# Photoprotection by 20-hydroxyvitamin D₃, a product of CYP11A1 in skin

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# ABSTRACT

Skin cancer is one of the most common health issues afflicting people in many countries, particularly in Australia. The major cause of skin cancer is ultraviolet radiation (UV), which is also needed for vitamin D synthesis. There is also evidence from the Mason group that the hormonally active form of vitamin D, 1α,25-hydroxyvitamin $D_3$ (1,25$(OH)_2D_3$), provides photoprotection. Vitamin D-related compounds having similar chemo-potential include lumisterol, a derivative of an over-irradiation product, which has been shown to be photoprotective in short term studies by the Mason group.

In previous studies, it had already been shown that the vitamin D receptor is critical to activate vitamin D induced photoprotection. Vitamin D receptor knockout mice were susceptible to carcinogenesis upon UV irradiation (Ellison et al., 2008). However, it was also noted that knockout of 1α-hydroxylase enzyme (CYP27B1), the enzyme essential for the production of 1,25$(OH)_2D_3$, does not increase susceptibility to photocarcinogenesis (Bikle, 2008). This led to the suggestion that other vitamin D compounds such as 20-hydroxyvitamin $D_3$ (20$(OH)D_3$) that are naturally made in irradiated skin without 1α-hydroxylation, through the action of the enzyme CYP11A1 on vitamin D itself could deliver photoprotective effects (Slominski et al., 2010).

Studies on the effects of 20$(OH)D_3$ on DNA damage and immunosuppression done in our laboratory by colleagues, revealed that 20$(OH)D_3$ is as effective

as $1,25(OH)_2D_3$ in reducing thymine dimer formation and UVR-induced immunosuppression in female hairless Skh:hr1 mice (Tongkao-on et al., 2015).

The aim of this project was to explore whether 20-hydroxyvitamin $D_3$ ($20(OH)D_3$), a product of vitamin $D_3$ hydroxylation, provides protection against UV-induced photocarcinogenesis. In order to investigate whether the protection against acute UV-induced damage translates into prevention of skin carcinogenesis, the major, *in vivo* component of this study involved a 40 week photocarcinogenesis study and contact hypersensitivity (CHS) response experiment, with $1,25(OH)_2D_3$ and $20(OH)D_3$ applied as topical treatments post UV irradiation. The results from the *in vivo* study showed that $1,25(OH)_2D_3$ was effective at inhibiting UV-induced tumour formation and progression, but $20(OH)D_3$ was not observed to be effective in reducing the following parameters: tumour incidence, tumour latency, tumour multiplicity and squamous cell carcinoma (SCC) incidence.

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# 1  INTRODUCTION

## 1.1  SKIN ANATOMY AND PHYSIOLOGY

The skin forms the outermost layer of the human body and serves as the organ of first line of defence, protecting the internal tissue organs from external sources of damage such as UV irradiation, chemical, bacterial and viral infections. Other than its protective effects, skin is also involved in important functions such as thermoregulation, sensory perception, control of fluid loss and vitamin D synthesis.

The skin is also known as the integument and consists of two layers; the epidermis and the underlying dermis. Beneath the dermis is a subcutaneous tissue known as hypodermis, which is theoretically not considered to be part of the skin.

### 1.1.1  EPIDERMIS
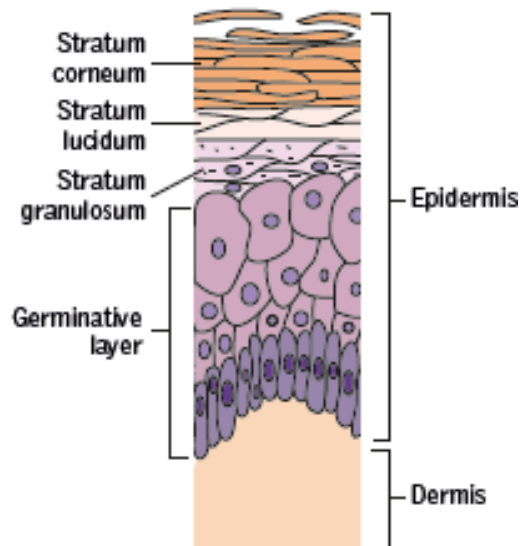


**Figure 1.1 Layers of epidermis (McLafferty et al., 2012)**

The epidermis is the outermost layer of the skin. It is continuously renewing, stratified, squamous epithelium that is further subdivided into five layers; stratum

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corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale. These layers of epidermis consist of four different cell types that are interdependent functional units. Each cell relies upon the modulation and regulation of surrounding tissue cells. The cells are called keratinocytes, melanocytes, Langerhans' cells and Merkel cells. Each layer of epidermis has a different composition of these cells, reflecting their different structure and roles.

The deepest layer, stratum basale, consists of single row of columnar keratinocytes that are capable of dividing as well as melanocytes. Stratum spinosum is the cell layer having rounder and 'spikier' appearance, where the keratinocytes are tightly held by desmosomes, contributing to the tensile strength and flexibility of the skin. This layer also contains Langerhans' cells, specialised dendritic cells of the immune system that are important in immune surveillance. Stratum granulosum consists of three to five layers of flattened keratinocytes that undergo apoptosis in this layer. These keratinocytes lose nuclei in this layer are left mostly with keratin. Stratum lucidum contains three to five layers of clear, dead keratinocytes, and is found only in areas where the skin is thick. Stratum corneum is the uppermost layer of the epidermis and consist of 25-30 layers of flattened, dead keratinocytes (McLafferty et al., 2012).

## 1.1.1.1 KERATINOCYTES
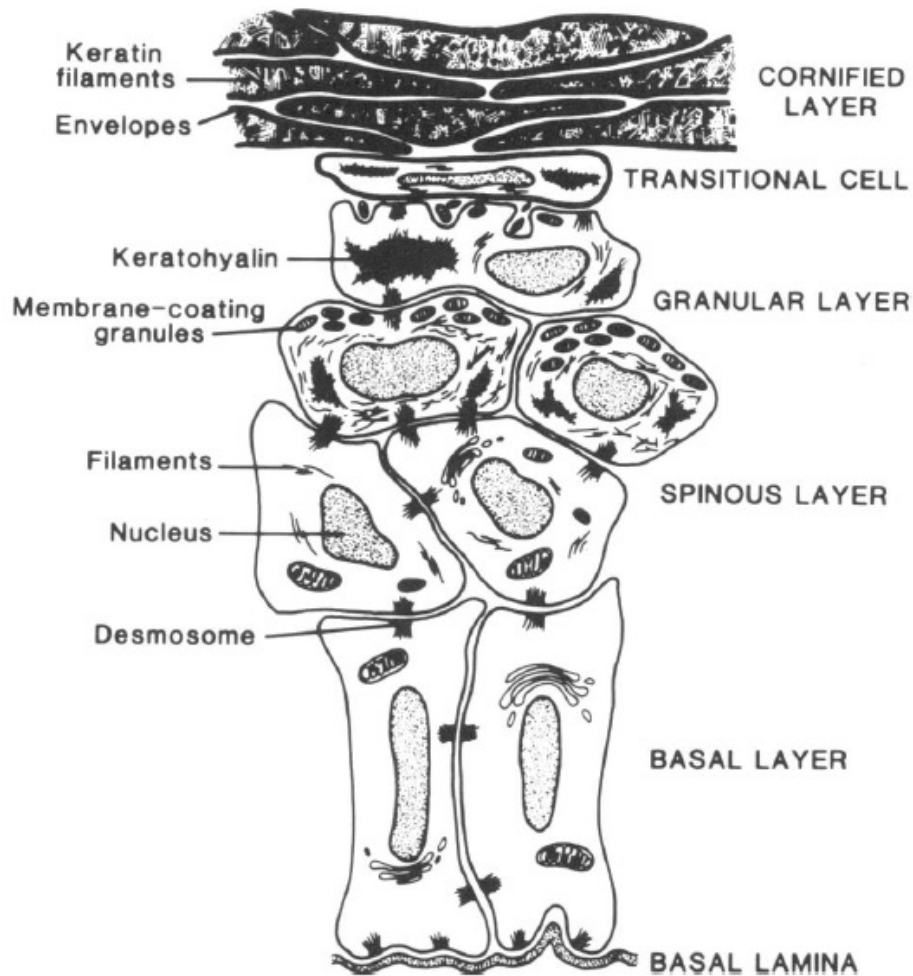
**Figure 1.2 Keratinisation in the epidermis. (Eckert, 1989)**

Keratinocytes are the most abundant type of cells in the epidermis, constituting about 80% of total population of cells. A keratinocyte is characterised by keratin filaments; proteins that serve a structural role.

Keratinocytes are spread over the five layers of epidermis, with each layer reflecting the state of differentiation and the synthetic properties of keratinocytes. The differentiation is also known as keratinisation, a process consisting of morphologic and metabolic changes in keratinocytes that occurs progressively as they move up the layers. Initially, keratinocytes arise at the basal layer of the epidermis and as they

migrate to the spinous layer, their shapes become more flattened and abundant of desmosomes, which promote cell-to-cell adhesion, thereby providing resistance to mechanical stress. Eventually, keratinocytes terminally differentiate to form a cornified layer, a dead surface layer of the skin (Figure 1.1).

During this process, the keratinocyte undergoes some major changes, including an increase in cell size, structural reorganization, eventual loss of organelles from the cell, dehydration and the synthesis of important structural and catalytic proteins. Some of important proteins synthesised include involucrin, the keratins, filaggrin, and transglutaminase, all of which are involved in maintaining integrity and cornification of the epidermis (Eckert and Rorke, 1989).

Mitotically active keratinocyte stem cells primarily reside in the stratum basale layer. These stem cells are clonogenic cells, cells that have a long life span and are able to undergo divisions while moving up towards the epidermal surface, where they are terminally differentiated (Haake and Holbrook, 1999).

## 1.1.1.2  MELANOCYTES

Melanocytes are dendritic cells responsible for pigmentation of the skin. They are derived from the neural crest, and reside mostly in the stratum basale. Melanocytes are characterised by a special organelle called melanosome, which synthesises melanin, a pigment responsible for colouring of the skin. Each melanocyte is thought to coexist with 36 basal and suprabasal keratinocytes, forming an 'epidermal melanin unit'. In this unit, melanocytes and keratinocytes interact with each other for functional and structural roles. The anatomical relationship of an epidermal melanin unit is important for melanogenesis.

Melanogenesis or cutaneous pigmentation, is a two step process. First of all, melanosomes within a melanocyte produce a pigment, melanin. Then, through the dendritic interaction by melanocytes with keratinocytes, melanin is transferred into the keratinocyte. The melanin covers over the nuclei of keratinocyte, acting efficiently as a protection against DNA damage. The distribution of melanin in the upper layers of the epidermis play a significant role in determining its photoprotective value to underlying cells. Lower layer of epidermis contains not only melanocytes but also keratinocyte stem cells, which are highly proliferative and are not lost via desquamation and thus are able to give rise to various types of skin cancers. However, with the melanin protecting the cells in the upper layer of epidermis from UVR, UVR-induced DNA damage in the lower layer of epidermis is prevented (Yamaguchi et al., 2006).

### 1.1.1.3 LANGERHANS CELLS

Langerhans cells have an immunosurveillance ability and mainly reside in the suprabasal epidermal layers but also in the spinous and granular layers of the skin. However, Langerhans cells are not exclusive to the skin but are also found in the other parts of the body such as oral cavity and the oesophagus (Haake and Holbrook, 1999).

As part of immune surveillance, Langerhans cells are antigen-presenting cells and thus are able to ingest antigens and present them for T cell stimulation. They are dendritic cells that do not form junctions with the neighbouring cells. Langerhans cells thus serve important functions and are necessary for the defence against diseases and UV radiation (Haake and Holbrook, 1999). Also, recent studies have found that resting Langerhans cells induce the proliferation of the skin resident regulatory T cell, cells that are thought to regulate inflammation in peripheral tissue

and possibly suppress cytokine production of effector T cells, though the role is incompletely characterised (Seneschal et al., 2012).

### 1.1.1.4  MERKEL CELLS

Similar to melanocytes, Merkel cells are primarily found in the stratum basale scattered among the keratinocytes. Merkel cells serve as mechanoreceptors with flattened process of sensory neuron called a Merkel disc, and process touch signals upon application of a tactile stimulus. When keratinocytes are deformed, they send signals to the Merkel cells and as a result chemical transmitters are released from the Merkel cells (Haake and Holbrook, 1999; McLafferty et al., 2012).

### 1.1.1.5  $\alpha\beta$ T CELLS

Human skin contains large number of $\alpha\beta$ T cells, which are memory T cells consisting of both CD4+ and CD8+ subsets. Of CD4+ subset, about 10-20% of T cells express Foxp3, a marker indicative of regulatory T cell phenotype (Treg). These regulatory T cells produced more of interleukin 17 (IL-17) in psoriatic skin than in normal skin, and also upon exposure to UVR.  The role of this population of cells has been highlighted in protection against cell injury, as IL-17 is known to induce molecules involved in cell apoptosis and growth arrest, processes that are important in DNA repair response (Heath et al., 2013; MacLeod et al., 2014).

### 1.1.1.6  OTHER CELLS

Recent evidence suggests important immunostimulatory role of dermal dendritic cells (DDCs) that are thought to arise from bone marrow HSC-derived lineage. They induce IL-10 secreting regulatory T cells and T cell hyperresponsiveness to antigenic stimulation. Cytokines such as IL-10 secreted by T-regs is known to suppress cellular

immune reactions such as contact hypersensitivity reactions (CHS). This suggests that DDCs and regulatory T cells have role in suppressing skin inflammation (Haniffa et al., 2015).

In addition, studies have found skin-invading immune cells known as $\gamma\delta$ T cells that may drive skin inflammation. The fact that this unconventional lymphocyte population, rather than the $T_H17$ subset, is the primary source of IL-17 in psoriatic patient suggests its potential role in cellular immune reactions upon exposure to UVR (Becher and Pantelyushin., 2012). Also, the role of $\gamma\delta$ T cells in epidermal homeostasis and tissue repair have been highlighted as they seem to produce growth factors such as fibroblast growth factor (FGF7), FGF9 and insulin-like growth factor, which all promote the survival of keratinocytes (Heath et al., 2013; MacLeod et al., 2014; Pasparakis et al., 2014).

### 1.1.2  DERMIS
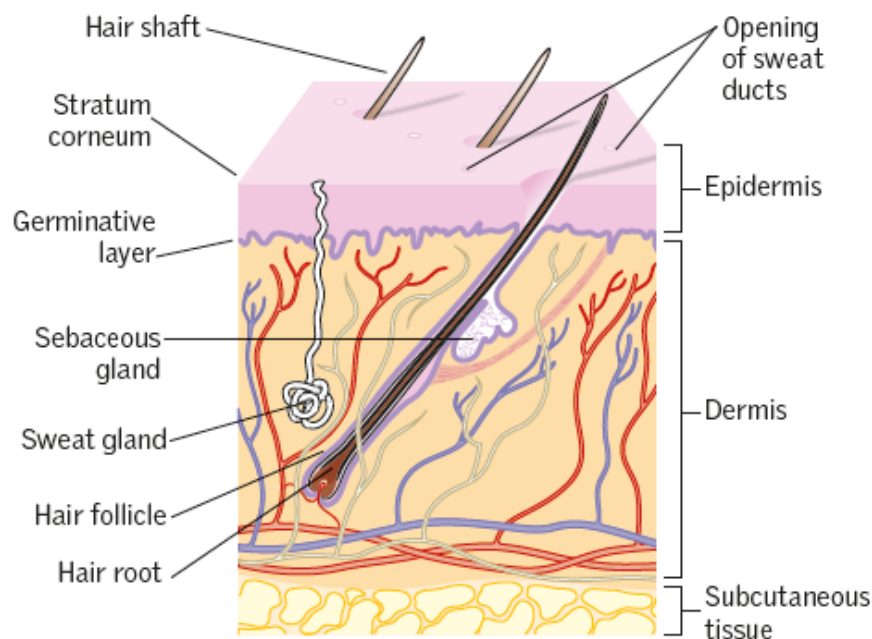


**Figure 1.3 Layers of the skin. (McLafferty, 2012)**

The dermis is the layer beneath the epidermis, which is further subdivided into two layers; reticular and papillary layers. The reticular layer contains the fibroelastic tissue, network of collagen, elastic fibres and the extracellular matrix, providing physical support for the epidermis. The papillary layer contains the blood vessels to provide nutrients for the epidermis, lymph vessels, nerve endings, glands and hair follicles (McLafferty et al., 2012).

## 1.1.2.1 FIBROBLASTS

Fibroblasts are the most abundant type of cells in the dermis. They are the cells that produce various different types of collagen. Synthesis of collagen and other components of the extracellular matrix are especially important in healing of the skin. Dermal fibroblasts also synthesise other components of the extracellular matrix such as elastin, proteoglycans and fibronectin (Rittie, 2005).

## 1.1.3 HYPODERMIS

The hypodermis is composed mostly of adipocytes. It is technically called a subcutaneous tissue in which fatty cells serve as energy reservoir and a mechanical shock absorber. Its thickness varies along different parts of human body (Haake and and Holbrook, 1999).

## 1.1.4  COMPARISON OF SKIN IN MICE AND HUMANS
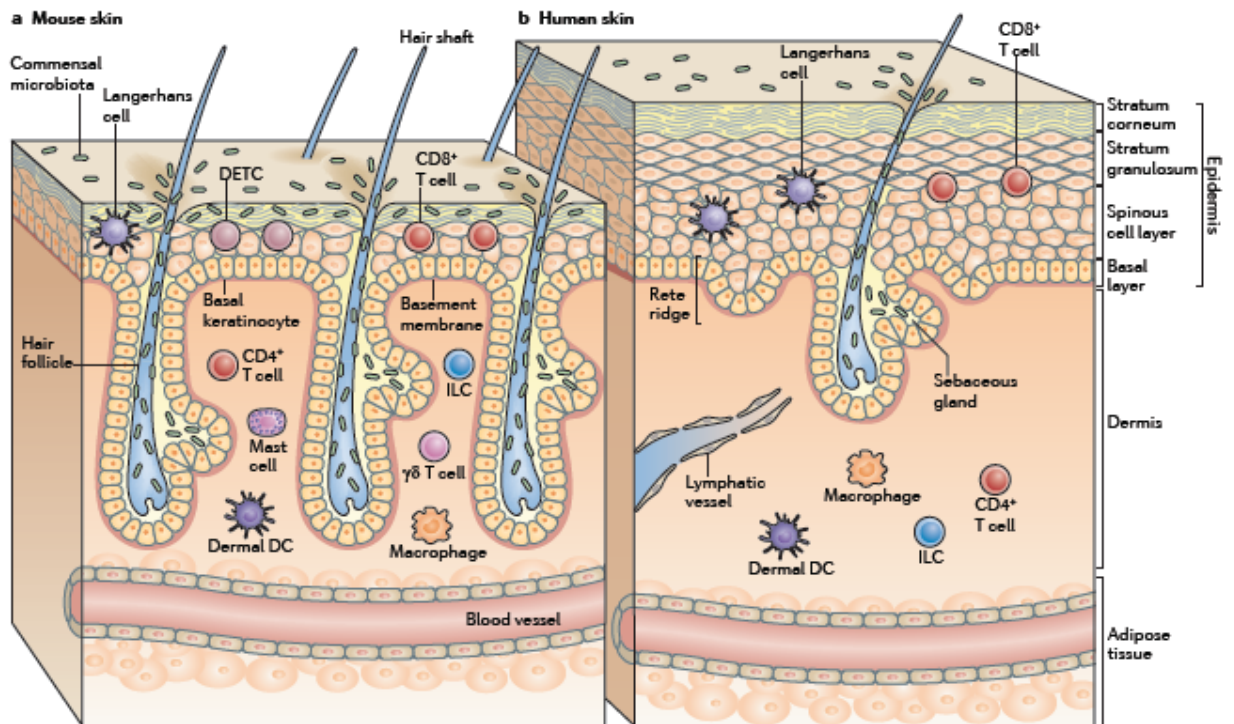


**Figure 1.4 Skin in mice and humans (**Pasparakis et al., 2014)

Structures and cellular components of the skin in mice and humans are largely similar. Like human skin, mouse skin also consists of the epidermis, dermis and hypodermis. Main differences between two species are that human skin has a thicker epidermis and dermis than mice skin, and a population of Vγ5+ dendritic epidermal T cells (DETCs) that are prominent in mice skin are absent in human skin. These differences should be taken into account when interpreting the cellular inflammation studies in mouse models in relation to mechanisms that drive inflammation in human skin (Pasparakis et al., 2014).

## 1.2 ULTRAVIOLET RADIATION (UVR)

Human skin is constantly under risk of DNA damage due to various environmental factors, of which UVR is known to be the most potent environmental factor to cause skin tumours (Brenner and Hearing, 2008).

### 1.2.1 ENERGY FROM UVR

UVR reaching the earth consists of three types, long wavelength UVA (315 to 400 nm), short wavelength UVB (290 to 315 nm) and UVC (100 to 290 nm) radiation (Figure 1.3). UVR that reaches the surface of the earth is mostly UVA and a small percentage (approximately 5%) UVB, whilst UVC is filtered out by atmospheric oxygen and the ozone layer. Thus, human skin is mostly exposed to wavelengths UVA and UVB. UVB is known to induce DNA damage directly because human DNA absorbs UVR maximally in the range of 245 to 290 nm. On the other hand, UVA induces indirect DNA damages by producing reactive oxygen species (ROS). (McGregor, 1999).
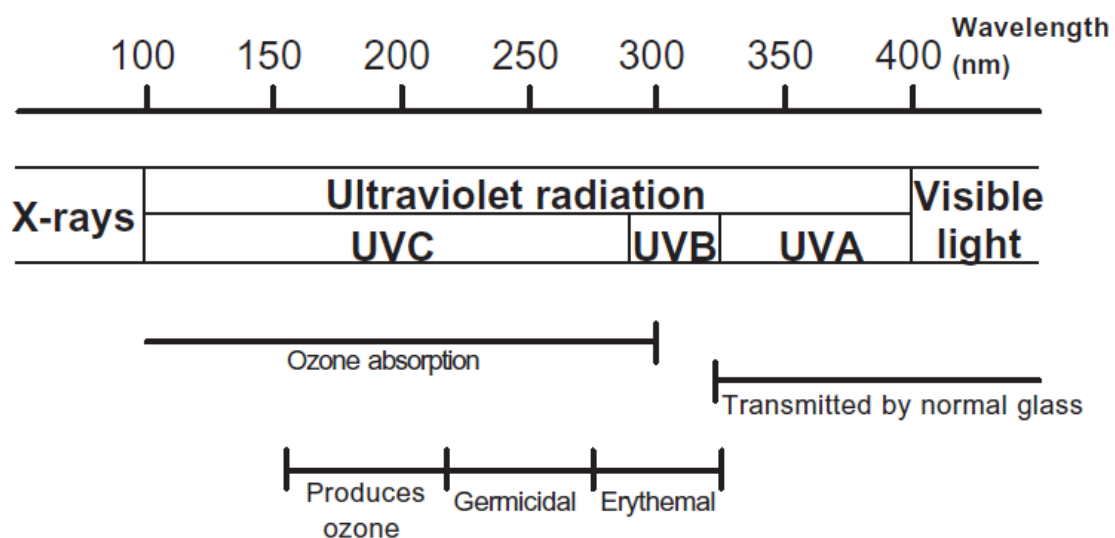


**Figure 1.5 UVR spectrum. (Matsumura and Ananthaswamy, 2004)**

## 1.2.2 CUTANEOUS PATHOLOGICAL EFFECTS

In general, UVR is responsible for various pathological effects of the skin. Some of the acute effects may include photodamage, erythema, mutation and immunosuppression. UVR can also induce chronic effects such as photocarcinogenesis (Brenner and Hearing, 2008).

## 1.2.3 GENERAL BIOLOGICAL EFFECTS OF UVR

As UVC is blocked by the Earth's stratospheric ozone layer, it is mainly UVA and UVB that have significant biological consequences on human skin and eyes. Although UVA is the more predominant type of solar UV radiation than UVB, it is known to be less carcinogenic than UVB, and is mainly responsible for aging and wrinkling of the skin (Matusumura and Ananthaswamy, 2004). However, there are some studies that suggest that UVA may have an important role in cutaneous melanoma (Moan et al., 2014). The effects of UVB from the sunlight are profound in that upon absorption into the skin, UVB is known to cause sunburn inflammation (erythema), oedema, skin pigmentation, photoaging, immunosuppression and skin cancer (Kochevar et al, 1999.; Mcgregor and Hawk 1999; Pathak et al, 1999). The extent of damage done by UVB is dependent on the depth of UVB penetration in the skin layers (Figure 1.4).
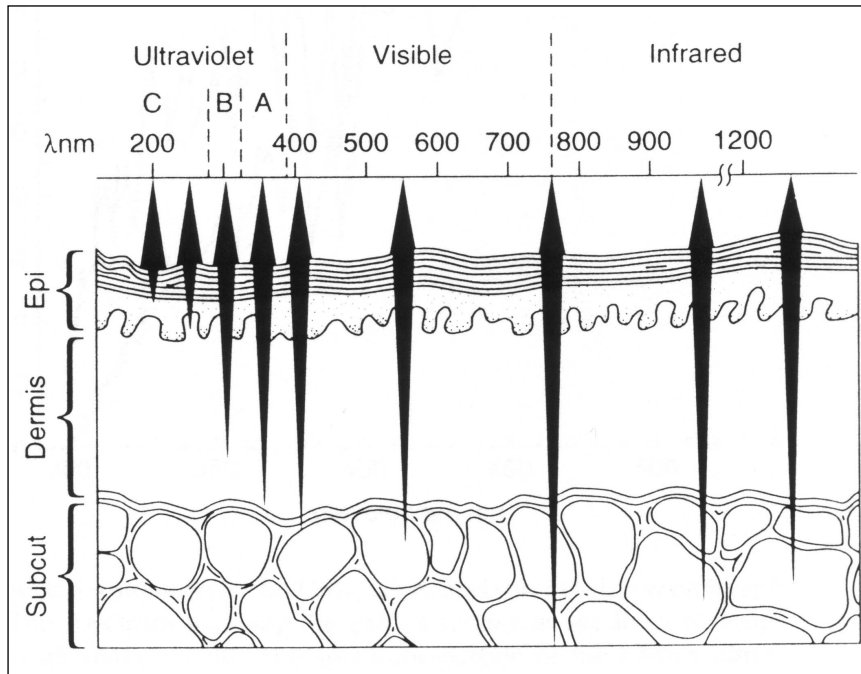
**Figure 1.6 UVR penetration through the skin. (Kochevar et al. 1999)**

As is seen from the Figure 1.4, radiation with shorter wavelengths travels less deeply than that with longer wavelengths. For this reason, UVA and UVB penetrate mainly the epidermal and the dermal layers of the skin, causing most damage in those areas.

