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Novel vitamin D compounds and skin cancer prevention

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As skin cancer is one of the most costly health issues in many countries, particularly in Australia, the possibility that vitamin D compounds might contribute to prevention of this disease is becoming increasingly more attractive to researchers and health communities. In this article, important epidemiologic, mechanistic and experimental data supporting the chemopreventive potential of several vitamin D-related compounds are explored. Evidence of photoprotection by the active hormone, 1α,25-dihydroxyvitamin D_3, as well as a derivative of an over-irradiation product, lumisterol, a fluorinated analog and bufalin, a potential vitamin D-like compound, are provided. The aim of this article is to understand how vitamin D compounds contribute to UV adaptation and potentially, skin cancer prevention.

Introduction

UVB irradiation from sunlight is the primary source for vitamin D synthesis in the epidermis. However UV can also cause skin cancer when excessive radiation is received. The same UVB that produces vitamin D also causes DNA and other damage that eventually results in skin cancers for large numbers of people. There is level 1 evidence from meta-analyses of randomized, controlled trials that adequate vitamin D status reduces parathyroid hormone levels, falls, fractures and overall mortality.[1] There is also increasing evidence for a range of other health benefits of adequate vitamin D status.[1,2] Considering the increase in skin cancer incidence worldwide, with up to approximately 450,000 new cases diagnosed per year in Australia alone, as reported by the Australian Institute for Health and Welfare,[3] it is vital to find a balance between vitamin D sufficiency and preventing the hazardous effects of UV that lead to skin cancers and photoaging.

There is compelling evidence that because vitamin D and its metabolites are made in skin, the damage from UV exposure is less than it would be otherwise.[4,5] Vitamin D compounds that do not cause hypercalcemia, are not photolabile but also protect skin cells from the hazardous effect of UV are promising alternatives to 1α,25dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) for therapeutic applications. The focus of this review is on the photoprotective actions and mechanisms of a number of natural and synthetic vitamin D compounds and vitamin D-like compounds, which might be used for protection from the hazardous effects of UV and for skin cancer prevention.

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Structure of Vitamin D

Technically speaking, vitamin D_3 is not a true vitamin but a seco-steroid, synthesized in skin.[6] The molecular structure of the vitamin D_3 compound family, including the active hormone 1,25$(OH)_2D_3$, is chemically related to that of classical steroid hormones through a steroid hormone specific cyclopentanoperhydrophenanthrene 4 ring carbon skeleton.[7] However, unlike its steroidal relatives, the 9-10 carbon-carbon bond of ring B is broken, making vitamin D_3 a seco-steroid (**Fig. 1**).

In comparison with their classical steroid counterparts, molecules of the vitamin D_3 superfamily are particularly conformationally flexible, due to three major structural regions of their carbon skeleton. First, simple rotation of the 8-carbon side chain about the 5 carbon-carbon single bonds allows for a plethora of differing molecular shapes. Additionally, as a result of the 9-10 carbon-carbon breakage, the cyclohexane-like A-ring is liberated from the B-ring and undergoes rapid interconversion between chair-chair conformers. The mobility of the A-ring results in orientation of the 1α-hydroxyl and 3α-hydroxyl to move between axial and equatorial planes. Finally, the seco B-ring rotates freely around its 6-7 single carbon bond, producing 6-*s-cis* and 6-*s-trans* variates of conformation, which may be locked or flexible.[7,8] It is the conformational flexibility of the vitamin D backbone, which allows for the generation of a vast number of ligand shapes that are able to generate different biological responses through the vitamin D receptor (VDR).

Photobiology and Metabolism of Vitamin D

Vitamin D is produced in skin by the photochemical conversion of 7-dehydrocholesterol in epidermis to pre-vitamin D. The energy for this reaction is provided by UV B radiation (UVB, 290–315 nm) which is the only part of the solar UV radiation

(UVR, 290–400 nm) that can split the B-ring of the precursor. Thermal conversion at body temperature forms the stable form, vitamin D.[9] Continued exposure of pre-vitamin D or vitamin D to UVB results in the formation of the so called "over-irradiation products" including lumisterol, tachysterol and suprasterols.[9] Vitamin D made in skin of animals is cholecalciferol or vitamin D_3, while vitamin D made from the plant precursor, ergosterol is vitamin D_2 or ergocalciferol. The metabolism and actions of D_2 or D_3 compounds are fairly, though not entirely, similar.[1] Subsequent to cutaneous production or intestinal absorption from food or supplements, vitamin D is transported in the circulation by serum vitamin D binding protein to the hepatocytes of the liver where the first hydroxylation occurs at the 25 position. As a result of this, 25(OH)D, the major circulating form of vitamin D is synthesized. This compound has a 15–50 day half-life and is widely used as the biological marker for vitamin D status in humans. This metabolite has little biological activity and needs to be hydroxylated again, in the proximal convoluted tubule cells of the kidney at the 1α-position, in order to yield the hormonally active metabolite, $1,25(OH)_2D_3$ or calcitriol, which enters the blood.[2] $1,25(OH)_2D_3$ is also able to be locally produced in many tissues including skin, which expresses both 25-hydroxylase and 1α-hydroxylase (CYP27B1) activity.[10,11] Local production of the hormone probably restricts its effects to those immediate areas.

Transduction Pathways

$1,25(OH)_2D_3$ is the predominant structural ligand of the vitamin D endocrine system. It exerts its biological effects via two well-known pathways; a genomic pathway mediated by the well-known nuclear vitamin D receptor (VDR), and a non-genomic/rapid response pathway via a receptor that has not yet been clearly characterized.[7,12-15]

Genomic Pathway

A nuclear receptor of the large steroid receptor family, the VDR is found in nucleated cells in most tissues of the human body including the intestine, kidney, bone, skin, parathyroid gland, pancreas, pituitary and cells of the immune and reproductive systems, among others.[16,17] The VDR is made up of six primary domains each designated a different functionality, specifically, a variable domain, a DNA binding domain, a hinge, a ligand binding domain and a transcriptional activation domain.[16]

The biological responses of $1,25(OH)_2D_3$ are mediated by the classic genomic pathway through stereospecific ligand binding to the nuclear vitamin D receptor (VDR). The lipophilic $1,25(OH)_2D_3$ molecule passes through the lipid bilayer of the plasma membrane and binds to the hydrophobic pocket in the ligand binding domain of the VDR. Upon activation, the VDR heterodimerizes with a retinoid X receptor (RXR) forming a VDR-RXR complex. Zinc fingers of the DNA binding domain recognize the VDR-RXR complex, allowing docking with vitamin D response elements (VDREs) in the DNA sequences of vitamin D target genes. These may be in the promoter region of target genes or at distant enhancer or repressor sites. Subsequent recruitment of protein co-modulators assists interaction with the general transcription apparatus wherein gene transcription is promoted or repressed.[18,19]

