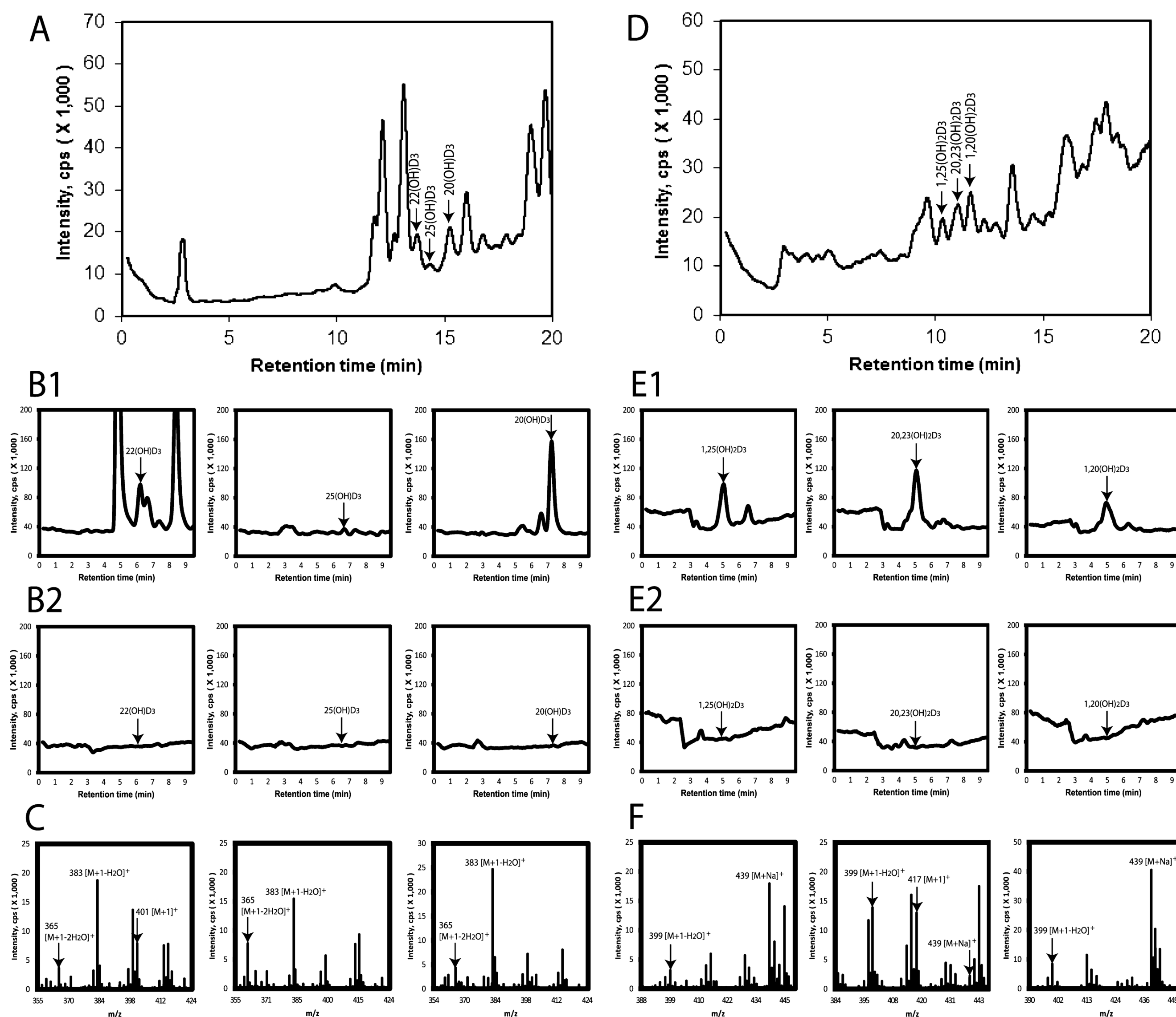

Fig. 2.