Although it is widely known that UVB delivers immunosuppressive effects, the potency of UVA on immune suppression has remained somewhat questionable as seen in various findings. This difference may be due to differences in subjects studied, differences in the irradiation sources, and especially the difference in the dosage and time points of irradiation (Kuchel et al, 2002). Firstly, a study from Poon *et al* showed that UVB and UVA induced immune suppression in a dose- and time-dependent manner in human subjects. It was found that UVB caused immune suppression within 24 hours, whilst UVA required 48 hours to induce suppression. Moreover, an interaction of the two caused immune suppression after 72 hours (Poon et al, 2005). Also, the results in Reeve *et al* suggested that an interaction

between UVA and UVB bands is more complex in terms of immunological function. Their results demonstrated that UVA exposure delivers photoprotective effects against UVB induced immune suppression in hairless mice. It was suggested in the study that UVA exposure possibly induce the formation of unidentified, yet possibly immunoprotective photoproducts (Reeve et al, 1998). In contrast, another study revealed that UVA does not induce a significant degree of immune suppression as with UVB in human subjects, but only augments solar-simulated ultraviolet-radiation induced immunosuppression (Kuchel et al, 2002).

## 1.2.4 ERYTHEMA

Erythema, or sunburn inflammation, is the body's initial response to UVR with signs of redness, warmth, pain and swelling. These signs seem to be biphasic in animals in response to UVB exposure, meaning that they could appear immediately after exposure or be delayed for minutes to hours. However, with UVA exposure, immediate erythema seems to appear more regularly.

The degree of erythema is largely dependent on the amount of exposure to UVR, but also on the types of UV sources, age, skin pigmentation, skin thickness and other environmental factors. Exposure to UVR is commonly expressed in terms of minimal erythemal dose (MED), the lowest radiation exposure to UVR sufficient to produce just perceptible erythema (JPE) on exposed skin after 24 hours. Although the onset of erythema in response to UVB and UVA is different, the intensity of erythema is higher in both cases following the exposure to multiple MEDs (McGregor, 1999).

## 1.2.5  UVR-INDUCED DNA DAMAGE

The types of UVR-induced DNA damage can be chiefly categorised into two types: DNA lesions caused by absorption of UVR by DNA and oxidative damage via formation of reactive oxygen species (ROS). The latter may be less important in mutagenesis and carcinogenesis.

## 1.2.6  DNA LESIONS

UVR is considered to be more detrimental when it induces DNA damage to the lower epidermal layers of skin than the upper layers. This is because the keratinocyte stem cells that reside in the lower layers are proliferative and are able to give rise to different types of skin cancers (Yamaguchi et al., 2006).

UVR-induced DNA lesions can occur due to the absorption of the UVR by DNA and hence the extent and the type of DNA damage are dependent upon the wavelength of the incident photons that strike the cells (Douki et al., 2003). The major type of DNA photolesion induced by the UVR, cyclobutane pyrimidine dimers (CPDs), occurs in adjacent pyrimidines (thymine and/or cytosine), resulting in the formation of dimers. Upon DNA absorption of UVB photons, the cyclobutane pyrimidine dimers, as shown in Figure 1.5a, are formed. These are dimers between the C-4 and C-5 carbon atoms of any two adjacent pyrimidine dimers, but mostly between thymine-thymine dimers (T<>T), with thymine-cytosine (C<>T or T<>C) and cytosine-cytosine (C<>C) occurring to a lesser extent (Matsumura and Ananthaswamy, 2002b; Cooke et al., 2003).

Although it is known that UVA is poorly absorbed by DNA, UVA may also contribute to the formation of CPDs. UVA-induced CPD formation occurs via photosensitisation that induces oxidative damage either by charge transfer from excited endogenous

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chromophores, or by reactions with reactive oxygen species (ROS) that are generated at UVA wavelengths (Perdiz et al., 2000). On top of that, it has recently been discovered that UVA has a role in inducing cyclobutane pyrimidine dimers in melanocytes. A new mechanism was suggested that the cyclobutane pyrimidine dimers are generated at least 3 hours after UVA exposure in murine melanocytes, at which point generation was offset by DNA repair. This implicated that the cyclobutane pyrimidine dimers continue to be generated in melanocytes long after UV exposure ends. This delayed production of CPDs in melanocytes was coined as "dark CPDs" (Premi et al., 2015).

The second type of photolesion caused frequently by UVR is the 6-4 photoproduct [(6-4)PP]. As shown in Figure 1.5b, (6-4)PPs are formed between the 5′ C-4 position and the 3′C-6 position of two adjacent pyrimidines, most frequently between thymine-cytosine (T<>C) and cytosine-cytosine (C<>C) residues (Matsumura and Ananthaswamy 2002b). The ratio of (6-4)PP formation is about 20-30 per cent that of CPDs, but this ratio appears to be dependent on the DNA sequence and on the chromatin environment (Tornaletti and Pfeifer, 1996). Both CPDs and (6-4)PPs affect the torsional flexibility of the DNA double helix at the site of damage, causing distortions and ultimately affecting the base-pairing ability. These in turn impede the key cellular processes such as transcription and replication, thereby inducing reduction of RNA synthesis, cell cycle arrest, and cell apoptosis (Tornaletti and Pfeifer., 1996; Schul et al., 2002). If not repaired before DNA replication, UVR-induced DNA lesions can lead to mutations in the DNA sequences. These mutations are in the form of C to T and CC to TT transitions, known as UV "signature" mutations (Matsumura and Ananthaswamy, 2002b).

**a** Formation of a cyclobutane pyrmidine dimer

Sugar—phosphate
backbone of DNA

Thymine          Thymine                    Thymine dimer
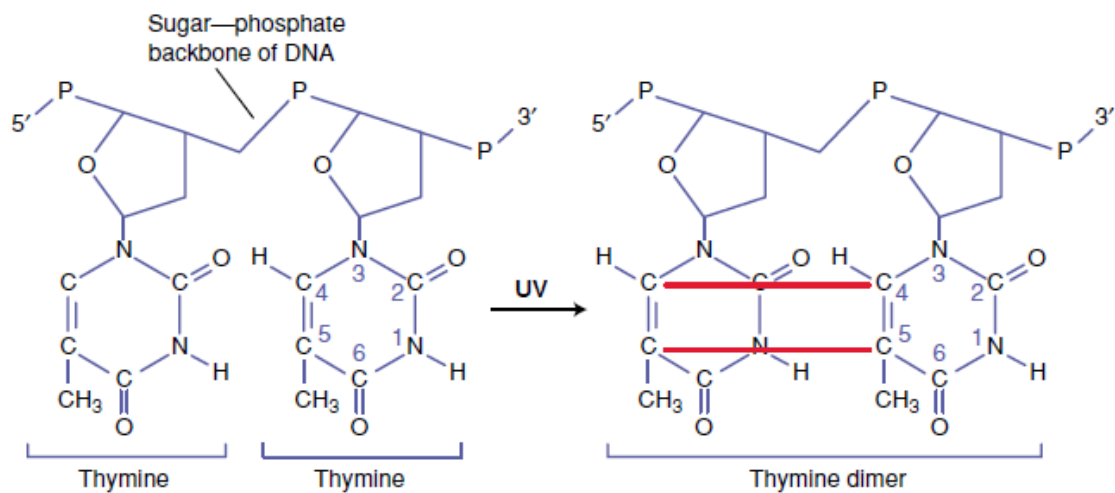
**b** Formation of a (6-4) photoproduct

Cytosine          Thymine                    (6-4) photoproduct
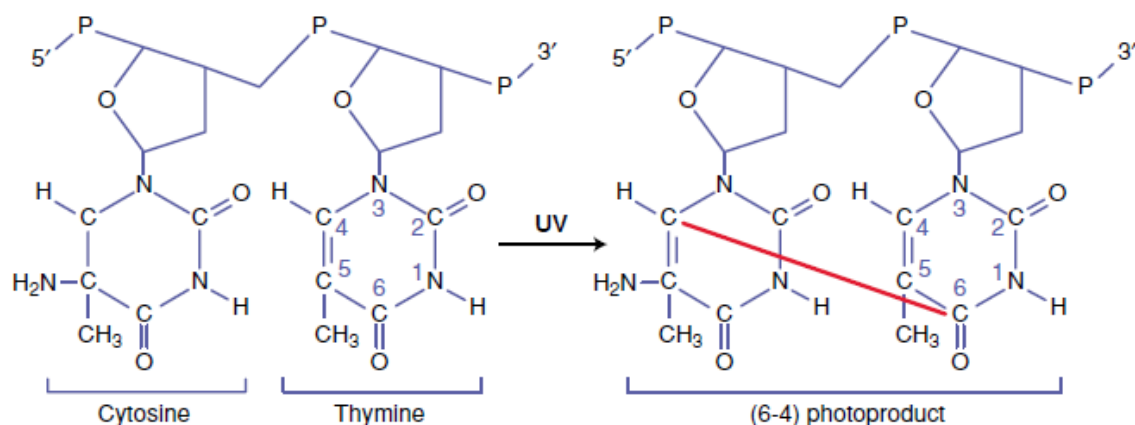
**Figure 1.7 a. Molecular structures of CPD and b. (6-4) photoproduct. (Matsumura and Ananthaswamy, 2002b)**

The signature mutations are caused by noninstructional insertion of adenine (A) nucleotide opposite the UV lesion in the newly synthesised DNA strand by DNA polymerase, a mechanism known as the 'A rule'. This results in the mispairing of cytosine with adenine residues, causing delay in the SOS repair of lesions whilst facilitating a spontaneous deamination of cytosine in CPDs. UVR-induced mutagenesis then occurs in the subsequent round of DNA replication including the strands with base-pair changes. This is the case in CC cyclobutane dimers, where a CC to TT transition occurs, as two A residues, rather than guanine (G) residues, are placed by default opposite the lesion, resulting in mutagenesis. On the other hand, in

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more common TT cyclobutane pyrimidine dimers, noninstructional insertion of adenine would not cause mutation as adenine residue is normally paired with thymine residues. Similar mutagenesis could also occur in (6-4)PPs. In (6-4)PPs between a pyrimidine and a C residue, the 5' residue base-pairs correctly but the 3' residue resembles a non-instructional site, meaning that a C to T transition occurs as an A residue is inserted opposite the C residue by default (Tessman et al., 1992; Matsumura and Ananthaswamy, 2004).

Therefore, UVR-induced mutagenesis can occur via both types of DNA lesions, CPDs and (6-4)PPs. Mutagenesis is detrimental to a cell as it disrupts controlled cell growth and proliferation, which may eventually lead to cancer formation. It is believed that the CPDs are the principal mutagenic lesions in mammalian cells, though (6-4)PPs are also mutagenic. Part of the reason for this is the fact that the repair of (6-4)PPs is generally far more rapid than that of CPDs (Mouret et al., 2006).

## 1.2.6.1 OXIDATIVE DNA DAMAGE

It was previously noted that UVR could directly cause DNA mutations by inducing the formation of CPDs and (6-4) photoproducts. On top of that, UVR can also cause DNA mutations indirectly via the formation of reactive oxygen species (ROS), These include the superoxide anion ($O_2^{\bullet -}$), hydroxyl radicals (HO·), singlet oxygen ($^1O_2$), and hydrogen peroxide ($H_2O_2$). Reactive oxygen species are normal byproducts of cellular metabolism, though they become mutagenic and are the potential initiators and promoters of carcinogenesis when produced in excessive amounts (Zhang et al., 1997). Typical targets of UVR-induced ROS include proteins, lipids and DNA. Of these, the guanine base of DNA seems to be a particular target of significance. ROS cause several types of DNA damage including strand breaks, modifications of

pyrimidine bases and DNA-protein cross-links (Hattori et al., 1996; Klaunig et al., 2010).

An appropriate biomarker for oxidative DNA damage both *in vitro* and *in vivo* is the oxidatively modified DNA base product 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) otherwise known as 8-hydroxy-2'-deoxyguanosine (8-OHdG). Out of reactive oxygen species, hydroxyl radicals seem to be the major culprit of formation of 8-OHdG, though singlet oxygen and photodynamic action also play a role. The mechanism by which HO· induces the formation is that it initially attacks the DNA strand, specifically the nucleobase guanine, forming a radical adduct and then through subsequent electron abstraction to form 8-OHdG (Zhang et al., 1997; Valavanidis et al., 2009). This oxidation product has been suggested to play a role in UVR-induced skin carcinogenesis and the suggestion is further supported by the fact that mutagens and carcinogens other than UVR, such as reducing agents and metals also modify guanine residues in DNA at the C-8 position (Kasai and Nishimura, 1984).

Although both UVA and UVB can cause oxidative damage, UVA is generally more associated with indirect oxidative damage whilst UVB is regarded to be primarily responsible for direct DNA damage via pyrimidine dimer formation (Halliday, 2005). Yet, the proportion of UVA and UVB contributing to oxidative damage remains controversial as it was found that upon repeated exposure to UVB, there is an increase in 8-OHdG in the epidermis of hairless mice (Hattori et al., 1996).

## 1.2.7 DNA REPAIR

Cells are equipped with special DNA repair machinery in order to counteract DNA-damaging compounds. Depending on the type of DNA damage, different repair systems are activated, including single or double stranded break repair, base excision repair, mismatch repair and nucleotide excision repair (NER). Despite various types of DNA damage being identified, it is known that more than 99% of UVR-induced DNA damage consists of chemical base modifications, with CPDs and (6-4)PPs being the most common types of photolesion (Schul et al., 2002).

Nuclear excision repair (NER) is a complex process involving more than 30 gene products in which the following five steps are identified (Aboussekhra et al., 1995): (1) recognition of a DNA lesion; (2) single strand incision at both sides flanking the lesion; (3) excision of a single stranded DNA nucleotide (possibly 24_/32 bases); (4) DNA repair synthesis to replace the excised DNA lesion; and (5) ligation of remaining single stranded nick (Ichihashi et al., 2003).

In the NER process, xeroderma pigmentosum (XP) factors are the best characterised components. These factors are proteins that are required for the initial steps of excision repair process, which correct the DNA deficiency of the seven complementation groups of XP cells, XP-A to XP-G. For instance, XPA is a DNA damage-binding protein and XPC is a single-stranded DNA-binding protein (Naegeli and Sugasawa., 2011).

UVR-induced DNA damage such as cyclobutane pyrimidine dimers and (6-4)PPs are normally repaired by the nucleotide excision repair (NER) pathway. NER is divided into two subpathways known as transcription-coupled repair (TCR) and global genomic repair (GGR). TCR is responsible for the repair of genes that are being

actively transcribed, whereas the GGR pathway is a relatively slower repair pathway and is mainly responsible for transcriptionally inactive parts of the genome (Berneburg, 2000). The two pathways differ only in the initial step. Mechanisms underlying the transcription-coupled repair pathway are not clear yet, but it requires the Cockayne syndrome (CS) A and B proteins (CSA and CSB) in the initial damage recognition. The subsequent steps for both pathways are identical and explained below and illustrated in figure 1.6.

a) Initially in damage recognition step, GGR begins with XPC gene first forming a complex with hHR23B, a human RAD23 homolog, and then binds to DNA lesion in the genome. In transcription coupled repair, however, a stalled RNA polymerase II along with CSA and CSB proteins serve as damage recognition signal.

b) In DNA unwinding step, the lesions are opened by the concerted action of XPA, replication protein A (RPA), and the bi-directional XPB/XPD helicase subunits of the transcription factor IIH (TFIIH) complex.

c) During incision of the damaged DNA, the exchange repair cross complementing (ERCC1)/XPF complex cuts at the single-strand to double-strand transition on the 5' side of the damage, and XPG cuts at the 3′ side of the open complex.

d) Finally, DNA excision and synthesis is accomplished by mammalian DNA replication factors such as the heterotrimeric replication protein A (RPA), replication factor C (RF-C), proliferating cell nuclear antigen (PCNA), and DNA polymerase δ and ε (Matsumura and Ananthaswamy 2002b; Ichihashi et al., 2003).
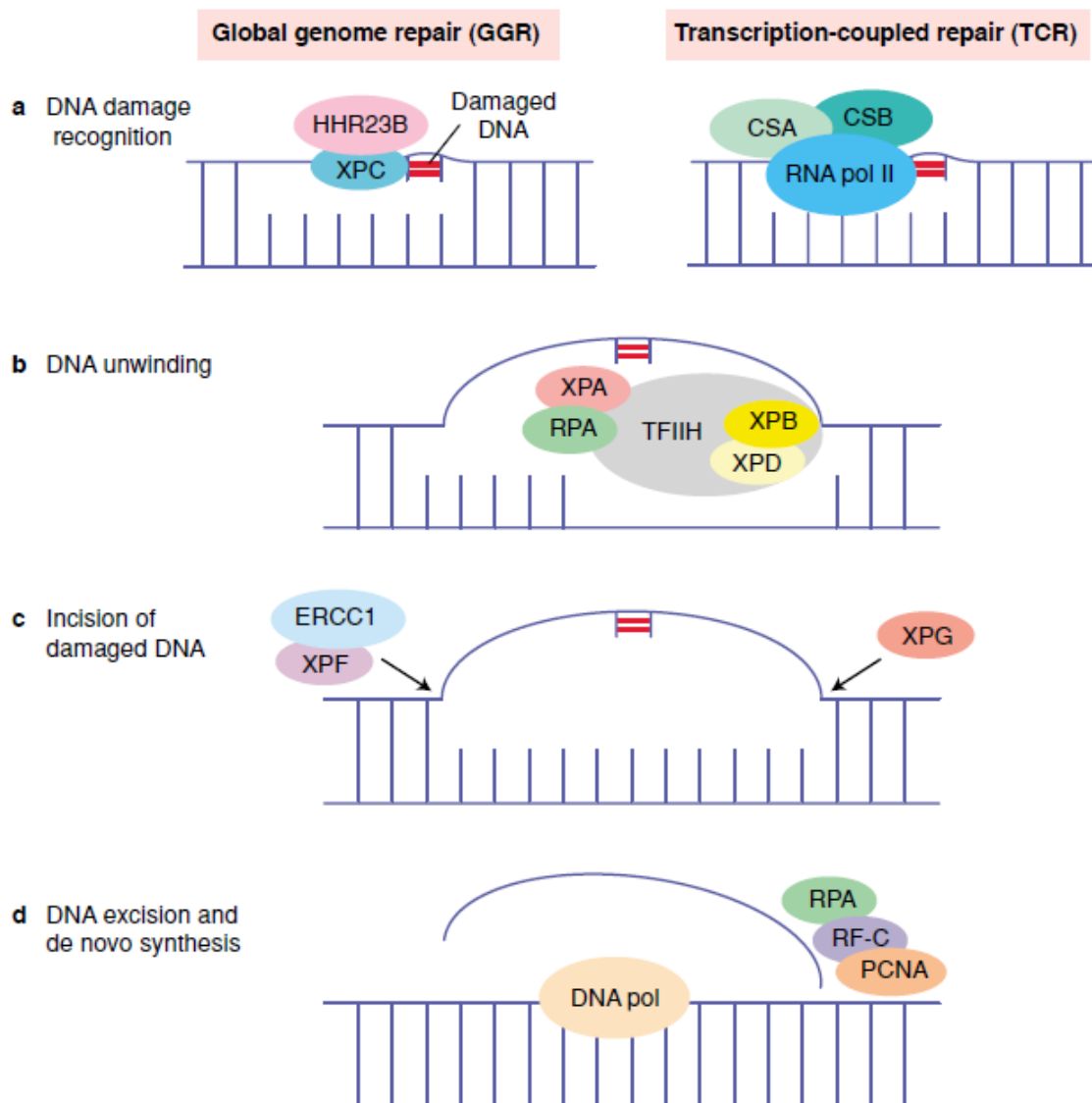
**Figure 1.8 Proposed model for nuclear excision repair subpathways: NER consist of two subpathways, global genomic repair (GGR) and transcription-coupled repair (TCR). (Matsumura and Anathaswamy, 2002b)**

Defects in both subpathways could lead to three distinct diseases: xeroderma pigmentosum (XP), the Cockayne syndrome (CS) and trichothiodystrophy (TTD). Of these, only XP patients have a predisposition to skin cancer and photosensitivity (Matsumura and Anathaswamy, 2002b).

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### 1.2.8 p53

Since its discovery in 1979 as a cellular Simian virus 40 large T antigen-binding protein, p53 has been proposed as the 'master regulator' of diverse cellular processes including cancer formation (Farnebo et al., 2010). Undoubtedly, p53 is one of the most frequently mutated genes in skin carcinogenesis (Nataraj et al., 1995; Decraene et al., 2001). The human p53 gene encodes a 53-kDa weight protein containing 393 amino acids, which is involved in the regulation of the cell cycle and facilitation of DNA repair (Harlow et al., 1985; Farnebo et al., 2010). A sequence-specific DNA binding domain is located within the central, conserved portion of the protein and encompasses amino acids 102-292, a region where the majority of missense mutations have been detected in tumours of cancer patients (Hollstein et al., 1991). As shown in Figure 1.7, p53 responds to many different cellular stressors including UV, and subsequently interacts with other genes including the proto-oncogene MDM2, cyclin-dependent kinase inhibitor WAF1/CIP1/p21 gene product, DNA damage inducible GADD45, and BAX and BCL2 to induce cellular processes such as cell cycle arrest, DNA repair and apoptosis.

The importance of the p53 gene in DNA repair was noted in earlier studies in which the removal of photoproducts in human cells by nuclear excision repair (NER) was demonstrated to be regulated by the p53 gene (Ford and Hanawalt, 1997). This occurs as UVR-induced photodamage induces an elevation of p53 expression in the skin, blocking the cell cycle at G1-S phase, and thereby allowing the repair of the damage and/or induction of apoptosis to eliminate cells containing severely damaged DNA (Ziegler et al., 1994; Ouhtit et al., 2000). The Mason group has reported a clear increase in nuclear p53 protein expression, observed 3 hours post UVB irradiation in irradiated keratinocytes treated for 24 hours with $1,25(OH)_2D_3$, thereby highlighting the role of p53 in photoprotection by $1,25(OH)_2D_3$ (Gupta Dixon et al., 2007). This

p53 accumulation and activation are believed to be triggered by DNA lesions, and are mediated through protein phosphorylations and acetylations.

At least 20 sites in the human p53 protein are modified in response to the activation of different stress signalling pathways (Saito et al., 2003). UVR leads to phosphorylation of p53 at multiple serine residues, including Ser15, Ser20, Ser33, Ser37, Ser46 and Ser392 (Ouhtit et al., 2000; Matsumura and Anathaswamy, 2004; Melnikova and Anathaswamy, 2005). Phosphorylation of p53 at various N-terminal serine residues, including Ser15, Ser20 and Ser37, has been reported to stabilize p53, whilst phosphorylation at the C-terminal residues Ser315 and Ser392 has been suggested to regulate the oligomerization state of p53 and its ability to bind DNA in a sequence-specific manner. Acetylation of lysine residues including Lys370, Lys372, Lys373, Lys381, and Lys382 within the C-terminal regulatory domain has been found to enhance sequence-specific DNA binding and play a critical role in Mdm2-mediated ubiquitination and subsequent degradation (Luo et al., 2003; Saito et al., 2003).

In normal conditions, p53 is a short-lived nuclear protein with a half-life of ~5–20 min in most cell types studied (Giaccia and Kastan, 1998). Under normal cellular conditions, p53 is bound to Mdm-2 protein, a 491-amino-acid phosphoprotein that is reported to be important in controlling the response of p53 to stress, whereby a phosphorylation of Mdm-2 or p53 itself by stress-activated protein kinases could prevent their interaction and hence allow the accumulation of p53 (Mayo et al., 1997). The Mdm-2 gene is a proto-oncogene, whose product promotes the rapid degradation of a phosphorylated form of p53 via ubiquitin proteosome pathway (Steele et al., 1998; Matsumura and Ananthaswamy, 2002a).
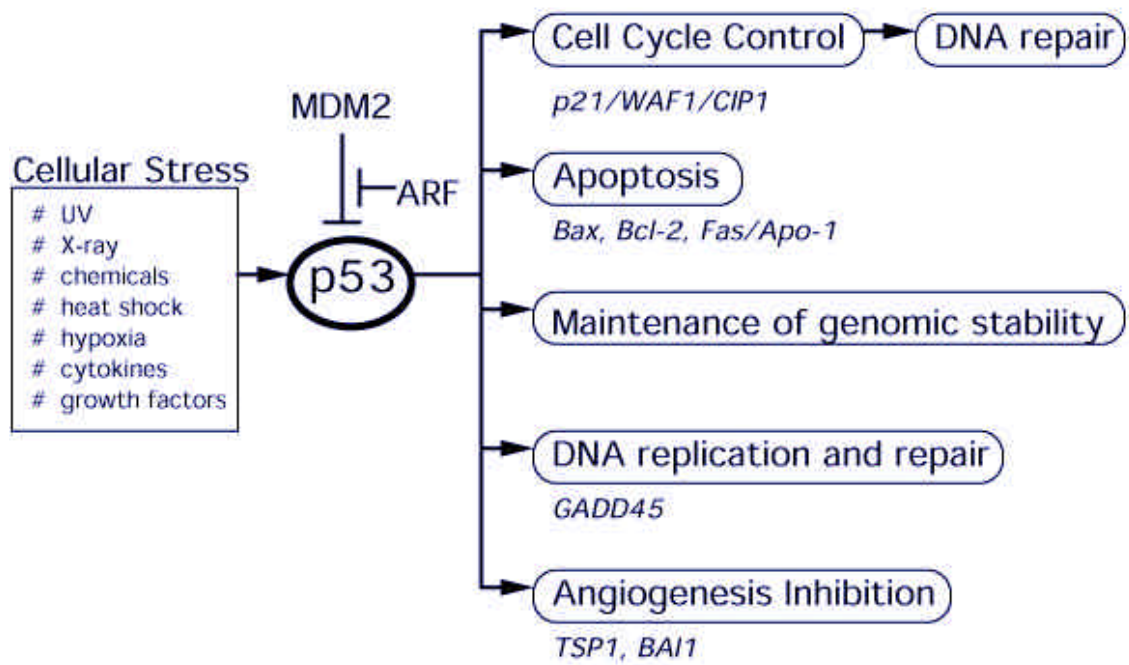
**Figure 1.9 The cellular response of p53 tumour suppressor gene. (Matsumura and Ananthaswamy, 2002a)**

### 1.2.9  UVR-INDUCED IMMUNOSUPPRESSION

UVR in the sunlight not only causes DNA damage but is also known to suppress the immune system (Kripke, 1974; Kripke and Fisher, 1976). The UVR-induced immunosuppression makes the cell more susceptible to skin carcinogenesis by depressing cell mediated immune reactions that normally destroy the developing skin tumours (Nghiem et al., 2002).