Non-Genomic Pathway

The non genomic pathway activated by $1,25(OH)_2D_3$ generates a biological response within seconds to minutes by triggering a number of intracellular signaling pathways.[7,16,18] These include the opening of chloride and calcium channels, mitogen activated protein kinases (MAPKs), protein kinase C, phosphatidylinositol 3-kinase (PI3K), phospholipase C and subsequent G-protein-coupled second messenger systems such as cyclic adenosine monophosphate (cAMP). Activated messenger systems may cross talk with the nucleus to contribute to gene transcription.[16]

The location of a binding protein/receptor through which $1,25(OH)_2D_3$ exerts its non-genomic effects is not yet well established. There is evidence that this is the classic vitamin D receptor, which is located in lipid rafts in the cell membrane.[12,20] There is also evidence for a rapid response binding protein for $1,25(OH)_2D_3$, first identified in the basolateral membrane of chick epithelium, involved in rapid calcium absorption from the duodenum.[13] Subsequent purification of the protein led to its definitive identification as the 66 kDa membrane-associated rapid response steroid binding (MARRS) protein, identical to ERp57/PDIA3.[21]

In the last decade, it has been proposed that the rapid actions of $1,25(OH)_2D_3$ may occur via a putative alternative ligand binding pocket (AP) within the ligand binding domain of the VDR.[22] Through molecular modeling, the natural 6 sec-*cis* locked analog of $1,25(OH)_2D_3$, $1\alpha,25$ dihydroxylumisterol$_3$ (JN) (**Fig. 1**), which has weak genomic activity but equivalent potency to $1,25(OH)_2D_3$ in non-genomic signaling, was shown to dock with the classic VDR through a pocket other than the genomic pocket (GP) to elicit cellular responses. The flexible $1,25(OH)_2D_3$ molecule was found to have a high affinity for both pockets, with differing modes of binding. It is thought that vitamin D sterol binding at the VDR occurs kinetically through the AP, while in a thermodynamic manner at the GP, and thus subsequent responses are highly dependent on the conformation and orientation of the ligand present.[15,22] The AP prefers the planar 6-s-*cis* locked non-genomic agonist vitamin D sterols, such as JN, while the bowl shaped GP binds 6-s-*trans* conformed isomers of $1,25(OH)_2D_3$. The conformationally flexible $1,25(OH)_2D_3$ molecule can take up both structures, thus making it the most potent agonist for both genomic and non-genomic biological responses. The AP forms complexes with an array of $1,25(OH)_2D_3$ molecule conformations, while the GP is more restricted with its binding affinity.[15]

Evidence for a bi-functional VDR comes from a study showing $1,25(OH)_2D_3$ induced rapid chloride fluxes in wild type mouse osteoblasts, while mouse osteoblasts lacking a functional VDR showed no response.[14] The VDR-KO mice used in the aforementioned study were previously reported to exhibit phenotypic defects similar those seen in human vitamin D-resistant rickets type II, such as alopecia, growth retardation, impaired

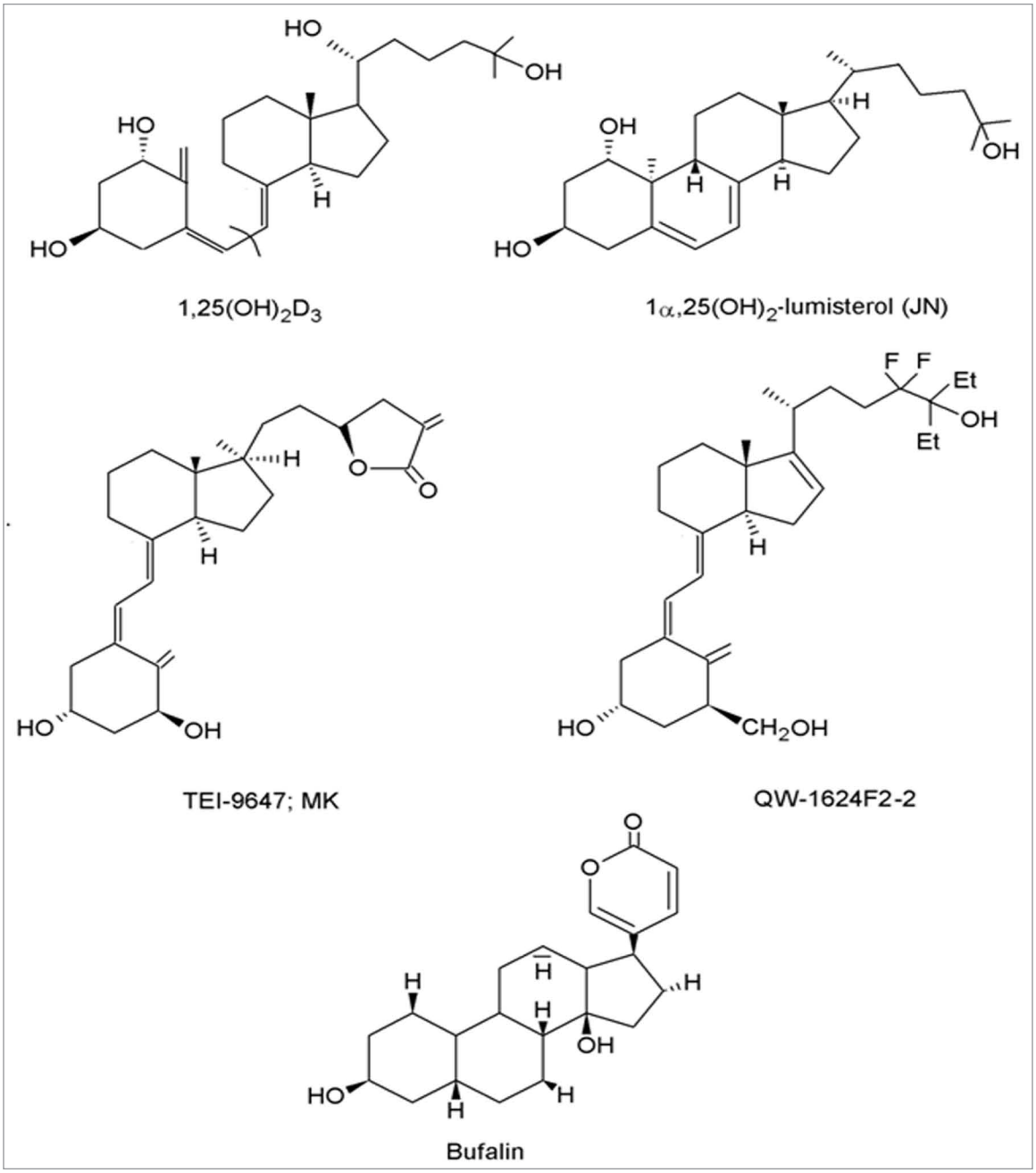


Figure 1. The chemical structures of the various D compounds.

