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Photoprotective properties of vitamin D and lumisterol hydroxyderivatives

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Abstract

We have previously described new pathways of vitamin D3 activation by CYP11A1 to produce a variety of metabolites including 20(OH)D3 and 20,23$(OH)_2$D3. These can be further hydroxylated by CYP27B1 to produce their C1α-hydroxyderivatives. CYP11A1 similarly initiates the metabolism of lumisterol (L3) through sequential hydroxylation of the side chain to produce 20(OH)L3, 22(OH)L3, 20,22$(OH)_2$L3 and 24(OH)L3. CYP11A1 also acts on 7-dehydrocholesterol (7DHC) producing 22(OH)7DHC, 20,22$(OH)_2$7DHC and 7-dehydropregnenolone (7DHP) which can be converted to the D3 and L3 configurations following exposure to UVB. These CYP11A1-derived compounds are produced *in vivo* and are biologically

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active displaying anti-proliferative, anti-inflammatory, anti-cancer and pro-differentiation properties. Since the protective role of the classical form of vitamin D3 ($1,25(OH)_2D3$) against UVB induced damage is recognized, we recently tested whether novel CYP11A1-derived D3- and L3-hydroxyderivatives protect against UVB-induced damage in epidermal human keratinocytes and melanocytes. We found that along with $1,25(OH)_2D3$, CYP11A1-derived D3-hydroxyderivatives and L3 and its hydroxyderivatives exert photoprotective effects. These included induction of intracellular free radical scavenging and attenuation and repair of DNA damage. The protection of human keratinocytes against DNA damage included the activation of the NRF2-regulated antioxidant response, p53-phosphorylation and its translocation to the nucleus, and DNA repair induction. These data indicate that novel derivatives of vitamin D3 and lumisterol are promising photoprotective agents. However, detailed mechanisms of action, and the involvement of specific nuclear receptors, other vitamin D binding proteins or mitochondria, remain to be established.

Keywords

Ultraviolet B; vitamin D; lumisterol; skin; oxidative stress; DNA damage; NRF2; NFκB; VDR; RORs; AhR; mitochondrion

Introduction to vitamin D biochemistry

7-Dehydrocholestrol (7DHC), the immediate precursor to cholesterol in the Kandutsch-Russel pathway, can absorb ultraviolet B radiation (UVB) producing pre-vitamin D3 which isomerizes to vitamin D3 (D3) or, after prolonged exposure to UVB, to lumisterol 3 (L3) and tachysterol 3 (T3)[1–3](Fig. 1). In the canonical pathway, D3 is activated by a 25-hydroxylase (CYP2R1 or CYP27A1) and 1a-hydroxylase (CYP27B1) to produce $1,25(OH)_2D3$. Both 25(OH)D3 and $1,25(OH)_2D3$ are degraded by sequential oxidations of the side chain catalyzed by CYP24A1, in the latter case producing calcitroic acid [4,5]. A similar sequence of reactions is involved in the activation of vitamin D2 (D2), a product of UVB induced transformation of fungi- or phytoplankton-derived ergosterol [2,3,5] A novel, non-canonical pathway of D3 activation is initiated by CYP11A1 [5,6] with initial hydroxylation at C20 [7,8] or C22 [9] and subsequent hydroxylations at C20, C22, C23 and/or C17 [7,8,10]. The resulting products can be selectively hydroxylated by CYP27A1, CYP24A1, CYP2R1 and/or CYP3A4, with additional hydroxylation at C1a occurring for all intermediates except those possessing a hydroxyl group at C17 [5,11–16](Fig. 1). Many of the above intermediates/products can be synthesized ex vivo in skin cells, placenta and adrenal glands and some are detectable in human serum and/or epidermis and in the pig adrenal gland [17–20]. Vitamin D2 is also hydroxylated by CYP11A1 producing 20(OH)D2, $17,20(OH)_2D2$ and $17,20,24(OH)_3D2$ [21,22], with 20(OH)D3 being hydroxylated by CYP27B1 to $1,20(OH)_2D2$ [23]. These D2 metabolites are also produced by human placenta, skin cells and a pig adrenal glands [24].

7DHC and L3 are also substrates for CYP11A1. 7DHC is converted to 22(OH)7DHC, $20,22(OH)_2$7DHC and 7-deydropregnenolone (7DHP) [7,25–27], while L3 produces 20(OH)L3, 22(OH)L3, $20,22(OH)_2$L3, 24(OH)L3 and pregnalumisterol (pL) [28,29]. 7DHP

and pL can be further metabolized to hydroxy-7DHP or 7-dehydroprogesterone, and hydroxy-pL compounds, respectively, by steroidogenic enzymes[26,27,29]. These pathways have been observed to operate in cultured skin cells, placenta and adrenal glands incubated ex-vivo, and the products have been identified in the human epidermis, serum, placenta and in pig adrenal glands [26,27,29]. Most recently, we have also shown that CYP27A1 can efficiently hydroxylate L3 to three major products, 25(OH)L3, (25R)27(OH)L3 and (25S)27(OH)L3 [30]. Finally, in the skin the 5,7-dienal compounds with a full-length or shortened side chain can undergo UVB-induced photoisomerization to give the corresponding D3, L3 or T3 products. The short side chain products include pregnacalciferol (pD), pL and pregnatachysterol (pT) [25,31–34].

Mechanism of action of vitamin D derivatives

Receptor mediated mechanisms

The major hormonally active form of vitamin D3, 1,25$(OH)_2$D3, not only regulates calcium homeostasis and the musculoskeletal system, but also exerts a vast spectrum of activities including stimulation of differentiation and inhibition of proliferation of cells of different lineage, anti-cancerogenic effects and stimulation of innate and inhibition of adaptive immunity and inflammation. It also regulates endocrine and central nervous systems and plays an important role in development [2,35–38]. In the skin it is involved in the formation of the epidermal barrier, regulates the functions of adnexal structures including hair follicles, and has a wide variety of ameliorating effects on skin cancer and proliferative and inflammatory cutaneous diseases [2,37,39–42]. The traditional view is that most, if not all of these pleiotropic effects are mediated through the interaction with the nuclear vitamin D receptor (VDR). After binding to 1,25$(OH)_2$D3 the receptor heterodimerizes with RXR and is translocated to the nucleus where it binds to the VDR responsive element (VDRE) in target genes to influence gene expression [36,39,43]. In addition, it has been proposed that 1,25$(OH)_2$D3 actions involve non-genomic activities associated with its binding to 1,25D3-MARRS (ERp57 or PDIA3), the endoplasmic membrane-associated protein that acts as thiol-disulfide oxidoreductase and also participates in the folding and quality control of newly synthesized glycoproteins [44–49].

