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Photoprotection by $1\alpha,25$-Dihydroxyvitamin D$_3$ and a vitamin D-like compound in human skin.

Eric Jung Yong Song

August 2014

A Thesis Submitted for the
Degree of Master of Philosophy (Medicine)
The University of Sydney
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Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 4 original papers published in peer reviewed journals, a chapter in a textbook and 1 manuscript yet to be submitted for publication. The core theme of the thesis is “Mechanisms of photoprotection by vitamin D and vitamin D-like compound in human skin”.

The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Physiology and Department of Dermatology at the University of Sydney and Bosch Institute, under the supervision of Professor Rebecca Mason and Professor Gary Halliday.

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: .................................................................

Date: ............................. 23th August, 2014............................
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Publications

$1\alpha,25$-Dihydroxyvitamin D$_3$ reduces several types of UV-induced DNA damage and contributes to photoprotection.


Vitamin D and death by sunshine. (Review)


Protection from UV damage and photocarcinogenesis by vitamin D compounds.

Clare Gordon-Thomson, Wannit Tongkao-on, Eric J. Song, Sally Carter, Katie Dixon, and Rebecca S. Mason. (Chapter in a Textbook)

Sunlight, Vitamin D and Skin Cancer (2nd Edition).
Editors: Jörg Reichrath

ISBN: 978-0-387-77573-9 (Print) 978-0-387-77574-6 (Online)

Novel vitamin D compounds and skin cancer prevention. (Review)


Primary oesophageal melanoma – a case report.

Eric J Song, Richard A Scolyer, Diona L Damian and John Thompson.

Accepted by World Journal of Surgical Oncology, March 2014.
Meetings and Presentations

2011 - Annual Molecular and Experimental Pathology Society of Australia Conference, The Institute of Health and Biomedical Innovation, Queensland Institute of Technology, Kelvin Grove, Brisbane, QLD.
Date 30/11/2011 Vitamin D session 1510-1645 Chair: Michael Kimlin
Oral Presentation: Photoprotection of Vitamin D and a Vitamin D like compound in human ex vivo skin.

2012 - Conjoint Australasian Society for Dermatology Research (ASDR) + Wound repair society of Australia, Cockle bay wharf, Sydney NSW
Photoprotection of Vitamin D and Vitamin D-like compound in human ex vivo skin.

International Academy of Pathology (Australian Division) 2014, Brisbane Convention Centre, Brisbane QLD.
Poster Presentation: Photoprotection of 1α,25-dihydroxyvitamin D₃ in human skin.

2013 - Australasian Society for Dermatology Research (ASDR) and Asian Society for Pigment Cell Research (ASPCR) Conjoint meeting, Mercure hotel, Sydney NSW.
Date 19/05/2013
Topical 1α,25-dihydroxyvitamin D₃, applied post-UV radiation to human volunteers, protects against three types of DNA damage, in part through XPC and DDB2 upregulation.
2013 - 6th Asia and Oceania Conference on Photobiology (AOCP). Nov 2013 Novotel, Sydney NSW

Oral Presentation: Photoprotection of Vitamin D and Vitamin D like compound in human skin – Randomised Clinical Trial.

Awarded best student presentation (Medicine Theme) for Asia and Oceania Conference on Photobiology.

2014 - International Academy of Pathology, Australian Division. May 30- June 1, 2014.

Brisbane Convention Centre, Brisbane QLD

Poster Presentation: Photoprotection of 1α,25-dihydroxyvitamin D3 in human skin.
Awards and Grants

2011 - Molecular and Experimental Pathology Society of Australia Annual Conference.
30/Nov-2/Dec. Travel grant received.

Oral Presentation: Photoprotection of Vitamin D and Vitamin D like compound in human skin – Randomised Clinical Trial.
Awarded best student presentation (Medicine Theme) for Asia and Oceania Conference on Photobiology.
# Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>1,25(OH)$_2$D$_3$</td>
<td>1α,25-dihydroxyvitamin D$_3$</td>
</tr>
<tr>
<td>6-4pp</td>
<td>6-4 photoproducts</td>
</tr>
<tr>
<td>8-NG</td>
<td>8-nitroguanosine</td>
</tr>
<tr>
<td>8-oxodG</td>
<td>8-oxo-7,8-dihydroguanine</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>25-hydroxyvitamin D</td>
</tr>
<tr>
<td>AMF</td>
<td>Advanced Microscopy Facility</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activation protein 1</td>
</tr>
<tr>
<td>BCC</td>
<td>basal cell carcinoma</td>
</tr>
<tr>
<td>BER</td>
<td>base excision repair</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>cyclin dependent kinase inhibitor 1A</td>
</tr>
<tr>
<td>CFC</td>
<td>chlorofluorocarbon</td>
</tr>
<tr>
<td>CPD</td>
<td>cyclobutane pyrimidine dimer</td>
</tr>
<tr>
<td>CUR</td>
<td>curcumin</td>
</tr>
<tr>
<td>DDB2</td>
<td>DNA damage binding protein 2</td>
</tr>
<tr>
<td>DIDS</td>
<td>4,4’-diisothiocyanatostilbene-2,2’-disulfonic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EB1089</td>
<td>Seocalcitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERp57</td>
<td>Endoplasmic reticulum stress protein 57</td>
</tr>
<tr>
<td>GAPD45</td>
<td>Glyceraldehyde phosphate dehydrogenase 45</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<tr>
<td>GGR</td>
<td>Global genomic repair</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Gamma interferon</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>JN</td>
<td>1α,25-dihydroxylumisterol3</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPKs</td>
<td>Mitogen activated protein kinases</td>
</tr>
<tr>
<td>MED</td>
<td>Minimal erythemaal dose</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Michigan cancer foundation-7</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OGG1</td>
<td>8-Oxoguanine glycosylase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>QW</td>
<td>1-hydroxymethyl-16-ene-24,24-F2-26,27-bishomo-25-hydroxyvitamin D3</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>ssUV</td>
<td>Solar simulated UV</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with Tween 20</td>
</tr>
<tr>
<td>TCR</td>
<td>Transcription coupled repair</td>
</tr>
<tr>
<td>TD</td>
<td>Thymine dimer</td>
</tr>
<tr>
<td>TFIIH</td>
<td>Transcription factor II H</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>THC</td>
<td>Tetrahydrocurcumin</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>UVR</td>
<td>Ultra violet radiation</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VDRE</td>
<td>Vitamin D response element</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>XPC</td>
<td>Xeroderma pigmentosum complementation group C</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

Ultraviolet (UV) radiation is an invisible part of the solar spectrum that is known to cause deleterious effects in skin such as skin cancer. Skin cancer incidence has progressively risen over the past several decades causing a significant burden on public health system, as well as considerable morbidity and mortality. The molecular pathways of how UV causes damage are reasonably well documented, however, it is still unclear what defence mechanisms are in place for protection and maintaining the normal integrity of our skin. In this study, one such defensive mechanism, the production of vitamin D compounds, will be examined to find how they provide photoprotection in human skin. At the same time, a novel vitamin D-like compound, tetrahydrocurcumin will be examined in parallel to see if it provides similar photoprotection as 1α,25-dihydroxyvitamin D₃. By doing so, important information may be gained not only on the mechanism of photoprotection, but also it may prove to be a very useful compound for use in sunscreens for the general public.

1.1 Ultraviolet radiation

Sunlight is made up of a continuous spectrum, including visible light (400-700nm), infrared (IR) (>700nm) and ultraviolet (UV) (<400nm) light (Diffey, 2002). Infrared gives us warmth, the visible spectrum is essential for photosynthesis where plants can produce energy for carbohydrate synthesis from atmospheric CO₂. UV, however, is known to cause deleterious effects in humans such as sunburn, DNA damage, photoageing and photocarcinogenesis.
Despite being only around 5% of terrestrial solar radiation, UV is subdivided into UVA (320-400nm), UVB (290-320nm) and UVC (100-290nm) (Diffey, 2002).

![Figure 1.1 Penetration of UV radiation into skin.](http://www.dermatology.ucsf.edu/skincancer/General/prevention/UV_Radiation.aspx)

The shorter wavelengths of UVB do not penetrate much deeper than the epidermis, but it is known to be much more effective in causing sunburn. UVA, in contrast, is made up of longer wavelengths which penetrate deeper, and cause more ageing effects such as breakdown of collagen through MMPs, as well as exerting oxidative stress through reactive oxygen species.
UVA is subdivided into UVA I (340-400nm) and UVA II (320-340nm). While UVB is thought to be more biologically active, and to be the main cause of UV induced erythema, epidermal burn, and photocarcinogenesis, it comprises only 5% of solar radiation reaching the earth (Diffey, 2002). UVB radiation is not the only cause of photocarcinogenesis, as UVA has also been reported to cause skin cancer and other harmful effects on skin (Halliday et al., 2011). UVC does not reach Earth’s surface since it is absorbed by the ozone layer in the stratosphere (Diffey, 2002). UVB also gets significantly reduced by the ozone layer, and this is of particular interest not only because UVB is the main cause of photocarcinogenesis, but the depletion of the ozone layer is allowing more of these harmful rays to reach the earth’s surface.