bone formation and rickets, among many others.[23] Importantly, VDR-KO mice are more susceptible to photocarcinogenesis as well as chemical skin carcinogenesis.[24-26]

Skin Carcinogenesis

UVR is classified as a class one carcinogen by the World Health Organisation.[27] Carcinogenesis is a multistep process which requires initiation, promotion and progression[28] and UVR is known to be both an initiator and a promoter. UVR is known to be responsible for more than 90% of all skin cancers in humans including non-melanoma skin cancers and malignant melanoma, through well characterized mechanisms including inflammation,[29] DNA damage,[30-32] mutagenesis[33] and immune suppression,[34] all of which are believed to be involved in combination or sequentially leading to photocarcinogenesis.

DNA damage. UV-induced DNA lesions, such as cyclobutane pyrimidine dimers (CPD) and 6–4 photoproducts[35] are thought to be the initiating events to photocarcinogenesis. UV radiation also causes other types of DNA damage through production of reactive oxygen species (ROS) and nitric oxide (NO), products which are known to be not only mutagenic but also carcinogenic on their own.[36-38] UV-induced DNA damage is known to be repaired at various rates through nucleotide excision repair

or base excision repair. CPDs are known to take many hours for nucleotide excision repair, with different rates of repair in different species.[39,40] Xeroderma pigmentosum is an autosomal recessive disorder with dysfunctional nucleotide excision repair and sufferers are known to have a very high incidence of skin cancer.[41]

p53. UVR induces the upregulation and accumulation of the tumor suppressor protein p53 in the nucleus, a process first described by Maltzman.[42-45] Lane proposed p53 to be a DNA guardian since it has been shown to cause growth arrest, which could allow enough time for adequate DNA repair to take place.[46] p53 is also believed to cause apoptosis to remove cells with severe and unrepairable DNA damage. This was evidenced by a markedly reduced number of sunburn cells after UVR in an inactivated p53 transgenic mouse model.[47] Inactivating mutations in p53 as a result of UVR therefore would prevent G1 arrest and the initiation of apoptosis, and therefore would lead to continued division of mutated cells and subsequent carcinogenesis.

Immunosuppression. Lewis Thomas proposed a new concept of immune surveillance in 1959 for the first time.[48] Since then, many studies have built on this proposal and confirmed that UV radiation causes immune suppression in skin.[49,50] Immunosuppression was suspected to contribute to carcinogenesis when observations were made showing increased cancer development, including skin cancers, in immunocompromised patients.[51] UV-induced immunosuppression is thought to be mediated through suppressor T cells and antigen presenting cells (APCs) or Langerhans cells.[52] UV is known to reduce contact and delayed type hypersensitivity, which would mean decreased skin immune surveillance.[53,54] Applegate and Kripke et al.[55] also suggested that UV-induced immunosuppression may be triggered or worsened by DNA damage proposing that UV-induced DNA damage and photoimmunosuppression play significant roles together in photocarcinogenesis.

Oxidative stress. Finally, UV-induced oxidative stress is known to contribute to photocarcinogenesis. Oxidative stress is believed to be caused mainly by UVA rather than UVB,[54] and when combined with the damaging effects of UVB, may have a very significant role in photocarcinogenesis.[56,57] The most frequent product from oxidative stress to the DNA guanine base is 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG).[37] 8-oxodG production was shown to be proportional to the degree of UV exposure[58] and hence believed to be caused by UVR. 8-oxodG is repaired through base excision repair (BER) and OGG1 has been identified as the important enzyme for the repair process.[54] Kunisada et al. showed OGG1 knockout mice had significantly increased incidence of basal cell carcinoma,[36] which supports the importance of ROS in photocarcinogenesis.

Notch signaling pathway. Notch signaling pathways are responsible for cell proliferation, migration, differentiation and apoptosis[59] and maintain homeostasis of epidermis. Aberrant notch signaling is implicated in carcinogenesis.[60] Although the exact mechanism of this is not fully understood, it is believed to have two contrasting sides where it may act either as a tumor promoter or a tumor suppressor depending on the cell type and tissue context, level of expression and potential contemporary cross talk with other signaling systems.[61] It is believed upregulation in Notch signaling is associated with melanoma while downregulation was observed in non-melanoma skin cancers,[60] except for basal cell carcinoma (BCC) where nodular or superficial subtypes showed overexpression of Notch-1 protein.[60]

Analogs of $1,25(OH)_2D_3$ and a Vitamin D-Like Compound

Previous studies have identified the photoprotective properties of $1,25(OH)_2D_3$, the active derivative of vitamin D. $1,25(OH)_2D_3$ has been shown to enhance survival of skin cells following exposure to UV radiation, by reducing the level of damage to DNA and thus UV-induced apoptosis.[62] Further, $1,25(OH)_2D_3$ has been shown to reduce post-irradiation edema and inflammation in mouse skin, as well as photocarcinogenesis.[62] These findings suggest that $1,25(OH)_2D_3$ might be beneficial as an addition to sunscreens or even as an after-sun topical therapy, to boost skin protection against the harmful effects of UV radiation. However, $1,25(OH)_2D_3$ is chemically unstable in the presence of light. Further, it is an extremely expensive compound to synthesize and potentially causes hypercalcemia when large areas of skin are covered.[63] Analogs of $1,25(OH)_2D_3$ are currently being studied for their potential commercial use in after-sun lotions; however, they are also expensive to produce.