The immune system in the skin consists of various cell types both in the epidermis and dermis, and the cells circulating in the blood and lymphatic system. The main cells involved in the skin's immune system include antigen-presenting Langerhans cells in the epidermis, T cells and the keratinocytes. In normal skin, Langerhans cells in the epidermis form a dendritic cell network that functions to capture invading microorganisms, internalise them, and then process their antigens into a form that could be recognized by T cells. They then migrate to the skin-draining lymph nodes where they present antigens to naïve T cells to initiate a protective immune response (Kripke et al., 1990). However, with UV exposure, the number, migration, antigen-presenting abilities, other functions and morphology of Langerhans cells are altered, resulting in reduced immunosurveillance capability of the skin (Wang et al., 2009). It has also been noted that following UVR, the capacity to present antigens on surface and the number of Langerhans cells decrease (McGregor, 1999). The role of Langerhans cell in UVR-induced immunosuppression is significant in that it was found to be essential for both the suppression of contact hypersensitivity (CHS) and the induction of regulatory T cells (Schwarz et al., 2010).

However, unlike the conventional idea that the epidermal Langerhans cells are the only langerin+ dendritic cells (DC) in the skin responsible for innate and adaptive immune responses, recent reports showed that langerin+ CD103+ dendritic cells also

reside in the dermis and induce immune response. In a study conducted in Wang et al, dermal langerin+ dendritic cells were found to have distinct biological functions to epidermal Langerhans cells and found to promote CD8 T cell response (Wang et al., 2009).

In addition, keratinocytes also play an important role in the skin immune system by producing cytokines including interleukin (IL)-1, IL-6, IL-7, IL-8, IL-10, interferon (IFN)-γ, tumour necrosis factor (TNF)- α and TGF-β that also participate in immune responses in the skin (Matsumura and Ananthaswamy, 2004).

There are two major types of immune responses that UVR is known to suppress: contact hypersensitivity reactions (CHS) (Jessup et al., 1978) and delayed type hypersensitivity reactions (DTH) to viral (Howie et al., 1986), bacterial (Jeevan et al., 1989) and fungal antigens (Denkins et al., 1989). Both CHS and DTH are the main experimental models used to study UVR-induced immunosuppression. Initially, CHS study begins with the epicutaneous application of a contact sensitizer in the induction phase. This is followed by an elicitation phase where a further application several days later induces the elicitation of CHS response, resulting in an inflammatory response at sites not directly exposed to UVR. CHS is usually measured experimentally either by observing a change in skin colour or measuring the skin thickness. In DTH the same protocol is followed except that the antigen is more complex and is usually injected subcutaneously so that antigen presenting cells other than those in the skin are involved (Norval, 2006).

UVR-induced immunosuppression is classified into local immune suppression and systemic immune suppression. Local immune suppression refers to the situation in which the antigen (or hapten) is applied directly to the UV-irradiated skin. Systemic immune suppression occurs when the immune response is elicited by antigen being

applied to a distant non-irradiated site as opposed to UV-irradiated site (Ullrich, 2005). Thus the two terms are used to describe the site of irradiation with respect to antigen exposure, but not to describe whether the immune response generated is local or systemic.

We have been interested in the mechanism by which *in vivo* UV irradiation initiates immunosuppression. Because the wavelengths responsible (250 to 320 nm) do not penetrate beyond the skin, the sequence of events leading to systemic immune suppression must be initiated by the absorption of UVR by some moiety in the skin (De fabo and Kripke, 1980; Noonan et al., 1981). Recent findings suggest the role of IL-10 in immunosuppressive effects. When cytotoxic T-lymphocyte associated protein (CTLA)- 4 (CD152) positive T cells were stimulated *in vitro*, a variety of cytokines including IFN-γ, TGF-β, IL-2, and IL-10 were secreted. However, only IL-10 monoclonal antibody neutralised the suppressive activity *in vivo*, suggesting that CTLA-4 positive suppressor T cells suppress contact hypersensitivity reactions by releasing IL-10 *in vivo* (Ullrich, 2005).

## 1.2.10 PHOTOCARCINOGENESIS

Carcinogenesis is the result of complex molecular and biochemical events that eventually lead to the development of cancer. It is most frequently caused by carcinogens in the environment ranging from chemicals, radiation, viral or one's own genetic susceptibility to cancer formation. These carcinogens are substances that interrupt the structure and function of the cell, ultimately leading to cancer development (McMillan, 1992).
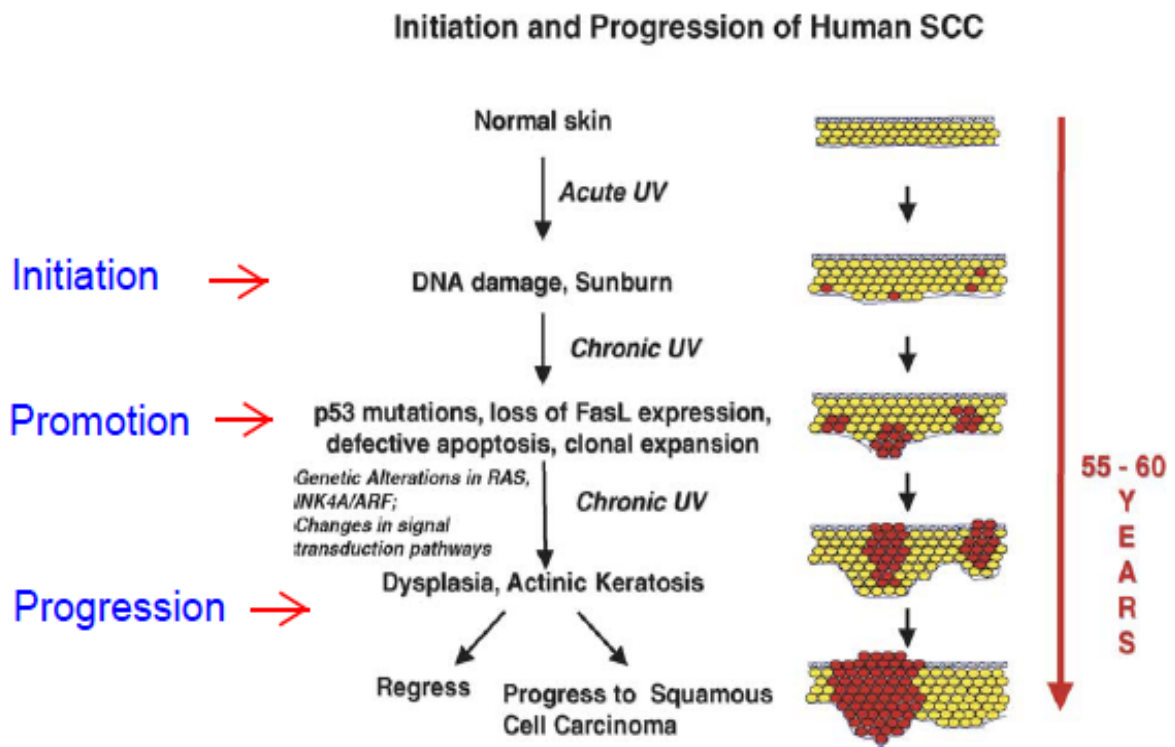


**Figure 1.10 Initiation and progression of human squamous cell carcinoma (SCC). (Melnikova and Ananthaswamy, 2005)**

As shown illustrated in the Figure 1.8, carcinogenesis occurs in three stages: initiation, promotion and progression. Initiation commences with any chemical, physical or biological agent causing DNA damage, which may be reversible or lead to genetic mutation if not repaired. However, whether or not the mutation of the gene results in malignancy depends on the promotion stage. The promotion stage is the

result of the second factor, known as promoter, acting on the initiated cell causing selective clonal expansion of the initiated cells due to altered expression of those genes associated with hyperproliferation. However, there are carcinogens called complete carcinogens that could both initiate and promote neoplastic transformation. The third stage is the progression that involves morphological and phenotypic changes in the cell, specifically the development of preneoplastic cells into tumours through clonal expansion, which is facilitated by altered gene expression and progressive genomic instability. It is also associated with increased malignancy, leading to invasion of surrounding tissue and metastasis to distant body parts (Hursting et al., 1999; McMillan, 1992; Melnikova and Ananthaswamy, 2005)

Unlike typical carcinogens, UVR acts as a complete carcinogen, having the ability to cause skin cancers without additional initiators or promoters (Melnikova and Ananthaswamy, 2005). Although UVR-induced skin carcinogenesis begins with DNA damage, it is the cascade of subsequent molecular and biochemical events that leads to cancer formation.

One important part of tumour progression is the inactivation of one or more of tumour suppressor genes or over activation of proto-oncogenes. Inactivation of tumour suppressor genes might occur due to the accumulation of proteins that bind to tumour suppressor genes, hindering their ability to regulate cellular growth and making the cells more susceptible to further mutations. On the other hand, oncogenes are genes that normally control cellular proliferation and differentiation, yet become 'proto-oncogenes' when expressed excessively. In both cases, the resultant altered gene product could cause cancer formation. The genes worthy of discussion in the context of photocarcinogenesis are p53 and Ras gene, of which Ras gene was outside the scope of this study. (Matsumura and Ananthaswamy, 2002).

Normally, when the cells have DNA damage there is an increase in p53 phosphorylation and translocation to the nucleus that facilitates DNA repair or causes apoptosis of cells with severely damaged DNA. However, with excessive UVR exposure, such DNA repair mechanisms are disrupted, leading to p53 mutations, thereby allowing damaged cells to resist apoptosis (Ziegler et al., 1994). Thus the p53 mutation is the key initiating step of skin carcinogenesis, and the significance of p53 gene mutation has been illustrated through the finding that the number of UVR-induced squamous cell carcinoma (SCC) is increased in the skin of p53 knockout (-/-) mice (Ziegler et al., 1994). By extrapolation, the cells containing inactivated p53 due to mutations would have a reduced capacity to remove potentially mutagenic cells. More specifically, p53 activates cell cycle arrest and programmed cell death to eliminate such cells prior to replication. This is a crucial step in preventing skin carcinogenesis as cells with DNA damage could survive otherwise and develop into mutagenic cells (Hartwell and Weinert, 1989). This is further supported by a study involving p53-/- and p53 +/- mice, which showed increased sensitivity towards UVR-induced skin tumour development compared with the wild-type mice. Heterozygous mice showed greater susceptibility to skin cancer induction compared with wild-type mice, and homozygous mice were even more susceptible (Jiang et al, 1999).

The p53 mutation has been found in more than 90% of SCC occurrences (Ziegler et al., 1994).


## 1.2.11 BODY'S DEFENCE AGAINST UVR

Stimulation of p53 tumour suppressor protein effectively inhibits photocarcinogenesis by enhancing the nuclear excision repair of UVR-induced damage (Yamaguchi, 2006).

## 1.2.11.1    SKIN PIGMENTATION

Following the exposure to UVR, pigmentation occurs in the skin, consisting of two phases: immediate pigment darkening (IPD) and a delayed formation of new melanin.

In immediate pigment darkening, pre-existing melanin undergoes oxidation and melanocytic melanosomes redistribute themselves from a perinuclear position into the peripheral dendrites, possibly providing a protection against damage to epidermal basal cell nuclei. This immediate pigment darkening may last from minutes to several days depending on the exposure dose.

Delayed formation of new epidermal melanin is generally called 'tanning' and appears about 72 hours after exposure to UVB. On the other hand, with UVA exposure, tanning occurs much earlier with almost no latency, suggesting that the mechanism of melanin synthesis is different to that after UVB exposure.

With shorter wavelengths UVB exposure, the number of melanocytes and melanocyte tyrosinase activity increase accelerates the melanin transfer to keratinocytes of upper epidermal layers. With UVA exposure, increase in melanin density is dependent on the wavelengths. For instance, UVA between 320 to 340 nm stimulates the synthesis and migration of melanised melanosomes to the upper layers, yet with UVA between 340 to 400 nm, increase in melanin density takes place in the basal cell layer (McGregor, 1999).

## 1.2.11.2 CORNIFICATION

Cornification is the final process of keratinocyte differentiation that occurs naturally yet can be regulated by various factors upon extrinsic and intrinsic stimulations. As mentioned before, corneocytes – terminally differentiated keratinocytes, form the outermost layer of the epidermis, stratum corneum, which provides natural defence against UVR. Cornification process takes about 2 weeks and as a result, the keratinocytes become metabolically inactive and the organelles are degraded. The resulting fully differentiated squames are flattened dead cells that resemble 'protein sacs' consisting of more than 80% keratins crosslinked to other cornified envelope proteins. These corneocytes are ultimately sloughed into the environment and are replaced by differentiating keratinocytes from the inward layers (Lippens et al., 2005).
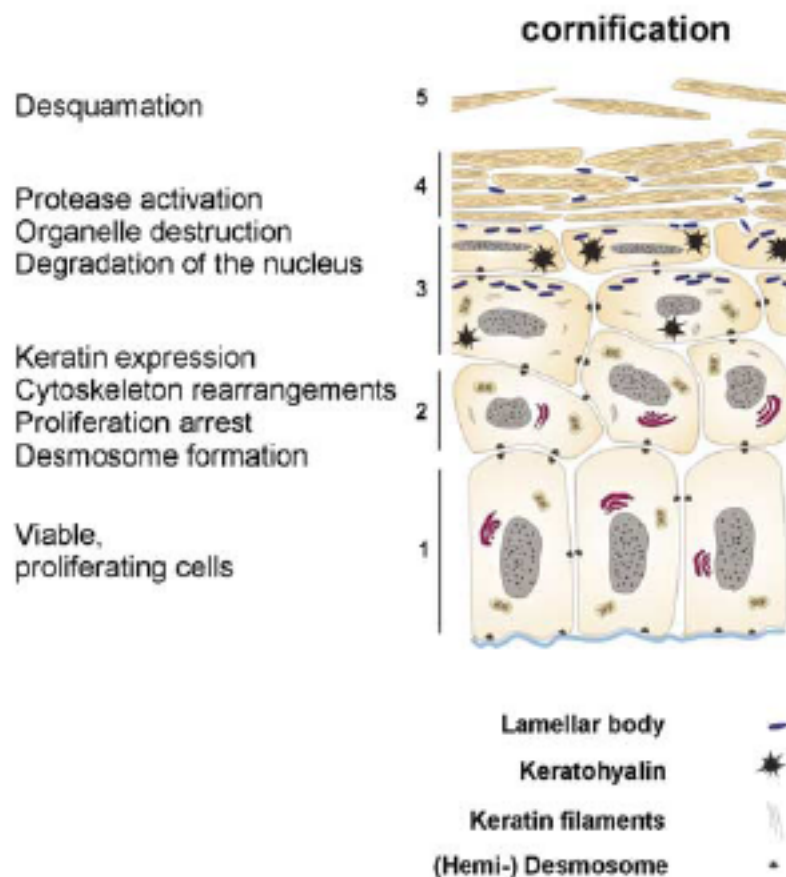


**Figure 1.11 Cornification process in skin. (Lippens et al., 2005)**

## 1.3 VITAMIN D

### 1.3.1 GENERAL BACKGROUND

Sir Edward Mellanby first recognized vitamin D2 in 1919, when his suggestion that rickets could be caused by a nutritional deficiency led to the isolation of a fat-soluble antirachitic substance in fish liver oil and other foods, which was identified as vitamin D2 (Mellanby, 1919). This work and continuing study led to the structural identification of vitamin D2 and vitamin $D_3$. Since the discovery, the physiological significance of vitamin D has been growing with particular attention to its regulatory role in bone and mineral metabolism on top of other endocrine functions in humans.

Vitamin D is a secosteroid cholesterol derivative in which one of its rings has undergone breakage (ring B in the case of vitamin D). This family has different geometric forms, where each member has its own unique structural features. The hormonally active form, 1α,25-hydroxy$(OH)_2$ vitamin $D_3$, otherwise known as $1,25(OH)_2D_3$ is the major metabolite of vitamin $D_3$ (Reichel et al., 1989).

### 1.3.2 STRUCTURE

As mentioned before, $1,25(OH)_2D_3$ is a secosteroid, having similar structure to classical steroid hormones. Vitamin D and all its metabolites, including the steroid hormone $1,25(OH)_2D_3$ are, in comparison to other steroid hormones, unusually conformationally flexible. This flexibility is due to three particular features of the molecule (shown in figure 1.10):

i. First is that the side chain has a 360° rotation around each of the 5 carbon-carbon single bonds.

ii. the A-ring undergoes a cyclohexane-like chair–chair immersion, which changes the orientation of 1α-hydroxyl and 3β-hydroxyl groups between the equatorial and axial orientations.

iii. the broken B-ring has a 360° rotation around the 6,7 single carbon bond.
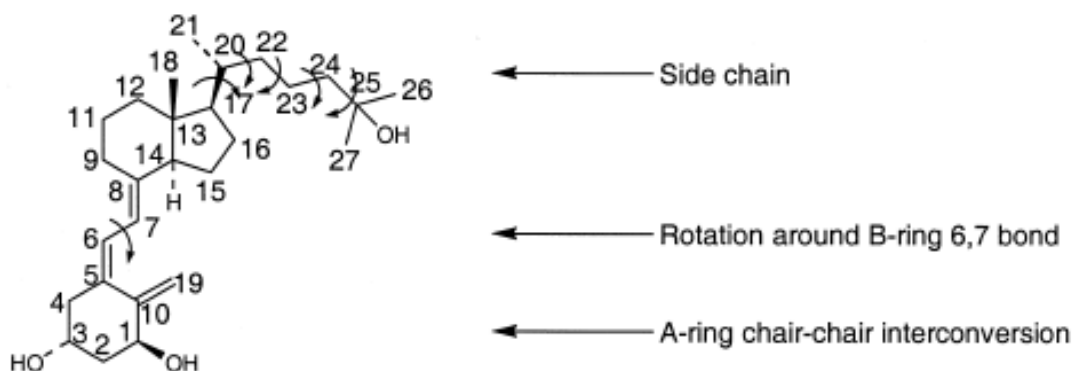


**Figure 1.12 Structure and the conformational flexibility of 1,25(OH)$_2$D$_3$. (Norman et al., 2002)**

This conformational flexibility allows a wide array of molecular shapes that are available for binding to receptors involved with 1,25(OH)$_2$D$_3$ mediated biologic responses (both genomic and non-genomic) as well as for binding to DBP and cytochrome P450 enzymes (Norman et al., 2002).

### 1.3.3  VITAMIN D RECEPTOR (VDR)

The Vitamin D receptor (VDR) is an intracellular nuclear receptor, which is a member of the steroid-thyroid hormone receptor superfamily (Petrazzuoli, 1999). As with other nuclear receptors, VDR has a transcriptional activation domain, a specific DNA-binding domain that is rich in cysteine, and a specific ligand binding domain (Fig 1.11).
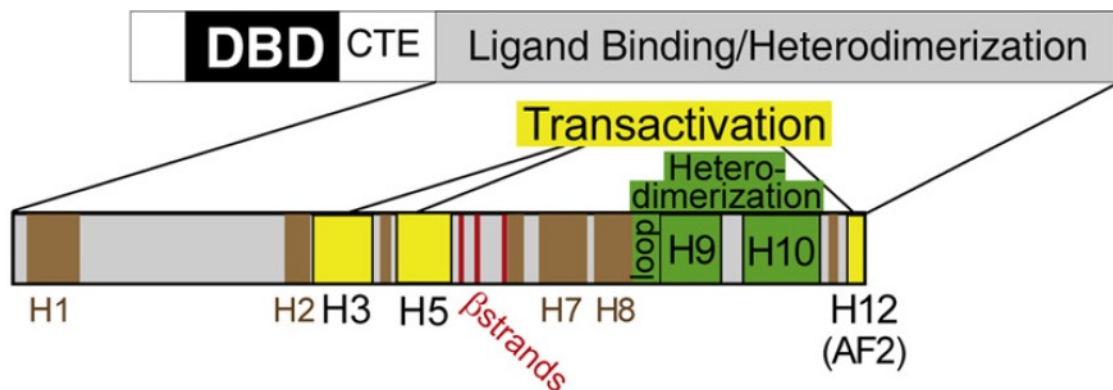


**Figure 1.13 The structure of Vitamin D receptor. (Haussler et al, 2011)**

DBD (DNA binding domain), CTE (C-terminal extensions).

The VDR is responsible for both the genomic and non-genomic pathways of action for which it contains a genomic pocket (VDR-GP) and an alternative pocket (VDR-AP). (Haussler et al., 2011)

### 1.3.4  BIOSYNTHESIS AND METABOLISM

Vitamin $D_3$ in its active form, $1\alpha,25(OH)_2D_3$, is synthesised after a number of reactions within human's body. Its precursor is 7-dehydrocholesterol (7-DHC) that is converted into the pre-vitamin $D_3$ upon UVB absorption. Then by thermal isomerisation at the body temperature, pre-vitamin $D_3$ is converted into vitamin $D_3$ over several hours. However, this is still an inactive form of vitamin $D_3$ that must be converted into the active form to induce any biological responses. First, vitamin $D_3$ binds to the vitamin D binding protein (DBP) in the blood to enter the circulation

(Norman et al., 2001a). Then vitamin $D_3$ undergoes two sequential hydroxylations by cytochrome P450 enzymes. It is firstly hydroxylated by 25-hydroxylase in the liver to form 25-hydroxyvitamin $D_3$ ($25(OH)D_3$). This is then hydroxylated again by 1α-hydroxylase, mostly in the kidney but also in other tissues, into $1,25(OH)_2 D_3$. This is illustrated in figure 1.12.

Vitamin D is an essential hormone that enables the absorption of calcium in the intestinal mucosa.
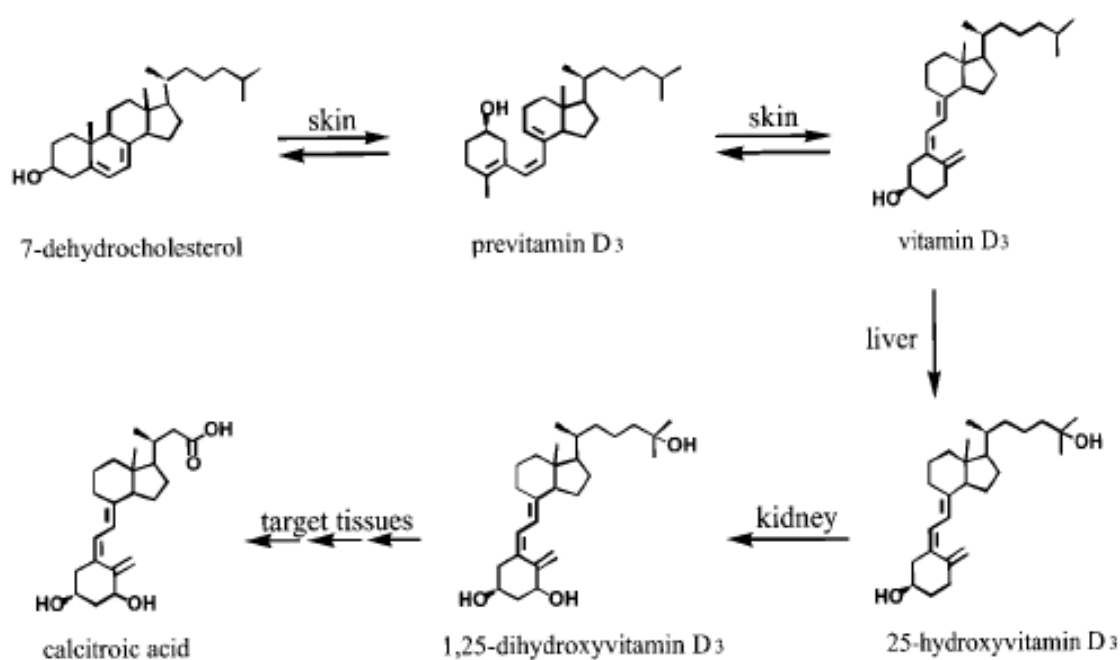


**Figure 1.14 Schema of vitamin D metabolism. (Brown et al., 1999)**

### 1.3.5 MECHANISMS OF ACTION

The active vitamin D metabolite, $1,25(OH)_2D_3$ generates physiologic effects at a cellular level, by binding to the ligand binding pockets on the nuclear vitamin D receptor (VDR). There are two mechanisms of its action, a genomic pathway that regulates gene transcription, and a non-genomic pathway that involves cellular cytoplasmic signalling pathways.