1.2 Ozone depletion

The ozone layer is part of Earth’s stratosphere, which consists of relatively high concentrations of ozone (O₃) (Gleason, 2008). The ozone layer absorbs more than 95% of the harmful lower to medium frequency ultraviolet (UV) radiation (200-315nm) while allowing the visible spectrum and heat to reach Earth (Gleason, 2008). The presence of ozone means that the lowest UV wavelength reaching the Earth’s surface is 290nm. Depletion of ozone has been a very significant cause of increasing levels of UV and subsequent rise in incidence of skin cancer globally. There are many causative agents responsible for ozone depletion, including chlorofluorocarbons (CFC), and bromofluorocarbons, which are not naturally occurring, but man-made organohalogen compounds developed in the 1920’s by Thomas Midgley Junior and his colleagues (Giunta, 2006). These were ideal substances to use as refrigerants, blowing agents, or propellants because of their low toxicity, reactivity and flammability (Sneader, 2005). The association of CFCs and ozone depletion was discovered...
by Sherry Rowland and Mario Molina in 1974 (Molina and Rowland, 1974). CFCs indeed have low reactivity, which give them lifespans of more than 100 years in the Earth’s stratosphere where ozone was found to be decreasing 4-5% per year, ultimately causing seasonal areas of complete ozone depletion over the Antarctica (Nash and Newman, 2001).
Figure 1.2 Ozone hole through the years. (NASA Earth observatory, 2011)

http://earthobservatory.nasa.gov/IOTD/view.php?id=49040

Ozone depletion at the Antarctica since 1979 demonstrating progressive worsening over the years despite efforts to reduce production and use of the harmful substances such as CFC. The maximum diameter of the ozone hole was recorded to be around 30 million square kilometres.
The Montreal protocol in 1987 was a landmark international agreement to prevent this ozone depletion by drastically reducing the production and use of the CFCs, such as halons, carbon tetrachloride, and methyl chloroform (den Elzen et al., 1992). Despite this treaty, it is projected that ozone depletion will continue over the next several decades and continue to worsen because of the low reactivity of CFCs. This has serious adverse biological effects since UV is known to cause deleterious effects on skin. Basal cell cancer and squamous cell cancers are two common types of skin cancer known to be caused by UV, and the incidence is thought to be increased by 2% with each percentage of decreased ozone layer (deGrujil, 1995). Malignant melanoma risk is also thought to be increased by 56% over period of 7 years with approximately 10% increase in UVB (Abarca and Casiccia, 2002).

1.3 Photoageing

Photoageing is a premature ageing process from overexposure to solar UV radiation, which is distinguished from the chronological, or natural ageing in that photochemical reactions initiate a cascade of effects including inflammation, oxidative stress, DNA lesions, mutagenesis, immunosuppression, collagen breakdown, melanogenesis, and carcinogenesis (Fischer et al., 2002). Upon UVB irradiation, TNF-α is upregulated by keratinocytes in the epidermis within a couple of hours, contributing to the acute inflammation (Bashir et al., 2009). Genotoxic photolesions such as cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts are also known to lead to the release of pro-inflammatory cytokines such as IL-1 and IL-6 (Setlow and Carrier, 1966, Mitchell, 1988, Mitchell and Nairn, 1989, Kondo et al., 1994) as well as to enhanced expression of inducible nitric oxide synthase (iNOS), which in turn increases nitric oxide products (Rhodes et al., 2001). UV exposure is also known to increase reactive oxygen species (ROS) and reactive nitrogen species (RNS) through
depletion of intracellular antioxidants such as glutathione, which in turn, add to the inflammatory process through peroxidation of membrane lipids and production of prostaglandin E2 (Wilgus et al., 2003).
Figure 1.3 Unilateral dermatoheliosis: A 69 year old gentleman with a 25 year history of gradual skin change over the left side hemiface, showing hyperkeratosis, accentuated ridging, multiple open comedones, and areas of nodular elastosis. Histopathology showed accumulation of elastolytic material in dermis and formation of milia within the villous hair follicles consistent with the Favre-Racouchot syndrome of photodamaged skin (dermatoheliosis). This was deemed to have been caused by 28 years of truck driving where his left hemiface had been exposed chronically to UVA transmitted through the window (Gordon and Brieva, 2012).
Another sign of photoageing is wrinkles and sagginess due to reduction in elasticity and collagen mediated through the activation protein 1 (AP-1) transcription factor and matrix metalloproteinases (MMPs), which cause both the breakdown of dermal collagen and inhibition of collagen synthesis (Fischer, 2005).

The mechanism of UV induced melanogenesis is not as clear since this is thought to be influenced by the individual’s Fitzpatrick skin type (Fitzpatrick, 1998). There is a constant basal melanogenesis which is determined by the individual’s genetic makeup. Since UV causes both sunburn and sun tan, it is generally believed they share a similar mechanism. It was suggested that UV induced DNA damage may be a cause for melanogenesis since severe dyspigmentation is commonly seen in patients with Xeroderma pigmentosum (XP). XP is a genetic condition with impaired nucleotide excision repair, which is important in repairing UV induced DNA damage in skin (Ichihashi and Funjiwara, 1981). Melanogenesis was once believed to be the negative feedback mechanism to control vitamin D synthesis since overproduction of vitamin D may lead to hypercalcaemia, however, now it is known that over-irradiation products produced as a result of continuing UV irradiation of pre-vitamin D or vitamin D are responsible for the inhibition of further vitamin D production (Holick, 2004).

### 1.4 Photocarcinogenesis

World Health Organisation (WHO) has defined UV radiation as a class one carcinogen (Twombly, 2003). Carcinogenesis is a multistep process which requires initiation, promotion and progression (Weinstein, 2000) and UV is known to be both an initiator and a promoter. UV is thought to be responsible for more than 90% of all skin cancers in humans including non-melanoma skin cancers and malignant melanoma, through well characterised
mechanisms including inflammation (Hruza and Pentland, 1993), DNA damage (Douki et al., 2000, Cooke et al., 2003, Mouret et al., 2008), mutagenesis (Ikehata and Ono, 2007) and immune suppression (Halliday, 2005), which are the steps leading to photocarcinogenesis.

1.4.1 DNA damage

UV radiation causes UV-induced DNA lesions, such as cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (Matsumura and Ananthaswamy, 2004) and these are thought to be caused by a photochemical reaction that induces dimerization of adjacent pyrimidine bases in DNA (Matsumura and Ananthaswamy, 2004). Thymine dimers (TD) are the main type of CPD, which is formed by a bond between adjacent thymine (T) residues. CPDs are mutagenic and carcinogenic (Hussein, 2005) unless promptly repaired before cell division.

UV radiation also causes other types of DNA damage indirectly through production of reactive oxygen species (ROS) or nitric oxide (NO) products which are known to be not only mutagenic, but also carcinogenic on their own (Kunisada et al., 2005, Niles et al., 2006, Huang et al., 2012). These indirect oxidative or nitrosative stresses result in production of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) (Kvam and Tyrrell, 1997, Agar et al., 2004, Halliday, 2005, Pattison and Davies, 2006) and 8-nitroguanine (8-NG) (Karasawa et al., 1991, Burney et al., 1999, Niles et al., 2006, Pacher et al., 2007). Cellular levels of ROS are increased by UV activation of endogenous photoreceptors in the cell (Epe, 2012). Excess levels of nitric oxide (NO) accumulate by UV upregulation of nitric oxide synthases (Deliconstantinos et al., 1996, Suschek et al., 2001, Cals-Grierson and Ormerod, 2004) and UVA decomposition of NO stores (Hess et al., 2001, Paunel et al., 2005, Mowbray et al., 2009). NO can act as a free radical on its own as well as react with ROS to form more
1.4.2 DNA repair

There are several DNA repair mechanisms but two major DNA repair mechanisms will be explored, which are nucleotide excision repair (NER) and base excision repair (BER) for this study. There are 2 subtypes of NER which are Global genome repair (GGR) and transcription coupled repair (TCR) (DeLaat et al., 1999). GGR is responsible for the repair of DNA lesions throughout the whole genome, and TCR repairs DNA lesions within the transcribed strand of the active genes (Hanawalt, 2002). They have 2 distinct repair initiating mechanisms. Firstly for TCR, RNA polymerase stalling when a DNA lesion is encountered initiates TCR (Fousteri et al., 2006), and for GGR, Xeroderma pigmentosum complementation group C (XPC) is the known trigger to initiate NER (Bergink et al., 2012). Once initiated, TCR and GGR share common cascade pathways including DNA unwinding, coordinated excision, single strand gap filling, and finally ligase action in sealing the injury site (Bergink et al., 2012).
Another important DNA repair mechanism is base excision repair (BER). This is responsible for repairing smaller and non-structurally distorting DNA base lesions unlike NER. BER is thought to be initiated by several DNA glycosylases (Liu et al., 2007) including oxoguanine glycosylase (OGG1). OGG1 is a DNA glycosylase enzyme encoded by OGG1 gene, with is involved in repairing 8-oxodG (Liu et al., 2007). Single base lesions can be caused by several insults including deamination, alkylation, or oxidation which may cause errors in base pairing and subsequent DNA mutation. BER is also considered important since impaired BER in patients result in significantly increased risk of various cancers (Starcevic et al., 2004). Although NER and BER are two distinct DNA repair pathways, there have been suggestions there are modulatory interactions between NER and BER (Shimizu et al., 2003, D'Errico et al., 2006). Parlanti et al also recently reported evidences that several NER factors played important roles with OGG1 for repairing 8-oxodG, in particular, XPC (Parlanti et al., 2012).