1α,25-dihydroxylumisterol$_3$ (JN). $1\alpha,25(OH)_2$-lumisterol$_3$ (JN) is a low-calcemic 6 sec-*cis*-locked analog (**Fig. 1**), which has been shown to be a full agonist of the non-genomic pathway that can only weakly bind to the VDR.[64] This compound is potentially a metabolite of the over-irradiation product, lumisterol.[65,66] JN was shown to generate non-genomic effects in pancreatic β cells and endothelial cells.[67,68]

1α-hydroxymethyl-16-ene-24,24-difluoro-25-hydroxy-26,27-bis-homovitaminD3 (QW; QW-1624F2–2). 1α-hydroxymethyl-16-ene-24,24-difluoro-25-hydroxy-26,27-bis-homovitaminD_3 (QW; QW-1624F2–2) has some transcriptional activity and is approximately 80–100 times less calciuric than $1,25(OH)_2D_3$.[69,70] This hybrid analog does not cause cachexia in animals[71] and has been shown to be non-genotoxic[71] (**Fig. 1**). QW has been demonstrated to have anti-proliferative and pro-differentiating activity and proved effective in the inhibition of skin tumor formation and latency in a model of chemical-induced skin tumorigenesis.[72]

Bufalin. Toad venom isolated from various Bufo species has been used in the preparation of Ch'an Su and Senso, traditional Chinese medicines, for centuries.[73] Extraction and characterization of this medicinal remedy identified bufalin from the family Bufadienolide as the most active component. Bufalin exhibits cardiotonic and local anesthetic properties via its action on the α subunit of Na^+, K^+-ATPase, inhibiting the movement of Na^+ and K^+ across the cell membrane.[73] Subsequent intracellular accumulation of Na^+ modulates the Na^+/ Ca^{2+} exchanger. This causes a build-up of Ca^{2+} ions within the cell, leading to enhanced cardiomyocyte contraction, preventing heart failure.[74]

Previous studies of bufalin have revealed it to have some effects in common with $1,25(OH)_2D_3$ in cancer cells.[75] The ability of $1,25(OH)_2D_3$ to differentiate human myeloid leukemia

cell lines toward monocyte/macrophage-like cells was significantly enhanced in combination with low doses of bufalin.[76] In these studies, bufalin did not appear to bind directly to the vitamin D receptor (VDR) but improved ligand-dependent activation of the VDR and upregulated VDR-mediated expression of endogenous target genes, such as CYP24.[75,77] In the presence of $1,25(OH)_2D_3$, bufalin maintained expression of the VDR in the nucleus of human leukemia cells, most likely through a reduction in nuclear VDR degradation or export.[77] Bufalin was also shown to modulate the relationship and enhance the interaction of the VDR with its recruited coactivators, such as SCR-1.[75] Bufalin treatment enhanced the association between the VDR and the corepressor N-CoR and abolished their dissociation in the presence of $1,25(OH)_2D_3$. Bufalin-induced modification of cofactor complexes enhanced $1,25(OH)_2D_3$-stimulated VDR transactivational activity.[75] Since the only known receptor for bufalin is the Na^+, K^+-ATPase and the plasma membrane is impermeable to bufalin, it has been proposed that bufalin must functionally modify the VDR through a Na^+, K^+-ATPase dependent mechanism.[78] Collectively, the findings suggest a new role for cardiotonic steroids, in particular bufalin, in the modulation of VDR function. More recently, it has been proposed that bufalin's structural similarity to 6 sec-*cis* conformation of $1,25(OH)_2D_3$ might allow it to bind to the alternative binding pocket of the VDR and activate the non-genomic pathway and thus confer protection to skin cells following UV-exposure (**Fig. 1**).

Effects of Vitamin D, Its Metabolites and Its Photoproducts on Skin Cells

Vitamin D and UV-induced DNA damage in skin cells. UV induces various types of DNA damage either photochemically or by UV activation of endogenous photoreceptors that create genotoxic free radicals that modify the DNA molecular structure. The most frequently occurring photolesion in sun-exposed human skin is the cyclobutane pyrimidine dimer (CPD),[79,80] particularly thymine dimers, which are induced primarily by UVB, and also by UVA to a lesser extent.[80-82] CPDs are produced by the dislocation of double bonds in two adjacent pyrimidines by UV absorption, resulting in a cyclobutane ring conformation linking the two nucleobases as a dimer.[83,84]

Our group and others have shown that $1,25(OH)_2D_3$ reduces thymine dimers in irradiated skin cells in vitro[85-90] and also in vivo in mouse[62,87,89,91] and human skin.[92,93] Thymine dimers are also reduced in irradiated skin cells in the presence of the low calcaemic rapid acting *cis*-locked non-genomic analogs, $1\alpha,25(OH)_2$-lumisterol$_3$ (JN) and $1\alpha,25(OH)_2$-7-dehydrocholesterol (JM) in vitro[85,87,94,95] and in mouse skin,[62] and also by the transcriptionally active hybrid QW.[85,95]

Evidence that the vitamin D photoprotective effect on reductions in thymine dimer DNA damage is via the rapid non-genomic pathway is demonstrated with various vitamin D-like compounds. As noted above, studies by our group have shown that that the transcriptionally non-active $1\alpha,25(OH)_2$-lumisterol$_3$ protects against UV-induced thymine dimers.[85,87,88] Of relevance to the mechanism of action of vitamin D compounds in photoprotection, the co-incubation of skin cells with $1,25(OH)_2D_3$ and 25-dehydro-1α-hydroxyvitamin D_3-26,23S-lactone (TEI-9647) (**Fig. 1**), an antagonist of the genomic action of $1,25(OH)_2D_3$,[96,97] did not alter the protective effects of $1,25(OH)_2D_3$ on thymine dimers.[87] In contrast, co-incubation with 1β,25-dihydroxyvitamin D_3 (HL), an antagonist of the non-genomic pathway, abolished the photoprotective effect of $1,25(OH)_2D_3$.[87,88]

As shown in **Figure 2**, bufalin caused a significant and dose-dependent reduction in thymine dimers in irradiated keratinocytes at nanomolar concentrations with 16 ± 1% to 18 ± 2% of nuclei carrying thymine dimers compared with 35 ± 3% of nuclei in vehicle-treated wells. This study shows for the first time that bufalin reduced UV-induced DNA damage. In Skh:hr1 mice given a single three minimal erythemal dose exposure of solar-simulated irradiation (as described in ref. 62) bufalin, at a concentration of 23 pmol/cm^2 applied topically immediately after irradiation, reduced at 24 h, the proportion of keratinocytes exhibiting thymine dimers from 4.8 ± 2.5% in mice treated with vehicle to 2.3 ± 1.6%. This figure was not significantly different to the thymine dimers measured at 24 h in skin of mice treated topically with a similar concentration of $1,25(OH)_2D_3$.

All the above studies were assessed by immunohistochemistry and image analysis. A reduction in thymine dimers (TD) by $1,25(OH)_2D_3$ has also been demonstrated by an entirely different method in irradiated keratinocytes by digestion with the site specific DNA repair enzyme T4 endonuclease IV for CPDs in the comet assay.[94,98]

Two other major UV-induced photolesions found in human skin are 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG)[84,99-101] and 8-nitroguanine.[102-105] These are induced indirectly by UV through oxidation or nitrosylation of guanine by increased levels of reactive oxygen (ROS) and nitrogen (RNS) species respectively. Cellular levels of ROS are increased by UV activation of endogenous photoreceptors in the cell.[106] Excess levels of nitric oxide (NO) accumulate by UV upregulation of nitric oxide synthases[107-109] and UVA decomposition of NO stores.[110-112] NO can act as a free radical on its own as well as react with ROS to form more powerful oxidating and nitrating intermediates, such as peroxynitrite.