## 1.3.5.1 GENOMIC PATHWAY

As mentioned earlier, genomic pathway of vitamin D signalling involves the activation of transcriptional complexes that target specific genes in the DNA sequence. As a DNA-binding transcriptional factor, $1\alpha,25(OH)_2D_3$ -bound VDR first binds to retinoid X receptor (RXR), making a heterodimeric protein. This liganded-VDR and RXR forms a complex that has a combined DNA binding domain (DBD) zinc fingers to recognize vitamin D responsive elements (VDREs) in the DNA sequence of vitamin D regulated genes. These may be in the promoter region of target genes or at distant enhancer or repressor sites. Following VDRE docking, protein co-activators (or co-repressors) are recruited to interact with VDR-RXR complex in order to either promote or repress gene transcription (Haussler et al, 2011; Zella et al, 2010).

## 1.3.5.2 NON-GENOMIC PATHWAY

Non-genomic pathway, or the rapid-acting pathway for the action of $1,25(OH)_2D_3$ has been known to induce various biological processes within seconds to minutes via intracellular signalling pathways, though the receptor involved in this pathway has not yet been definitively identified. The pathway is often referred to as 'rapid-acting' because the responses are generated within 1-2 min to 15-45 min, which is in contrast to genomic responses that take hours to days to be fully apparent (Haussler et al, 2011).

The binding of $1,25(OH)_2D_3$ to its proposed rapid-response receptor can stimulate various intracellular signalling pathways including protein kinase C (Nemere et al. 1998), cAMP, intracellular calcium, and MAP kinase, phosphatidylinositol 3-kinase (PI3K), phospholipase C and subsequent G-protein coupled second messenger systems (Norman, 2008).

There is evidence of a membrane-associated rapid response steroid-binding protein (1,25D$_3$ -MARRS), one that is identical to endoplasmic reticulum stress protein 57 (ERp57), as the receptor involved in non-genomic actions by 1,25(OH)$_2$D$_3$. Previous studies have shown that in the presence of Ab099, a neutralizing antibody against the N terminus of ERp57, 1,25(OH)$_2$D$_3$-mediated effects such as augmented intracellular calcium and protein kinase C activity were abolished in chick intestinal epithelial cells (Nemere et al., 2000; Nemere et al., 2004). Also, recent studies have reported that when normal fibroblasts were pretreated with Ab099, protective effect of 1,25(OH)$_2$D$_3$ against UVR-induced thymine dimers was abolished (Sequeira et al., 2012). This evidence implicates ERp57 as a necessary component of photoprotection by 1,25(OH)$_2$D$_3$, suggesting that ERp57 mediates at least some of the nongenomic actions 1,25(OH)$_2$D$_3$.

In addition, opening of chloride channels has recently been reported to be another possible non-genomic pathway of 1,25(OH)$_2$D$_3$-induced protection against thymine dimers formation in keratinocytes. Treatment with DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid), a chloride channel blocker, which was shown to have no effects on thymine dimers on its own, completely prevented the protective effects of 1,25(OH)$_2$D$_3$ when they were used together (Sequeira et al., 2013).

As mentioned previously, the conformational flexibility of 1,25(OH)$_2$D$_3$ plays an important role as it allows 1,25(OH)$_2$D$_3$ a wide variety of different shapes to serve as ligands for available receptor(s) in the vitamin D endocrine system.

The evidence reported support the view that the VDR and the receptor/binding domain involved in rapid responses recognize different shapes of 1,25(OH)$_2$D$_3$, thereby enabling 1,25(OH)$_2$D$_3$ to interact with its receptors in all of its many target

cells. By extrapolation, this hypothesis also suggests that conformationally flexible analogues of vitamin D can theoretically, initiate either genomic or rapid, non-genomic biological responses.

## 1.3.6 VITAMIN D$_3$ REGULATED KERATINOCYTE DIFFERENTIATION

Vitamin D$_3$ in its active form is also known to have various other biological roles. One important process that it regulates is keratinocyte homeostasis. Through genomic and non-genomic events, 1,25(OH)$_2$D and calcium are known to regulate a decrease in keratinocyte proliferation whilst inducing an increase in morphologic differentiation of keratinocytes (Haake and Holbrook, 1999).

Keratinocytes in the basal layer are able to synthesise 1,25(OH)$_2$D$_3$ from 7-DHC, yet at the same time, they are also the target for hormone. In addition, intracellular calcium is known to have potent effects on proliferation and differentiation as well. In number of *in vitro* studies, calcium has been shown to induce various signs of keratinocyte differentiation including cell to cell contact, desmosome formation and the upregulation of involucrin, transglutaminase, keratins and filaggrin expressions. Together, 1,25(OH)$_2$D$_3$ and calcium have their own mechanisms to create synergistic regulation of keratinocyte differentiation (Bikle, 2004).

### 1.3.7  PREVIOUS FINDINGS ON PHOTOPROTECTION BY VITAMIN D$_3$

It has been shown in the current research laboratory that 1,25(OH)$_2$D$_3$ protects against UVR-induced cell loss when it is applied to keratinocytes, melanocytes and fibroblasts prior to and/or immediately after UVR. In those skin cells, application of 1,25(OH)$_2$D$_3$ diminished the level of cyclopyrimidine dimers, a major marker for photodamage, substantially in a dose-dependent manner and the survival rate of keratinocytes following UVB was improved upon addition of 1,25(OH)$_2$D$_3$ (Wong et al., 2004; Song et al., 2013).

Although it has previously been found that the photoprotection by 1,25(OH)$_2$D$_3$ is chiefly due to its inhibitory effect on the formation of cyclopyrimidine dimers in human skin after UV exposure, it was found by our research group that DNA damage is not solely dependent on direct absorption of UVR by DNA. The damage is also found to be dependent on metabolic pathways, particularly the nitric oxide (NO) pathway. Nitric oxide is known to react with the UVR-induced reactive oxygen species that combine to excite an electron in fragments of the pigment melanin, forming "dark CPDs" previously mentioned in chapter 1.2.5.1 (Premi et al., 2015). This was also evident in Mason group's finding, which demonstrated that 1,25(OH)$_2$D$_3$ suppresses various forms of promutagenic DNA damage, including thymine dimers, in part through a reduction of genotoxic reactive nitrogen species (RNS). This study further reinforced the suggestion that a reduction in reactive nitrogen species by 1,25(OH)$_2$D$_3$ is a likely mechanism for its photoprotective effect against oxidative and nitrative DNA damage, as well as CPDs (Gordon-Thomson et al., 2012).

There are other potential mechanisms including 1,25(OH)$_2$D$_3$ induced expression of metallothionein (MT), a potent radical scavenger. 1,25(OH)$_2$D$_3$ neither inhibited peroxidation of plasma lipids nor interacted with superoxide, nor removed hydrogen

peroxide as an antioxidant (Lee and Youn, 1998). These findings suggest that $1,25(OH)_2D_3$ has photoprotective effect not related with glutathione or its endogenous antioxidant property. Rather, it could be attributed to $1,25(OH)_2D_3$-induced metallothionein and its capacity to prevent radical-related damage in UVB irradiation (Lee and Youn, 1998).

## 1.3.8  VITAMIN D LIKE COMPOUNDS

The discovery of the newer non-genomic functions of $1,25(OH)_2 D_3$ led to a growing interest in the search for new vitamin D like compounds or analogues (Jones et al., 1998). Some of the analogues discussed below include known agonists and antagonists of the rapid response pathway, and an antagonist of the genomic pathway. No specific agonist of the genomic pathway has been reported.

## 1.3.8.1  JN: NON-GENOMIC AGONIST

JN ($1\alpha,25(OH)_2$ -lumisterol) mediates its biological functions via the rapid, non-genomic pathway. JN, a low-calcemic 6-s-cis-locked analogue, has been shown to be a full agonist of the non-genomic pathway and can only weakly bind to the VDR. It has also been known to be effective inhibitor of UVR-induced damage in an immunocompetent mouse (Skh:hr1) model susceptible to UVR-induced tumours. Photoprotection by JN was shown through reduction in UVR-induced cyclobutane pyridimine dimers, apoptotic sunburn cells, and immunosuppression (Normal et al., 1997; Dixon et al., 2011). However, these markers of photoprotection do not necessarily imply that they inhibit skin carcinogenesis as they are the results of acute UVR exposure.

## 1.3.8.2 QW: LOW CALCEMIC ANALOGUE

QW (1α-hydroxymethyl-16-ene-24,24-difluoro-25-hydroxy-26,27-bis-homovitaminD$_3$ or QW-1624F2-2) is transcriptionally active and is approximately 80-100 times less calciuric than 1,25(OH)$_2$ D$_3$ (Posner et al., 2004; Reddy et al., 2006). QW has high anti-proliferative and pro-differentiating activity and has been shown to inhibit chemical-induced skin tumourigenesis and tumour latency (Kensler et al., 2000). Furthermore, this hybrid analogue does not cause cachexia in animals (Posner et al., 1998) and has been shown to be non-genotoxic (Posner et al., 2004). QW has been fast tracked by the United States Food and Drug Administration (U.S.F.D.A.) for approval for clinical use in humans. Recent studies have revealed that QW significantly reduced UVR-induced DNA lesions (CPD) in skin fibroblasts and keratinocytes and reduced cell death after UV exposure (Dixon et al., 2012).

## 1.3.8.3 HL: NON-GENOMIC ANTAGONIST

HL (1β,25(OH)$_2$ D$_3$ ) has been shown to selectively inhibit the functioning of the non-genomic pathway (Norman et al., 1999). The employment of a non-genomic pathway antagonist in this project was crucial in the isolation of the pathway through which photoprotection is mediated.

## 1.3.8.4 TEI-9647: GENOMIC ANTAGONIST

TEI-9647 (25-dehydro-1α-hydroxyvitamin D$_3$-26,23S-lactone; MK), acknowledged for its antagonistic effect on the genomic action of 1,25(OH)$_2$D$_3$ in human tissues (Bula et al., 2000; Ishizuka et al., 2001), was used in this study to assess the effects of inhibition of the genomic pathway on photoprotection by 1,25(OH)$_2$D$_3$. At high concentrations it is known to have some agonist activity. When used in combination with 1,25(OH)$_2$D$_3$, TEI-9647 did not inhibit the effect of 1,25(OH)$_2$D$_3$, as the degree of

UVR-induced cell loss was not significantly different to the level of loss in melanocytes treated with $1,25(OH)_2D_3$ only. Also, TEI-9647 did not show significant antagonistic effect on photoprotection by $1,25(OH)_2D_3$ in terms of UVR-induced cyclobutane pyrimidine dimers (CPDs). The proportion of positive CPDs staining in melanocytes treated with a combination of TEI-9647 and $1,25(OH)_2D_3$ did not differ from that in cells treated with $1,25(OH)_2D_3$ alone. Similar results were observed in fibroblasts, where a significant reduction in cell loss and photoprotective action by $1,25(OH)_2D_3$ were maintained in the presence of TEI-9647 (Dixon et al., 2005).

## 1.4  20-HYDROXYVITAMIN D$_3$ (20(OH)D$_3$ )

### 1.4.1  BACKGROUND

20-hydroxyvitamin D$_3$ is a product of vitamin D$_3$ hydroxylation by an enzyme called cytochrome P450scc (or CYP11A1), which normally performs cholesterol side chain cleavage on 7-dehydrocholesterol or cholesterol (Slominski et al., 2005b). The expression of CYP11A1 enzyme was shown to be increased in both human keratinocytes and melanocytes after UVR exposure (Skobowiat et al., 2011).

### 1.4.2  STRUCTURE
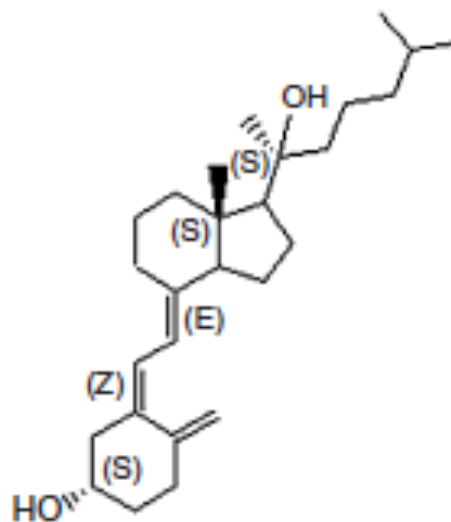


**Figure 1.15 Structure of 20(OH)D$_3$. (Zbytek et al., 2008)**

### 1.4.3  EVIDENCE SUGGESTING PHOTOPROTECTIVE EFFECTS OF 20-HYDROXYVITAMIN D$_3$.

It has been shown that the active form of vitamin D, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) protects skin cells from mutagenic DNA damage and photocarcinogenesis (Dixon et al., 2011). In experiments involving vitamin D receptor knockout mice, it was shown that these mice became more susceptible to carcinogenesis upon UV irradiation (Ellison et al., 2008). However, it was also found

that knockout of 1α-hydroxylase enzyme (CYP27B1), an enzyme essential for the production of $1,25(OH)_2D_3$, does not increase the susceptibility to photocarcinogenesis (Teichert et al., 2011), This led to the question whether other vitamin D metabolites, specifically 20-hydroxyvitamin $D_3$ ($20(OH)D_3$), which is made naturally in irradiated skin from vitamin D by the CYP11A1 enzyme, could play a role in photoprotection.

Also, a study has been done on human epidermal keratinocytes to observe the effect of $20(OH)D_3$ on the genes involved in keratinocyte differentiation. For instance, a study has been done to show that the expression of involucrin mRNA increased upon treatment with $20(OH)D_3$. The stimulating effect of $20(OH)D_3$ did not stop at the transcriptional level but was found that this stimulation was reflected in the keratinocyte phenotype, hence confirming that $20(OH)D_3$ is a stimulant of involucrin expression (Zbytek et al., 2008). As involucrin is an important protein involved in cornification of keratinocyte, one of protective mechanisms within human's body, it can be inferred that $20(OH)D_3$ could affect genes that could provide photoprotective capacity to skin cells.

In addition, antitumour activities of $20(OH)D_3$ has been shown previously on cancer cell lines such as breast carcinoma cells and hepatocellular carcinoma cells. Although these cell lines have different metabolism and machinery in nature to skin carcinoma cells, these evidences strongly suggest potential antitumour activity of $20(OH)D_3$ on skin cell lines as well (Wang et al., 2012).

Lastly, it was shown in the short terms studies by our colleagues that $20(OH)D_3$ like $1,25(OH)_2D_3$ and at similar concentrations, reduced CPD and 8-oxodG in UV-irradiated human keratinocytes. They also found that $20(OH)D_3$ reduced UVR-induced skin oedema measured as dorsal skin-fold thickness in UV irradiated mice

(p<0.01) and significantly reduced UVR-induced immunosuppression in these mice, measured by contact hypersensitivity from 35% in vehicle treated mice, to under 5% in mice treated with $20(OH)D_3$, to a similar extent as $1,25(OH)_2D_3$ (p<0.001 vs vehicle). (Tongkao-on et al., 2015).

On the other hand, studies on photoprotective effect of $20(OH)D_3$ on HaCaT cell line are incompletely understood. There are however, some studies suggesting that there is a limitation of HaCaT cell line as a model to study photoprotection. Firstly, HaCaT cell lines have mutated p53, tumour suppressor gene that is known to play a significant role in regulating DNA repair after UVR exposure in keratinocytes as explored by the Mason group (Section 1.2.7). Although we do not have direct evidence suggesting that $20(OH)D_3$ increases nuclear p53 expression, it is reasonable to assume that DNA repair pathways following UVR exposure in HaCaT cell lines will be different, and thus difference in photoprotective mechanisms between primary keratinocytes and HaCaT cell line. In addition, recent study found that gene transcriptional profile of cornified envelope-associated proteins, such as filaggrin, loricrin, involucrin and keratin 10, in response to major helper T cell cytokines such as IL-17 and IFN-$\gamma$, are different from that in normal human keratinocytes. This also suggests a limitation in HaCaT cell line as a model to study chronic inflammation and skin barrier function as they respond differently to inflammatory cytokines when compared to normal keratinocytes (Seo et al., 2012).

## 1.5 PROJECT

### 1.5.1 AIMS AND HYPOTHESES

**Aims:**

1) To examine the effects of $20(OH)D_3$ and $1,25(OH)_2D_3$ on the viability of the immortalised human keratinocyte cell line, HaCaT, and of primary keratinocytes in culture after UV exposure.

2) To examine whether $20(OH)D_3$, with $1,25(OH)_2D_3$ as a positive control, would alter UV-induced skin tumour formation and chronic immunosuppression in Skh:hr1 albino female mice.

**Hypotheses to be tested:**

a) That $20(OH)D_3$, like $1,25(OH)_2D_3$, protects human keratinocytes against UVR-induced damage, but that this protection is not observed in HaCaT cells, often used as a model for primary keratinocytes.

b) That $20(OH)D_3$, like $1,25(OH)_2D_3$, will reduce skin tumour formation, and chronic immune suppression in a mouse photocarcinogenesis model.

# 2 MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 EQUIPMENT

A sterile experimental environment for cell culture was provided by a Class II biohazard laminar flow cabinet obtained from Gelman Sciences Pty Ltd (Sydney, Australia).

Cells were stored in a liquid nitrogen Dewar purchased from Taylor-Wharton Australia (Albury, Australia). The centrifuge was purchased from Hettich Zentrifugen (Tuttlingen, Germany). Cells were incubated in a water-jacketed incubator with controlled $CO_2$ atmosphere from Forma Scientific (Sydney, Australia). Cells were regularly monitored under a phase-contrast inverted microscope (Olympus model IMT-2) from Olympus Optical Co Ltd (Tokyo, Japan). Cells were manually counted using a Neubauer chamber haemocytometer from Superior (Germany). Solutions were filtered using Sterivex™ 0.22 µm filter unit from Millipore (Billeria, MA, USA).

### 2.1.2 GENERAL CHEMICALS AND REAGENTS

Aqueous solutions were prepared using MilliQ purified water, provided by the Bosch Molecular Biology Facility (University of Sydney, Australia).

Chemicals and solvents including 1,2-propanediol were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

## 2.1.3 VITAMIN D COMPOUNDS

1,25(OH)$_2$D$_3$ was purchased from Sigma Aldrich (St Louis, MO, USA). 20(OH)D$_3$ – a gift from Dr. Robert Tuckey, UWA. These compounds were dissolved in spectroscopic ethanol and were stored under argon at -80°C.

The quality of vitamin D compounds was checked by measuring absorbance peaks using a spectrophotometer. This is shown in Figure 2.1 and Figure 2.2.
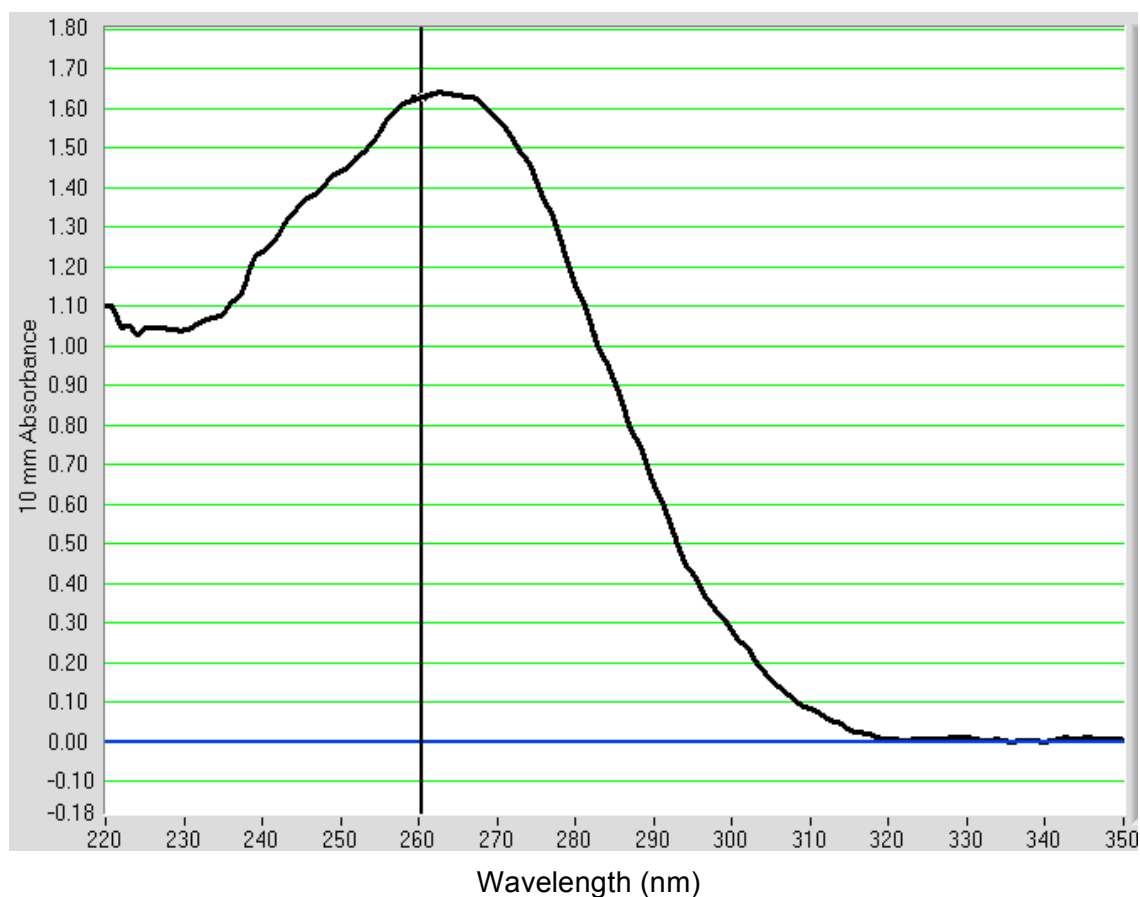


**Figure 2.1 Spectrophotometric analysis of 1,25(OH)$_2$D$_3$**
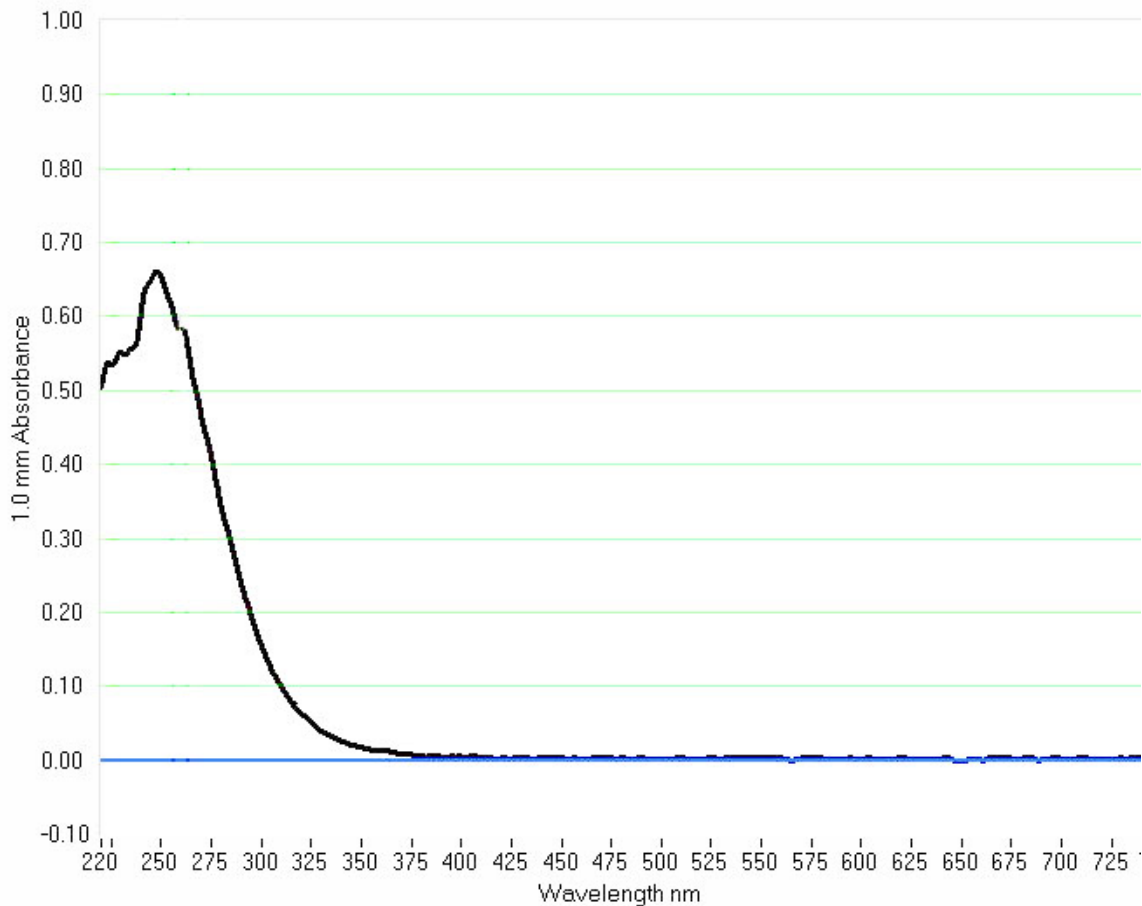
**Figure 2.2 Spectrophotometric analysis of 20(OH)D$_3$. (image courtesy of colleague, Bianca McCarthy)**

### 2.1.4  COMPOSITION OF MEDIA AND SOLUTIONS

HaCaT Growth Medium

HaCaT cells were grown in a medium consisting of normal calcium and glucose Dulbecco's Modified Eagle Media (DMEM).

Keratinocyte growth medium (KGM)

Keratinocytes were grown in Dulbecco's modified Eagle's medium (DMEM) 2.2g/L NaHCO3, 0.293g/L glutamine, 2.385g/L HEPES, 0.03g/L penicillin, 0.05g/L streptomycin, and 5% v/v FBS, without calcium, pH 7.4. Growth supplements added to KGM included 10ng/mL epidermal growth factor (EGF), 500µg/L Hydrocortisone and $10^{-10}$ M cholera toxin.

Martinez Solution

8.4738g/L NaCl, 0.41g/L KCl, 0.244g/L $MgCl_2.6H2O$, 0.1872g/L $NaH_2PO_4.2H_2O$, 1.9523g/L NaHEPES, 1.7873g/L HHEPES, 0.147g/L $CaCl_2.2H2O$, 1.8016g/L D-glucose, pH 7.4.