1.4.2.1 DNA damage binding protein 2 (DDB2)

DNA damage causes expression of DNA damage binding proteins (DDB), heterodimers responsible for p53 dependent GGR (Keeney et al., 1993). DDB2 in particular, is responsible for detection and subsequent NER of UV induced DNA damage and the deficiency of DDB2 results in xeroderma pigmentosum type E, with increased photosensitivity and development of skin cancers (Ichihashi and Funjiwara, 1981, Nichols et al., 1996, Hwang et al., 1998). The exact function of DDB2 or the coordination of NER is not fully understood, however, it is generally believed DDB2 functions as the initial sensor to recognise UV induced DNA damage by Cullin 4A (CUL4A)- mediated ubiquitylation and proteolysis which promotes recruitment of XPC to the site of DNA damage which subsequently initiates subsequent NER cascade (Fei et al., 2011). It has been suggested the role of DDB2 in DNA damage is largely
in the early phase since it is degraded before DNA is fully repaired (Rapic-Otrin et al., 2002).

**1.4.2.2 Xeroderma pigmentosum complementation group C (XPC)**

Xeroderma pigmentosum (XP) is an autosomal recessive disorder resulting from a mutation in one of the XP genes subsequently causing defective NER (Rochette et al., 2003). There have been 7 different complementation groups identified (Group A through G) and one additional form which is considered to be a variant form (Group V) (Kraemer et al., 1987). XPC is one of these groups of XP which is critical for the early steps in NER such as DNA damage recognition and initiation of global genomic nucleotide excision repair (Wang et al., 2004). It was also suggested to be the rate determining component of NER (Luijsterburg et al., 2010). Stability and the tightness in binding of XPC to the DNA lesion is critical for the initiation, and it requires RAD23 homolog B (RAD23B) (Bergink et al., 2012). It is believed that p53 and DDB2 are important in both the recruitment of XPC to the site of DNA damage, and the more intimate binding of XPC-RAD23B complex to chromatin (Wang et al., 2004). Once initiated, transcription factor II H (TFIIH) with XPB and XPD subunits open the DNA helix around the damage (Evans et al., 1997, Berdal et al., 1998, Yokoi et al., 2000), and it is verified by XPA and replication protein A (RPA) (Sugasawa et al., 2001) before endonucleases such as XPF-excision repair cross-complementation group 1 (ERCC1) and XPG cleave the damaged section (Matsunaga et al., 1995, Evans et al., 1997) and subsequent restoration.

**1.4.2.3 p53**

It is known that p53 is selectively involved in GGR but not in TCR (Wang et al., 2004). It is
also involved in repair of CPD but not 6-4pp (Ford and Hanawalt, 1997, Wani et al., 1999, Zhu et al., 2000, Wang et al., 2003). p53 is required for rapid recruitment of XPC and TFIIH where DDB2 was suggested to mediate this recruitment (Hwang et al., 1999). It is unlikely p53 is directly involved in NER since it does not associate physically with DNA lesions, but it has been suggested that p53 influences p21, GAPD45, and in particular, DDB2 (Hwang et al., 1999). Furthermore, XPC is upregulated not only by increased DDB2, but also directly by p53 (Batista et al., 2007). Our group has shown that 1,25(OH)2D3 reduced UV-induced damage with decreased nitric oxide products and increased p53 expression (Gupta et al., 2007) and this is likely to improve efficiency of NER through effective recruitment of XPC through p53 mediated DDB2 upregulation.

1.4.3 Mutagenesis
DNA damage may lead to mutagenesis, and indeed, most DNA damages are known to be mutagenic including CPD, 8-oxodG, and nitrosative DNA damage caused by reactive nitrogen species (RNS) (Agar et al., 2004).

The predominant type of mutation from UV-induced damage involve sequence changes of C to T transitions associated with CPDs, and this is due to the fact that CPD are common UV induced DNA lesions and also because the repair of CPD is relatively slow. These are termed solar signature mutations (Mouret et al., 2008). Currently, much attention is given to the contribution of UVA since recent evidence suggests that UVA radiation also contribute to CPD production (Kuluncsics et al., 1999, Perdiz et al., 2000, You and Pfeifer, 2001, Rochette et al., 2003, Reelfs et al., 2011, Tewari et al., 2012). Mutations once generated, are implicated in skin cancer development (Agar et al., 2004). Evidence for this is that mutations in the p53 tumour suppressor gene are common in many skin cancers and in actinic keratosis cells.
The latter is a common premalignant UV-induced skin lesion that is a precursor of squamous cell carcinoma, and both UVA and UVB have been implicated in the production of p53 lesions (Nataraj et al., 1995, Matsumura and Ananthaswamy, 2002, Pfeifer et al., 2005).

1.4.4 Oxidative stress

UV radiation causes oxidative stress through the generation of reactive oxygen species (ROS) that are known to contribute to photocarcinogenesis. UVA rather than UVB seems to be more important in causing ROS and subsequent DNA damage (Halliday et al., 2011). The damaging effects of UVA appear to work synergistically with UVB, which is more biologically active in the epidermis, and therefore plays a significant role in photocarcinogenesis (Black, 1993, Vile and Tyrrell, 1995). The most abundantly produced DNA lesion from the generation of ROS is 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG) (Huang et al., 2012). 8-oxodG production is known to be linearly related with the dose of UV (Floyd et al., 1988). 8-oxodG, once produced, is rapidly repaired through BER, and OGG1 has been identified as the enzyme responsible for this (Halliday et al., 2011).

1.4.5 Nitrosative stress

UV radiation causes an increase in intracellular nitric oxide (NO) level (Deliconstantinos et al., 1995, Bruch-Gerharz et al., 1998, Hess et al., 2001, Cals-Grierson and Ormerod, 2004, Paunel-Gorgulu et al., 2005, Mowbray et al., 2009) through increased expression of inducible nitric oxide synthase (iNOS) which promotes conversion of L-arginine to NO. Internal stores of NO are also known to be released by UV, adding to the increase in NO levels (Kubaszewski et al., 1994). High levels of NO are responsible for nitrosylation of DNA repair enzymes (Jaiswal et al., 2000), and inhibit DNA repair (Bau et al., 2001), and react
with ROS to produce toxic RNS intermediates such as peroxynitrite to cause oxidative and nitrosative damage to DNA (Pacher et al., 2007).

### 1.4.6 Photoimmunosuppression

UV radiation is known to cause immunosuppression in skin (Daynes et al., 1981, Kripke et al., 1992). This concept evolved from a report that described immune surveillance by Lewis Thomas in 1959 (Thomas, 1959). Kripke et al revealed that mice exposed to UV shortly before tumour cell implantation showed suppression of T cell responses to the tumour (Kripke and Fisher, 1976). The observation that the incidence of skin malignancies in immunocompromised patients, such as patients who have had organ transplants taking immunosuppressants or patients with HIV infection, was significantly higher than the general population has suggested photoimmunosuppression may also play an important role in photocarcinogenesis (Kinlen et al., 1979).

The mechanisms by which UV causes immunosuppression, locally as well as systematically are still not fully understood, but it is known to involve multiple pathways, cell types and mediators. A review by Gibbs et al listed the cutaneous chromophores of UV which are involved in the initiation of photoimmunosuppression including DNA, trans-urocanic acid, membrane phospholipids, 7-dehydrocholesterol, and tryptophan and the probable mechanisms of how they act, which include DNA damage such as CPD and oxidative damage, prostaglandin pathways, and several cytokines such as IL-6, IL-10, IL-33, TNF-a, platelet activating factor (PAF) and involvement of suppressor T cells, Langerhans cells, dermal dendritic cells, mast cells and monocyte/macrophages (Gibbs and Norval, 2011).