Novel CYP11A1-derived D3-hydroxyderivatives also exert anti-proliferative, pro-differentiation, and anti-inflammatory effects *in vitro* that are comparable or better than those of 1,25$(OH)_2$D3 [9,50–60]. They also show antifbrotic activities both *in vitro* [54–56] and *in vivo* [56]. In addition, they demonstrate anti-melanoma and anti-tumor properties that are cell-type lineage dependent [9,55,57,61–66]. Importantly, CYP11A1-derived hydroxymetabolites of D3 that have not been acted on by CYP27B1 and therefore lack a C1α hydroxyl group, are non-calcemic [56,61,63,67]. Thus, hydroxylation at C1α determines the mode of signal transduction, e.g., CYP11A1-derived ligands without a C1α hydroxyl group act as biased agonists on the VDR, producing some but not all responses, while the D3-derivatives with a C1α (OH) act as full agonists on this receptor [58,68–70]. Importantly, alternative nuclear receptors for CYP11A1-derived D3-hydroxyderivatives (and also likely for classical 1,25$(OH)_2$D3 to some extent) have been discovered. These include the retinoid-related orphan receptors (ROR)α and γ and the arylhydrocarbon receptor (AhR) on which they act

as inverse agonists and agonists, respectively [60,68,71]. The interaction of different D3-derivatives with genomic and non-genomic binding sites of the VDR, ROR α and γ and AhR, and possibly 1,25D3-MARRS, is dependent on the structural localization of hydroxyl groups which defines their specificity and affinity (Fig. 2).

It has recently been demonstrated that lumisterol hydroxyderivatives are produced and exert biological activity in epidermal and dermal skin cells [29]. Their mechanism of action appears to include functioning as inverse agonists of RORs and acting on the non-genomic pocket of the VDR [29]. Based on their structural similarities they are also likely to interact with other nuclear receptors that interact with sterols.

Recently, Holick and coworkers, 2019 presented the microarray data from "buffy coat" of volunteers supplemented with 600, 4000 or 10,000 IU of vitamin D for 6 months [72]. This treatment resulted in modulation of expression of 162, 320 and 1289 genes, respectively. It this paper the authors postulated that supplementation with a high dose of vitamin D (10,000 IU) upregulates pathways involved in histone and chromatin modifications and functions as a key factor of transcriptional regulation at the epigenomic level. This finding partially explains the pleiotropic effects of vitamin D and is consistent with our hypothesis that these effects are secondary to production of multiple D3-hydroxyderivatives that act on the VDR and alternative nuclear receptors [6,34,71].

Mitochondria as a switchboard for vitamin D and lumisterol actions

Crucial enzymes involved in the activation (CYP11A1, CYP27A1, CYP27B1) and inactivation (CYP24A1) of vitamin D are located within the mitochondrion and require NADPH as an energy source. CYP27A1 and CYP11A1 also hydroxylates L3 and in studies currently underway also appear to hydroxylate T3 (Figs. 1, 2). Therefore, reactions catalyzed by them may affect mitochondrial functions and their hydroxy-D3, L3 and T3-derivatives could act in the mitochondrial environment as regulators (Fig. 2). In support of this hypothesis there are reports showing the presence of the VDR in mitochondria [73] as well as on the inhibitory effect of $1,25(OH)_2D3$ on energy yielding metabolism and electron transport [74–77] and the role of the VDR in this process [78]. We have data to support these findings, identifying co-localization of the VDR, StAR and CYP11A1 within mitochondria of keratinocytes (Fig. 3) and showing inhibition of mitochondrial respiration and ATP production by $1,25(OH)_2D3$, and to a lesser degree by 20(OH)D3 (Figs 4, 5).

We have previously reported Ingenuity's toxicity pathway analyses of microarray data deposited by us at NCBI GEO (GSE117351[71]) Additional bioinformatics analysis (ingenuity canonical pathway analysis (IPA) with cut-off 2.0 and gene set enrichment analysis (GSEA)) of this microarray data shows that $20,23(OH)_2D3$ downregulates the citric acid (TCA) cycle and respiratory electron transport, glucose metabolism, fatty acid metabolism, ATP synthesis, glycolysis, mitochondrial fatty acid beta-oxidation, fatty acyl-CoA biosynthesis, pyruvate metabolism, mitochondrial calcium ion transport, and mitochondrial biogenesis with increased mitochondrial protein import (Table 1). Similar effects on glucose and fatty acid metabolism were seen for $1,25(OH)_2D3$ with some differences [71](Table 1). The GSEA analysis shows downregulation of fatty acyl-CoA biosynthesis, glycolysis and glucose metabolism. In contrast to $20,23(OH)_2D3$, $1,25(OH)_2D3$ upregulated several pathways,

including mitochondrial fatty acid beta-oxidation and pyruvate metabolism. This suggests a protective role for $1,25(OH)_2D3$ against diseases associated with lipid storage. Similar to $20,23(OH)_2D3$, $1,25(OH)_2D3$ decreased the activity of respiratory electron transport and mitochondrial calcium ion transport pathways. These findings indicate genetic alterations that have long-term impact on mitochondrial bioenergetics.

Interestingly, detailed analyses of upregulated genes from the microarray data published by Holick and coworkers, 2019 (supplementary data Table 2[72]), showed vitamin D3-mediated upregulation of the expression of several genes involved in mitochondrial transcription and translation. These included genes encoding transcription factor B1 *(TFB1M),* mitochondrial RNA processing *(RMRP),* mitochondrial ribosomal proteins *(MRPS15, MRPL55, MRPL22, MRPL51),* and mitochondrial import and protein folding proteins *(TIMM17A, TOMM20, TIMM23, GRPEL1).* In addition, vitamin D3 increased the expression of genes coding for several enzymes involved in fatty acid metabolism including peroxisomal acyl-coenzyme A oxidase 1 *(ACOX1),* mitochondrial 2,4-dienoyl-CoA reductase 1 *(DECR1),* mitochondrial uncoupling protein 2, the mitochondrial complex III component ubiquinol-cytochrome c reductase core protein II *(UQCRC2),* and cytochrome c oxidase assembly factor 3 (*COA3*). Although the microarray data requires further confirmation by other methods, it can be postulated that vitamin D supplementation affects mitochondrial fonctions which is already validated by the analysis performed above.

Based on the results above, we conclude that mitochondria are involved in the regulation of the cellular phenotype in response to the actions of vitamin D3 and L3 hydroxyderivatives, secondary to their involvement in the synthesis and metabolism of these compounds. We suggest that the intramitochondrial actions of D3-, L3- and possibly T3-hydroxyderivatives may be through receptor independent mechanisms or involve nuclear receptor(s) in a non-genomic manner, localized to mitochondria (Fig. 2). Notably, they also can alter mitochondrilal function related to oxphos and TCA cycle activities with expected inhibitory effects.