0.1 % Trypsin / 0.2% EDTA

0.2 g/L EDTA, 1g/L D-glucose, 0.4g/L KCl, 8g/L NaCl, 0.580g/L $NaHCO_3$, 1g/L trypsin in MilliQ water.

Cell lysis buffer

Cell were lysed using a lysis buffer containing 1.21g/L Tris buffer, 8.77g/L NaCl, 0.5% SDS, 1% Triton X-100, 292.2g/L EDTA, 0.0168g/L NaF, $H_2O$ and protease inhibitor which was added immediately prior to lysing the cells. Cell lysis buffer was made fresh on the day prior to use.

Base lotion for *in vivo* treatments

The base lotion for *in vivo* treatments consisted of spectroscopic ethanol, and 1,2-propanediol (propylene glycol) and MilliQ water to a final solvent ratio of 2:1:1, respectively. Base lotion was prepared in a 50 mL container and stored at room temperature under normal laboratory conditions.

## 2.2 METHODS FOR *IN VITRO* STUDIES WITH HACAT CELLS AND KERATINOCYTES

### 2.2.1 SOURCE OF TISSUE

Primary keratinocytes used for cell culture studies were obtained from neonatal foreskins donated after circumcision, with parental consent. Skin samples arrived at 4ºC in a serum-free medium containing streptomycin and penicillin, and were refrigerated at 4ºC upon receipt. Primary keratinocyte collection was conducted within 48 hours of circumcision surgery. All studies were conducted with the approval of the University of Sydney Human Research Ethics Committee (protocol number 14751) and Sydney South West Area Health Service (protocol number X09-0069).

HaCaT cells were donated from Professor Gary Halliday, University of Sydney (with permission from the originator).

### 2.2.2 GENERAL CELL CULTURE

Cell culture was conducted in a Class II biohazard laminar flow cabinet under sterile conditions (by UV radiation). Solutions including media, PBS and trypsin were pre-warmed in a heating water bath to room temperature prior to use.

### 2.2.3 PRIMARY KERATINOCYTE CULTURE

Primary keratinocytes were processed as previously described by Gupta and Dixon *et al.* (2007) from donated neonate foreskin samples under aseptic conditions in a Class II biohazard laminar flow cabinet, and all solutions used were sterile. Each foreskin sample was washed with 70% ethanol twice, after discarding the original media. The samples were further washed in PBS containing penicillin and streptomycin for 3 minutes, followed by another 3 minutes in PBS without the added

antibiotics. Following the initial washing of the samples, the subcutaneous tissue was removed and each sample was cut into small pieces approximately 3x3mm, before briefly submerging the pieces in betadine antiseptic solution and rinsing in PBS. The skin pieces were incubated at 4°C in 0.1% dispase (w/v) in PBS for approximately 24 hours to facilitate the separation of the epidermal and dermal layers.

The following day, the epidermis was removed with forceps and incubated briefly at 37°C in a solution of 0.1%/0.02% Trypsin/EDTA (w/v) in PBS in order to form a cell suspension. The trypsin was neutralised using a medium containing serum and calcium, the cell suspension was then centrifuged at 2000 rpm for 8 minutes. The resulting cell pellet was resuspended in KGM containing 5% FBS and the cells were cultured in a 75 cm$^2$ culture flask. Cells were grown at 37°C and 5% $CO_2$ in a humidified cell culture incubator. The medium in each culture flask was aspirated three times weekly and replaced with fresh medium. The condition of cells was monitored regularly via a phase-contrast microscope.

## 2.2.4  CULTIVATING CELLS

For cell growth and proliferation, HaCaT cells were cultured in DMEM with 10 % FBS (v/v) and the keratinocytes were cultured in KGM with 5% FBS. These cells were incubated under 37°C and 5% $CO_2$ gas and the growth medium was replaced every second or third day since culturing (or subculturing). Cell cultures were monitored regularly by using a phase contrast microscope to ensure growth, the absence of contaminants and to check for cell confluency. HaCaT cultures usually reached 90-100% confluency within 2-3 days.

## 2.2.5  CELL HARVESTING BY TRYPSINIZATION

Cells were harvested by applying trypsin, allowing cells to be detached from the flask surface by enzymatic cleavage. Prior to trypsinizing the cells, all culture medium was aspirated and the inside of the flask was washed with Dulbecco's PBS (D-PBS) in order to remove remaining traces of medium that could inhibit trypsinization. Trypsin stock solution containing 4 mL of D-PBS and 1 mL of 0.1% trypsin was added to the flask, prior to incubation for approximately 5-10 minutes at 37°C for trypsin activation. Incubation time may vary depending on the degree of cell adhesion and confluency. Cells were viewed under the microscope after approximately 5 minutes in order to examine the degree of cell detachment. The trypsinization period should be kept to a minimum to prevent potential damage to the cell membrane. The flask was gently tapped to help detach and suspend cells.

The cell suspension was then transferred to a centrifuge tube in a laminar flow hood, before being centrifuged at 1200 rpm for 5 minutes at room temperature. The supernatant was discarded and the cell pellet was resuspended with the appropriate cell culture medium.

After trypsinizing and resuspending the cells, the resulting cell suspension could be used to perform subculturing, cell freezing and cell plating. Before these processes were performed, a manual cell count was conducted using Neubauer Chamber (hemocytometer) when necessary.

In general, the cell trypsinizing process could be repeated several times according to the cell passage number limits. However, as HaCaT cells are an immortalized cell line, they do not undergo cell senescence and as a consequence, cell passage could be conducted numerous times. Primary cells have a lower number of permitted passages, in the case of keratinocytes a maximum passage number of 5 is

permitted, and cells grown past this passage number have a lower viability. For this reason, experiments with keratinocytes were conducted at low passage number.

## 2.2.6  CELL PASSAGING (SUBCULTURE)

Typically, when cells reach approximately 70% or greater confluency, subculturing was necessary in order to prevent cell apoptosis, cell death associated with high cell density within the culture flask. This process was performed after trypsinizing and resuspension of the cells as described in Section 2.3.4. To subculture the cells 8 mL of cell suspension was discarded and the remaining 1 mL resuspended with DMEM (or KGM with keratinocytes) to make up a total of 10 mL growth medium in the culture flask.

## 2.2.7  CRYOPRESERVATION

Before cryopreservation (freezing), cells were first trypsinized as described in Section 2.3.4.and then resuspended in an ice-cold freezing solution containing DMSO, and frozen down in a cryovial at –80°C. Keratinocytes were frozen in 5% DMSO (v/v) diluted in KGM with 20% FBS (v/v).

Cells treated in this manner can be stored at –80°C for no less than 24 hours and up to 7 days before transferring to a liquid nitrogen Dewar (-196°C) for long term storage.

## 2.2.8  RECOVERY FROM CRYOPRESERVATION

Frozen down cells stored in a liquid nitrogen dewar were thawed and transferred directly into a centrifuge tube containing approximately 4 mL of appropriate growth medium. The suspension was then centrifuged and the supernatant containing DMSO was discarded. The cell pellet was then resuspended with the appropriate

growth medium and was immediately placed in a culture flask containing the appropriate growth medium.

## 2.2.9  PLATING CELLS FOR EXPERIMENTATION

Keratinocytes of passage 1-3 were seeded onto plates upon reaching 70-80% confluence, whilst HaCaT cells were seeded regardless of their passage number. Cells were plated in the appropriate plates and allowed 48 hours to attach prior to irradiation and treatment.

Two plate sizes were used in *in vitro* studies: sterile 96 well-plate for used for cell viability assays. One 96 well plate containing both sham-irradiated and UVR-irradiated groups was used for conducting the cell viability assay. Keratinocytes were seeded at a density of approximately 20,000 cells per well whilst HaCaT cells were seeded at approximately 10,000 cells per well.

The respective medium without supplements but containing the appropriate concentration of FBS for each cell type was replaced in all wells 24 hours before irradiation. This allows the cell signalling pathways to stabilise.

## 2.2.10 ULTRAVIOLET RADIATION SOURCE

The UVR source for *in vitro* studies consisted of one UVA and one UVB lamp, with irradiance of 203 mJ/cm$^2$ UVB and 1168 mJ/cm$^2$ UVA (Philips, Amsterdam, Holland), filtered through 0.5 mm cellulose tri-acetate (Eastman Chemical Products, Kingsport, TN) to remove wavelengths below 290 nm as previously described (Dixon et al., 2011). Irradiance was checked regularly with an OL754 spectroradiometer (Optronics Laboratories Inc., Orlando, FL).
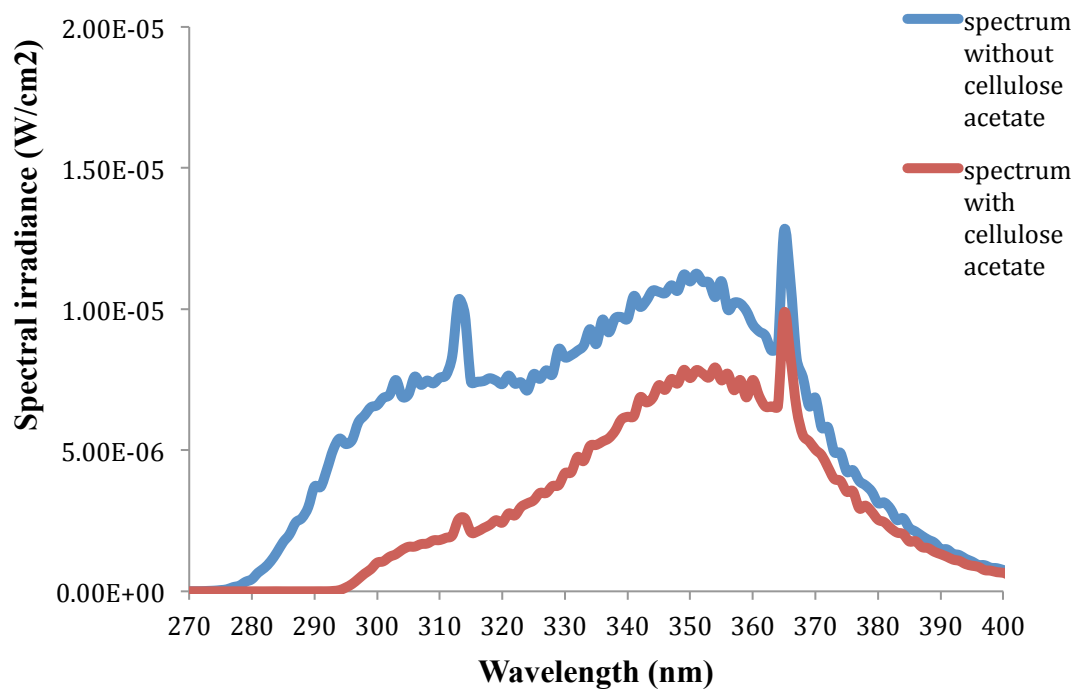


**Figure 2.3 Irradiance output of UV lamps used in cell culture studies with and without cellulose acetate filter**

Output of UV lamps is shown with and without cellulose acetate filter, as measured with the OL754 spectroradiometer.

## 2.2.11 ULTRAVIOLET IRRADIATION

When the plated cells reached appropriate confluency (approximately 70-80%), they were ready to be irradiated; this was typically within 48 hours of replacing medium with medium without supplements.

Equipments for UVR were set up in a laminar flow hazard hood to allow sterile conditions and temperature stability as shown in Figure 2.4. Prior to irradiation, cell medium was replaced with Martinez buffer solution containing D-glucose, a solution that does not absorb light in the UV spectrum whilst maintaining necessary nutrient levels for the cells during the period of irradiation. As shown in Figure 2.4, both plates equally received cellulose acetate filtered UVR and cells were irradiated on a turntable and plates rotated 180° halfway through the irradiation procedure to ensure even UVR exposure throughout the multi-well plate. For sham-irradiated group, the plate was covered with aluminium foil whilst UVR-irradiated group was not.

Immediately following the irradiation, Martinez solution was aspirated from each well and replaced with media containing vehicle, $1,25(OH)_2D_3$ or $20(OH)D_3$.
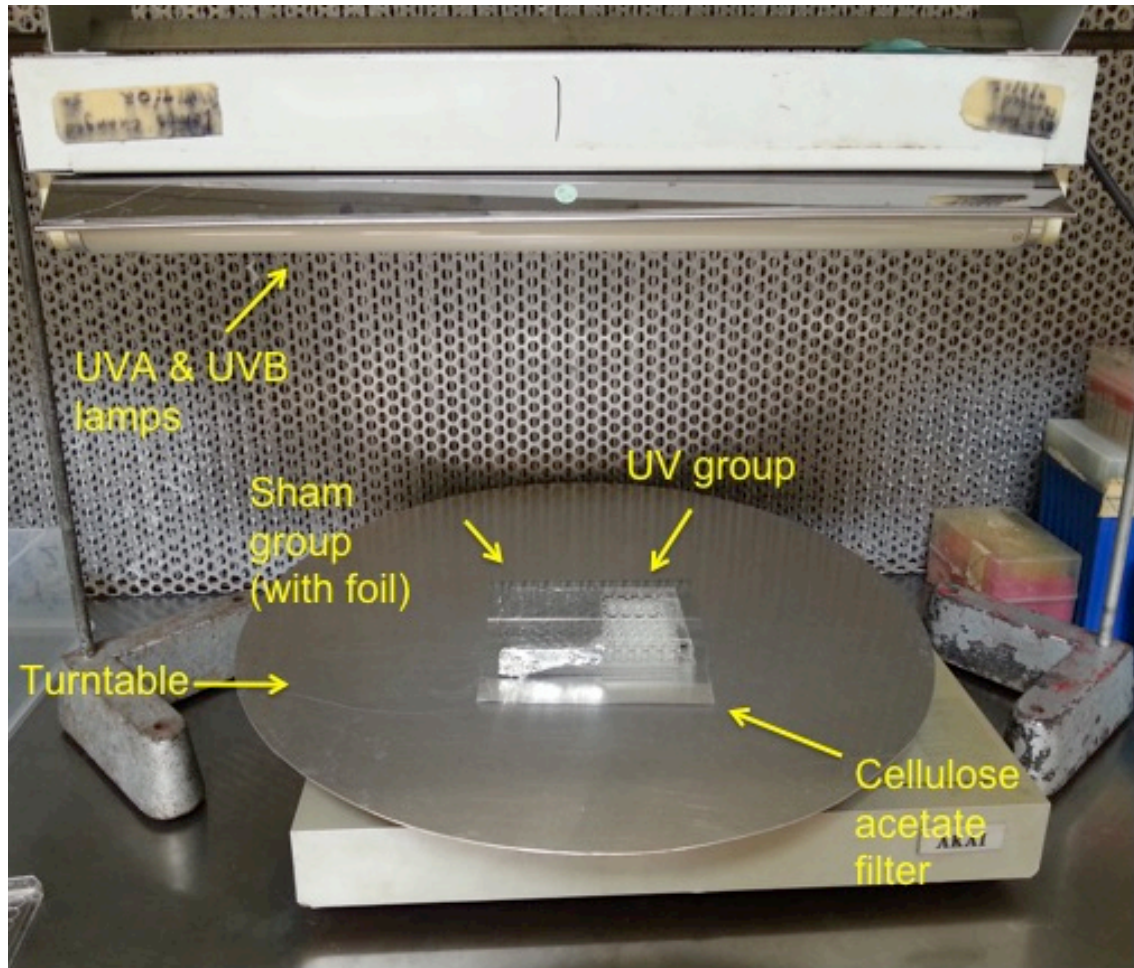
**Figure 2.4 UV irradiation set up for cell culture studies**

Equipments include, turntable, cellulose acetate filter, UV lamps, aluminium foil, cell culture plate.

## 2.2.12 TREATMENTS

Vehicle treatment was made by mixing 1 µL of spectroscopic ethanol with 999 µl cell growth medium. $1,25(OH)_2D_3$ treatment was made by mixing 1 µL of 10-6 M $1,25(OH)_2D_3$ stock solution with 999 µL cell growth medium to make a final concentration of 10-9 M. $20(OH)D_3$ treatment was made by mixing 1 µL of 10-6 M $20(OH)D_3$ stock solution with 999 µl cell growth medium.

## 2.2.13 CELL VIABILITY ASSAY

In order to assess cell viability, cell titer blue assay was chosen, an assay that provides a homogenous, fluorescent method for monitoring cell viability. This assay is based on the ability of metabolically active cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Nonviable cells would not generate fluorescence as they have lost metabolic activity. The cell titer blue assay was conducted according to the manufacturer's protocol (Promega, Madison, WI, USA).

First, the cells that were grown in culture flasks were trypsinized as explained in Sections 2.3.4 and 2.3.5. Then these cells were seeded onto 96-multi well plates as shown in Section 2.3.10. Then the cells were handled for UVR protocol as explained in Section 2.3.12. Once the UV irradiation protocol was finished, cells were immediately treated with the treatments and incubated for at least 24 hours at 37°C. Then the cell titer blue solution was thawed, and 10 μL of it was added to each well containing the cells. This was incubated for at least 90 minutes and up to 4 hours at 37°C. After the incubation period, the cell medium in each well was pipetted and transferred to a black well plate for fluorescence reading.

The fluorescence reading was achieved using the BMG Fluostar Galaxy Plate reader in Bosch Molecular Biology Facility (University of Sydney, Australia).

## 2.2.14 STATISTICAL ANALYSIS

For *in vitro* studies with HaCaT cells and keratinocytes, results are based on quadruplicates of each treatment, and are presented as either single experiments, expressed as mean ± 1 standard deviation, or as normalized data from more than one experiment, expressed as mean ± SEM. Standard error of mean and coefficients of variation of percentage cell survival were calculated using the coefficients of variation of each mean by the method of Colquhoun (Colquhoun, 1971).

For both *in vitro* studies, comparisons between treatment groups were made by one-way analysis of variance (ANOVA) followed by Dunnett's test using the GraphPad Instat statistical program (GraphPad Software Inc., San Diego, CA).

## 2.3  METHODS FOR *IN VIVO* STUDIES WITH SKH:HR-1 MICE

### 2.3.1  ANIMALS AND MAINTENANCE

*In vivo* studies were conducted on a total of 100 inbred female Skh:HR-1 albino hairless mice, aged 6 weeks at commencement of the experiment. These mice were provided by Associate Professor Vivienne Reeve from the Faculty of Veterinary Science (University of Sydney, Australia). Animal husbandry was provided by Laboratory Animal Services (University of Sydney, Australia). Animals were supplied water and standard mouse chow Gordon Rat and Mouse Pellets (Yandeera, Australalia) ad libitum. Compressed paper bedding was obtained from Fibrecycle Pty Ltd (Mudgeeraba, Australia). Animal studies were carried out according to a protocol approved by the Animal Ethics Committee of the University of Sydney (protocol number 5457) and complied with the Australian code of practice for the care and use of animals for scientific purposes.

Each mouse weighed, on average, 24 g initially. Animals were randomly allocated to each treatment group and were allowed about a week to adapt to the environment prior to the commencement of experimentation.

Initially, hundred mice were allocated to five different treatment groups, each group consisting of twenty mice. Mice in each group were ear-tagged with identification numbers for future weighing and monitoring purposes. Each group of mice was housed in an individual opaque plastic box with wired tops, compressed paper pellets for bedding and a red translucent plastic igloo. The climate for the animals was maintained at a temperature of 24 (±1) °C with a photoperiod cycle of 12 hours provided by yellow fluorescent lamps, without any UVR emission. Dry-pelleted commercial feed was provided to the mice and the tap water was provided through the gravity-feed bottle. Feed, water and bedding were regularly changed by

Laboratory animal services (LAS) staff of the University of Sydney. Mice were regularly monitored and weighed once a week for 36 weeks. This is shown in Figure 2.5 and Figure 2.6.

❖ NOTE: 1 mouse from group 1 (vehicle group) died in week 4 for unknown reasons, leaving a total of 99 mice for *in vivo* studies.
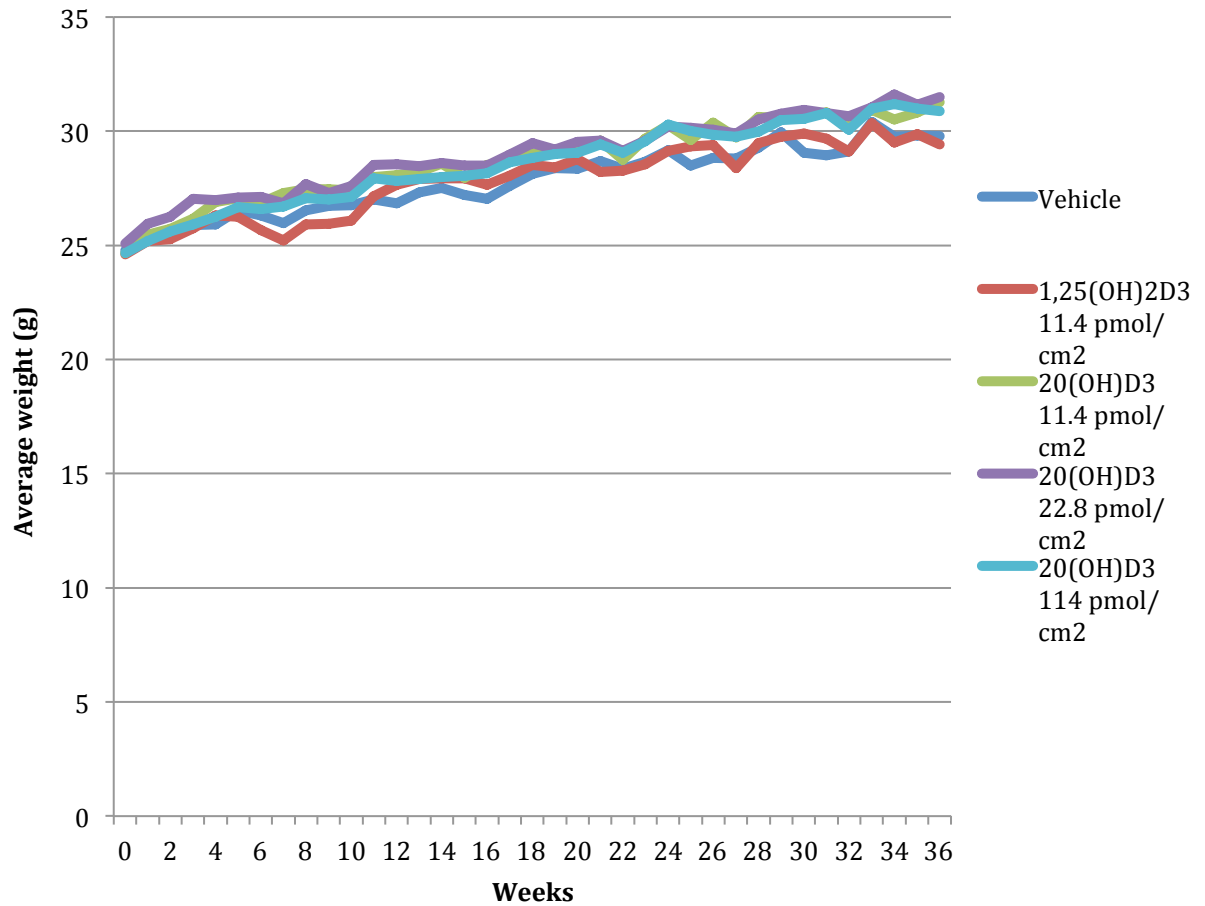


**Figure 2.5 Average weight of mice**
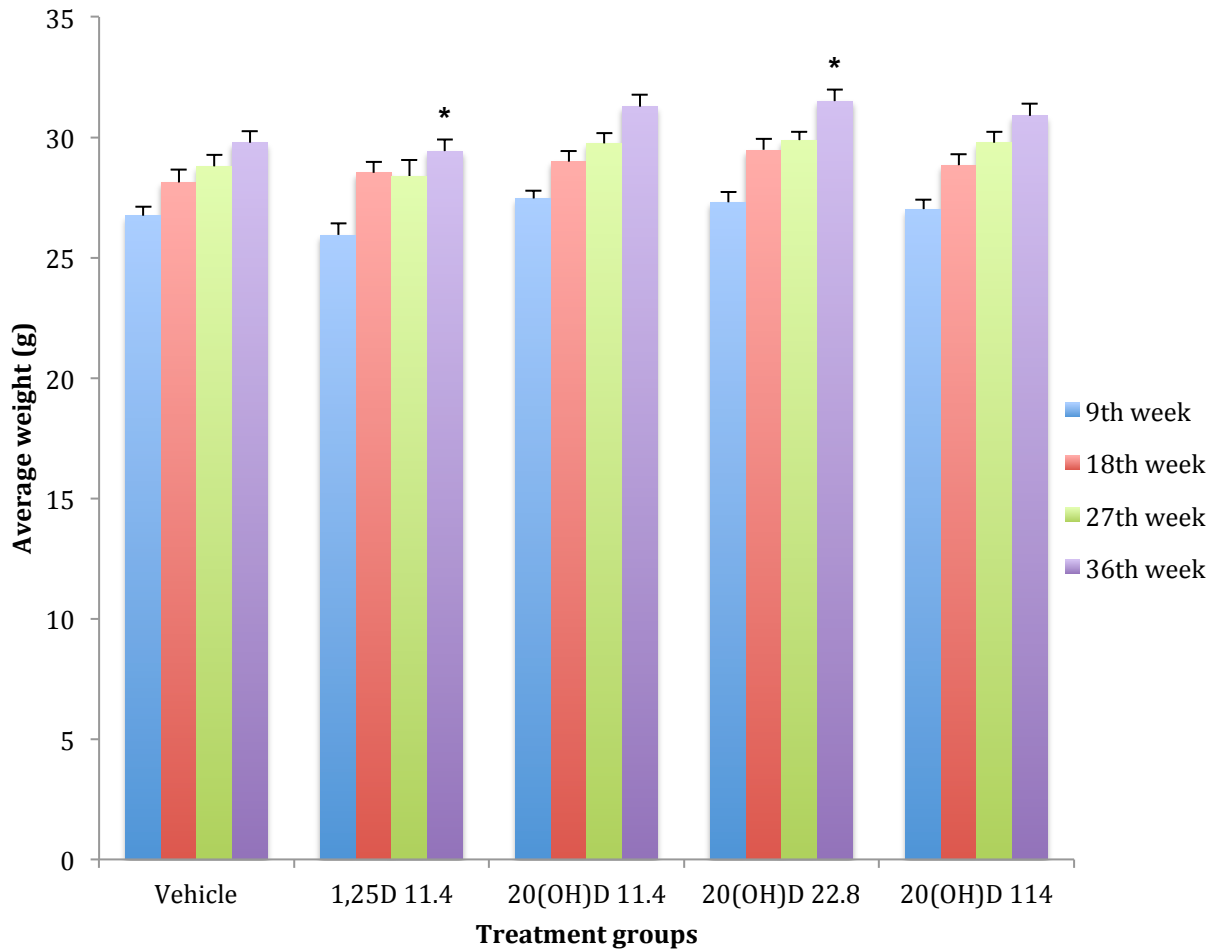
**Figure 2.6 Average weight of mice at different time points**

For the purpose of comparison between groups, 4 different time points (9th, 18th, 27th and 36th week) were selected to show average weight of each treatment group. . Data presented as mean ± SEM. Each treatment group results significantly different to each other group at each time point is expressed as: * $p<0.05$, otherwise p = NS.