It is interesting to note, that immunosuppression may be localised post-UV, and also systemic. UV induced immunosuppression involves migration of dendritic cells, antigen presenting
cells or mast cells back to local draining lymph nodes and then further migration to bone marrow (Hart et al., 1998b, Halliday et al., 2011). Ullrich reported UV induced photoimmune suppression as an important factor in photocarcinogenesis (Ullrich, 2002).
1.5 Vitamin D

Vitamin D has gained much attention recently not only because of the health risks when people are deficient, but because of the numerous health benefits it may provide (Bouillon et al., 2008). It is ironic that vitamin D production requires exposure to UV, which is the source of many skin problems today. The following describes the basics of vitamin D and some of the current controversies.

1.5.1 Structure of Vitamin D

Vitamin D is commonly known to be a fat soluble vitamin together with Vitamin A, K and E. In fact, it is not a true vitamin in that it is produced in most mammals and many other species exposed to sunlight. The molecular structures of vitamin D and its analogues are related to classical steroid hormones with a steroid hormone specific cyclopentanoperhydrophenanthrene 4 ring carbon skeleton (Norman et al., 1999a, Norman et al., 1999b), but with a broken 9-10 carbon-carbon bond of ring B, which makes vitamin D a seco-steroid (Malloy et al., 1999).
It is worthwhile to note a peculiar characteristic seen in the structure of vitamin D in comparison with classical steroid molecule in terms of their molecular structures. Vitamin D and its family are known to be flexible in their structure mainly due to the three structural regions of the carbon skeleton. First of all, the 8-carbon side chain can rotate around the 5-carbon-carbon single bond, which allows several different molecular shapes. Secondly, due to the broken 9-10 carbon-carbon bond, the cyclohexane like A ring is free from the B-ring allowing rapid interconversion between chair-chair conformers. This mobility of the A ring results in its ability for 1α-hydroxyl and 3α-hydroxyl to move between axial and equatorial planes. Thirdly, the seco B-ring is capable of rotating around the 6-7 single carbon bond, which produces 6-s-cis and 6-s-trans conformation variants, in both rigid and flexible manner.
(Norman et al., 1999b, Norman et al., 2001). This conformational flexibility of vitamin D allows a vast number of ligand shapes and this is the probable reason why vitamin D produces many different biological effects through VDR.

1.5.2 Photobiology and Metabolism of Vitamin D

Vitamin D production is initiated in skin by photochemical conversion of 7-dehydrocholesterol to pre-vitamin D (Holick et al., 1980). UVB (290-320nm), the more epidermally active shorter wavelength UV can split the B-ring from the precursor (Holick et al., 1980). The next step is thermal isomerisation that converts pre-vitamin D to the more stable vitamin D (Holick et al., 1980). Excessive or continued UVB exposure to pre-vitamin D or vitamin D can lead to the production of over-irradiation products, which include lumisterol, tachysterol, and suprasterols (Holick et al., 1980). The production of over-irradiation products reduces vitamin D concentrations and thus prevents hypercalcaemia from excess sun exposure (Holick et al., 1980).

Vitamin D produced in skin or derived from diet or supplements and absorbed in the intestine are carried in the blood bound to the vitamin D binding protein and transported to the liver where the first hydroxylation at position 25 produces 25-dihydroxyvitamin D (25(OH)D). Although 25(OH)D is not biologically active, it constitutes the major circulating form of vitamin D, which we measure in blood tests to determine vitamin D levels. A final hydroxylation step in 1alpha position that occurs in the proximal convoluted tubular cells of the kidney converts 25(OH)D to the active metabolite, 1a,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) also known as calcitriol (Malloy et al., 1999). It is important to note that the human epidermis has both 25 hydroxylase and 1α hydroxylase (CYP27b1) and therefore is capable of producing this active metabolite locally (Bikle et al., 1986, Lehmann et al., 2001).
1.5.3 Biological Effects of Vitamin D

1.5.3.1 Calcium and phosphate homeostasis.

The main role of vitamin D is to regulate calcium homeostasis and phosphate metabolism. Vitamin D maintains the normal concentration of calcium and phosphate in plasma through facilitating the absorption of calcium and phosphate in small intestine causing bone resorption it deeded and reducing renal excretion (Jones et al., 1998, Friedman, 2006). Furthermore, 1,25(OH)$_2$D$_3$ is a known inhibitor of parathyroid hormone (PTH) which increases bone resorption, renal calcium reabsorption and urinary phosphate excretion, hence contributes to calcium and phosphate homeostasis (Jones et al., 1998).

Deficiency of vitamin D causes decreased calcium and phosphate absorption which reduces plasma concentration of calcium which in turn stimulates PTH secretion. If vitamin D levels are very low, bone resorption is increased and so is the PTH level. PTH will cause resorption of calcium from bones to normalise serum calcium level, and at the same time, will increase renal phosphate excretion resulting in hypophosphataemia leading to clinical conditions such as rickets due to defective bone and cartilage mineralisation or osteomalacia due to under-mineralised bone matrix which may lead to bone pain and muscle weakness (Holick, 2003).

Excessive vitamin D on the other hand, causes hypercalcaemia, which can cause result in neurocognitive dysfunction, cardiac dysrhythmia, and also may cause complete growth arrest in children (Friedman, 2006).
1.5.3.2 Biological effects on immune system

1,25(OH)₂D₃ was shown to inhibit antigen presenting cell or Langerhans cell maturation *in vitro* (Kowitz et al., 1998) and an animal study in mice showed that 1,25(OH)₂D₃ inhibited Langerhans cell migration (Suzuki et al., 2000). Dam et al demonstrated that 1,25(OH)₂D₃ and calcipotriol inhibited antigen induced T cell proliferation *in vitro* (Dam et al., 1996). Furthermore, 1,25(OH)₂D₃ was shown to inhibit dendritic cells such as Langerhans cells from maturation, production of IL-12, but promoted IL-10 production which resulted in reduced T cell activation (Lehmann et al., 2004). These data therefore, suggested that 1,25(OH)₂D₃ may cause immunosuppression in skin (Dam et al., 1996).

It was shown that 1,25(OH)₂D₃ was capable of modulating several genes in purified CD4+ cells (Mahon et al., 2003), and inhibit antigen specific T cell proliferation (Bhalla et al., 1984), as well as to decrease helper T cell activity *in vitro* (Lemire et al., 1985). An analogue of 1,25(OH)₂D₃, Ro-26-2198 was shown to enhance proliferation of regulatory T cells in mice and suppress autoimmune diabetes (Gregori et al., 2002). These modulatory effects on T cells by 1,25(OH)₂D₃ are thought to be enhanced by cytokines. In particular, IL-2 was required for 1,25(OH)₂D₃ to reduce inflammation in mice, and to reduce production of IL-10 and IFN-γ *in vitro* (Bemiss et al., 2002).

In experimental autoimmune encephalomyelitis (EAE) model, mice treated with 1,25(OH)₂D₃ showed complete suppression of the disease through decrease in number of lymphocytes in the lymph nodes and increase in IL-4 and transforming growth factor-beta1 (TGF-β1) (Cantorna et al., 2000). It has been suggested that the immunomodulatory effect of 1,25(OH)₂D₃ was comparable to other established immunosuppressants (vanEtten and
Mathieu, 2005, Di Rosa et al., 2011), since it was capable of inducing full tolerance to mismatched pancreatic islet allografts in mice, when introduced with mycophenolate mofetil (Gregori et al., 2001).

1,25(OH)$_2$D$_3$ has been shown to improve autoimmune diseases in several animal studies. It prevented type I diabetes, and improved inflammatory bowel disease, rheumatoid arthritis and autoimmune encephalomyelitis as mentioned earlier (Shah et al., 2014). 1,25(OH)$_2$D$_3$ helped transplant rejection, and improved transplant survival in mice (Hullett et al., 1998, Redaelli et al., 2001). There have been suggestions that high intake of vitamin D may reduce risk of multiple sclerosis (Munger et al., 2004), and rheumatoid arthritis (Merlino et al., 2004) in humans through regulation of T helper cells (Cantorna and Mahon, 2004). 1,25(OH)$_2$D$_3$ was also shown to reduce IgE production and hence suppress IgE mediated skin reactions in vitro (Heine et al., 2002), which is consistent with the findings from an epidemiological study by Solvoll et al where vitamin D deficiency was more common in the patients with atopic dermatitis (Solvoll et al., 2000).

Hart et al showed that dermal mast cells were necessary for UVB induced systemic immunosuppression (Hart et al., 1998a) and Byrne et al showed that mast cell migration to the local lymph nodes post UV irradiation was the key step in the induction of systemic immune suppression (Byrne et al., 2008). Grimbaldeston et al also suggested that IL-10 derived from mast cells were likely to be involved in UVB induced immune suppression (Grimbaldeston et al., 2007). Yu and Biggs helped our understanding in this area of photoimmune suppression and vitamin D by showing that the regulatory interactions between 1,25(OH)$_2$D$_3$ and dermal mast cells thereby reducing pathogenic outcomes
associated with chronic UVB irradiation (Biggs et al., 2010, Yu et al., 2011).