Photoprotective properties of canonical vitamin D3 derivatives

UVR is a key physical agent inducing photocarcinogenesis and skin damage through mechanisms that depend on its wavelength, for example, chromophore-based absorption of UVB energy and/or generation of reactive oxygen and nitrosyl species (ROS and RNS) by UVA [59,79–86]. UVB directly induces DNA damage predominantly via the formation of mutagenic and cytotoxic cyclobutane-pyrimidine dimers (CPDs) and 6–4 pyrimidine photoproducts (6–4PP), with some contribution from the generation of ROS [79–81,85,87–89] CPDs and 6–4PP play a crucial role in cutaneous carcinogenesis and contribute to mutations in the *p53* gene, an important tumor suppressor [90–92]. The nucleotide excision repair (NER) system, nuclear factor E2-related factor 2 (NRF2) with its downstream antioxidant elements, and p53 signaling are crucial mechanisms protecting against mutations and photocarcinogenesis [91,93–98].

The photoprotective functions of vitamin D3 and its active forms are well documented [99–110]. In addition, high doses of vitamin D3 given orally can reverse UVB-induced skin

damage with attenuation of inflammation and induction of barrier repair mechanisms [110]. In this context, the work from Dr Mason's group was fundamental in establishing the protective functions of natural and synthetic hydroxyderivatives of vitamin D3, including $1,25(OH)_2D3$, against UVR [49,77,99,104,111]. In addition to $1,25(OH)_2D3$, a synthetic L3 hydroxyderivative, $1,25(OH)_2L3$, protected against UVR-induced DNA damage and reduced proinflammatory responses in human and mouse skin and human keratinocytes. These effects were connected with increased expression of p53 and accompanied by attenuation of UVB-induced immunosuppression [104,112]. Because $1,25(OH)_2L3$ binds to the A-pocket of the VDR, a nongenomic mechanism of action was suggested [112]. This is consistent with a mechanism proposed by others [113–115], and the interaction with the A-pocket by 25(OH)D3, $1,25(OH)_2D3$, $1,25(OH)_2L3$ [112,114] and some CYP11A1-derived hydroxylumisterol derivatives [29], but not CYP11A1-derived vitamin D3 hydroxyderivatives [68]. In addition, the same group proposed that other receptor-dependent pathways can counteract UVB damage [49,105,106,116]. Interestingly, they proposed that UVB effects on epidermal keratinocytes are directly linked to $1,25(OH)_2D3$ action on mitochondria to enhance glycolysis and energy-conserving processes such as autophagy and mitophagy, which would help in the repair of CPDs and would decrease oxidative DNA damage [77].

Through studies of cutaneous carcinogenesis in $VDR^{-/-}$ and $RXR^{-/-}$ mice, several groups indicated the importance of the VDR and its partner, RXR, in the mechanism of attenuation of photocarcinogenesis [39,42,112,117–122]. This led to the proposal that that the VDR acts as a tumor suppressor in epidermal phocarcinogenesis[123124,125]. This concept also appears to be valid for melanoma, since a decrease or loss of the VDR is associated with a negative outcome for the disease as measured by tumor stage and overall and disease-free survival [126]. However, $1,25(OH)_2D3$ can inhibit BCC in an animal model acting through both the VDR and by inhibition of Hedgehog (Hh)-signaling which is independent on the VDR [127]. Moreover, vitamin D3 by itself inhibited the growth of BCC *in vitro* and *in vivo* through inhibition of Hh-signaling acting independently of the VDR [128]. Recent studies on UVB-induced BCC (basal cell carcinoma) carcinogenesis in mice show that inhibition of UVB-induced transformation of 7DHC to D3 in the skin accelerates BCC carcinogenesis. Topical application of vitamin D3 inhibited BCC photocancerogenesis, with orally delivered vitamin D3 having no effect [109]. Thus, it is possible that the sterol binding site in Smoothened [129,130] can be a target for non-canonical D3-derivatives and L3-derivatives (Fig. 1) in cancer therapy [131].

Thus, vitamin D3 as the prohormone, its active form $1,25(OH)_2D3$ and low calcemic-chemically synthesized derivatives of D3 and $1,25(OH)_2L3$, promise to serve as excellent protectors of the skin against the damaging effect of UVR and cutaneous cancerogenesis.

Photoprotective properties of novel (non-cannonical) vitamin D3 derivatives and L3 photoproducts

Previously, we showed that 20(OH)D3, $20,23(OH)_2D3$ and $1,25(OH)_2D3$, and to some extent 20(OH)7DHC and 25(OH)D3, can protect cultured human keratinocytes and melanocytes against UVB damage [18]. The effects included attenuation of ROS, H_2O_2 and

NO production and upregulation of the expression of genes encoding enzymes responsible for defense against oxidative stress [18]. 20(OH)D3, 20,23$(OH)_2$D3 and 1,25$(OH)_2$D3 showed strong protection against UVR-induced DNA damage with activation of p53 by 20(OH)D3 and 1,25$(OH)_2$D3 [18]. Interestingly, these effects were seen at a concentration of 10^{-7} M which is 100–1,000 fold lower that of melatonin and its metabolites that produce similar effects [59,83,132]. Melatonin and metabolites are recognized photoprotectors [133–135]. Importantly, topical application of 20(OH)D3, the first major metabolite ofCYP11A1 action on vitamin D3, protected mouse skin against UVB-induced DNA damage, reduced sunburn edema and protected against UVR-induced immunosuppression to a similar degree to 1,25$(OH)_2$D3 [136]. We are currently testing 20(OH)D3 for inhibition of UVB induced cancerogenesis in a Ptch1$^{+/-}$/SKH-1 hairless mouse model [137] with very promising results.