## 2.3.2  EXPERIMENTAL DESIGN

Animals were divided into treatment groups of five for a photocarcinogenesis study. The test substances were, vehicle, $1,25(OH)_2D_3$ (11.4 ρmol/cm$^2$), $20(OH)D_3$ (11.4 ρmol/cm$^2$), $20(OH)D_3$ (22.8 ρmol/cm$^2$) and $20(OH)D_3$ (114 ρmol/cm$^2$) .

## 2.3.3  UV SOURCE AND IRRADIATION

The solar simulated UVR source was one fluorescent UVB tube flanked by six UVA tubes. The output of these lamps is shown in Figure 2.5. Before commencing irradiation, UV lamps were warmed up for about 20 minutes. Then the broadband measurement was taken using a calibrated broadband radiometer to estimate the output of UVR. The highest measurement along different parts of the lamps was used to calculate the time period for a dose of 1 MED using the following formula: Time of exposure (1 MED) = 25.43 / UVB output.

Whilst the UV lamps were being warmed up, mice in each group were transferred from their original boxes into the perforated boxes, in order to permit enough airflow during UV irradiation. At the same time, a sticker with identification details for each box was put onto the perforated boxes respectively. Then a film of cellulose triacetate filter was put on to each box in order to eliminate UVR of wavelengths below 290 nm. Then the boxes were directly exposed to UVR source and were irradiated for a time period, depending on the UVR output each day. On average, mice were all exposed to about 15 minutes of UVR. A cooling fan was turned on during irradiation to assist ventilation for the mice.
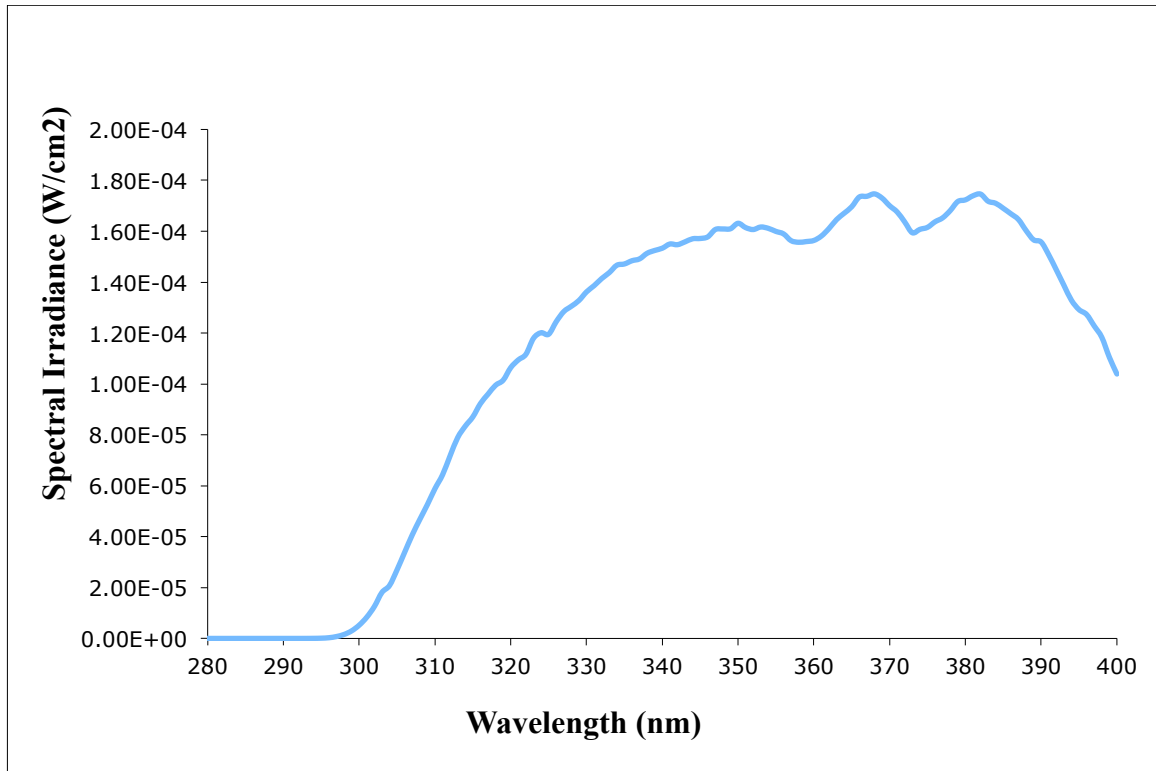
**Figure 2.7 Output of UV lamps used in animal studies**

Output of solar simulated UVR measured in May, 2013.

## 2.3.4  TOPICAL TREATMENTS

Mice were treated topically on the dorsum (Figure 2.6) with the following treatments immediately after UV irradiation: vehicle only, $1,25(OH)_2D_3$ (11.4 pmol/cm$^2$), $20(OH)D_3$ (11.4 pmol/cm$^2$), $20(OH)D_3$ (22.8 pmol/cm$^2$) and $20(OH)D_3$ (114 pmol/cm$^2$)

.



**Figure 2.8 Application of topical treatments**

Treatments were applied to the dorsum of mice using a pipette.

The mice were treated with 100 µL of treatments immediately after each irradiation. Topical treatment involved pipetting the treatments from vials and gently applying them on the dorsum of the skin of each mouse, covering approximately 7 cm$^2$ (Figure 2.6). Applied lotions were left on cages to air-dry prior to returning them to their home boxes. Topical treatments consisted of a base lotion containing the test

compound. The stock solutions of $1,25(OH)_2D_3$ and $20(OH)D_3$ were dissolved in a spectroscopic ethanol and diluted in the base lotion consisting of ethanol, propylene glycol and water to a final solvent ratio of 2:1:1 respectively.

### 2.3.5  PHOTOCARCINOGENESIS

Groups of 20 mice were exposed to a suberythemal dose of UVR on five days per week for 10 weeks. The daily dose provided 0.658 $kJ/m^2$ UVB and 20.30 $kJ/m^2$ UVA, which is approximately one minimal erythemal dose (MED) for these mice. Immediately after irradiation mice were treated topically on the irradiated dorsal skin with 100 microlitres of either vehicle only, $1,25(OH)_2D_3$ (11.4 $pmol/cm^2$), $20(OH)D_3$ (11.4 $pmol/cm^2$), $20(OH)D_3$ (22.8 $pmol/cm^2$) or $20(OH)D_3$ (114 $pmol/cm^2$). Mice receiving $1,25(OH)_2D_3$ (11.4 $pmol/cm^2$) showed non-significant weight reductions compared with mice in other treatment groups. This was likely to be due to dehydration caused by hypercalcemia/hypercalciuria. Hypercalcemia results in increased urinary calcium, which then causes a diuresis and subsequent weight loss. Calcium levels were not directly measured but mice were monitored for weight loss as it is a sensitive sign of hypercalcemia. Weight loss was not observed in mice treated with either vehicle group or $20(OH)D_3$ group. In order to improve dehydration in $1,25(OH)_2D_3$ group, sipper bottles were replaced with bigger holes to facilitate drinking. Following this change, no significant weight loss was observed - average mouse weight did not differ significantly from that of other groups (Figure 2.6).

The time of appearance, type of tumour (whether papilloma, cyst, hematoma or SCC) and the location of tumours were mapped for each mouse over the next 30 weeks. Skin tumour formation was confirmed by a tumour outgrowth of 1 mm or greater in diameter, which was expressed as the tumour incidence, the average tumour multiplicity (average number of tumours per tumour-bearing mouse), tumour

latency, and tumour regressions for each treatment group. SCCs were assessed visually by experienced researchers (Nicole Painter and Vivien Reeve) and then confirmed histologically.


## 2.3.6  IMMUNOSUPPRESSION STUDIES

The contact hypersensitivity response was tested to investigate the effects of $1,25(OH)_2D_3$ and $20(OH)D_3$ on UVR-induced systemic immunosuppression. Mice were sensitised approximately one week after the photocarcinogenesis study finished, with 100 µL of 2% oxazolone (Sigma, St. Louis, MO, USA) (w/v) in absolute alcohol, applied to the non-irradiated abdominal skin. A group of age-matched, non UV-irradiated mice were used as a control. Sensitisation was repeated on the subsequent day with the same treatment to ensure sensitisation in all areas of abdominal skin. The sensitised mice were challenged one week after by application of 5 µL 2% oxazolone to each surface of the pinnae of both ears so that each mouse received 20 µL in total. Ear thickness measurements were recorded to estimate the amount of oedema before challenge and at the designated time points (18 and 20 hours) after challenge.

## 2.3.7 SKIN BIOPSIES AND TUMOUR COLLECTION

Mice with SCCs greater than 1 cm in diameter were euthanized by $CO_2$ asphyxiation and the skin sample was harvested with scissors and forceps. The collected sample was stored in formalin for future histological analyses and record.

## 2.3.8 STATISTICAL ANALYSIS

For mass monitoring of mice, results are expressed as mean ± 1 SEM. Comparisons between treatment groups were made by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using the GraphPad InStat statistical program (GraphPad Software Inc., San Diego, CA).

For *in vivo* studies, results are based on 19-20 mice per group for the photocarcinogenesis study and 11 mice in age matched control group. Results are expressed as mean ± 1 standard deviation (SD) or ± 1 SEM as indicated. Comparisons between treatment groups were made by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test using the GraphPad InStat statistical program (GraphPad Software Inc., San Diego, CA).

The Mantel-Haenszel log rank test (Mantel and Haenszel 1959) was used to analyze incidence data in the photocarcinogenesis study. Using this test, all treatments were compared to vehicle-treated group over the entire time course of the experiment, specifically by testing whether there was a difference in the risk of developing a tumour.

# 3  RESULTS

## 3.1  *IN VITRO* STUDIES WITH HACAT CELLS AND KERATINOCYTES

### 3.1.1  CELL VIABILITY AFTER UVR AND TREATMENT WITH 20(OH)D$_3$ AND 1,25(OH)$_2$D$_3$

Treatment of HaCaT cells or keratinocytes with 20(OH)D$_3$ or 1,25(OH)$_2$D$_3$, immediately after UVR for incubation period of 24 hours did not show significant photoprotection, as determined by an increase cell viability after UVR (Figure 3.1; p = NS).
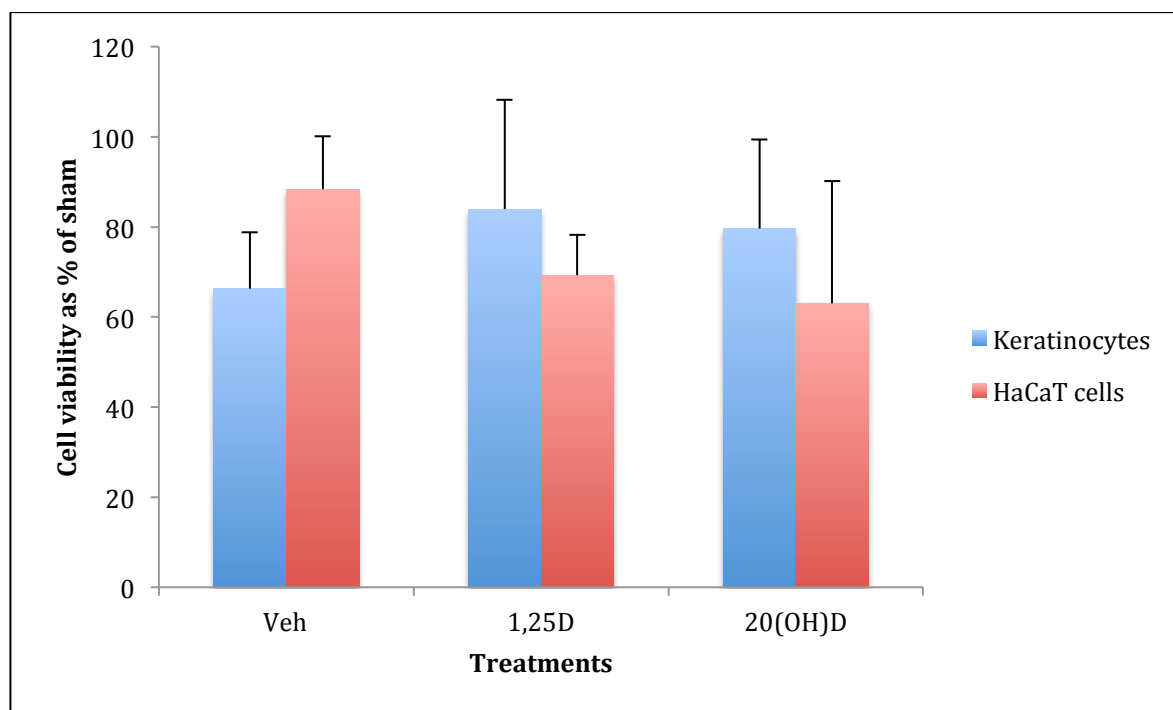


**Figure 3.1 Cell viability after UVR**

Viability of HaCaT cells and keratinocytes after UV exposure and various treatments was expressed as a percentage of sham-irradiated group. Results are expressed as mean ± SD. n = 4 replicates per group.

## 3.2  *IN VIVO* STUDIES

### 3.2.1  PHOTOCARCINOGENESIS

Figure 3.2 shows representative photographs of the papillomas and squamous cell carcinomas (SCC), which developed in mice subjected to the photocarcinogenesis protocol, while Figure 3.3 shows photomicrographs of tumour histology.
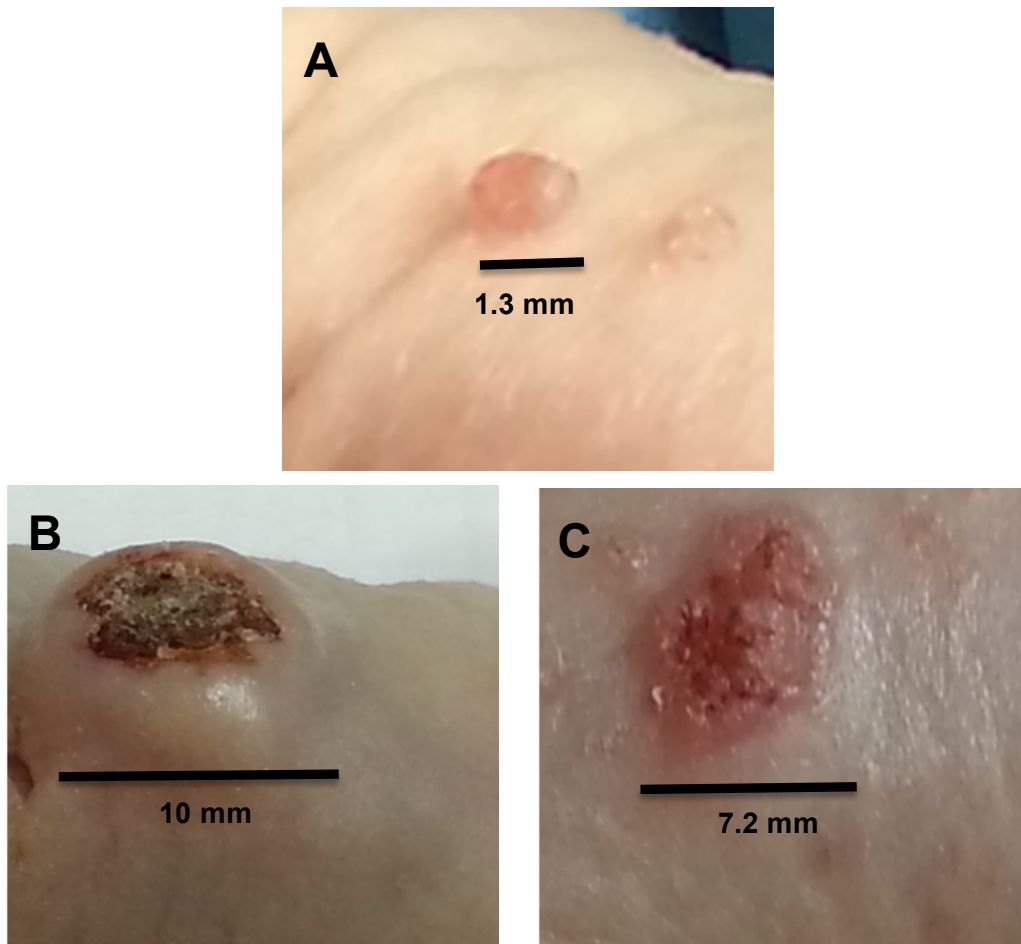


**Figure 3.2 Papilloma and SCCs**

Photographs of papilloma (A) and SCCs (B and C) are taken from different mice. Photograph B shows the SCC of mouse that was sacrificed at 35 weeks and C shows SCC of mouse at 40 weeks. Note the difference in shapes of SCCs between B and C; B is an elevated above the most superficial skin layer and contains a "crater", whilst C is more flattened. This is usually suggestive of the difference in degree of cancer cell invasion into deeper layers of skin.

**A** — Epidermal hyperplasia. Scale bar: 400 um
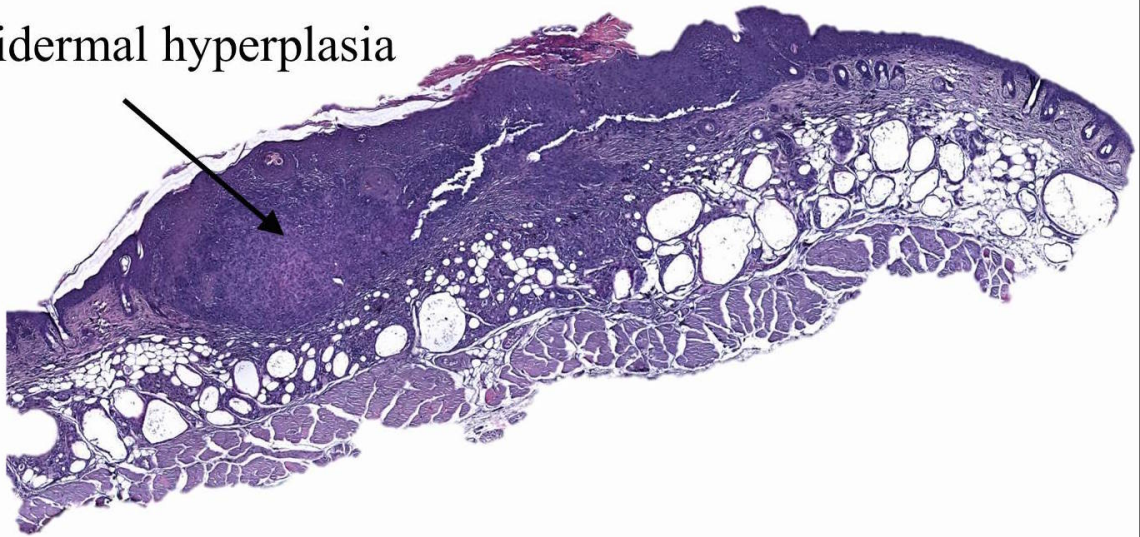


**B** — Keratinisation, "Horn pearls", Epidermal invasion. Scale bar: 400um

Keratinisation

400 um

C
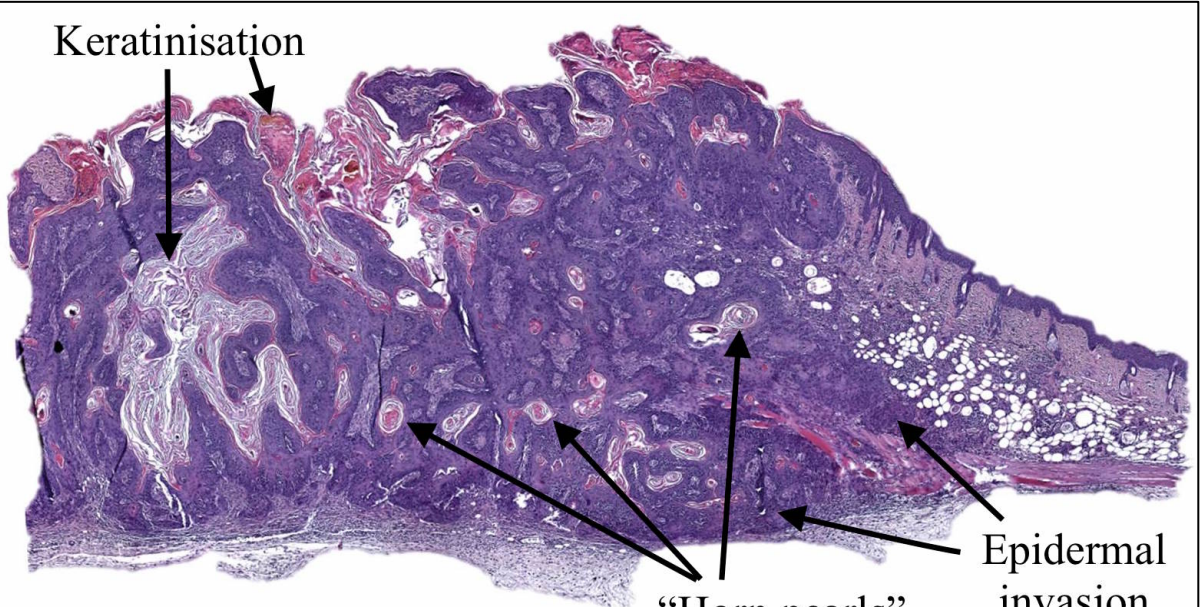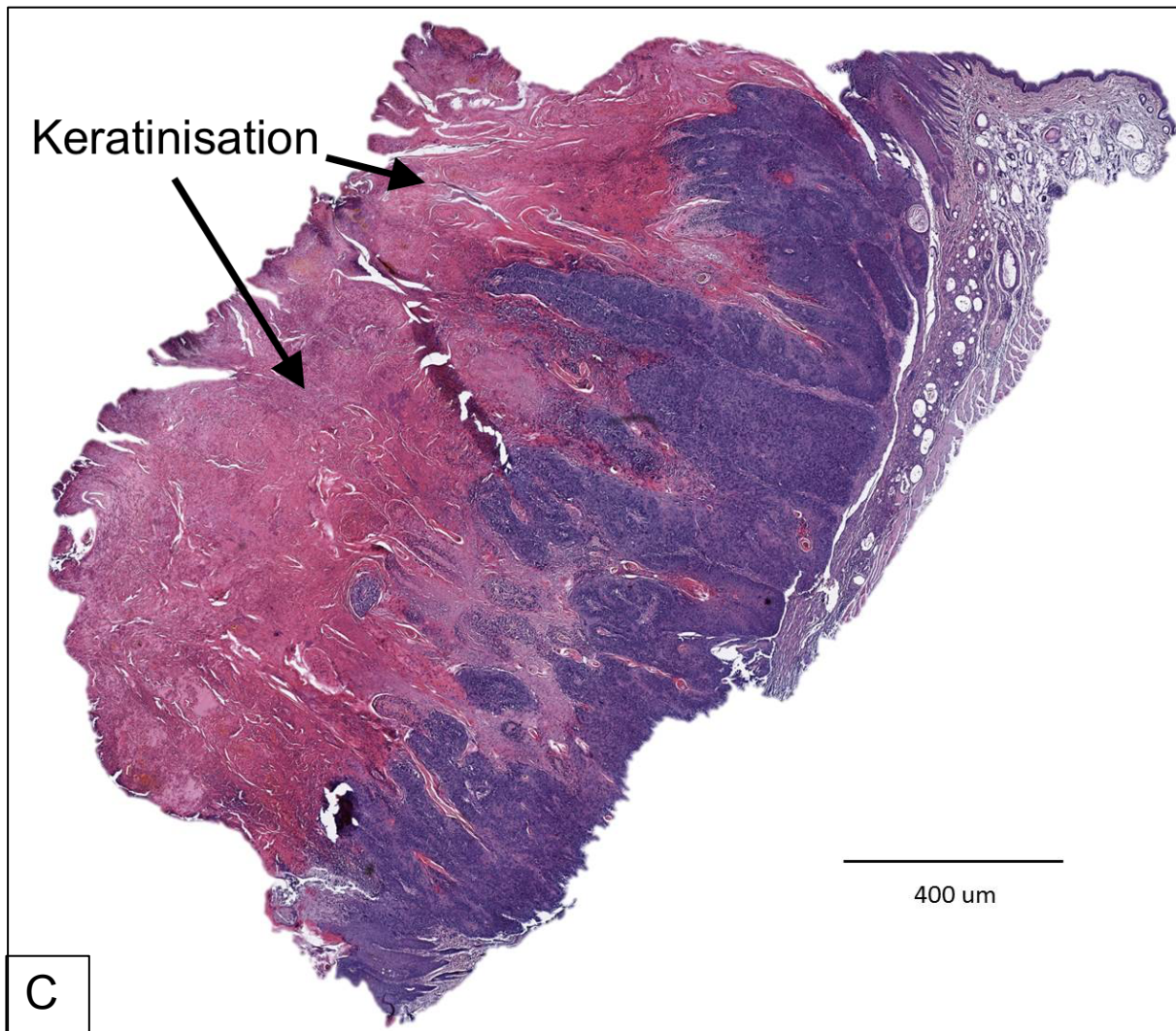
**Figure 3.3 Photomicrographs of papilloma and SCCs**

a)  Papilloma showing epidermal hyperplasia with no significant dermal invasion (10x).

b)  "Horn pearls" typically seen in SCC, keratinisation on tumour surface and within abnormal cells in the epidermis. A lump of cells of epidermal origin invading into dermis (pale mauve colour) is typical characteristic feature of SCC (10x).

c)  SCC showing extensive keratinisation (10x).