Gorman et al showed that the photoimmune suppression was unlikely due to UV induced vitamin D through a novel approach utilizing both contact hypersensitivity model mediated through T helper type 1 and 17 cells, as well as allergic airway disease model mediated through T helper type 2 cells. (Gorman et al., 2007).

1.5.3.3 Effects on cancer

There are evidences $1,25(\text{OH})_2\text{D}_3$ may exert biological effects on several types of malignancies. Firstly, skin cancers including malignant melanoma which was briefly mentioned above. The speculation that $1,25(\text{OH})_2\text{D}_3$ may protect against development of melanoma was based on its inhibitory effects on melanoma cell proliferation (Mason et al., 1988), and the invasiveness (Yudoh et al., 1999). Other observations made include the association of cumulative sun exposure with melanoma survival (Berwick et al., 2005), and low serum vitamin D level seen more frequently in patients with melanoma (Cornwell et al., 1992). Hutchinson et al also reported the association between the incidence and prognosis of melanoma with vitamin D receptor polymorphism (Hutchinson et al., 2000, Zeljic et al., 2014).

$1,25(\text{OH})_2\text{D}_3$ was shown to promote differentiation and inhibit growth of several cancers including solid tumours such as colon, prostate, breast, and bladder, as well as leukaemia (Osborne and Hutchinson, 2002). The risk of prostate cancer was higher with decreases in serum vitamin D level (Corder et al., 1993), and the prostate cancer cells expressed lower $1\alpha$-hydroxylase activity compared to the normal prostate cells in vitro, which suggests local
production of $1,25(\text{OH})_2\text{D}_3$ may play an important role in prostate cancer biology (Whitlatch et al., 2002).

Breast cancer related mortality was less with cumulative sun exposure and adequate serum vitamin D level (Garland et al., 1990). Patients suffering from breast cancer had significantly slower disease progression when the tumour cells expressed VDR (Colston et al., 1989), and again Mawer et al showed the breast cancer progression was associated with lower serum vitamin D levels (Mawer et al., 1997).

Colon cancer risk was significantly lower with adequate serum 25-hydroxyvitamin D levels (Garland et al., 1989), and another study by Bostick et al also reported similar findings where reduced vitamin D intake significantly increased the risk of colon cancer (Bostick et al., 1993). More recent studies have also suggested possible reduction in risk of colon cancer with adequate vitamin D levels (Pereira et al., 2012, Jacobs et al., 2013).

There are controversies surrounding $1,25(\text{OH})_2\text{D}_3$ as chemotherapeutic agent. It is believed that the anticancer activity of $1,25(\text{OH})_2\text{D}_3$ is mediated through inhibition of cell proliferation or apoptotic cell death of tumour cells (Beer and Myrthue, 2004). Ravid et al showed $1,25(\text{OH})_2\text{D}_3$ enhanced cytokines including TNF, IL-1, and IL-6 in human breast cells without affecting the killer lymphocytes or cytotoxic interferon-a activity (Ravid and Koren, 2003).

Anticancer effects of $1,25(\text{OH})_2\text{D}_3$ have been tested in several studies. A clinical trial have shown that $1,25(\text{OH})_2\text{D}_3$ decreased the rate of PSA rise in prostate cancer patients (Gross et
al., 1998). On the other hand, EB1089, an analogue of calcitriol, did not improve patients with breast cancer and bowel cancer in phase I trial (Guilford et al., 1998). A phase II trial has shown that calcitriol and docetaxel in combination improved PSA level, and disease progression, as well as patient survival in metastatic prostate cancer compared to docetaxel alone (Beer et al., 2003), which imply \(1,25(\text{OH})_2\text{D}_3\) in itself may not have sufficient anticancer activity. More recently, synthetic vitamin D analogues (24R)-1,24-dihydroxyvitamin D3 (PRI-2191) and 5,6-trans calcipotriol (PRI-2205) both showed favourable outcome when combined with 5-fluorouracil (5-FU) in treating human colon HT-29 cancer compared to 5-FU alone both in vitro, and in vivo animal studies (Milczarek et al., 2014). Calcitriol was found to restore estrogen receptor in breast cancer cells and improve response to antiestrogen therapy for breast cancer treatments (Santos-Martinez et al., 2014).

### 1.5.4 Signal Transduction Pathways

An active metabolite of vitamin D, \(1,25(\text{OH})_2\text{D}_3\) is known to be the main but not the only structural ligand of vitamin D receptors (VDR). Its biological effects are conveyed through two well described pathways, including the genomic and non-genomic pathways. The genomic pathway is mediated by vitamin D receptor (VDR), which is well known, but the non-genomic pathway or the rapid response pathway has not yet been clearly described (Nemere et al., 1994, Norman et al., 1999a, Zanello and Norman, 2004a, Mizwicki et al., 2010, Sequeira et al., 2012).

#### 1.5.4.1 Genomic Pathway

The genomic pathway of \(1,25(\text{OH})_2\text{D}_3\) involves VDR, a member of the steroid nuclear receptor superfamily. VDR is known to be expressed in almost all tissues in the human body including the intestine, kidney, bone, parathyroid gland, pituitary, parathyroid glands, and the
haematopoietic system (Norman, 2008, Wang et al., 2012). There are 6 primary domains that have been identified in the VDR including the variable domain, a DNA binding domain, hinge, ligand binding domain, and a transcriptional activation domain and they are known to have different functions (Norman, 2008). The classical or genomic pathway is responsible for mediating the main biological effects of $1,25(OH)_2D_3$ through binding to the ligand binding domain and activation of VDR. The $1,25(OH)_2D_3$ molecule is lipophilic hence it easily passes through the plasma membrane lipid bilayer into the cytoplasm of cells where it then binds to the hydrophobic pocket in the ligand binding domain of the VDR.

Once bound, it activates VDR to heterodimerise with retinoid X receptor (RXR) to form a VDR-RXR complex. Zinc fingers of the DNA binding domain sense this VDR-RXR complex, which triggers the vitamin D response elements (VDRE) in the DNA sequences of vitamin D target genes. These vitamin D target genes may be in the promoter region or, distance enhancer or repressor sites, which are determined by further recruitment of co-modulators that assist the transcription apparatus to either promote or repress gene transcription (Zella et al., 2010, Haussler et al., 2011).

1.5.4.2 Non-Genomic Pathway

The active hormone, $1,25(OH)_2D_3$ is a conformationally flexible secosteroid hormone, which is also known to initiate processes by a mechanism that does primarily not involve altered transcription some of these are rapid biological responses much quicker than the classic genomic response. The onset of rapid responses were seen within minutes and these were too fast to be explained through the classical VDR mediated genomic response such as gene transcription regulation, which is expected to take several hours to days (Norman et al.,
Examples of rapid response of $1,25(OH)_2D_3$ include:

1. Photoprotective effects in human cell cultures and hairless mice which will be described in more detail in next chapter.
2. Transcalcitachia which is a rapid hormonal stimulation of calcium absorption (Norman et al., 1993, Norman et al., 1997).
3. Insulin secretion from pancreatic cells (Kajikawa et al., 1999).
4. Rapid opening of calcium and chlorine channels seen and measured in mouse osteoblasts (Zanello and Norman, 1997, Zanello and Norman, 2004a) and human sertoli cells in the seminiferous tubules of testis (Norman et al., 1993).
5. Rapid migration of human endothelial cells (Rebsamen et al., 2002).

It is important to note that 6-s-cis locked $1\alpha,25(OH)_2$-lumisterol$_3$ (JN), a known VDR ligand with no ability to modulate transcription, was also seen to activate rapid responses and this effect was inhibited by an antagonist, $1\beta,25(OH)_2D_3$ (Norman et al., 1993). The mechanisms of these actions have been described to involve opening of voltage gated chloride and calcium channels, and the activation of mitogen activated protein kinases (MAPKs), protein kinase C, phosphatidylinositol 3-kinase (PI3K), phospholipase C, and G protein coupled second messenger system such as cyclic adenosine monophosphate (cAMP) (Norman, 2008).