Most recently, we tested several CYP11A1-derived vitamin D3 (20(OH)D3, 1,20$(OH)_2$D3, 20,23$(OH)_2$D3, 1,20,23$(OH)_3$D3) and L3 (20(OH)L3, 22(OH)L3, 20,22$(OH)_2$L3, and 24(OH)L3) hydroxyderivatives and compared the photoprotective effects to those of 1,25$(OH)_2$D3 and L3 (precursor) in human epidermal keratinocytes [138]. They attenuated UVB-induced oxidative stress and DNA damage as measured by CPD levels and the tail moment of comets, and induced DNA repair measured from 6–4PP production. These effects correlated with enhanced expression of antioxidant response genes downstream of NRF-2 *(GR, HO-1, CAT, SOD1,* and *SOD2),* and the expression of HO-1, CAT, and MnSOD proteins [138]. Hydroxyderivatives of D3 and L3 not only stimulated the phosphorylation of p53 at Ser-15, but also induced p53 and NRF2 translocation into the nucleus. Importantly, treatment of keratinocytes with D3 and L3 hydroxyderivatives after UVB-treatment was able to reverse the radiation-induced damage [138], similar to what was reported for melatonin and its metabolites [83,132] Ofparticular note is the observation that UVB can upregulate CYPP11A1 expression in human and murine skin. [139,140,141]

Of great interest is that the above effects by D3 and L3 hydroxyderivatives correlated with stimulation of the NRF2- and p53- dependent responses[71] and stimulation of the DNA repair system. This is consistent with microarray analyses reported previously showing a highly significant effect of 20,23$(OH)_2$D3 on p53 signaling, GADD45 signaling, mismatch repairs in eukaryotes, the protein folding response and the NRF2-mediated oxidative stress response [71] (supplemental figure 1). These responses were less pronounced than those of 1,25$(OH)_2$D3 [71]. The GSEA analysis of these data (GSE117351) [71] re-emphasized the induction of protective mechanisms by 20,23$(OH)_2$D3. This analysis showed upregulation of *p53* transcriptional activity and downregulation of *p53* activity through methylation, and downregulation of oxidative stress induced senescence and of the SUMOylation of proteins involved in the repair of damaged DNA (Table 2). Similarly, 1,25$(OH)_2$D3 downregulated oxidative stress-induced senescence and of SUMOylation of proteins involved in the DNA damage response, while upregulating detoxification of ROS (Table 2).

Most recently, we tested the protective effects of 20(OH)D3, 1,20$(OH)_2$D3, 20,23$(OH)_2$D3 and 1,20,23$(OH)_3$D3 in comparison to 1,25$(OH)_2$D3, against UVB-induced keratinocyte inflammatory responses. We observed that there is modulation of the expression of *IL-17, NFκBp65, IκB-α* and other genes. There was also the reduction of NFκB-p65 activity and attenuation of proinflammatory mediators IL-17, TNF-α and IFN-γ that were stimulated by

UVB [142]. In addition, 1,25(OH)$_2$D3, 20(OH)D3, 1,20(OH)$_2$D3, and 20,23(OH)$_2$D3 stimulated the expression of the major markers of epidermal differentiation in UVB-irradiated cells. This led to the proposal that D3-hydroxyderivatives protect the epidermis from UVB-induced damage via activation of IκB-α expression and suppression of NFκB-p65 activity and its downstream cytokines including TNF-α, and IFN-γ, inhibition of IL-17 production, and stimulation of keratinocyte differentiation [142]. This concept is further supported by previous data showing that both calcemic and non-calcemic D3-derivatives cause downregulation of NFκB activity and production of Th1 and Th17 cytokines and inverse agonist activity of RORs[51,52,58–60,62,68,69]. The anti-inflammatory activities of 20,23(OH)$_2$D3 and 1,25(OH)$_2$D3 also had this signature in the microarray data (GSE117351) [71]. Again, the IPA and GSEA of the data showed downregulation of interferon (INF) signaling, TNFR2 non-canonical and FCER1-activated and NIK-noncanonical NFκB pathways, downregulation of IL-1 and IL-12 pathways, downregulation of arachidonic acid metabolism and upregulation of IL-4 and IL-13 signaling (Table 2). Similarly, 1,25(OH)$_2$D3 downregulated INF signaling, TNSFF non-canonical and TRAF-activated NFκβ pathways, downregulated inflammasomes and upregulated IL-4, IL-10 and IL-13 signaling.

In summary, it has been demonstrated by a variety of methods that calcemic and non-calcemic D3-hydroxyderivatives induce several protective pathways against UVB damage, including p53, NRF2 and DNA damage response systems, and downregulate proinflammatory responses via downregulation of NFκB signaling. The future challenge is to determine which pathways are affected by L3-hydroxyderivatives in addition to their stimulation of p53, NRF2 and DNA damage responses systems.

Conclusions and future directions

Based on the information above it is clear that the phenotypic effects of products of the canonical and non-canonical pathways of D3 activation will depend on their chemical structure (location of hydroxyl group and presence of C1α(OH), subcellular localization (plasma membrane, mitochondrion cytoplasm and nucleus), concentration and ability to interact with various regulatory proteins (nuclear receptors, transporters, CYP enzymes).The list of nuclear receptors these compounds may interact with include the VDR, RORs and AhR with each particular compound potentially displaying different affinities (Fig. 2). Although 1,25(OH)$_2$D3 is believed to have the highest affinity for VDR, involvement of these other receptors in photoprotection is expected because they are expressed in the epidermis, where D3 is activated and thus the local concentrations of active products should be high, allowing them to act on multiple receptors at once. Hence the next challenge is how to connect the particular receptor activity with other transcriptional master regulators such as NRF2, p53 and NFκB and others mentioned above, to coordinate anti-oxidative, DNA repair and anti-inflammatory mechanisms to attenuate the UVB induced damage (Fig. 6). Another challenge is to identify other receptors in addition to RORs and A pocket of the VDR mentioned above, that may interact with L3- and putative T3-hydroxyderivatives (Figs. 1, 2). For the AhR our recent molecular modeling predicts that vitamin D3 derivatives share the same ligand binding pocket with the corresponding native ligand in the LBDs for AhR (Fig. 7).

Emerging from these studies is the concept of mitochondria as the hub for D3 and L3 activation and metabolism, with products that may directly or indirectly affect the mitochondrion and cellular bioenergetics through biochemical (non-receptor) and receptor-depend mechanisms of action that regulate homeostasis at the cellular (keratinocytes) and organ (epidermis, skin with adnexa) levels (Figs. 1–6). The role of mitochondria in such process could be crucial [77], as already proposed for another endogenous molecule, melatonin [135].