    Scale bar = 400 µm

## 3.2.1.1 INCIDENCE

Tumour incidence was calculated as the per cent of mice in each group bearing one or more tumours, recorded on a weekly basis throughout the 40 week *in vivo* study. Table 3.1 shows statistical data generated by a Mantel-Cox log rank analysis of incidence data (GraphPad, Prism) in which all treatments were compared to vehicle group over the entire time course of the experiment, specifically by testing whether there was a difference in the risk of developing a tumour. Treatment with $1,25(OH)_2D_3$ (11.4 ρmol/cm$^2$) resulted in a significantly lowered risk of tumour development compared with vehicle treatment. However, treatment with the CYP11A1 metabolite, $20(OH)D_3$ (11.4 and 22.8 ρmol/cm$^2$) resulted in a significantly higher risk of developing a tumour compared with vehicle treatment. Figure 3.4 clearly demonstrates the reduction in tumour incidence over the 40 week time course by $1,25(OH)_2D_3$ (11.4 ρmol/cm$^2$). Figure 3.5 (A-D) shows individual group "survival" by using Kaplan-Meier method as a comparison between each group and the vehicle group in relation to the percentage of mice bearing tumour across 40 weeks.
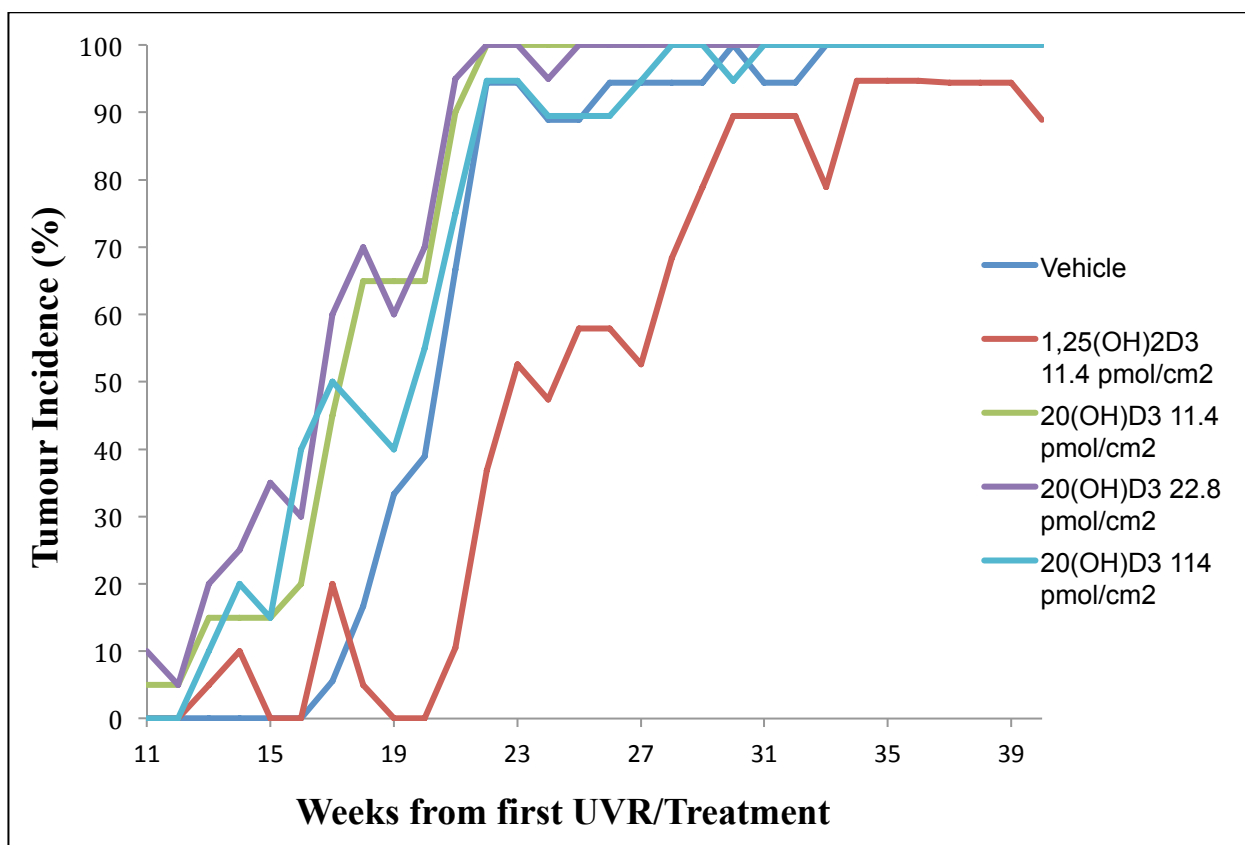
**Figure 3.4 Tumour incidence**

Tumour incidence, including both papillomas and SCCs. Incidence calculated at each weekly time point as per cent of mice in each group bearing at least one tumour. In all groups except the $1,25(OH)_2D_3$ group, the tumour incidence reached 100% by the end of the 40 week study.

**Table 3-1 Summary of Mantel-Cox log rank analysis of papilloma incidence data**

| Comparison by Mantel-cox Log Rank Analysis | P value | Significance and risk of developing tumour |
|---|---|---|
| Vehicle Vs. $1,25(OH)_2D_3$ 11.4 pmol/cm$^2$ | 0.0001 | *** ; reduced risk |
| Vehicle Vs. $20(OH)D_3$ 11.4 pmol/cm$^2$ | 0.0287 | * ; increased risk |
| Vehicle Vs. $20(OH)D_3$ 22.8 pmol/cm$^2$ | 0.0050 | **; increased risk |
| Vehicle Vs. $20(OH)D_3$ 114 pmol/cm$^2$ | 0.4050 | Not significant |

**A**

Survival proportions: Survival of Veh Vs 1,25D 11.4
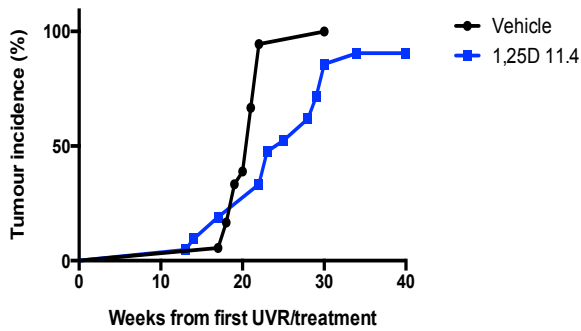
**B**

Survival proportions: Survival of Veh Vs 20OHD 11.4
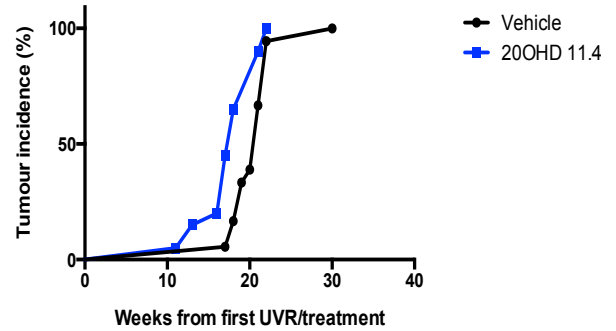
**C**

Survival proportions: Survival of Veh Vs 20OHD 22.8
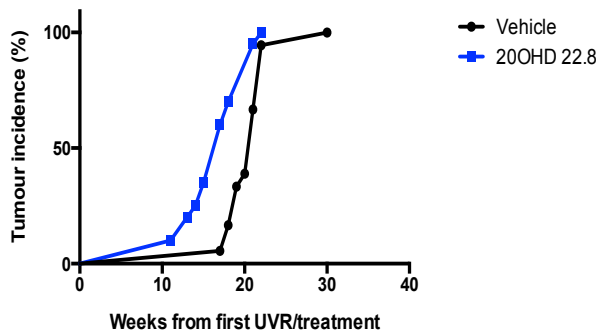
**D**

Survival proportions: Survival of Veh Vs 20OHD 114
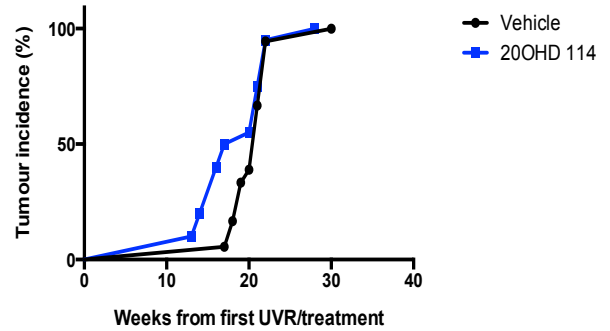
**Figure 3.5 Survival analysis of tumour incidence**

In conventional survival analysis, y-axis is expressed as percentage deaths. For this analysis, survival curves show time until each mouse present any tumour (papillomas and/or SCCs), expressed as a percentage of total number of mice at specific time point. Note that in the $1,25(OH)_2D_3$-treated group (A), mice developed tumours more gradually over 40 weeks, whereas all mice in vehicle group presented tumours by week 30. In all other groups treated with $20(OH)D_3$, mice developed more tumours at earlier time points similar to the vehicle-treated group. In the $1,25(OH)_2D_3$-treated group, the tumour incidence did not reach 100% at the end of the study.

### 3.2.1.2 MULTIPLICITY

Tumour multiplicity was calculated as the average number of tumours per tumour-bearing mouse. Figure 3.6 shows the average tumour multiplicity throughout the 40 week course of study. Significant reductions in tumour multiplicity were observed with $1,25(OH)_2D_3$ (11.4 pmol/cm$^2$). Average tumour multiplicity was significantly reduced from $1.43 \pm 0.18$ in vehicle-treated mice to $0.00 \pm 0.00$ ($p < 0.001$) in mice treated with $1,25(OH)_2D_3$ (11.4 pmol/cm$^2$) at 20 weeks following the first UVR exposure, and remained significantly reduced for the entire study thereafter. At week 30, a further reduction in multiplicity was noted in the $1,25(OH)_2D_3$-treated group, from $7.61 \pm 0.94$ in the vehicle group to $2.82 \pm 0.44$ ($p < 0.001$) and an increase in multiplicity was noted in $20(OH)D_3$-treated group (22.8 pmol/cm$^2$), $13.17 \pm 1.23$ ($p < 0.01$). Tumour multiplicity at three time points is shown in Figure 3.7. Statistical information was obtained by one-way ANOVA followed by Dunnett's multiple comparisons test.
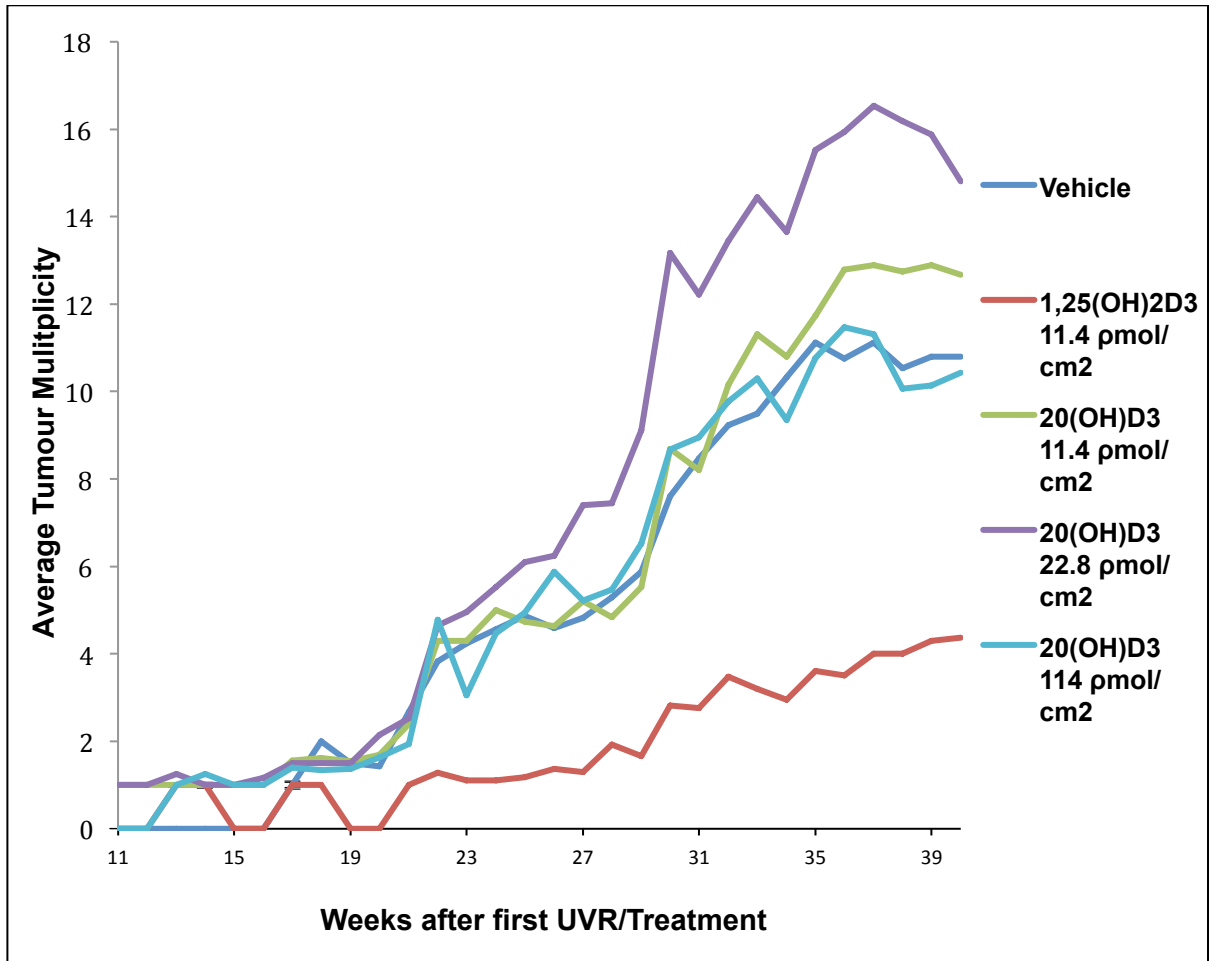
**Figure 3.6 Tumour multiplicity**

Average tumour multiplicity, including both papillomas and SCCs. Multiplicity calculated at each weekly time point as the average number of tumours per tumour-bearing mouse.
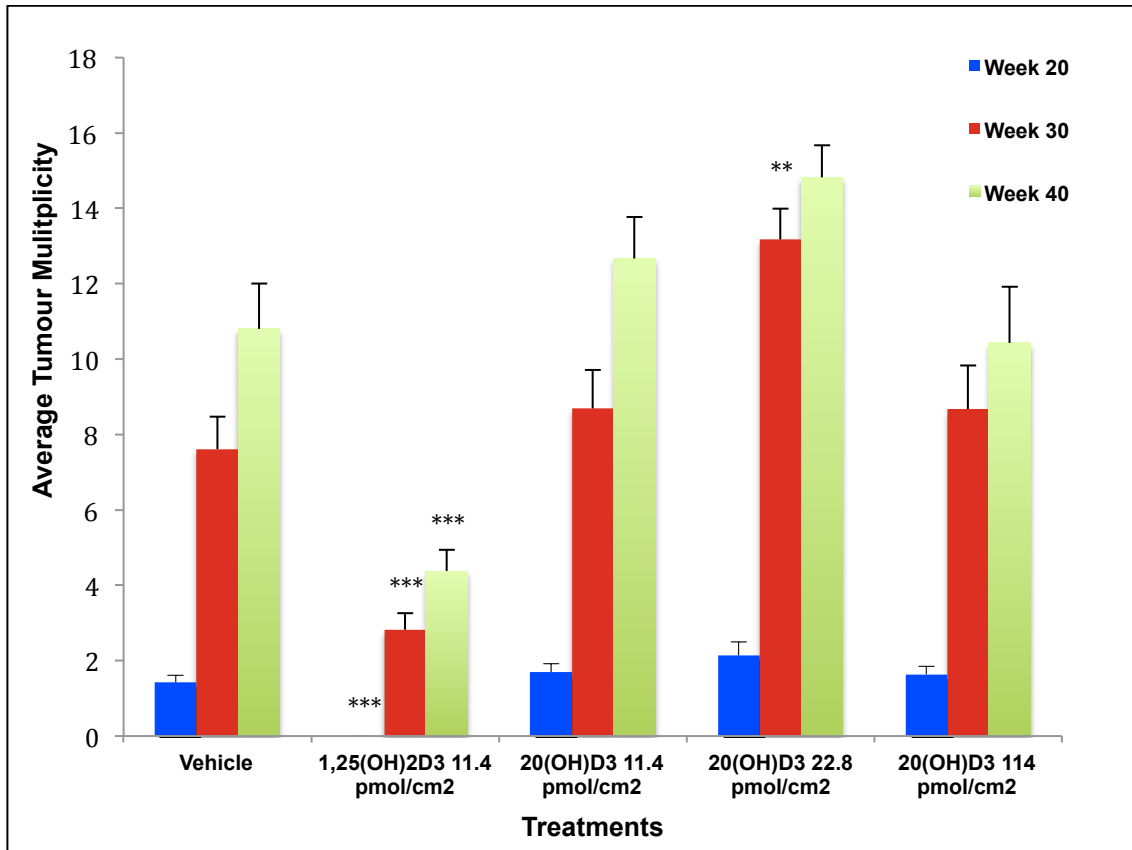
**Figure 3.7 Tumour multiplicity; 10 weeks interval**

Average tumour multiplicity at 20, 30 and 40 weeks for each treatment group. Data presented as mean ± SEM. Each treatment group results significantly different to vehicle group at each time point is expressed as: ** $p < 0.01$, *** $p < 0.001$.

### 3.2.1.3 LATENCY

Latency is the period to onset of first tumour (or papilloma) formation in mice and this varied between the treatment groups. As shown in Figure 3.8, the average latency in the vehicle-treated group was $20.8 \pm 2.8$ weeks from the first UVR exposure and topical treatment. This was similar to $1,25(OH)_2D_3$-treated mice ($11.4$ pmol/cm$^2$) which had an average latency of $20.8 \pm 6.9$ weeks. On the other hand, all three $20(OH)D_3$ groups had significantly lower average latency compared to the vehicle-treated group. The average latency for the three doses of $20(OH)D_3$ from the lowest to highest were $17.9 \pm 3.1$ ($p<0.01$) , $16.9 \pm 3.4$ ($p<0.001$) and $17.9 \pm 3.3$ ($p<0.01$) weeks respectively, all significantly lower in comparison to the vehicle-treated group. Statistical information was obtained by one-way ANOVA followed by Dunnett's multiple comparisons test.
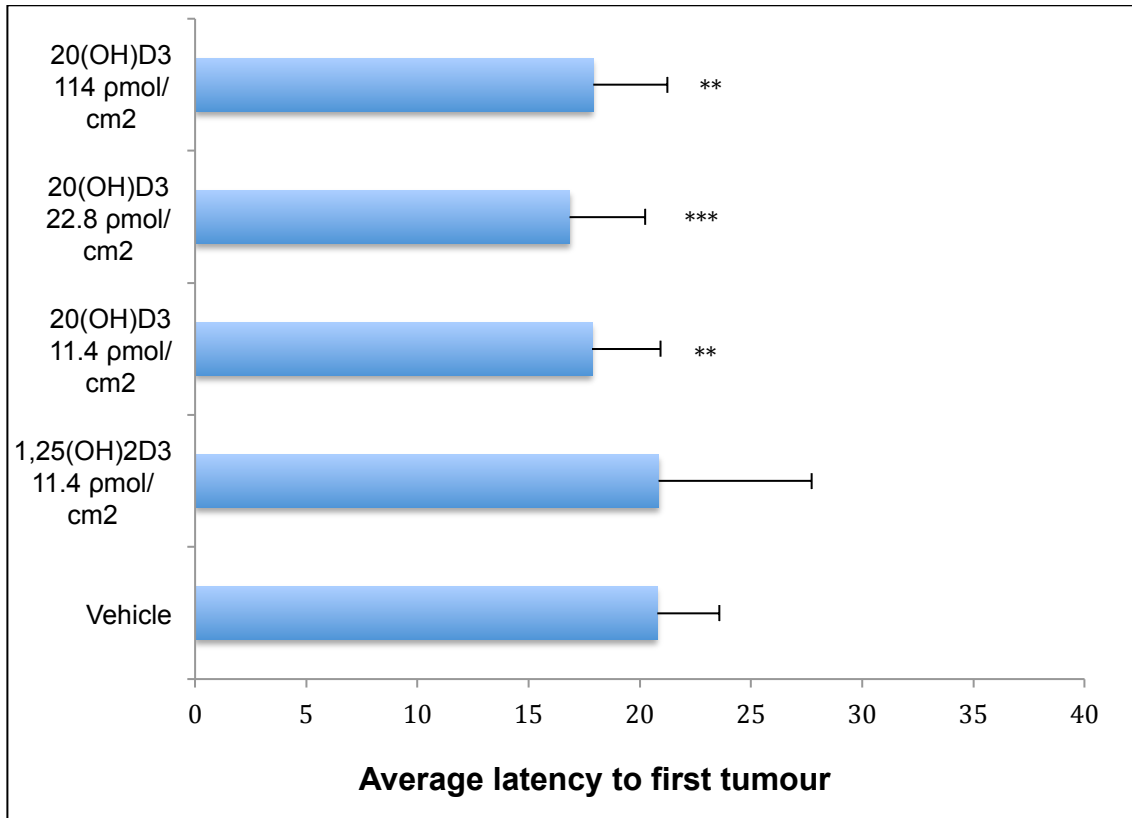
**Figure 3.8 Tumour latency**

Average tumour latency, calculated as mean time period from first UVR exposure to first tumour outgrowth. Significantly different from vehicle: ** $P < 0.01$, *** $P<0.001$, n = 20, (except vehicle n =19), mean ± SD.

## 3.2.1.4  REGRESSIONS

A regression was recorded when a previously noted tumour totally disappeared. In the vehicle group there was a total of 76 tumour regressions; 47% of tumours completely regressed. There was a total of 55 regressions in $1,25(OH)_2D_3$ 11.4 $\rho mol/cm^2$ group, and percentage of tumour regressions in this group was 79%. It should be noted that there were fewer tumours overall in this $1,25(OH)_2D_3$-treated group. Table 3.2 shows the regression data for each treatment group.

**Table 3-2 Tumour regression data**

| Treatment group | No. regressions | Total no. of tumours at 40 weeks. | Regression incidence |
|---|---|---|---|
| Vehicle | 76 | 162 | 47% |
| $1,25(OH)_2D_3$ 11.4 $\rho mol/cm^2$ | 55 | 70 | 79% |
| $20(OH)D_3$ 11.4 $\rho mol/cm^2$ | 92 | 228 | 40% |
| $20(OH)D_3$ 22.8 $\rho mol/cm^2$ | 112 | 237 | 47% |
| $20(OH)D_3$ 114 $\rho mol/cm^2$ | 114 | 146 | 78% |

### 3.2.1.5 SQUAMOUS CELL CARCINOMAS (SCCS)

Some of the papillomas progressed to squamous cell carcinomas (SCCs) throughout the course of the study. This was later confirmed histologically by extensive invasion into the dermis (as shown in figure 3.3). Table 3.3 shows statistical data generated by a Mantel-Cox log rank analysis of incidence data in which all treatments were compared to control over the entire time course of the experiment, specifically by testing whether there was a difference in the risk of SCC development.

**Table 3-3 Summary of SCC incidence data**

| Treatment group | No. of mice with SCC | No. SCCs | SCC Incidence |
|---|---|---|---|
| Vehicle | 8 | 9 | 42% |
| $1,25(OH)_2D_3$ 11.4 pmol/cm$^2$ | 3 | 4 | 15% |
| $20(OH)D_3$ 11.4 pmol/cm$^2$ | 9 | 9 | 45% |
| $20(OH)D_3$ 22.8 pmol/cm$^2$ | 10 | 11 | 50% |
| $20(OH)D_3$ 114 pmol/cm$^2$ | 6 | 9 | 30% |

**Table 3-4 Summary of Mantel-cox log rank analysis of SCC incidence data**

| Comparison by Mantel-cox Log Rank Analysis | P value | Significantly lower |
|---|---|---|
| Vehicle Vs. $1,25(OH)_2D_3$ 11.4 pmol/cm$^2$ | 0.041 * | yes |
| Vehicle Vs. $20(OH)D_3$ 11.4 pmol/cm$^2$ | 0.711 | no |
| Vehicle Vs. $20(OH)D_3$ 22.8 pmol/cm$^2$ | 0.358 | no |
| Vehicle Vs. $20(OH)D_3$ 114 pmol/cm$^2$ | 0.616 | no |

* The log rank analysis between the vehicle and the $1,25(OH)_2D_3$ treatment groups showed that mice in the $1,25(OH)_2D_3$ group were at significantly lower risk of developing a SCC than those in the vehicle treatment group.
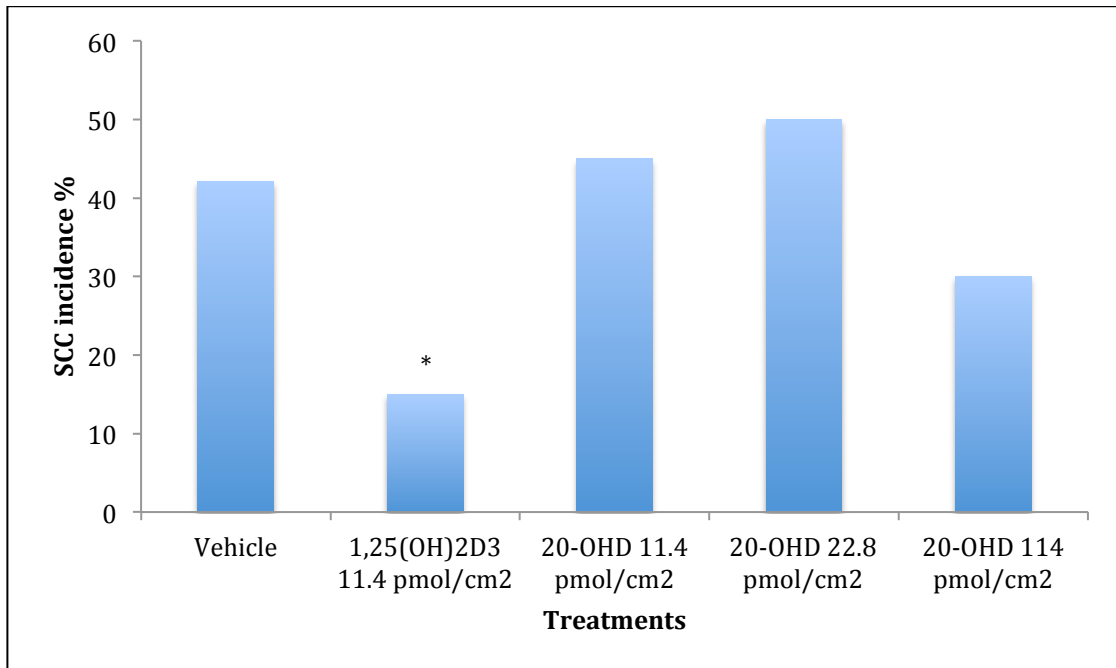
**Figure 3.9 Percentage SCC incidence at 40 weeks**

Mice that received $1,25(OH)_2D_3$ treatment showed significantly less risk of developing SCC (refer to table 3.4).