The exact location where $1,25(OH)_2D_3$ mediated rapid signal transduction pathways occurs is not clear, but some evidence suggested that a rapid response binding proteins exist in the basolateral membrane of the chick duodenal epithelium, which was involved in rapid calcium
absorption (Nemere et al., 1984). Other studies have identified that caveolae are the source of many rapid response signalling pathways (Huhtakangas et al., 2004, Bula et al., 2005, Razani et al., 2005). Caveolae are flask shaped invaginations in the plasma membrane that are rich in sphingolipids, and cholesterol (Huhtakangas et al., 2004). Confocal immunofluorescence microscopy of ROS 17/2.8 cells showed caveolae marker protein, caveolin-1 was in close association with the VDR (Huhtakangas et al., 2004). Also, when vitamin D deficient chicks were administered 1,25(OH)₂D₃, it was seen to localise in the plasma membrane caveolae (Huhtakangas et al., 2004). Caveolar binding of 1,25(OH)₂D₃ was absent in VDR-null transgenic mice (Zanello and Norman, 2004b), and the osteoblasts from VDR-null mice failed to show the rapid response of chloride channel opening or stimulation of exocytosis (Mizwicki and Norman, 2009). Furthermore, oestrogen receptors, and androgen receptors were found to be present in caveolae as well, which is strongly suggestive of the presence of the traditional nuclear steroid receptors in this region (Lutz et al., 2003, Levin, 2005).

The existence of another protein, ERp57, synonymous with PDIA3 or membrane associated rapid response steroid binding receptor (MARRS) has been reported to be important in this response. Sequeira et al showed that ERp57 co-immuno precipitated with VDR in non-nuclear fractions of skin fibroblasts (Sequeira et al., 2012). They also showed that antibody to ERp57 which would affect only membrane proteins, abolished the actions of 1,25(OH)₂D₃ in human skin fibroblasts (Sequeira et al., 2012). An earlier problem with this concept was that the flexible 1,25(OH)₂D₃ must be in a 6-s-trans shape to be able to activate the genomic response, while the 6-s-cis shape was required for rapid responses in a VDR with a single ligand binding domain (Whitfield et al., 1996). Mizwiki et al demonstrated in an “in silica” computational model that in fact, the VDR contained an alternate ligand binding pocket,
which could accommodate the primary ligand, \(1,25(\text{OH})_2\text{D}_3\) in the 6-s-cis conformation, or analogues that had been identified as agonists for the rapid response pathways (Mizwicki et al., 2010).

1.5.5 Biological effects in skin

The keratinocytes in epidermis are capable of producing \(1,25(\text{OH})_2\text{D}_3\) (Bikle et al., 1986, Lehmann et al., 2001) and also express VDRs which suggest \(1,25(\text{OH})_2\text{D}_3\) has both intracrine and autocrine effects on keratinocytes and also may have paracrine effects on surrounding cells (Lehmann et al., 2004).

\(1,25(\text{OH})_2\text{D}_3\) showed dose dependent effects on keratinocytes where proliferation was promoted at low dose, but at high dose, it was inhibited (Lehmann et al., 2004). This is not clearly understood, but there have been suggestions that \(1,25(\text{OH})_2\text{D}_3\) arrests cells at the G0/G1 to S transition, as well as increased transforming growth factors \(\beta1\) and \(\beta2\) by keratinocytes (Bikle, 2004). \(1, 25(\text{OH})2\text{D}3\) also induces keratinocyte differentiation through modulating intracellular calcium (Bikle, 2004).

More importantly, there have been numerous reports on the photoprotective effects of \(1,25(\text{OH})_2\text{D}_3\) in skin which are discussed in more detail in the next chapter.

There are other numerous reports on the effects of \(1,25(\text{OH})_2\text{D}_3\) in skin. Mason et al have suggested that \(1,25(\text{OH})_2\text{D}_3\) may protect from malignant melanoma development since it had inhibitory effect on proliferation (Mason et al., 1988), and Osborne et al have also suggested serum level of \(1,25(\text{OH})_2\text{D}_3\) was important in spread of melanoma cells (Osborne and Hutchinson, 2002). Also an epidemiological study has suggested an association between
1,25(OH)<sub>2</sub>D<sub>3</sub> level with atopic dermatitis (Solvoll et al., 2000).

There are several case reports, observational studies, and small randomised controlled trials which have described the therapeutic effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> in other skin disorders. Complete remission was seen in some cases of ichthyosis, confluent and reticulated papillomatosis, Grover’s disease, pityriasis rubra pilaris, seborrheic dermatitis, and erythema annulare centrifugum with calcipotriol treatment, and analogue of 1,25(OH)<sub>2</sub>D<sub>3</sub> (Holm and Jemec, 2002). Partial or symptomatic improvements were reported in some cases with disseminated superficial actinic keratosis, inflammatory linear verrucous epidermal naevus, ichthyosis, lichen amyloidosis, morphoea, vitiligo, and pityriasis rubra pilaris (Harrison and Stollery, 1994, Holm and Jemec, 2002, Kullavanijaya and Lim, 2004). It is likely that 1,25(OH)<sub>2</sub>D<sub>3</sub> is beneficial to many skin conditions caused by increased proliferation and decreased terminal cell differentiation, even though the underlying mechanisms are not completely understood (Holm and Jemec, 2002).

VDR was implicated for activating hundreds of target genes and Moll et al reported RNA profiling array study in human keratinocytes, where DNA damage binding protein 2 (DDB2) and xeroderma pigmentosum complementation group C (XPC) were two out of many that were suggested to be upregulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> (Moll et al., 2007) but this has not been shown in any biological study as yet. In addition to this postulation, Demetriou et al suggested VDR was seen to mediate DNA repair, in particular NER which strongly suggests a pivotal role of 1,25(OH)<sub>2</sub>D<sub>3</sub> in NER where XPC and DDB2 are responsible for the early phase of NER (Demetriou et al., 2012).
1.5.5.1 $1,25(\text{OH})_2\text{D}_3$ Protection from UV induced DNA damage

UV radiation causes several types of DNA damage through either direct photochemical reaction, or indirect means with generation of reactive oxygen species and reactive nitrogen intermediates as described earlier. Our group and also others have shown that $1,25(\text{OH})_2\text{D}_3$ reduces CPDs in UV exposed skin cells in vitro (Wong et al., 2004, De Haes et al., 2005, Dixon et al., 2005, Dixon et al., 2007, Gupta et al., 2007, Sequeira et al., 2012) and also in mice in vivo (Lee and Youn, 1998, Dixon et al., 2005, Gupta et al., 2007, Dixon et al., 2011) and human skin in vivo (Damian et al., 2010) through immunohistochemistry and image analysis. Thymine dimers, a major form of CPD, were also reduced when treated with the low calcaemic cis-locked non genomic analogue, $1,25(\text{OH})_2$-lumisterol$_3$ (JN) and $1,25(\text{OH})_2$-7-dehydrocholesterol (JM) in skin cells in vitro (Dixon et al., 2005, Dixon et al., 2012, Sequeira et al., 2012), in mice in vivo (Dixon et al., 2011) as well as by $1\alpha$-hydroxymethyl-16-ene-24,24-difluoro-25-hydroxy-26,27-bis-homovitaminD$_3$ (QW), which is a low calcaemic transcriptionally active hybrid analogue of $1,25(\text{OH})_2\text{D}_3$ (Wong et al., 2004, Dixon et al., 2005).

The reduction in UV induced TDs with $1,25(\text{OH})_2\text{D}_3$ were not only measured through immunohistochemistry but also through other methods as well. Comet assay with the site specific DNA repair enzyme T4 endonuclease IV for CPDs also demonstrated the reduction in UV induced CPD with $1,25(\text{OH})_2\text{D}_3$ (Gordon-Thomson et al., 2012, Sequeira et al., 2013). Gordon-Thomson et al also examined an indirect form of UV induced DNA damage, 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG), which was found to be reduced with $1,25(\text{OH})_2\text{D}_3$ in human keratinocytes in vitro through Comet assay, incorporating digestion with the site specific DNA repair enzyme, in this case, human 8-oxoguanine DNA glycosylase (hOGG1).
1.5.5.2 Protection from UV induced cell death by 1,25(OH)$_2$D$_3$

Cell survival of UV-irradiated skin cells was improved with treatment with vitamin D compounds together with increased expression of p53 (Gupta et al., 2007, Dixon et al., 2011). This was shown in studies in cultured human keratinocytes treated with 1,25(OH)$_2$D$_3$ (Lee and Youn, 1998, Mason, 2000, Manggau et al., 2001, Mason et al., 2002, De Haes et al., 2003, De Haes et al., 2004, Wong et al., 2004, Gupta et al., 2007, Mason et al., 2010), and its analogue JN (Wong et al., 2004), JM (Wong et al., 2004), QW (Dixon et al., 2007), and calcipotriol (Youn et al., 1997). Again, there is evidence suggesting that this improved cell survival is facilitated by a non-genomic rapid response pathway. The improved cell survival with 1,25(OH)$_2$D$_3$ was abolished in the presence of a non-genomic antagonist HL (Wong et al., 2004). Sunburn cells, or apoptotic cells characterised by dense darker nuclei, and eosinophilic cytoplasm seen with conventional haematoxylin and eosin staining post-UV were also reduced by systemic administration of 1,25(OH)$_2$D$_3$ (Hanada et al., 1995), as well as topical application (Dixon et al., 2007, Gupta et al., 2007, Dixon et al., 2011) in mice, and also in human subjects (Damian et al., 2010).