Production of vitamin D3 hydroxy-derivatives by human Caco-2 colon cells. Cells were incubated with 50 μM vitamin D3 for 16 h as described before [52]. After extraction of cell suspensions with dichloromethane the products were analyzed by LC–MS using single ion monitoring (SIM) mode [52] for mono-hydroxyvitamin D3 (**A**) and di-hydroxyvitamin D3 (D) (top panels). Peaks with retention times corresponding to authentic standards were collected and subjected to further analysis by LC–MS for mono-hydroxyvitamin D3 (B1) and di-hydroxyvitamin D3 (**E1**) (upper panels). Middle panels of **B2** and E2 show negative control (no substrate added), while bottom panels show mass spectra for each tested fraction of hydroxyvitamin D3. Arrows identify retention times corresponding to 20(OH)D3, 22(OH)D3, 25(OH)D3, 1,20(OH)$_2$D3, 20,23(OH)$_2$D3 and 1,25(OH)$_2$D3 standards. For A and D, LC was run with a gradient of methanol in water (85–100%) for 20 min and 100% methanol for 10 min at a flow rate of 0.5 ml/min. MS was performed using an ESI source and SIM at m/z = 383.3 [M+1-H_2O] for mono-hydroxyvitamin D3 (A, B1 and B2) and

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399.3 [M + 1-H_2O] for dihydroxyvitamin D3 (D, E1 and E2). The fractions with retention times corresponding to 20(OH)D3, 22(OH)D3, 25(OH)D3, 1,20$(OH)_2$D3, 20,23$(OH)_2$D3 and 1,25$(OH)_2$D3 standards were collected and analyzed separately (B1, B2, E1 and E2) by LC–MS isocratically using 96% methanol in water at a flow rate of 0.05 ml/min for 10 min, on a Zorbax Eclipse Plus C18 column.

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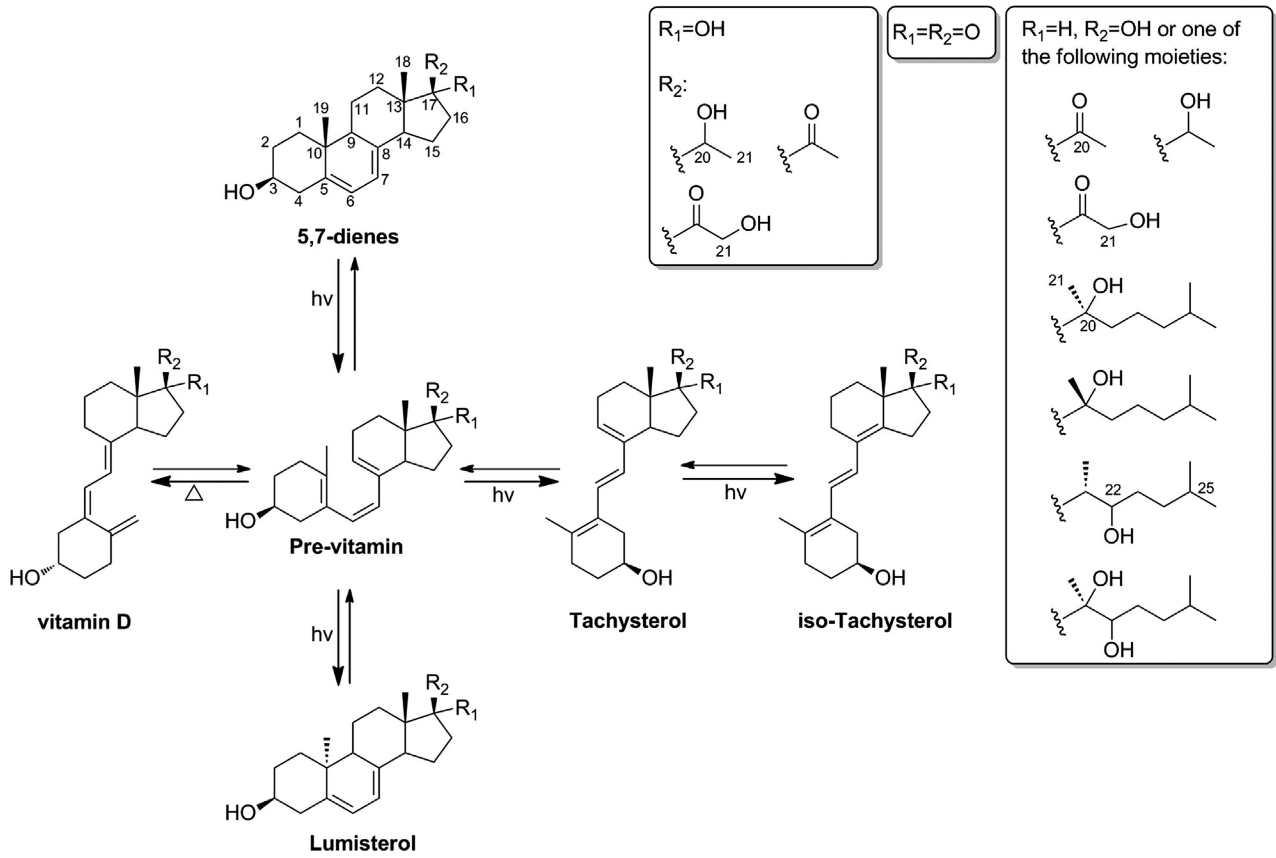

Fig. 3.