## 3.2.1.6 IMMUNOSUPPRESSION

Neither $1,25(OH)_2D_3$ nor $20(OH)D_3$ were effective at inhibiting UVR-induced immunosuppression measured by the contact hypersensitivity reaction at the end of the 40 week photocarcinogenesis study. Figure 3.10 shows the average change in ear thickness for non-irradiated age matched mice and UV-irradiated mice treated with vehicle, $1,25(OH)_2D_3$ or $20(OH)D_3$. Average change in ear thickness was calculated as the difference between pre- and post-challenge ear thickness. Note that the mice in this study were all UV-irradiated as they were involved in the photocarcinogenesis study except for the mice in the age-matched control (AMC) group and all had reduced ear swelling in comparison to the age-matched control group ($p < 0.0001$).
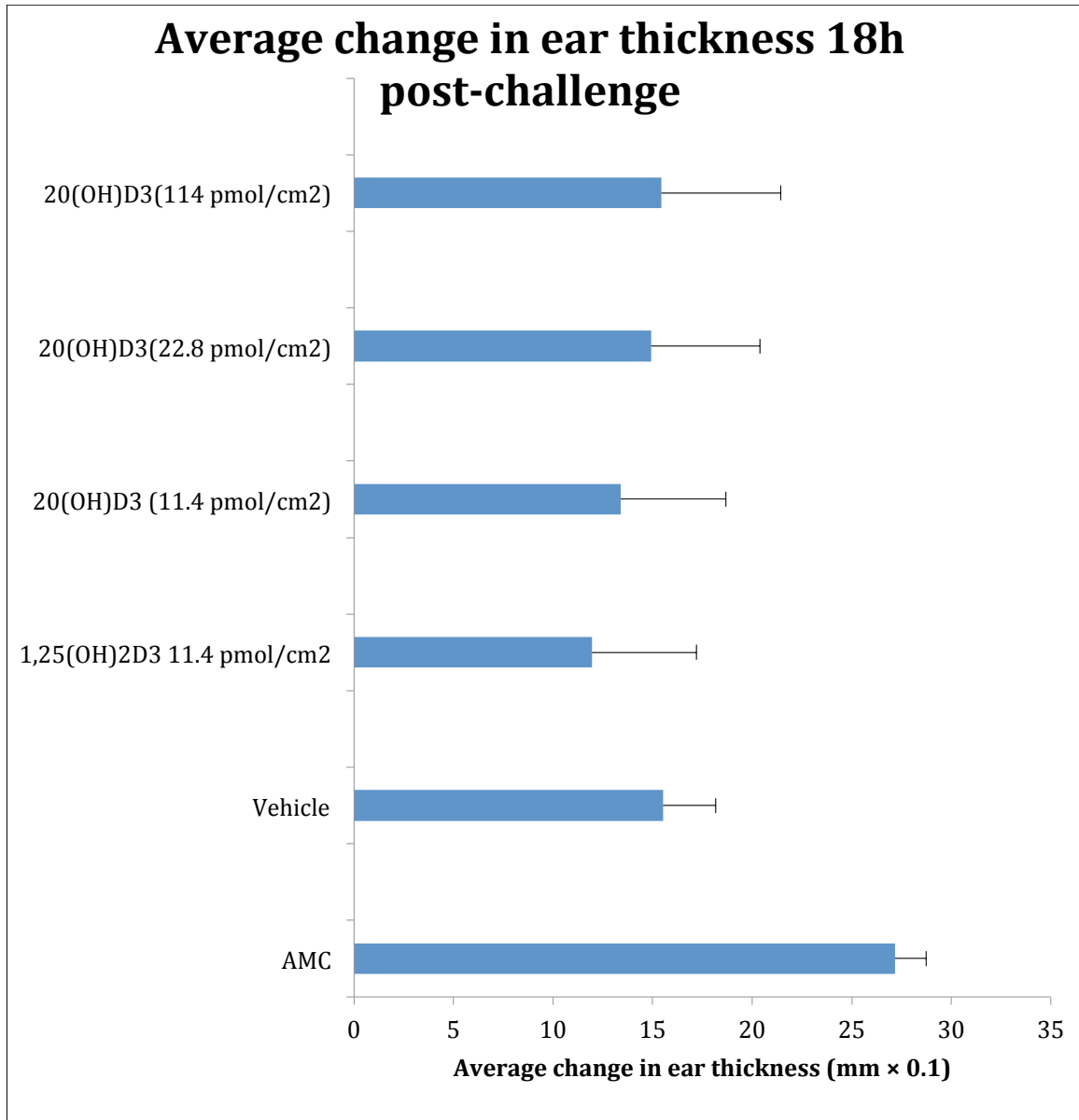
**Figure 3.10 Average change in ear thickness 18 hours post-challenge**

*AMC refers to Age-matched control mice. n = 11 for AMC, n = 12 for vehicle, n = 16 for $1,25(OH)_2D_3$ 11.4 pmol/cm$^2$, n = 15 for $20(OH)D_3$ 11.4 pmol/cm$^2$, n = 13 for $20(OH)D_3$ 22.8 pmol/cm$^2$ and n = 14 for $20(OH)D_3$ 114 pmol/cm$^2$ group, mean $\pm$SD.

**Table 3-5 Comparison of ear swelling against age-matched control group**

| Comparison by GraphPad prism analysis | P value |
|---|---|
| AMC Vs. Vehicle | <0.0001 *** |
| AMC Vs. 1,25(OH)$_2$D$_3$ 11.4 $\rho$mol/cm$^2$ | <0.0001 *** |
| Vehicle Vs. 20(OH)D$_3$ 11.4 $\rho$mol/cm$^2$ | <0.0001 *** |
| Vehicle Vs. 20(OH)D$_3$ 22.8 $\rho$mol/cm$^2$ | <0.0001 *** |
| Vehicle Vs. 20(OH)D$_3$ 114 $\rho$mol/cm$^2$ | <0.0001 *** |

## 3.3  DISCUSSION

The results from short term *in vitro* studies showed that $20(OH)D_3$ was not photoprotective in HaCaT cells. Neither $20(OH)D_3$ nor $1,25(OH)_2D_3$ were found to be protective against UV-induced cell death in primary keratinocytes either as examined by a cell viability assay. This protection against acute UV damage in primary keratinocytes is in contrast with what my colleagues (Tongkao-on et al., 2015) found; $20(OH)D_3$ and $1,25(OH)_2D_3$ reduced UVR-induced DNA damage in primary keratinocytes and hence are photoprotective (Tongkao-on et al., 2015). Since photoprotection was not observed in my studies in primary keratinocytes, it is not possible to interpret the results in HaCaT cells. One possible reason that it was predicted that HaCaT cells would not be a good model for keratinocytes for this functionality is that there could be differences between HaCaT cells and normal keratinocytes that could affect the vitamin D non-genomic pathway or other intracellular mechanisms indirectly associated with photoprotection such as apoptosis. HaCaT cells express the vitamin D receptor (VDR) at equal levels to keratinocytes, which is essential for genomic actions of vitamin D compounds, but whether these cells express ERp57, the other protein required for photoprotection (Sequeira et al, 2012) is unknown. Furthermore, recent findings revealed that UVB increases apoptosis and autophagy-related genes such as bcl2, caspase 3 and LC3 in HaCaT cells at the same time (Ryu et al., 2011). In addition, it was found that HaCaT cells have different transcriptional profiles of cornified-envelope associated genes such as filaggrin, loricin and involucrin to normal human keratinocytes (Seo et al, 2012). These proteins may only play a minor role in photoprotection but the difference in the level of expression of these proteins suggests a limitation of HaCaT cells as a model to study UVR-induced damage. Furthermore, previous studies conducted by the Mason group showed that $1,25(OH)_2D_3$ significantly increases the expression of p53 protein in keratinocytes after UVR exposure, which plays an

important role in photoprotection (Gupta et al., 2007). However, photoprotection by $1,25(OH)_2D_3$ in HaCaT cells is likely to be limited as they have p53 mutation.

The reasons for the lack of demonstrated photoprotection by $1,25(OH)_2D_3$ in keratinocytes in this study are unclear. The most likely reason is inexperience of the experimenter, since this positive finding has been observed by numerous other researchers in the lab and by other groups (Dixon et al., 2012; Haes et al, 2003). Dixon *et al* (2012) reported that $1,25(OH)_2D_3$ reduced UVR-induced cell loss significantly in keratinocytes in comparison to vehicle group. Also keratinocytes are derived from different donors and do vary on this basis in terms of how susceptible they are to photodamage and how much they are protected by a given concentration of $1,25(OH)_2D_3$ (Gupta et al., 2007).

The results from our photocarcinogenesis study reinforced the findings in previous studies by the Mason group that demonstrated the photoprotective ability of $1,25(OH)_2D_3$ against acute UVR-induced events, such as DNA damage, which is associated with an initiation of carcinogenesis (Dixon et al., 2011). For the long term *in vivo* photocarcinogenesis study, novel data, outlined in this thesis, one dose of $1,25(OH)_2D_3$ and three different doses of $20(OH)D_3$ were tested. The single dose of $1,25(OH)_2D_3$ was used as a positive control, as it had been shown in previous studies by my group to inhibit photocarcinogenesis in these mice (Dixon et al., 2011). In my study, mice that received $1,25(OH)_2D_3$ (11.4 pmol/cm$^2$) showed markedly reduced tumour multiplicity and significantly lower incidence of SCCs. These results confirmed that $1,25(OH)_2D_3$ at 11.4 pmol/cm$^2$ was very effective at reducing UVR-induced skin carcinogenesis and was aligned with the previous study. These results also provided a good positive control for the other test agent. On the other hand, in spite of predictions made from the studies of my colleagues (Tongkao-on et al., 2015), $20(OH)D_3$ was found not to be protective against UV-induced

carcinogenesis in female Skh:hr1 mice at any of three doses – 11.4, 22.8 and 114 $pmol/cm^2$. Instead, the three doses, in general, showed significantly lower average tumour latency and higher average tumour multiplicity compared to the vehicle-treated group. More specifically, the lower doses of $20(OH)D_3$ – 11.4 and 22.8 $pmol/cm^2$, showed significantly increased risk of tumour incidence. Also, $20(OH)D_3$ at 22.8 $pmol/cm^2$ showed significantly increased average tumour multiplicity at 30 weeks against the vehicle-treated group (Figure 3.7), and the lowest average tumour latency, compared to other doses of $20(OH)D_3$ (Figure 3.8). In contrast, $20(OH)D_3$ at 114 $pmol/cm^2$ did not confer significantly increased risk of developing tumours (Table 3-1), and showed the lowest SCC incidence amongst other doses. Although the results are inconclusive, it can be proposed from the parameters explored in this *in vivo* study that $20(OH)D_3$ at 22.8 $pmol/cm^2$ provides the least protection from UV-induced carcinogenesis whilst the highest dose of 114 $pmol/cm^2$ showed the most protection amongst other doses. These results suggest the need for future investigation into various doses of $20(OH)D_3$ in delivering protective effects against carcinogenesis. This is possible with $20(OH)D_3$ as it does not cause hypercalcaemia in mice at a dose as high as 30 $\mu g/kg$, whilst $1,25(OH)_2D_3$ causes severe hypercalcaemia at a dose of 2 $\mu g/kg$, thereby suggesting that $20(OH)D_3$ can potentially have greater therapeutic window (Wang et al., 2012).

Thus the results did not support the use of $20(OH)D_3$ as photoprotective agent since it was not shown to be effective in reducing tumour incidence, multiplicity or progression of premalignant lesions to squamous cell carcinomas (SCCs). This is in stark contrast to the previous results, using acute UV exposure, from our colleagues (Tongkao-on., 2015), who found that there were significant reductions in UVR-induced DNA damage such as thymine dimers in human keratinocytes (by approximately 70%) and in male and female Skh:hr1 mice (by 98%) at both 23 and 46 $pmol/cm^2$ doses of $20(OH)D_3$. My colleagues also conducted a contact

hypersensitivity (CHS) study on both $20(OH)D_3$ and $1,25(OH)_2D_3$ in these mice, and found that they provided protection to UV-induced immunosuppression in female hairless mice, but not in male hairless mice. The photocarcinogenesis studies involved only female mice because fighting amongst male mice cause skin lesions, which may interfere with a long term photocarcinogenesis study. In their studies, the ear swelling in female hairless mice decreased in the vehicle-treated group following UVR. On the other hand, in $1,25(OH)_2D_3$ and $20(OH)D_3$ treated groups, the immune response following UVR measured as increase in ear swelling, was close to that of non-irradiated treated mice, suggesting immune protection.

Previous CHS studies from colleagues (Wannit Tongkao-on, personal communication) have shown that the $1,25(OH)_2D_3$-treated group showed significantly less immune suppression following the 40 week photocarcinogenesis study. Although from our CHS study, $1,25(OH)_2D_3$ was not shown to be immune protective at 40 weeks , there could be several reasons why this maybe the case. First, since the CHS experiment was conducted after the completion of 40 weeks of carcinogenesis study, the number of mice in each group varied due to animal ethics requirements (mice were culled when tumours reached 1 cm), reducing the n value. Secondly, as these mice were tagged for identification using ear tags that had been in place for 40 weeks, the majority of mice had one side of their ears with tags already swollen, preventing accurate measurement. Although both ears were measured in this study, it may be necessary to exclude ears with tags from the measurement for future studies.

The conflicting evidence for $20(OH)D_3$ between the early studies by colleagues (Tongkao-on et al., 2015) that showed protection against acute effects of UV exposure, and its inefficacy in our long term skin carcinogenesis study suggest that a clear relationship between its acute and long term effects does not exist or at least

that other factors may play a role in UV-induced tumour formation. Other factors may include the ability to stimulate the growth of blood vessels, ability to stimulate local invasion and metastasis, and the ability to regulate proteins involved in tumour growth. Recent study about the effect of $1,25(OH)_2D_3$ on E-cadherin expression showed that it increases the expression of E-cadherin in breast cancer cells, thereby decreasing the aggressiveness of cancerous cells (Lopes et al., 2012). It could be worthwhile to investigate the effects of $20(OH)D_3$ on proteins involved in cell-cell adhesion and hence cancer invasion. Also, $20(OH)D_3$ may play a role in formation of tumour vasculature. Formation of tumour vasculature is regulated by the balance of anti-angiogenic and pro-angiogenic molecules. However, recent report revealed that $1,25(OH)_2D_3$, mediated by VDR, has antiproliferative actions on tumour-derived endothelial cells (Chung et al., 2009). Examination of blood vessels in tumours from the mice given different treatments was not within the scope of this study, but could be undertaken in the future.

All in all, it can be concluded that reduction in photocarcinogenesis may not be adequately determined just by the ability to reduce DNA damage or immunosuppression from acute UV exposure.

It is interesting to note that QW (1α-hydroxymethyl-16-ene-24,24-difluoro-25-hydroxy-26,27-bis-homovitaminD$_3$ or QW-1624F2-2), another analogue of $1,25(OH)_2D_3$ showed a similar pattern of protection against UV exposure as $20(OH)D_3$. The results in Dixon *et al* (2012) showed that QW significantly ($p < 0.05$–0.01) reduced UV-induced DNA lesions (CPD) in skin fibroblasts and keratinocytes and reduced cell death after UV exposure. Also, in the mouse model, they found that QW (22.8 pmol/ cm$^2$) reduced immunosuppression in a short term UV exposure model. Yet these results did not translate well into the photocarcinogenesis study. In the photocarcinogenesis study, which followed the same protocol as was used in our

study, the same dose of QW that reduced UV-induced immunosuppression, did not reduce tumour incidence nor did it reduce tumour multiplicity. In fact, the average tumour multiplicity measurement of QW was similar to that of the vehicle-treated group and was significantly higher than that of the $1,25(OH)_2D_3$-treated group, suggesting that QW was not photoprotective in a chronic UV exposure experiment, at least at the concentration that was used (Dixon et al., 2012). Thus the results of our *in vivo* study using $20(OH)D_3$ paralleled the recent studies on QW, suggesting that vitamin D-like compounds may have different mechanisms in response to acute or chronic exposures to UVR.

Albino hairless mice are reliable model of carcinogenesis for a number of reasons. Firstly, being hairless, these mice do not need to be shaved and thus artefacts due to skin damage from shaving could be avoided. Their lack of tyrosinase activity results in reduced production of melanin pigment in the skin and retina (Reeve and Ley, 2004; Saran et al., 2004), leading to acceleration in tumour development and thus a convenient method for induction and analysis of carcinogenesis. In such accelerated models of skin cancer, results may be more informative with respect to the protective capacity of vitamin D compounds. It has been proposed that inbred mouse strains differ in their susceptibility to the promotional and malignant conversion stages of carcinogenesis (Brown and Balmain, 1995), but it is also arguable that the use of inbred mice for carcinogenesis studies is advantageous due to the reduced genetic variability between treatment groups, allowing for more meaningful comparisons. In addition, species-specific differences in immune cell populations of skin in mice and humans pose problems in interpreting results from cellular immune reactions. As mentioned earlier, mouse skin has a prominent population of Vγ5+ dendritic epidermal T cells (DETCs) that secrete pro-inflammatory cytokines. However, this cell population is absent in human skin and

can thus be a limitation in mouse model to study mechanisms that drive inflammation in human skin upon exposure to UVR (Pasparakis et al., 2014).

To conclude, there are numerous limitations to be considered about this study. Firstly, for *in vitro* experiments, cell viability assay conducted for the investigation of photoprotection of keratinocytes provided only superficial data. Other experiments to investigate the barometer of cellular damage such as caspase assay for apoptosis could be done in the future. Western blots for proteins involved in DNA repair such as XPC and OGG1 were conducted but the results were not shown due to technical difficulties. Secondly, the limitations of *in vivo* study involve investigating immunosuppression using the same mice previously used for the photocarcinogenesis study. These mice had ear tags on one ear since the beginning of *in vivo* experiment, and this may have affected tests on the other ear by the time of immunosuppression studies. For future studies, tail marking may eliminate this concern.

Aside from the effects of compounds, UVR and other experimental environments mentioned above, it has been suggested through a recent study that difference in experimenter's gender could induce different physiological stress in mice and rats. A study has found that male-related olfactory stimuli induce a robust physiological stress response that results in stress-induced analgesia (Sorge et al, 2014). This suggests that male handling can interfere with correct representation of immune response in mice. As both a male and a female engaged in this photocarcinogenesis study, there is a possibility that male handling could have caused immune suppression in mice and hence misrepresentation of the effects of vitamin D compounds on immune response. In addition, the fact that painting of treatments was done by inexperienced experimenter further compromises the effects of compounds in all degrees.

Nevertheless, it is reasonable to conclude overall that while 20(OH)D$_3$ protects against acute UV damage, it is not protective, long term, for photocarcinogenesis. The reasons for this discrepancy are unclear. The implications for the importance of the CYP11A1 pathway in photoprotection are also not clear, since there are many other metabolites of vitamin D and overirradiation products which are produced by this enzyme in skin (Tuckey at el., 2008).

# 4 CONCLUSION AND FUTURE DIRECTIONS

In conclusion, neither $1,25(OH)_2D_3$ nor $20(OH)D_3$ were photoprotective in primary keratinocytes in the short term *in vitro* studies, presented here, although both compounds reduced DNA damage and improved keratinocyte viability in studies undertaken by other members of the Mason group.

Despite the photoprotective effects of $20(OH)D_3$ in the short term preliminary studies (Tongkao-on et al., 2015), $20(OH)D_3$ was not protective in long term *in vivo* carcinogenesis. Only $1,25(OH)_2D_3$ was found to be photoprotective in the long term *in vivo* studies. The fact that the short term photoprotective effects of $20(OH)D_3$ did not translate into long term photocarcinogenesis protection suggests a number of things. It is possible that $20(OH)D_3$ may have effects to reduce cell apoptosis or it may enhance angiogenesis in early tumours. Although none of these were tested, it would be possible to examine blood vessel formation in the micrographs of the papillomas and SCCs. These methods could include the Verhoeff-Van Gieson stain for vasculature, Ki-67 stain for lymphocyte invasion, caspase assay or TUNEL for apoptosis, and LC3-II immunoblot for autophagy. As previously mentioned in chapter 1, photocarcinogenesis is a multistage, multistep process. Thus, in the future, it could be worthwhile investigating the effects of $20(OH)D_3$ not only when the tumour is fully developed but examining the tumours at different time points. For future studies, it would be worthwhile to investigate the difference in the expression levels of specific proteins such as XPC and p53, after treatment with both compounds, as these are crucial in protection against UVR.

Much attention has focused on UVB-induced DNA damage such as pyrimidine dimers but not much on reactive oxygen species (ROS). Although UVB is thought to be the main culprit of photocarcinogenesis, effects of UVA on skin cannot be neglected. The effects of UVA on photocarcinogenesis have been controversial but it is known to cause DNA damage indirectly by inducing ROS and lipid peroxidation (Nishigori, 2005). Future studies could examine whether $20(OH)D_3$ reduces oxidative damage to DNA or lipids.

As described in chapter 1, there are two distinct pathways of action for vitamin D compounds; a classical/genomic pathway and a rapid-acting/non-genomic pathway. It is known that $20(OH)D_3$ is a partial agonist of VDR but pathways mediating the actions of this compound remain uncertain. A recent study has reported that $20(OH)D_3$ is capable of activating non-genomic pathway. The study revealed that $20(OH)D_3$ and $20,23(OH)_2D_3$, act as antagonists of RORα and RORγ mediated transactivation. RORα and RORγ are nuclear receptors involved in global and local homeostasis. They are expressed in major skin cell populations including epithelial cells of the epidermis, hair follicles, sebaceous glands, sweat glands, dermal fibroblasts and immune cells. The fact that $20(OH)D_3$ can act as antagonists on immune cells expressing these nuclear receptors suggests the possibility of $20(OH)D_3$ acting as a regulator of local or systemic immune response via RORα and RORγ receptors (Slominski et al., 2014). Even more recently, in the Mason group, it has been demonstrated that the reduction in UV-induced thymine dimers by $20(OH)D_3$ requires both the classical vitamin D receptor and the protein ERp57 (Jeremy Han, personal communication). ERp57 has been shown by Sequeira *et al* (2012) to co-immunoprecipitate with the vitamin D receptor and to be essential for the photoprotective effects of $1,25(OH)_2D_3$.

In chapter 1, it was mentioned that the knockout of 1α-hydroxylase enzyme (CYP27B1), enzyme essential for the production of $1,25(OH)_2D_3$, does not increase the susceptibility to photocarcinogenesis (Teichert et al., 2011). For this reason, $20(OH)D_3$, a vitamin D metabolite by CYP11A1 enzyme in the skin, was investigated. Thus, it could be interesting to examine the effects of the knock out of CYP11A1 enzyme in mouse skin, if these mice could be bred. Recently, a genetically modified mouse with a potential LoxP site flanking the CYP11A1 site has been generated by the Sanger Institute in the UK.

CYP11A1 (cytochrome P450scc) is an enzyme that catalyzes the conversion of cholesterol into progenolone, the first and rate-limiting step of steroidogenesis. It is expressed in the gonads and the adrenals under the control of pituitary peptide hormones. Knock out of CYP11A1 gene could result in pseudohermaphroditism. In order to avoid any side effects, tissue-specific factors would be necessary. This is possible in conditional gene knockout method using the Cre-lox system. With a keratin-14 promoter for Cre-, an epidermis-specific knockout of CYP11A1 gene is possible, allowing us to explore the physiological effects of other metabolites of CYP11A1 cleavage other than $20(OH)D_3$ (Scharfenberger et al., 2014). Although, $20(OH)D_3$ is the major metabolite of CYP11A1 induced metabolism of vitamin D3, there are also other metabolites such as $20,23(OH)_2D_3$, $20,22(OH)_2D_3$, $17,20(OH)_2D_3$ and $17,20,23(OH)_3D_3$. Of these metabolites, $20,23(OH)_2D_3$ has shown to inhibit proliferation of keratinocytes, and its inhibitory effect on DNA synthesis was shown to be comparable to that of $1,25(OH)_2D_3$ and $20(OH)D_3$, suggesting a potential role in anti-carcinogenic activity (Slominski et al., 2014; Janjetovic et al., 2010).

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