The mechanism for this improved cell survival in the presence of vitamin D compounds is probably due to reduced DNA damage, possibly as a result of improved DNA repair. The improved cell survival may also be mediated by the reduced catalytic action of caspase 3, which is involved in apoptosis on poly(ADP-ribose) polymerase (De Haes et al., 2003), and possibly through reduced effects of sphingosine-1-phosphate, a breakdown product of membrane sphingolipid and second messenger in the apoptosis signalling pathway (Manggau...
1.5.5.3 Protection from Nitric Oxide derivatives by 1,25(OH)\(_2\)D\(_3\)

1,25(OH)\(_2\)D\(_3\) was shown to reduce nitrite (Gupta et al., 2007), and 3-nitrotyrosine (Dixon et al., 2011) which are two end products of nitric oxide. Similarly aminoguanidine and L-N-monomethylarginine, inhibitors of nitric oxide synthase, reduced UV induced nitrite and TDs in skin cells (Wong et al., 2004, Gupta et al., 2007), as did a selective inhibitor of the inducible NOS isoform, which reduced both CPD and 8-oxodG (Gordon-Thomson et al., 2012). It is therefore, likely a reduction in UV induced NO products may be one of the mechanisms by which 1,25(OH)\(_2\)D\(_3\) provides protection from oxidative and nitrosative DNA damage and also enhances DNA repair. However, it is not clear if the reduction of NO products contributes to reduction of TD since a study in human keratinocytes showed no association between a reduction in NO products and TD. Sequeira et al showed that 4,4’-diisothiocyanatostilbene-2,20-disulfonic acid (DIDS) antagonised the reduction in UV induced TD by 1,25(OH)\(_2\)D\(_3\), but the reduction in nitrotyrosine by 1,25(OH)\(_2\)D\(_3\) remained (Sequeira et al., 2013). In this study, treatment of keratinocytes with the chloride channel blocking DIDS blocked the protective effect of 1,25(OH)\(_2\)D\(_3\) on UV induced CPD but did not block the reduction in nitrotyrosine (Sequeira et al., 2013). One explanation is that a reduction in NO products is necessary but not sufficient for 1,25(OH)\(_2\)D\(_3\) to reduce UV induced DNA damage.
1.5.5.4 Increase in p53 by 1,25(OH)$_2$D$_3$

In addition to the reduction in NO products, Gupta et al also showed that one of the mechanism of photoprotection by 1,25(OH)$_2$D$_3$ was due to increase in p53 (Gupta et al., 2007). As mentioned earlier, p53 is responsible for the cell cycle arrest and modulation of DNA repair pathway (Smith et al., 1995) or apoptosis with excessive unrepaired DNA damage (Decraene et al., 2001). Vitamin D analogues were reported to increase p53 and p21 in xenograft model of human retinoblastoma in 2003 (Audo et al., 2003). On the contrary, De Haes et al in 2004 showed increased Bcl-2 expression together with decrease in p53 when keratinocytes were treated with a high dose of 1,25(OH)$_2$D$_3$ (De Haes et al., 2003). It was also shown that p53 and its family genes, such as p63 and p73, directly upregulated VDR gene expression in several cancer cell lines, suggesting close relationship between the two entities (Maruyama et al., 2006). More recent evidenced also suggested increase in p53 with 1,25(OH)$_2$D$_3$ in testicular germ cell cancer derived cell lines (Jorgensen et al., 2013).

1.5.5.5 Protection from UV induced immunosuppression by 1,25(OH)$_2$D$_3$

UV radiation is known to cause suppression in skin immunity (Kripke and Fisher, 1976). Suppression in cell mediated immune reactions would mean reduced defence against carcinogenesis through impaired immune surveillance (Nghiem et al., 2002). Many of these cells of the immune system such as monocytes, macrophages, T cells and B cells are known to express VDR, and in fact, it has been suspected that the UV induced immunosuppression was mediated through VDR. It is important to note, 1,25(OH)$_2$D$_3$ was reported to increase mRNA expression for interleukin-10 (IL-10), which is a cytokine responsible for immunosuppression, as well as induce IL-10 from dermal mast cells which can result in localised immunosuppression (Biggs et al., 2010). However, Gorman et al have found that
1,25(OH)₂D₃ itself caused a degree of immunosuppression in skin as well as systemic suppression through CD4+CD25+ cells to modulate T helper type 2 cells (Gorman et al., 2007, Gorman et al., 2010), although not directly responsible for UV induced immunosuppression (Gorman et al., 2012).

The UV induced immune suppression and the role of vitamin D compounds on the immune system is complex and likely dose related. High concentration of 1,25(OH)₂D₃ produced immunosuppressive effects (Yang et al., 1993a) but lack of 1,25(OH)₂D₃ is also known to cause immunosuppression (Yang et al., 1993b). Our group have shown that vitamin D compounds including the active metabolite, 1,25(OH)₂D₃ and other analogues, JN, and QW, inhibit UV induced immune suppression in hairless mice using contact hypersensitivity model (Dixon et al., 2005, Gupta et al., 2007, Dixon et al., 2011). We have also shown this in other species of mice (Ryan, 2010). In contrast to this, human studies showed that 1,25(OH)₂D₃ resulted in no protection against UV induced suppression in recall delayed type hypersensitivity when tested in subjects reacted positive for Mantoux reaction, and in fact, caused immunosuppression when a higher dose was used (Damian et al., 2010). A parallel study in human subjects also showed calcipotriene, a vitamin D analogue, suppressed contact hypersensitivity response to dinitrochlorobenzene by 64%, which was comparable to that caused by solar simulator generated UV (Hanneman et al., 2006).

1.5.5.6 Protection against photocarcinogenesis by the vitamin D system

Vitamin D system including VDR is known to play a significant role in resisting carcinogenesis in skin as shown by studies which demonstrated VDR knockout mice were
predisposed to skin tumour formation by either chemical (DMBA) or UV induced causes (Zinser et al., 2002, Ellison et al., 2008, Teichert et al., 2011). Vitamin D and its photoproducts provide photoprotection in UV-induced CPD as described earlier (Wong et al., 2004, Dixon et al., 2005, Dixon et al., 2011, Sequeira et al., 2012). It was also reported that 1,25(OH)$_2$D$_3$ provided resistance to cancer development through upregulation of p53 and reduction in NO products (Gupta et al., 2007). As mentioned earlier, VDR knockout mice developed significantly higher number of skin cancers compared to the wild type (Ellison et al., 2008, Quigley et al., 2009, Teichert et al., 2011). It was observed the reduction in UV induced CPD with 1,25(OH)$_2$D$_3$ was also abolished in human VDR null fibroblasts (Sequeira et al., 2012).

Furthermore, VDR null mice showed significantly higher CPD levels when exposed to UVB, and these failed to reduce over time while the wild type mice showed the gradual reduction in CPD level post-UV suggesting VDR may be important in DNA repair (Teichert et al., 2011). Our group recently reported a role for the ERp57/MARRS/PDIA3 in photoprotection mediated by 1,25(OH)$_2$D$_3$ (Sequeira et al., 2012). When ERp57 was blocked by neutralizing antibody or siRNA, the reduction in UV induced TDs with 1,25(OH)$_2$D$_3$ was abolished (Sequeira et al., 2012). It was also found that VDR and ERp57 co-immunoprecipitated in non-nuclear cell extracts which strongly suggest association (Sequeira et al., 2012). It has been shown that treatment with 1,25(OH)$_2$D$_3$ or its photoproduct, JN, reduced photocarcinogenesis in mice (Dixon et al., 2011). Bikle et al noted that 1alpha-hydroxylase knockout mice were not more susceptible to photocarcinogenesis suggesting that vitamin D derivatives activated by other pathways may contribute to photoprotection (Bikle, 2009). Another enzyme which metabolizes vitamin D to 20-hydroxyvitamin D and subsequent
metabolites, CYP11A1, has also been reported (Slominski et al., 2013a, Slominski et al., 2013b). Preliminary results from the Mason group have indicated that products of CYP11A1, such as 20OHD may also contribute to photoprotection (Tongkao-On et al, JSBMB submitted).
1.5.6 Curcuminoids

1.5.6.1 Definition

Curcumin (CUR) is a naturally occurring polyphenol derived from Turmeric. Tumeric has been one of the traditional dietary spices commonly and widely used in India. There are also records of the medicinal use of curcumin in India and Japan since the second millennium BC (Brouk, 1975). CUR is known to have a molecular structure of 1,7-bis-(4-hydroxy-3-3methoxyphenyl)-1,6-heptadiene-3,5-dione (Figure 1.4). It has gained much attention in modern medicine due to several medicinal properties including anti-inflammatory and antioxidant properties (Sharma et al., 2005).