UVB-induced transformation of steroidal 5,7-dienes with a short or full-length side chain to novel secosteroids.

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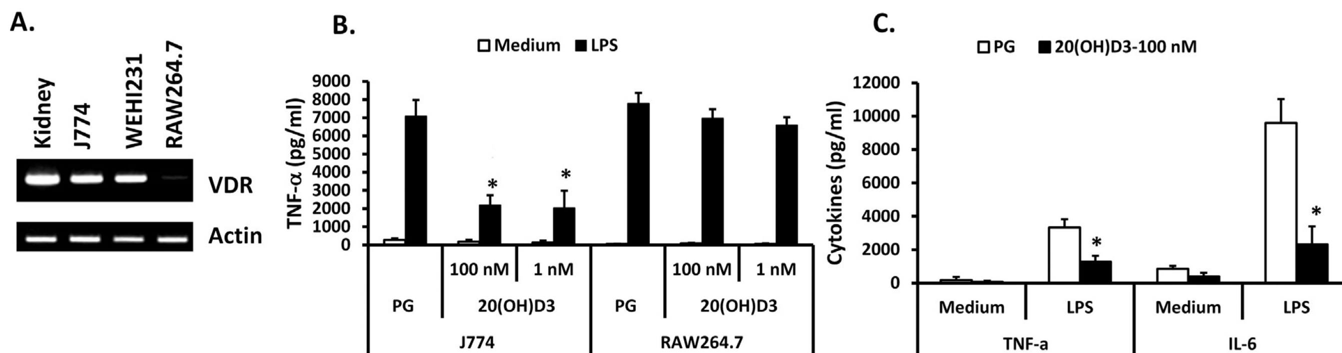

Fig. 4.

20(OH)D3 suppress TLR4 ligand (LPS)-induced proinflammatory cytokine production in mouse macrophages in a VDR-dependent manner. (A) RT-PCR demonstrates that kidney, J774 cells and WEHI-231 cells express VDR. In contrast VDR mRNA in RAW264.7 cells was only just detectable. The sequences of RT-PCR primers for murine VDR and PCR conditions have been previously described [93]. (B) J774 cells (1×10^6 cell/ml) and RAW264.7 cells (1×10^6 cell/ml) were pre-treated with vehicle (propylene glycol; PG) or the indicated dose of 20(OH)D3. Forty-eight h later, these cells were stimulated with LPS (25 ng/ml) for 24 h and then TNFα in the culture supernatants was measured by ELISA as described previously [94]. Data represent mean (pg/ml) ± S.D. of triplicates. LPS-induced TNFα production was significantly suppressed in J774 cells, but not in RAW264.7 cells (**$p < 0.005$, *$p < 0.05$). C. Peritoneal macrophages were isolated from CIA mice (CII-immunized DBA1 mice that fully developed arthritis) as previously described [95]. Peritoneal macrophages (1×10^6 cell/ml) were pre-treated with vehicle (PG) or the indicated dose of 20(OH)D3. Forty-eight h later, cells were stimulated with LPS (5 ng/ml) for 24 h and then levels of TNFα and IL-6 in the culture supernatants were measured by ELISA. Data represent mean (pg/ml) ± S.D. of triplicates. LPS-induced production of TNFα and IL-6 was significantly suppressed by 20(OH)D3 compared to that in vehicle-treated cells (*$p < 0.05$).