Recently, we reported that $1,25(OH)_2D_3$ reduces 8-oxodG in irradiated keratinocytes in culture, in mouse skin[98] and in irradiated ex vivo human skin explants.[93] This was demonstrated by reduced nuclear staining with a monoclonal antibody to 8-oxodG, immunohistochemistry and image analysis, and also by Comet assay incorporating digestion with the site specific DNA repair enzyme, human 8-oxoguanine DNA glycosylase (hOGG1) in cultured keratinocytes.[93,98] Reduction of hOGG-sensitive sites by $1,25(OH)_2D_3$ occurred within 30 min of UV irradiation.[98] The 8-nitroguanosine lesion, considered a marker for inflammation and carcinogenesis,[113] was also reduced by $1,25(OH)_2D_3$. This was demonstrated by reduced nuclear staining with a monoclonal antibody to 8-nitroguanine in irradiated human ex vivo skin explants.[93] The 8-nitroguanosine lesion is rapidly removed by depurination to leave an abasic site.[114] We have also demonstrated that $1,25(OH)_2D_3$ reduced abasic sites in irradiated keratinocytes

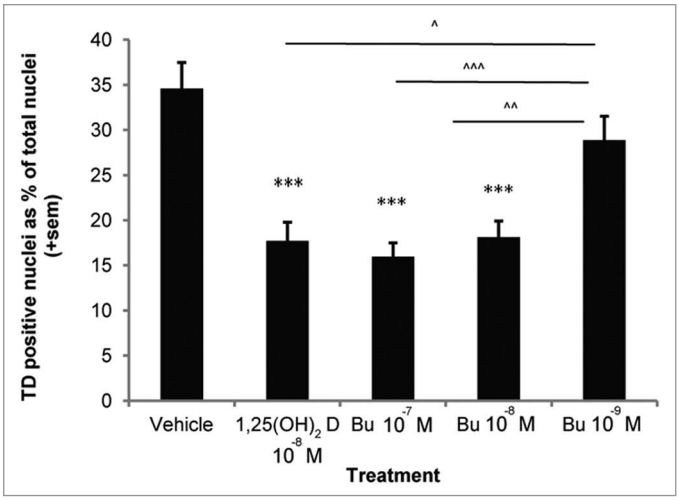


Figure 2. Photoprotection against thymine dimers by $1,25(OH)_2D_3$ and Bufalin in human keratinocytes. DNA damage was assessed using a monoclonal antibody to thymine dimers,[182] immunohistochemistry and image analysis as previously described[89,94] at 3 h after UV irradiation, in human keratinocytes treated with vehicle, $1,25(OH)_2D_3$ or three concentrations of bufalin and expressed as positively stained nuclei as a percent of total nuclei. The graph illustrates pooled data from a minimum of four independent experiments. *** denotes a significant difference compared with vehicle (***$p < 0.001$), ^, ^^, ^^^ denotes a significant difference compared with 10^{-9} M bufalin (^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$).

by Comet assay with endonuclease IV digestion, which cleaves DNA at abasic sites.[98]

Vitamin D compounds reduce UV-induced cell death in skin cells. Cell death is suppressed in UV-irradiated skin cells treated with vitamin D compounds despite a further increase in expression of p53.[62,89] This has been demonstrated in human skin cell cultures treated with $1,25(OH)_2D_3$[85,89,91,115-119] and the vitamin D analogs calcipotriol,[120] JN,[85] JM[85] and QW.[88] Evidence suggests that this is facilitated by a non-genomic pathway as the non-genomic antagonist HL suppressed improved cell survival with $1,25(OH)_2D_3$.[85] Apoptotic cells (sunburn cells) in the epidermis of UV irradiated skin were also reduced by $1,25(OH)_2D_3$ when applied systemically in mice,[121] and topically in mice[62,88,89] and human subjects.[92] When applied to an in vivo model bufalin reduced the number of sunburn (apoptotic) cells as well as the aforementioned thymine dimers in the epidermis of Skh:hr1 hairless mice. At a concentration of 115 pmol/cm^2, topical bufalin significantly ($p < 0.01$) reduced apoptotic keratinocytes (sunburn cells) in skin of Skh:hr1 mice irradiated with a single exposure of three minimal erythemal doses of solar-simulated UV radiation as described in ref. 62 compared with vehicle treatment and to a similar extent as 23 pmol/cm^2 of $1,25(OH)_2D_3$.

The mechanism for this improved cell survival in the presence of vitamin D compounds is almost certainly due to reduced DNA damage, and may be mediated by a reduced catalytic action of caspase 3 on poly(ADP-ribose) polymerase[118] and through effects on sphingosine-1 phosphate, a breakdown product of membrane sphingolipid and second messenger in the apoptotic signaling pathway.[116]

Vitamin D compounds reduce nitric oxide derivatives. Nitric oxide (NO) levels are increased in irradiated skin[112,122-126] as noted in the above section. High levels of NO cause nitrosylation of DNA repair enzymes,[127] inhibit DNA repair[128] and combine with excess levels of ROS to produce more toxic RNS intermediates that cause oxidative and nitrosative damage to DNA and proteins and peroxidation of lipids.[102] The vitamin D hormone, $1,25(OH)_2D_3$, has been shown to reduce nitrite[89] and 3-nitrotyrosine,[62] two stable end products of the nitric oxide pathway. Furthermore, similar to $1,25(OH)_2D_3$, the inhibitors of nitric oxide synthase, aminoguanidine and L-N-monomethylarginine, reduced UV-induced nitrite and thymine dimers in skin cells,[85,89] while a selective inhibitor of the inducible isoform (1400 W) reduced both CPDs and 8-oxodG.[98] It is likely that a reduction in post-UVR NO products is a mechanism whereby $1,25(OH)_2D_3$ protects skin cells from oxidative and nitrative DNA damage, as well as enhancing DNA repair and improving cell survival. Whether a reduction in NO products contributes to a reduction in thymine dimers as well, is unclear, since in one study in human keratinocytes, the reduction in NO products with $1,25(OH)_2D_3$ was dissociated from the reduction in thymine dimers.[94]