In summary, there are multiple signaling pathways activated by different D3 and L3-hydroxyderivatives acting in concert to protect the skin from or reverse UVB-induced damage. Thus, the epidermal ecosystem involves many different but related molecules, with different receptors, which surprisingly produce a similar phenotypic effect, photoprotection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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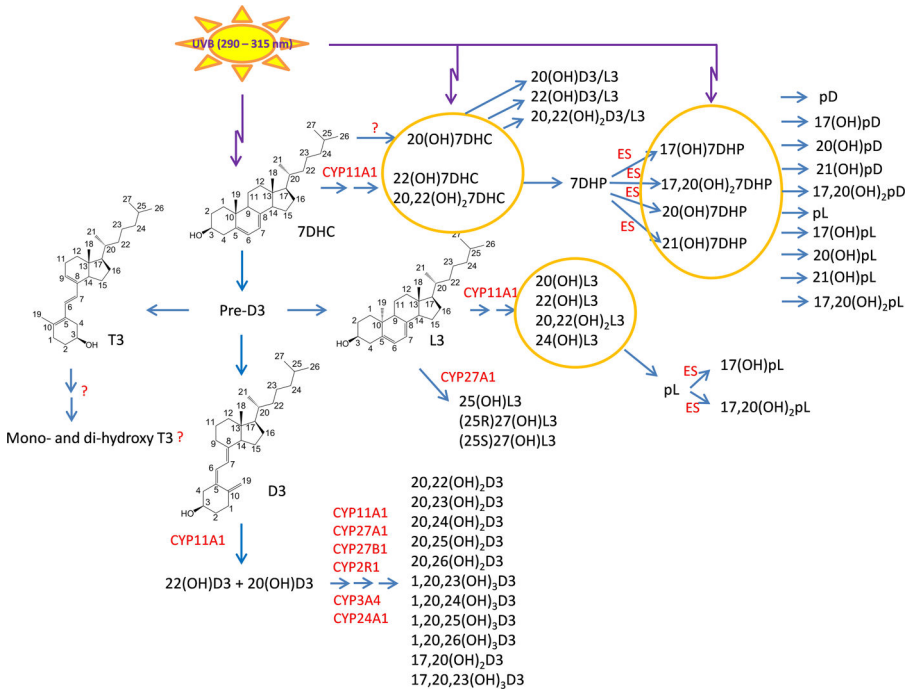

Figure 1.

Noncanonical pathways of vitamin D3 and lumisterol (L3) activation. D3, L3 and 7DHC are substrates for CYP11A1 activity that by itself or in cooperation with other CYPs enzymes produces the corresponding hydroxyderivatives. In the case of L3 and 7HDC, the side chain can be cleaved by CYP11A1 to produce 7DHP or pL that can be further metabolized by steroidogenic enzymes (ES). In the skin UVB acting on 5,7-dienes can lead to production of D3, L3 and T3 derivatives with a full-length side chain, and pD, pL and pT derivatives with a shortened side chain. While the cut-off for UVC/UVB is 280 nm, we show the range 290–315 nm because wavelengths below 290 nm are filtered by the ozone layer and no additional pre-D3 is produced above 315 nm [143].

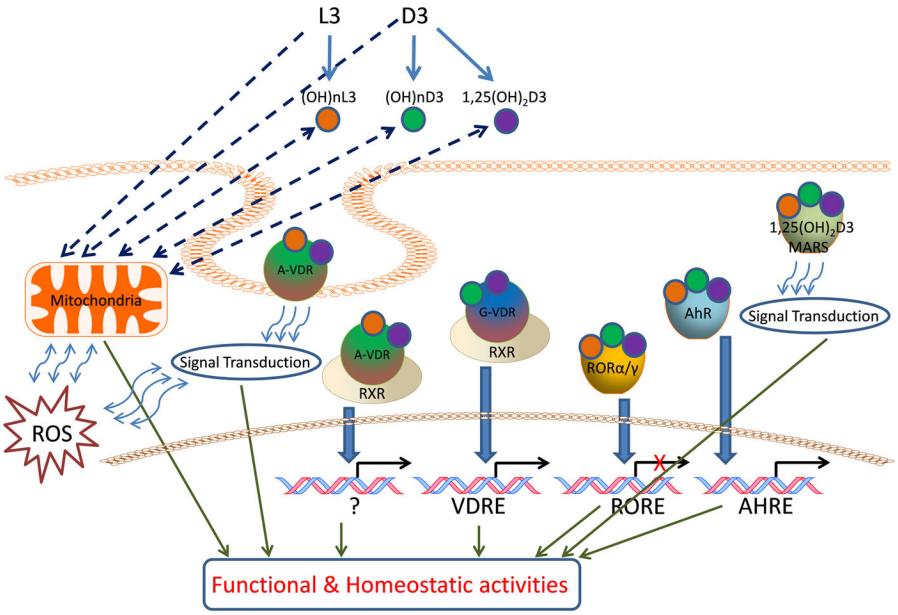

Figure 2.

Vitamin D3 and L3 target receptors and mitochondria for regulation of the cell phenotype and homeostatic activities.

D3 and L3 precursors are hydroxylated by either microsomal or mitochondrial CYPs to generate (OH)nD3, (OH)nL3 and classical 1,25(OH)$_2$D3, which can bind to the A or G site of VDR, or to RORs, AhR or 1,25D3 MARRS to activate genomic or non-genomic signal transduction pathways. The mitochondrion is also a target for these hydroxyderivatives.

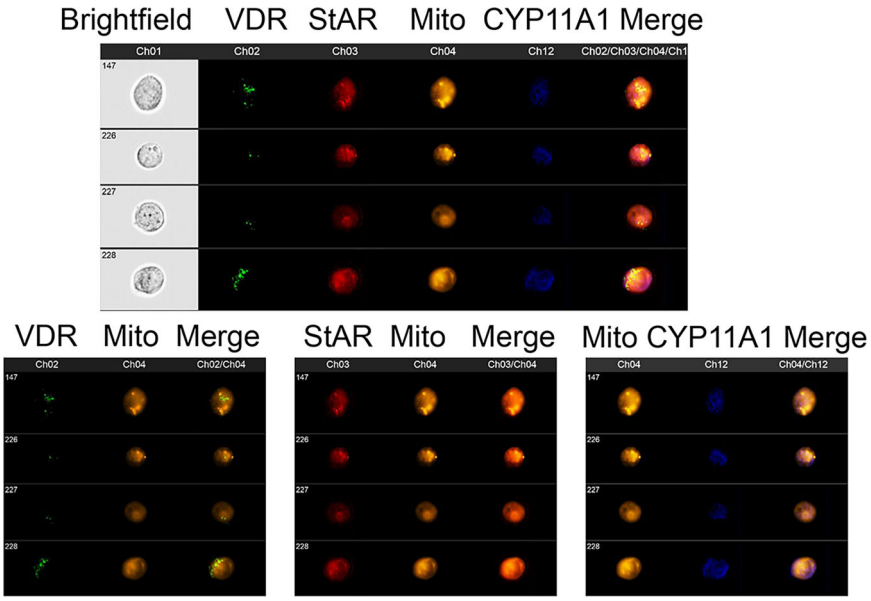

Figure 3.