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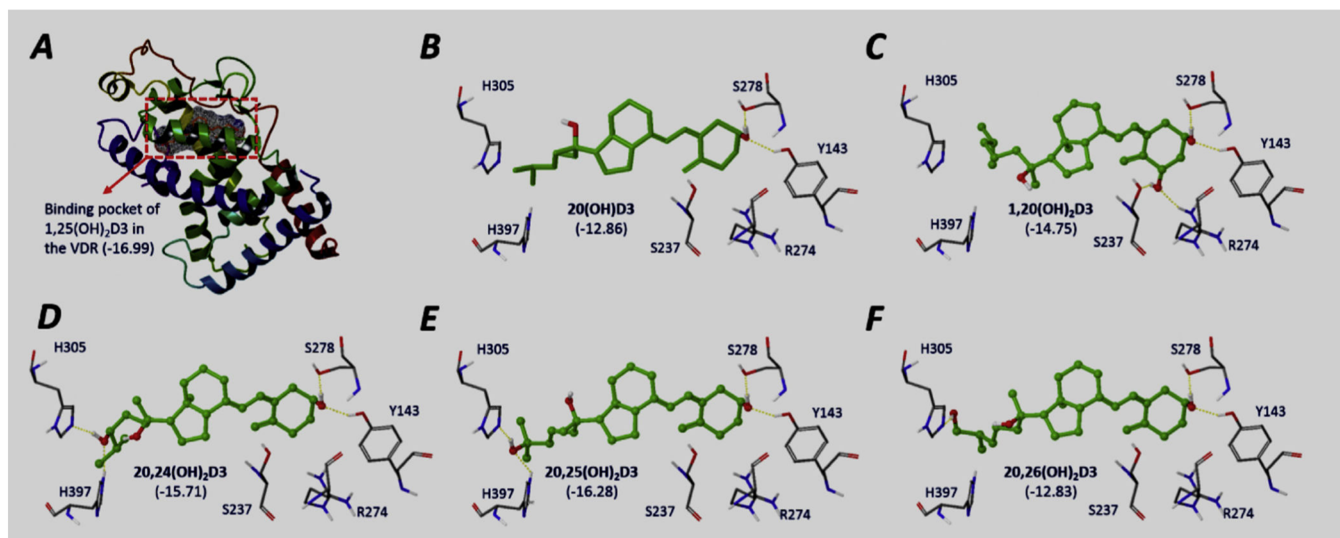

Fig. 5.

Potential binding poses of novel secosteroids, docking scores, and hydrogen bonding interactions with the VDR based on molecular modeling. (A) Crystal structure of VDR and its native ligand, 1,25$(OH)_2$D3. The dotted surface within the rectangle box shows the ligand surface. Docking score (–16.99) is shown in the parenthesis. (B)–(F), predicted binding poses for each of the novel secosteroid in VDR and their hydrogen bonding interactions (dotted line between the atoms in the ligand and the VDR) to the six residues that can form hydrogen bonds to 1,25$(OH)_2$D3. Molecule modeling studies were performed using the Extra Precision (XP) Glide function within the Schrodinger's Small Molecule Drug Discovery Suite 2012 (Schrodinger Inc., New York, NY, USA). Due to the large number of rotatable bonds in these secosteroids, it is challenging for the software to predict the most favorable binding poses in one run since XP requires a good starting conformation. Thus, for each novel secosteroid, we performed docking calculations iteratively, using the best pose generated from the previous run as the new starting pose for the next run until there was no improvement in the docking score. The optimized docking scores obtained from this process are shown in parenthesis for each panel, along with the predicted hydrogen bonding interactions between the secosteroid and the VDR.

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Table 1

In vitro phenotypic effects of CYP11A1-derived hydroxyderivatives of vitamin D on skin cells.

Vitamin D hydroxyderivatives	Keratinocytes	Melanocytes	Fibroblasts	Melanoma cells
20(OH)D3	↓Proliferation, colony formation in monolayer↑Differentiation, ↓NFκB≡	↓Proliferation	↓Proliferation, ↓Fibrosing effect*	↓Proliferation, ↓Colony formation in monolayer and soft agar
20,23(OH)D3	↓Proliferation, colony formation in monolayer↑Differentiation, ↓NFκB≡	↓Proliferation	↓Proliferation, ↓Fibrosing effect*	↓Proliferation, ↓Colony formation in monolayer and soft agar
22(OH)D3 and 20,22$(OH)_2$D3	↓Proliferation, ↑Differentiation,	ND	↓Proliferation	ND
17,20,23$(OH)_3$D3	↓Proliferation	ND	↓Proliferation	↓Proliferation
20,24$(OH)_2$D3, 20,25$(OH)_2$D3 and 20,26$(OH)_2$D3	ND	ND	ND	↓Colony formation in soft agar
1,20$(OH)_2$D3	↓Proliferation, ↑Differentiation,	↓Proliferation, ↓Dendrite formation	↓Proliferation	↓Proliferation, ↓Colony formation in monolayer and soft agar
1,20,25$(OH)_3$D3 and 1,20,26$(OH)_3$D3	ND	ND	ND	↓Colony formation in soft agar
20(OH)D2	↓Proliferation, colony formation in monolayer↑Differentiation, ↓NFκB≡	↓Proliferation	↓Proliferation	↓Proliferation, ↓Colony formation in monolayer
1,20$(OH)_2$D2	↓Proliferation, ↑Differentiation	ND	ND	↓Proliferation
17,20$(OH)_2$D2	↓Proliferation, ↑Differentiation	ND	ND	ND

↓ – inhibition, ↑ – stimulation, * – defined as an inhibition of TGF-β induced hyaluronate and collagen production, colony formation in monolayer indicates plating efficiency, while colony formation in soft agar indicates tumorogenic potential. ND, not determined. See description in text for references.