Mutagenesis. Most DNA damage is promutagenic, including that induced by RNS.[100,129-131] If the lesions are not perfectly repaired before DNA replication, the damaged nucleobases are replaced with an adenine during transcription. This may alter the coding sequence.[35] Mutations with sequence changes C to T transitions are associated with unrepaired CPDs, which are the predominant mutation due to the relatively high frequency and slow repair of CPD. These are now termed "solar signature mutations" rather than "UVB signature mutations," as the contribution of UVA to mutations in unrepaired bipyrimidines is currently being debated (refer to refs. 80, 132–135). The hallmark of mutations from UV- induced 8-oxodG are G to T transversions, found predominantly in the basal layer of the epidermis where keratinocyte stem cells and melanocytes are situated. These mutations have implications for skin cancer development.[100,136] The presence of mutations in the p53 tumor suppressor gene in many skin cancers, including premalignant precursors of SCC, correlate with both UVA- and UVB-induced lesions, providing evidence of their involvement in photocarcinogenesis.[137-140] Although there is no direct data that $1,25(OH)_2D_3$ reduces mutagenicity in UV-irradiated skin, the observation that photocarcinogenesis is substantially reduced by topical application of this compound suggests that this is indeed the case.[62]

D compounds and UV-induced immune suppression. The UVR component of sunlight causes immune suppression.[141] These immunosuppressive effects suppress cell-mediated immune reactions that normally destroy developing skin tumors.[142] The VDR is present in several immune cells, including monocytes, macrophages and activated T and B cells.[143] In fact, it has been suggested that the UVB-induced immune suppression from sun exposure is mediated through vitamin D. Interestingly, $1,25(OH)_2D_3$ was reported to increase mRNA expression of interleukin-10 (IL-10), an immune suppressive cytokine, and induce IL-10 secretion by mouse mast cells, thereby adding to

the mast cell's ability to suppress inflammation and skin pathology at sites of UV irradiation.[144]

The effects of vitamin D compounds on the immune system are complex and depend on many factors including concentration. Interestingly, high concentrations of vitamin D have been shown to be immunosuppressive,[145] while vitamin D deficiency also causes immunosuppression.[146] Studies by our group have shown that vitamin D compounds including $1,25(OH)_2D_3$, JN and QW inhibit UV-induced immune suppression (contact hypersensitivity reaction to oxazolone) in hairless mice.[62,87,88] Conversely, studies in human subjects showed that $1,25(OH)_2D_3$ provided no protection against UV-induced suppression of a recall delayed-type hypersensitivity (DTH) (Mantoux) reaction and that topical $1,25(OH)_2D_3$ (doses of 1 μg or higher) was immunosuppressive in a recall DTH model.[92] This is supported by findings in another human study in which the vitamin D analog calcipotriene suppressed contact hypersensitivity responses to dinitrochlorobenzene by 64%, a level similar to that caused by solar simulated UV.[147]

Photoaging. Photoaging, also known as dermatoheliosis,[148] is a premature aging process caused by excessive exposure to solar UV radiation determined by the degree of sun exposure and the level of skin pigmentation.[149] This extrinsic aging process is distinguished from the natural chronological or intrinsic aging, in that it produces several complex and sequential cellular and molecular phenomena from photochemical reactions that are not seen in chronological aging including inflammation, oxidative stress, DNA lesions, mutation and immunosuppression caused by UV.

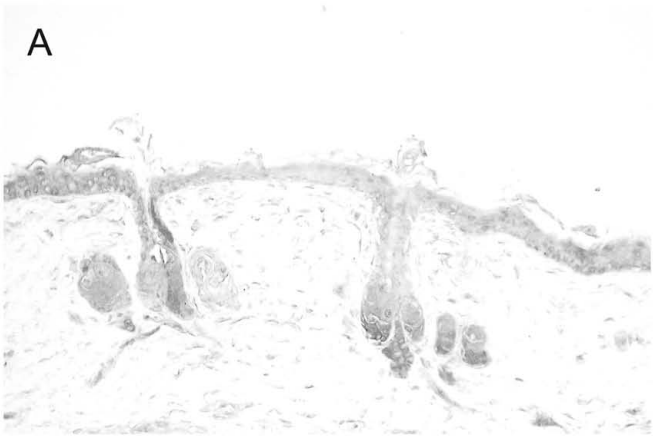
The acute effects of UVB on skin include the upregulation of TNFα by both keratinocytes and dermal fibroblasts within a couple of hours after UV exposure,[150] which is an important step for development of the inflammatory cascade in skin. This is augmented by release of pro-inflammatory cytokines such as IL-1, and IL-6[151] due to UV-induced DNA lesions such as CPDs and 6–4 photoproducts.[152-154] These DNA lesions are also known to enhance expression of inducible nitric oxide synthetase (iNOS) that increases nitric oxide products.[155] UVA is known to induce IL-10 in dermal macrophages and neutrophils which are, at least in part, responsible for UV-induced immunosuppression. Reeve and Tyrrell also reported the induction of IL-12 by UVA, which they proposed would counteract the UVB- induced immunosuppression through the hemoxygenase 1 pathway.[156] UV exposure is also known to deplete cellular antioxidants such as glutathione,[157] which increases reactive oxygen species (ROS) and reactive nitrogen species (RNS), which adds to the inflammatory process through peroxidation of membrane lipids and production of prostaglandins E_2.[158]

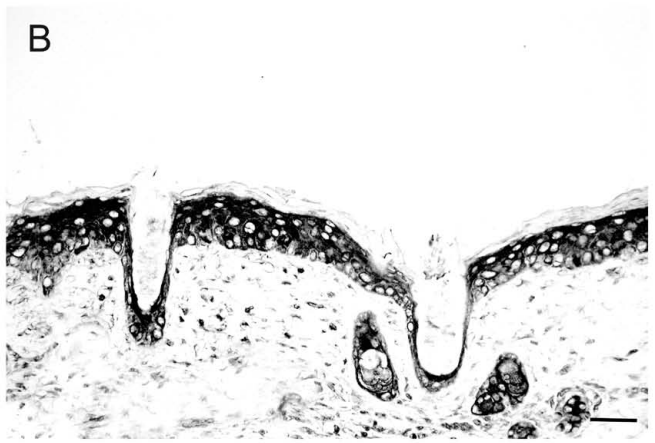
The signs of photoaging include wrinkles, reduced elasticity, dyspigmentation, telangiectasia and the development of benign or malignant tumors.[149] Wrinkles and reduced elasticity are produced, in part, through activation protein 1 (AP-1) transcription factor, which is known to be a critical mediator of photoaging and also involved in the overexpression of matrix metalloproteinases (MMPs) that in turn cause both the reduction of dermal collage and inhibition of collagen synthesis.[159] Interestingly, MMPs are also known to be responsible for collagen breakdown during invasion of various malignancies.[160]