Co-localization of VDR, StAR and CYP11A1 with mitochondria in human keratinocytes. Fixed and permeabilized HaCaT cells (human keratinocytes) were stained for expression of VDR (Ch2:green), StAR (Ch3:red), mitochondria (Ch4:Orange) and CYP11A1 (Ch12:blue) and analyzed using an ImageStream II (Amms, Seattle, WA, USA) cytometer as described previously[144]. The composite image of four different cells (upper panel) shows that all of the StAR colocalizes with mitochondria. VDR and CYP11A1 have different subcellular distributions but both of them are partially found colocalized with mitochondria. This is observed with greater clarity when the same four cells are analyzed for VDR and mitochondria (lower left panel), StAR and mitochondria (lower middle panel) and CYP11A1 and mitochondria (lower right panel). HaCaT cells were detached and processed as previously described[52]. The cells were fixed, permeabilized and stained with antibodies to VDR (Santa Cruz; Dallas, TX, USA), CYP11A1 (Cell signaling technology; Danvers, MA, USA), StAR (Santa Cruz; Dallas, TX, USA), and Mitotracker Red (10 nM - CMX Ros Invitrogen; Carlsbad, CA, USA) as described previously[144]. Data were analyzed using IDEAS software (Amms, Seattle, WA, USA).

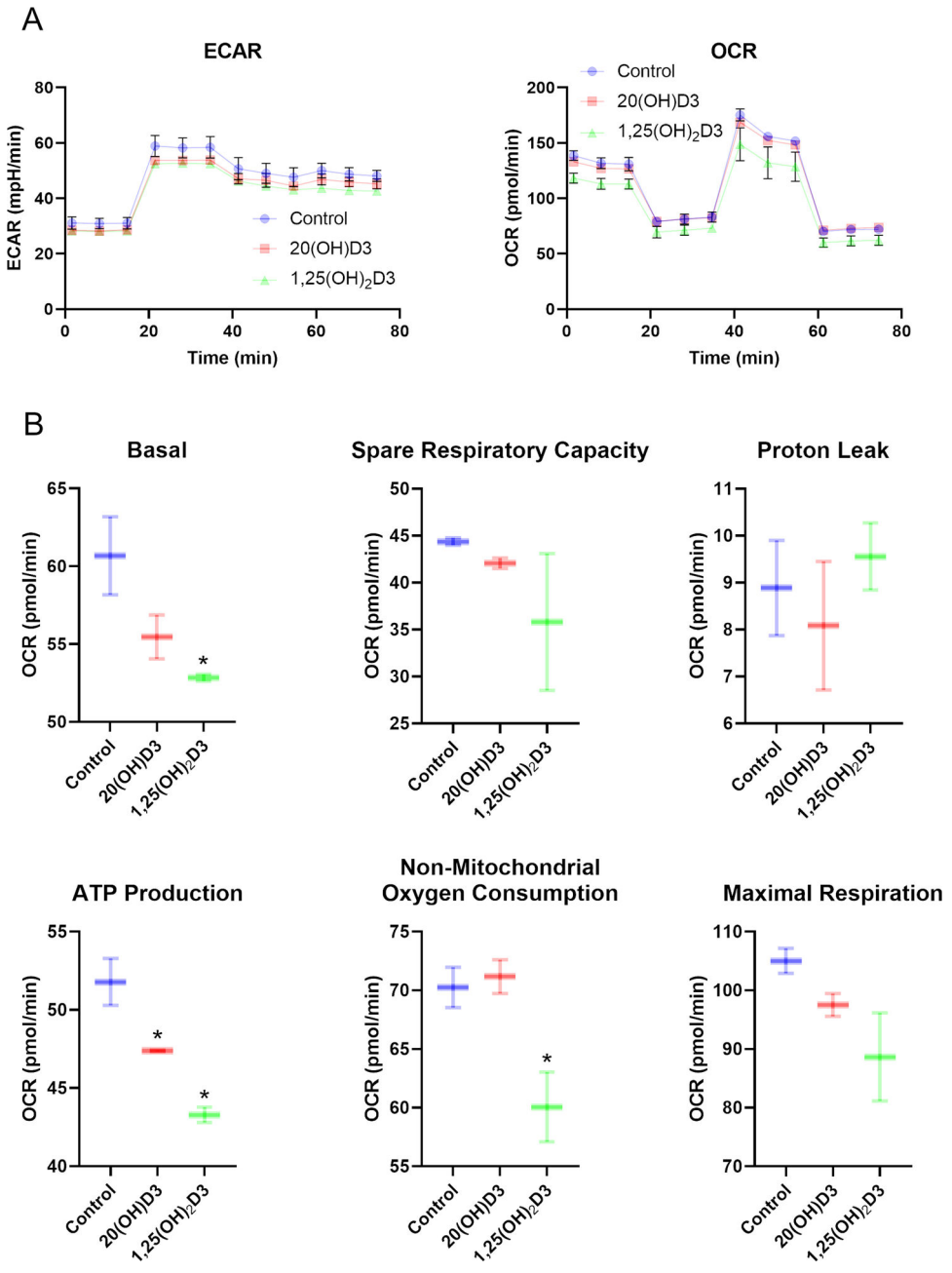

Figure 4.

The effects of vitamin D3 derivatives on mitochondrial function. A, Representative traces of mitochondrial oxygen consumption rates (OCR) and, extracellular acidification rates (ECAR) in control (vehicle), 100n M 20(OH)D3 or 1,25$(OH)_2$D3-treated HaCaT cells. B, Indices of mitochondrial function include basal, ATP-linked, maximal, reserve capacity, proton leak, and nonmitochondrial oxygen consumption rates. Data are presented as mean ± SD, $n = 2$. *$P < 0.05$ (Student's t-test).

A Seahorse XFe24 Analyzer (Agilent Technologies, Inc., Santa Clara, CA) was used to determine ATP production rates, extracellular acidification rates (ECAR) and oxygen consumption rates (OCR). An XF Real-Time ATP Rate Assay Kit and an XF Cell Mito

Stress Test Kits were used according to the manufacture's protocol. HaCaT cells were cultured on a XF Cell Culture Microplate in DMEM media containing 5% charcoal-stripped FBS for 24 h followed by treatment with vitamin D3 derivatives, as indicated, for 24 h. Next, cells were washed with assay media and incubated for 60 min prior to OCR and ECAR assays.