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Table 2

Natural noncalcemic vitamin D3 analogs modulate Th1, Th2 and Th17 cytokine production in vitro*

Experiment 1

Additions	**IFNγ (*p*-value)**	**IL-2 (*p*-value)**	**IL-4 (*p*-value)**	**IL-10 (*p*-value)**
PG + PBS	0.62 ± 0.3	2.3 ± 0.1	0.12 ± 0.01	2.5 ± 0.1
PG + $CII_{259-273}$	**2557 ± 156 (<0.001)**	**557 ± 28 (<0.001)**	**84 ± 5 (<0.001)**	**320 ± 34 (<0.001)**
1,25(OH)2D3 + $CII_{259-273}$	**1278 ± 154 (0.004)**	**176 ± 10 (<0.001)**	**162 ± (0.005)**	**695 ± 43 (0.003)**
20(OH)D3 + $CII_{259-273}$	**1323 ± 119 (0.003)**	**436 ± 6 (0.014)**	**144 ± 6 (0.001)**	**687 ± −47 (0.003)**
20,23(OH)2D3 + $CII_{259-273}$	**1454 ± 55 (0.003)**	455 ± 48 (0.06)	105 ± 6 (0.06)	283 ± 31 (NS)

Experiment 2

Additions	**IFNγ (*p*-value)**	**IL-2 (*p*-value)**	**IL-4 (*p*-value)**	**IL-10 (*p*-value)**	**IL-17 (*p*-value)**
PG + PBS	0.1 ± 0.001	0.5 ± 0.01	0.9 ± 0.005	14 ± 0.6	0.14 ± 0.03
PG + anti-CD3	**340 ± 20 (0.003)**	**101 ± 4 (0.002)**	**35 ± 8 (0.049)**	**74 ± 13 (0.044)**	**104 ± 01 (<0.001)**
1,25(OH)D3 + anti-CD3	**25 ± 6 (0.004)**	**62 ± 4 (0.008)**	15 ± 3 (NS)	34 ± 5 (NS)	**19 ± 2 (<0.001)**
20(OH)D3 + anti-CD3	**68 ± 8 (0.006)**	**5 ± 5 (0.028)**	26 ± 3 (NS)	46 ± 6 (NS)	**44 ± 5 (0.008)**
20,23(OH)2D3 + anti-CD3	**63 ± 4 (0.005)**	**62 ± 4 (0.018)**	27 ± 1 (NS)	56 ± 4 (NS)	**68 ± (0.007)**

*Vitamin D3 analogs were solubilized is sterile propylene glycol (PG) at 10^{-4} M then diluted to 10^{-7} M stocks in RPMI 1640 medium containing 9% charcoal-stripped FCS. PG vehicle control was similarly diluted 1:1000. Spleen cells from 8 weeks old DBA/1 Lac J CII24TCRTg mice (Experiment 1) or DBA/1 Lac J mice (Experiment 2) were added at 2×10^{6} cells/ml to 24 well COSTAR plates. The PG and Vitamin D 3 analogs at 10^{-7} M stock were added at a 1:10 dilution (10^{-8} M final concentrations) in duplicate wells 2 h prior to addition of PBS or PBS containing 25 μg/ml $CII_{259-273}$ peptide (Experiment I) or rat anti-mouse anti-CD3 monoclonal antibody (0.2 μg/ml final concentration (Experiment 2)). After 72 h of culture, supernatants were harvested and analyzed on a Luminex Instrument using Custom Mouse Kit and values in pg/ml and were analyzed for significant difference by ANOVA. In Experiment 1, values for each vitamin D analog were compared to values for PG + $CII_{259-273}$ while in Experiment 2, values for each vitamin D analog were compared to PG + anti-CD3 values. Significantly different values are shown in bold. $CII_{259-273}$ is the synthetic peptide with the sequence of the arthritogenic $Cll_{259-273}$ epitope of type ll collagen [87].