The mechanism of UV-induced melanogenesis is unclear. There is a constant basal melanogenesis determined by genetic makeup, and this is increased with exposure to UV.[161] Since UV is responsible both for sunburn and tanning, it is believed the mechanism may be similar. Certainly, pigmentary disorders such as solar lentigo are commonly seen in photoaged skin, and it was postulated they were caused by UV-induced DNA mutations of keratinocytes and melanocytes with subsequently increased melanogenesis and transfer.[162] The evidence of this is seen in patients suffering from xeroderma pigmentosum with excessive development of severe dyspigmentation and skin tumors due to impaired nucleotide excision repair, which is important for repair of UV-induced cyclobutane pyrimidine dimers and other photoproducts.[41] Melanogenesis was once thought to be the negative feedback mechanism to control vitamin D synthesis to prevent hypercalcemia, but this is now unlikely and it has been proposed that the "over-irradiation" derivatives of the vitamin D pathway from excessive UVR reduce vitamin D synthesis in skin.[163]

To prevent photoaging, the most important and obvious strategy is to minimize UV exposure through lifestyle changes and using effective UV filters to prevent penetration of UVR into skin. Further to this, cellular and molecular protection to minimize some of the molecular events described may be attempted. In particular, prevention of UV-induced ROS has been suggested to be an important aspect in photoaging prevention, although these claims may be difficult to substantiate since there still are many challenges in accurately assessing the antioxidant activities required to provide cellular protection.[164] Endogenous, naturally occurring cellular protective anti-oxidants such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase are known to be depleted after UV exposure in animals[165] as well as in photoaged human skin.[166] There is some evidence that topical and systemic administration of antioxidants such as vitamin E, vitamin C, polyphenols and carotenoids provide some photoprotection in human skin.[167-171] Several plant based polyphenolic compounds such as green tea, grape seed and pomegranate showed some protection in vitro, and it has been suggested that they may reduce photoaging and photocarcinogenesis.[172] Topical all-trans-retinoic acids showed more convincing evidence of photo-aging protection through inhibition of UV-induced inflammation mediated by AP-1 and NFkB transcription factors.[173,174] It does this through reduction in UV-induced c-jun both by reducing the accumulation and also stimulating the breakdown of c-jun through ubiquitin-proteasome degradation.[173,174]

There is some evidence that suggests vitamin D compounds may also contribute to reductions in photoaging. As noted earlier, vitamin D metabolites and other photoproducts reduce several types of UV-induced DNA damage including thymine dimers, 8-oxodG and 8-nitroguanosine.[62,85,87,89,90,92,93,175] They also reduce NO products (8-nitrotyrosine and nitrite) and augment p53 expression,[89] thereby probably reducing inflammation and improving DNA repair. Both $1,25(OH)_2D_3$, and an analog, $1,24(OH)D_2$, reduced TNFα in human macrophages[176] and





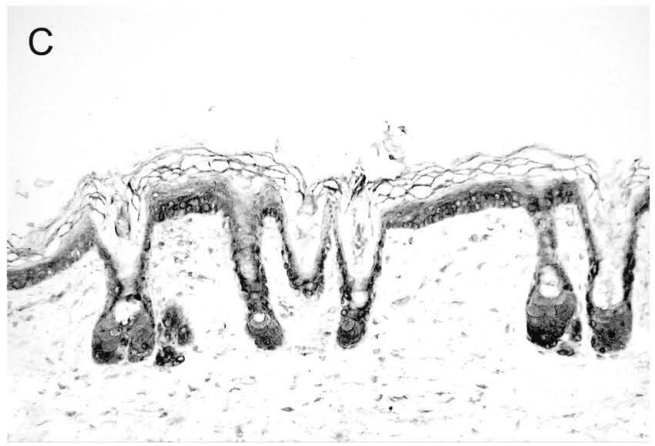


Figure 3. Reduction of UV-induced IL-6 expression by topical application of $1,25(OH)_2D_3$ in mouse skin. Immunohistochemical detection of IL-6 in Skh:hr1 hairless mice skin was with a monoclonal antibody to IL-6 and a biotinylated secondary rabbit anti-goat IgG. Figures are representative dorsal skin sections (**A**) non-irradiated skin or (**B**) after solar simulated radiation followed by 48-h treatment with vehicle or (**C**) after solar simulated radiation followed by 48-h treatment with 1,25D (22.8 pmol/cm^2). Reprinted from The Journal of Steroid Biochemistry and Molecular Biology. R.S. Mason, V.B. Sequeira, K.M. Dixon, C. Gordon-Thomson, K. Pobre, A. Dilley, M.T. Mizwicki, A.W. Norman, D. Feldman, G.M. Halliday, V.E. Reeve (2010).

also caused upregulation of IkappaBalpha levels by increased mRNA stability thereby reducing nuclear translocation of NFkB and downgrading its activity. We have also reported that $1,25(OH)_2D_3$ reduced expression of the inflammatory marker IL-6 in mouse skin (**Fig. 3**),[177] similar to the reduction in IL-6 production by irradiated keratinocytes treated with this compound in vitro.[118] Treatment of mice with topical $1,25(OH)_2D_3$ also reduced post-UV irradiation skin edema (**Fig. 4**).[62]

$1,25(OH)_2D_3$ was found to have regulatory effects on AP-1, and MMPs via the VDR[178] and a recent study suggested that $1,25(OH)_2D_3$ attenuated TNFα induced MMP3, hence probably also reducing UV-induced collagen degradation in skin.[179]

In summary, vitamin D and photoproducts are likely to provide protection against photoaging by protecting keratinocytes and fibroblasts from UV-induced DNA damage, oxidative stress and excessive nitric oxide products and also protecting against dermal collagen breakdown through MMP regulation.

Vitamin D system and photocarcinogenesis. Vitamin D and its photoproducts provide photoprotection in several types of UV-induced DNA damage, as described earlier, including CPD,[62,85,87,89,90,92,93,177] 8-oxodG[93,98] and 8-nitroguanosine.[93,98] The further increase in p53 with $1,25(OH)_2D_3$, which is known to promote DNA repair and thus reduce mutagenesis, is also thought to be an important mechanism of photoprotection by $1,25(OH)_2D_3$.[89] Skin cancers induced by UV or chemically were significantly increased in VDR knockout mice.[24-26] Interestingly, the absence of a vitamin D receptor also blocked the ability of $1,25(OH)_2D_3$ to reduce UV-induced thymine dimers in human skin fibroblasts.[90] VDR is also implicated in the DNA repair process where VDR-/- mice showed significantly higher CPD levels when subjected to UVB, which failed to reduce over time compared with the wild-type mice.[24] We also reported the key role of the alternate receptor ERp57/MARRS/PDIA3 in $1,25(OH)_2D_3$-mediated photoprotection. Blockade of this protein by a neutralizing antibody or downregulation with siRNA, abolished the reduction in post-UV thymine dimers in skin cells treated with $1,25(OH)_2D_3$.[90] In this model the VDR and ERp57 co-immunoprecipitated in non-nuclear cell extracts.[90]