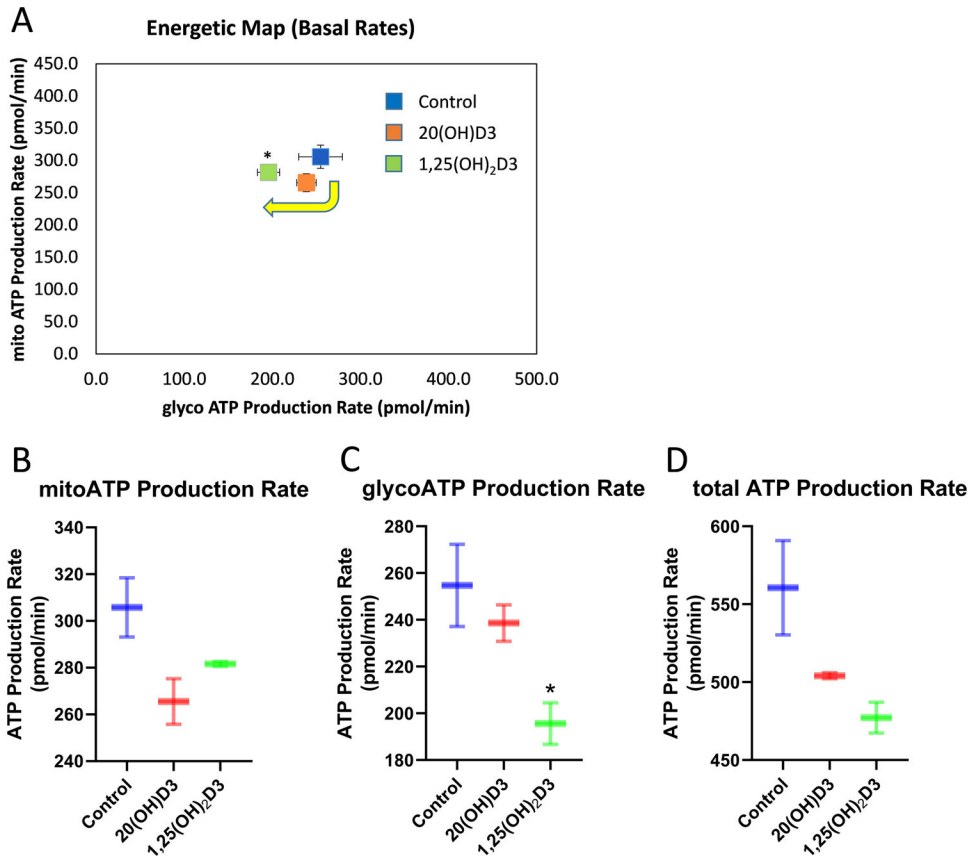

Figure 5.

The effects of vitamin D3 derivatives on OXPHOS and glycolytic flux. A, Energetic map indicates that there is a shift from mitochondrial to glycolytic metabolism after treatment HaCaT cells with 100 nM 20(OH)D3 or $1,25(OH)_2D3$ for 24 h. The effects of vitamin D3 derivatives on ATP production by mitochondria (B), glycolysis (C), and total amounts (cytoplasm) (D) are shown. Data are means ± SD, $n = 2$). $^*P < 0.05$ (Student's t-test).

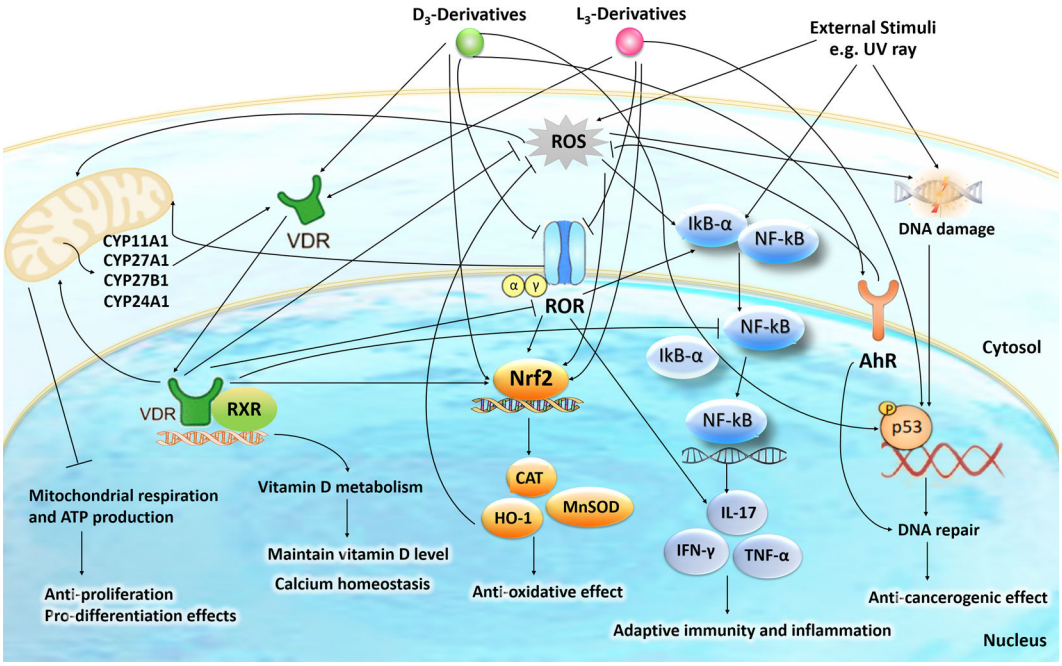

Figure 6.

The intracellular action of vitamin D3 (D3)- and lumisterol (L_3)-hydroxyderivatives in photoprotection against UVR.

Signal transduction includes activation of nuclear receptors including the VDR, RORα/γ, and AhR and the action of D3- and L3-hydroxyderivatives on mitochondrial processes. The nuclear receptors activities are linked with the transcriptional master regulators Nrf2, p53 and NFκB to coordinate anti-oxidative, DNA repair, anti-inflammatory, and anti-proliferative as well as anti-carcinogenesis mechanisms.

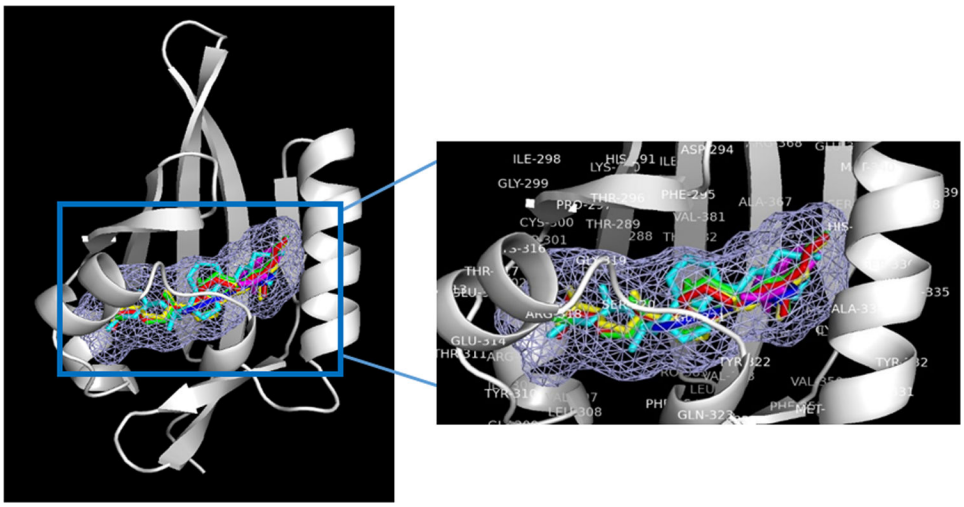

Figure 7.