It has now been demonstrated that treatment with $1,25(OH)_2D_3$ or the derivative of the over-irradiation product lumisterol, JN, reduced photocarcinogenesis in mice[62] (**Fig. 5**). The low calcemic analog, QW, also protected against UV-induced cell death, DNA damage and immunosuppression,[85,87] but at a comparable dose to $1,25(OH)_2D_3$, did not confer protection in a model of chronic UV-induced skin carcinogenesis in hairless mice.[180] It is possible that this analog might prove effective at higher doses. The non-steroid compound bufalin, also protected against UV-induced thymine dimers in vitro and in vivo, but has not yet been tested in a photocarcinogenesis model. Bufalin is more likely to be stable in the presence of light than vitamin D compounds, as it does not feature the broken B-ring seen in the molecular structure of $1,25(OH)_2D_3$. Provided that bufalin can mimic the photoprotective properties of other low-calcemic analogs being trialled, it could provide a cheap and efficient way to increase the level of protection against UV-induced skin damage.

Summary and Perspectives

It has been noted with some curiosity that while vitamin D receptor knockout mice are more susceptible to photocarcinogenesis, mice with a knockout of the 1α-hydroxylase enzyme and so unable to make $1,25(OH)_2D_3$ are not.[181] Given that a range of vitamin D compounds, and even the non-steroidal compound bufalin, appear to have some protective properties against UV-damage, it is likely that further metabolites of vitamin D, or even other over-irradiation products, will be found to contribute to endogenous photoprotection by the vitamin D system. The production of many vitamin D compounds in skin takes several hours, so it is likely that this system mostly protects against the next exposure to UV, in a similar manner to increased pigmentation and increased epidermal thickness, both well-known adaptive responses to UV. By applying vitamin D or vitamin D like compounds during or even immediately after UV exposure, it seems possible to enhance endogenous protection of skin from the various changes that lead to photocarcinogenesis. The search is now on to find relatively cheap and stable vitamin D-like compounds to add to sunscreens and possibly even after sun lotions to reduce the risk of UV damage.

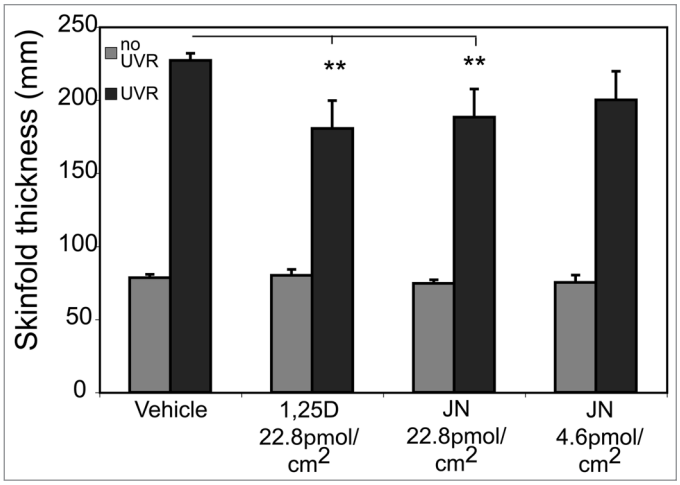


Figure 4. $1,25(OH)_2D_3$ and JN protect against UV-induced edema. $1,25(OH)_2D_3$ and JN reduce UV-induced edema in Skh:hr1 mouse skin. Mice were exposed to 1 × 3 MEDs (minimal erythemal doses) of solar-simulated UVR (3.98 kJ/m² UVB and 63.8 kJ/m² UVA) and were treated topically on the UV-irradiated dorsal surface immediately after UVR exposure with 100 mL of vehicle only, $1,25(OH)_2D_3$, or JN. Edema was measured as dorsal skin-fold thickness in the mice 48 h after UVR exposure. Significantly different from vehicle-treated UV-irradiated mice. **$p < 0.01$; $n = 5$. Reproduced from Cancer Prevention Research. K.M. Dixon, A.W. Norman, V.B. Sequeira, R. Mohan, M.S. Rybchyn, V.E. Reeve, G.M. Halliday, R.S. Mason (2011).

Disclosure of Potential Conflicts of Interest

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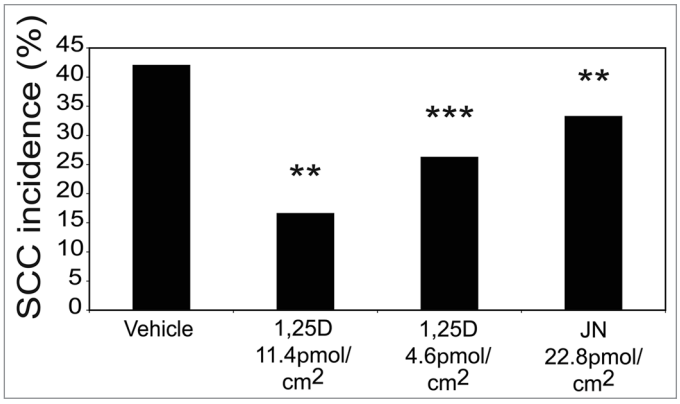

Figure 5. 1,25(OH)$_2$D$_3$ and JN protect against chronic UV-induced squamous cell carcinomas (SCC). Groups of 20 mice were exposed to a suberythemal dose of UVR on 5 d/wk for 10 wk. The solar-simulated UVR source was identical to that used in previous studies,[88] as described earlier for acute exposures, but the daily dose was lowered to mimic chronic human sunlight exposure without burning. The daily dose provided 0.658 kJ/m^2 UVB and 20.30 kJ/m^2 UVA, which is approximately one MED (minimal erythemal dose). Incidence of SCC was reduced by 1,25(OH)$_2$D$_3$ and JN at 40-wk time point. SCC incidence was calculated at each weekly time point as the percentage of mice in each group bearing at least 1 SCC. The Mantel-Haenszel log-rank test was performed to determine the difference in risk of developing an SCC between treatment groups and vehicle control. There was a significantly reduced risk of SCC development in both the 1,25(OH)$_2$D$_3$ and JN treatment groups compared with vehicle. The asterisks refer to the incidence risk assessed by the Mantel-Haenszel log-rank test over the whole time course. Significantly different from vehicle. ***$p < 0.001$; **$p < 0.01$; n = 20. Reproduced from Cancer Prevention Research. K.M. Dixon, A.W. Norman, V.B. Sequeira, R. Mohan, M.S. Rybchyn, V.E. Reeve, G.M. Halliday, R.S. Mason (2011).

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