Binding modes for 1^{α}, 20S(OH)$_2$D3(red), 1^{α},25(OH)$_2$D3 (yellow), 20S(OH)D3 (green) and 20S,23R(OH)$_2$D3 (cyan), indirubin (native ligand, blue) and indole acetic acid (native ligand, magenta) to the ligand binding domain (LBD) of AhR (in white, Homology model from previous study[145]). The light blue meshing area shown in the figure is the hydrophobic binding pocket in AhR. Vitamin D3 derivatives share the same ligand binding pocket with the corresponding native ligand in the LBDs for AhR.

Table 1.

Gene Set Enrichment Analysis for signalling associated with mitochondrial metabolism based on microarray data obtained after 24 h of *in vitro* incubation of primary human epidermal keratinocytes with $1,25(OH)_2D3$ or $20,23(OH)_2D3$.

Gene Set Enrichment Analysis for $1,25(OH)_2D3$					Gene Set Enrichment Analysis for $20,23(OH)_2D3$			
Reactome Pathway	**Normalized Enriched Score**	**P-value**	**FDR**	**Direction**	**Normalized Enriched Score**	**P-value**	**FDR**	**Direction**
Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins	−1.368	0.068	0.258	Down	−3.195	0.00	3.496	Down
Respiratory electron transport	−1.352	0.091	0.270	Down	−3.095	0.00	6.049	Down
Fatty acyl-CoA biosynthesis	−0.720	0.745	0.871	Down	−1.980	0.035	0.029	Down
Glucose metabolism	−1.842	0.00	0.037	Down	−3.081	0.00	6.189	Down
Glycolysis	−2.000	0.00	0.016	Down	−2.726	0.016	0.012	Down
Mitochondrial calcium ion transport	−1.343	0.077	0.277	Down	−2.527	0.00	0.014	Down
Glucagon signaling in metabolic regulation	−1.837	0.00	0.038	Down				
Digestion and absorption	−0.676	0.886	0.904	Down				
Mitochondrial Fatty Acid Beta-Oxidation	1.207	0.224	0.455	Up	−2.051	0.00	0.024	Down
Citric acid cycle (TCA cycle)	0.948	0.480	0.693	Up	−2.354	0.00	0.015	Down
Pyruvate metabolism and Citric Acid (TCA) cycle	1.044	0.333	0.597	Up	−2.441	0.00	0.014	Down
Pyruvate metabolism	0.621	0.913	0.927	Up	−1.916	0.039	0.035	Down
Glycogen breakdown (glycogenolysis)	2.290	0.00	0.146	Up				
Glycogen metabolism					−2.271	0.020	0.017	Down
Glycogen synthesis					−2.335	0.00	0.015	Down
Mitochondrial protein import					1.559	0.071	0.158	Up
Mitochondrial biogenesis					−1.462	0.089	0.139	Down
Fatty acid metabolism					−2.817	0.00	0.012	Down
Peroxisomal lipid metabolism					−2.432	0.00	0.014	Down

Table 2.

Gene Set Enrichment Analysis (GSEA) for signalling connected with DNA repair and oxidative stress based on microarray data obtained after 24 h of *in vitro* incubation of primary human epidermal keratinocytes with $1,25(OH)_2D3$ or $20,23(OH)_2D3$.

Gene Set Enrichment Analysis for $1,25(OH)_2D3$					**Gene Set Enrichment Analysis for $20,23(OH)_2D3$**			
Reactome Pathway	**Normalized Enriched Score**	**P-value**	**FDR**	**Direction**	**Normalized Enriched Score**	**P-value**	**FDR**	**Direction**
Oxidative Stress Induced Senescence	−1.931	0.00	0.024	Down	−2.509	0.00	0.013	Down
SUMOylation of DNA damage response and repair proteins	−3.236	0.00	0.00	Down	−2.213	0.00	0.018	Down
Cellular response to heat stress	−2.889	0.00	1.616	Down				
DNA Damage/Telomere Stress Induced Senescence	−1.382	0.140	0.249	Down				
TNF receptor superfamily (TNFSF) members mediating noncanonical NF-kB pathway	−1.231	0.245	0.356	Down				
TNFR2 noncanonical NF-kB pathway					−1.999	0.020	0.028	Down
TRAF6 mediated NF-kB activation	−1.362	0.174	0.262	Down				
FCERI mediated NF-kB activation					−2.604	0.00	0.014	Down
NIK-noncanonical NF-kB signaling	−2.761	0.00	0.012	Down				
Interferon gamma signaling	−1.890	0.00	0.030	Down	−2.706	0.00	0.013	Down
Interferon Signaling	−3.851	0.00	0.00	Down	−2.706	0.00	0.013	Down
Interleukin-20 family signaling	−1.591	0.022	0.118	Down				
Interleukin-1 signaling					−1.888	0.016	0.038	Down
Interleukin-1 family signaling					−1.796	0.035	0.050	Down
Inflammasomes	−1.593	0.057	0.117	Down				
Interleukin-12 signaling					−1.773	0.043	0.054	Down
Oxygen-dependent proline hydroxylation of Hypoxia-inducible Factor Alpha					−2.626	0.00	0.013	Down
AURKA Activation by TPX2	−2.409	0.00	1.876	Down				
Regulation of Hypoxia-inducible Factor (HIF) by oxygen					−2.275	0.021	0.017	Down
Arachidonic acid metabolism					−2.245	0.00	0.018	Down
TP53 Regulates Transcription of Genes Involved in Cytochrome C Release	0.838	0.686	0.768	Up	2.115	0.00	0.062	Up
TP53 Regulates Metabolic Genes	1.392	0.102	0.307	Up				
TP53 Regulates Transcription of Cell Cycle Genes					1.982	0.020	0.074	Up

Gene Set Enrichment Analysis for 1,25(OH)$_2$D3					Gene Set Enrichment Analysis for 20,23(OH)$_2$D3			
Reactome Pathway	**Normalized Enriched Score**	**P-value**	**FDR**	**Direction**	**Normalized Enriched Score**	**P-value**	**FDR**	**Direction**
Regulation of TP53 Activity through Methylation					−1.796	0.00	0.050	Down
Detoxification of Reactive Oxygen Species	1.522	0.041	0.280	Up				
Interleukin-4 and Interleukin-13 signaling	2.109	0.024	0.152	Up	2.859	0.00	9.922	Up
Interleukin-10 signaling	1.748	0.036	0.201	Up				