![Curcumin molecule](image)

Figure 1.6 Curcumin (Sharma 2005).

CUR has also been discussed in countless numbers of published reports for chemopreventative and possible chemotherapeutic properties (Ammon and Wahl, 1991, Aggarwal et al., 2003, Joe et al., 2004, Lee et al., 2013). CUR is known to induce the powerful tumour suppressor gene p53 and promote apoptosis in basal cell skin cancer cells.
(Jiang et al., 1996). Furthermore, research shows that curcumin can make mutant, treatment-resistant malignant melanoma cell lines more responsive to chemotherapy (Simon et al., 1998).

While several studies also suggest that CUR when used topically at millimolar or micromolar doses may be beneficial in protecting the skin, the staining properties of its bright yellow pigment have prevented its widespread use in topical pharmaceutical products. One of the major metabolite of curcumin, tetrahydrocurcumin (THC) does not possess the staining characteristics of native curcumin, while demonstrating biological effects that are similar to CUR. In current study, the biological effects of much lower concentrations of THC, in the nanomolar range will be examined in parallel to 1,25(OH)₂D₃ in terms of the photoprotective effects it may have in human skin.

### 1.5.6.2 Metabolism of curcuminoids

CUR itself is known to be relatively unstable especially in a high pH environment, with oxygen, and under UV or visible light (Tonnesen and Karlsen, 1985). Wang et al described the half-life of curcumin to be around 10 minutes in phosphate buffer at 37oC, and around 8 hours in 10% serum or in human blood, but that it is more or less stable below pH 6.4 (Wang et al., 1997). CUR is degraded into a major degradation product, bicyclopentadiones, and minor degradation products, including vanillin, ferulic acid and feruloyl methane (Roughley and Whiting, 1973, Tonnesen and Karlsen, 1985, Wang et al., 1997).

The metabolism of CUR was first studied in 1978, and it was suggested that approximately 50-60% of intravenously injected CUR was excreted in bile, of which greater than 95% were
glucuronides (Holder et al., 1978). Major metabolites of CUR are THC and hexahydrocurcumin (Holder 1978; ) (Holder et al., 1978) and the minor metabolites of CUR included dihydrocurcumin and octahydrocurcumin (Hoehle et al., 2006). It was suggested CUR was metabolised by alcohol dehydrogenases, but not through cytochrome p450 (Hoehle et al., 2006).

*In vivo* metabolism of curcumin is known to be a stepwise reduction process of olefinic hetaloid chain followed by conjugation of the parent compounds and reductive metabolites with glucuronic acid and sulphate (Metzler et al., 2013).

In an early study in rats, oral or intraperitoneal doses of CUR labelled with deuterium or tritium resulted in the faecal excretion of most of the radioactivity. The major biliary metabolites were glucuronides of tetrahydrocurcumin and hexahydrocurcumin. A minor biliary metabolite was dihydroferulic acid together with traces of ferulic acid. These metabolites were identified using chemical ionization mass spectrometry (Holder et al., 1978). A recent pharmacokinetic study has revealed that when CUR was administered intra-peritoneally (0.1g/kg) to mice, it appeared in the plasma at concentration around 2.25 g/ml within the first 15 minutes. Treatment of the plasma with beta-glucuronidase resulted in a decrease in the concentrations of two putative conjugates and the concomitant appearance of tetrahydrocurcumin (THC) and CUR, respectively. To investigate the nature of these glucuronide conjugates *in vivo*, the plasma was analysed by an electrospray liquid chromatography technique. The chemical structures of these metabolites, determined by mass spectrometry, suggested that CUR was first biotransformed to dihydrocurcumin and THC and that these compounds subsequently were converted to monoglucuronide conjugates. CUR is not only unstable but also extremely poorly absorbed in intestine, which severely limits
bioavailability despite huge doses consumed (Metzler et al., 2013).

Interestingly, there have been many reports of beneficial biological effects of CUR. CUR at relatively high concentration was shown in rodent models to prevent cancers of the gastrointestinal system, breast, and sebaceous glands (Sharma et al., 2005, Lopez-Lazaro, 2008). Also clinical pilot studies showed CUR consumption caused regression of premalignant lesions in the gastric mucosa, bladder, cervix and skin (Cheng et al., 2001). Furthermore, reports have suggested protective role of CUR against various malignancies, arthritis, allergies, cardiovascular diseases, as well as anti-Alzheimer effects (Anand et al., 2007, Zhou et al., 2011). Considering the extremely low bioavailability of CUR post oral ingestion, these biological effects may be mediated through congeners, or metabolites of CUR.

1.5.6.3 Biological effects of curcuminoids

Although curcumin has been a merely a dietary spice which had been consumed for centuries, its biological effects such as anti-oxidant and anti-inflammatory effects were studied in in vivo studies (Sharma, 1976, Mukhopadhyay et al., 1982). More recently, laboratory in vitro studies have also shown anti-oxidant and anti-inflammatory effects of curcumin, which were suggested to be mediated through down regulating the activity of cyclooxygenase-2, lipoxygenase, and inducible nitric oxide synthase (Abe et al., 1999, Goel et al., 2008).

Curcuminoids at 25 mM concentration supressed TNF-induced NF-κB activation hence have anti-inflammatory and anti-proliferative effects (Sandur et al., 2007). The anti-proliferative effects were tested on U937, KBM-5, Jurkat, H1299, Caky-6, A549, SCC-4, Panc-1, MCF-7,
and DU145 in a dose dependent manner, although the major in vivo metabolite, THC showed reduced potency when compared to CUR (Sandur et al., 2007).

CUR is also known to modulate the immune system. Jagetia et al described modulatory functions of CUR on T cells, B cells, NK, dendritic cells as well as cytokines such as TNF, IL-1, IL-2, IL-6, IL-8 and IL-12 (Jagetia and Aggarwal, 2007) which may be an explanation for some of the medicinal uses of CUR including in arthritis, asthma, cardiovascular, and other allergy related disorders.

CUR was shown to activate p38 MAP kinase but reduce p44/42 MAP kinase activity, which means CUR is capable of differential regulation of MAPKs by activation of stress-induced MAPK, while inhibiting mitogen-induced MAPK (Johnston and DeMaster, 2003). CUR also was found to promote phosphorylation of p53 and increase cellular level of p21, which is a known p53 transcription target (Johnston and DeMaster, 2003).

CUR has also been shown to activate p21 in a p53-independent fashion in breast (MCF-7), prostate (PC-3) and colon (Colo-205) cancer cells (Hour et al., 2002, Su et al., 2006, Aggarwal et al., 2007). The up-regulation of p21 by 1,25(OH)2D3 and its analogues has also been documented in MCF-7 and PC-3 cells (Campbell et al., 1997, Bratland et al., 2000).

These biological effects of CUR strongly suggest there is a significant overlap among the molecular targets of 1,25(OH)2D3 and CUR, including their effect to prevent TNF-induced degradation of IκB, leading to attenuated activity of NF-κB, a well known cancer promoter (Singh 1995; Szeto 2007; Inoue 2007).

CUR was shown to have chemopreventative effects as well as anti-cancer effects in several types of malignancies. Firstly, CUR was proposed for colon cancer chemoprevention,
Interestingly, through direct binding to and activation of VDR (Bartik et al., 2010). Also, there have been several reports of chemopreventative effects of curcumin against cholangiocarcinoma (Suphim et al., 2010, Prakobwong et al., 2011a, Prakobwong et al., 2011b), hepatoma (Huang et al., 2008), and lung cancer (Chen et al., 2008).

CUR was reported to protect from UV induced skin cancer developments in recent studies by Heng et al, and Philips et al (Heng, 2010, Phillips et al., 2013). Heng et al conducted open label placebo controlled clinical studies for subjects with burn injuries or marked photodamage which showed topical CUR gel treatments at unspecified concentration, helped burn wounds to heal better and reduced the number of photodamage lesions including solar lentigines or actinic keratosis (Heng, 2010). The inhibitory effects on serine/threonine kinase dependent pathways and tyrosinase kinase dependent pathways were suggested to be the mechanism of such beneficial effects of CUR (Heng, 2010).

Furthermore, Philips et al conducted a photocarcinogenesis study in hairless mice and found mice fed oral CUR of 15mg daily dosage, or mice treated topically with CUR at 15mg/100ml concentration showed statistically significant reduction in UV induced tumour formation (Phillips et al., 2013). CUR was also used in the treatment of various cancers such as BCC (Jee et al., 1998) and also may be useful as an adjunct therapy in malignant melanoma (Odot et al., 2004).
1.5.6.4  *Tetrahydrocurcumin*

Tetrahydrocurcumin (THC) is a major metabolite of CUR, with similar antioxidant and anti-proliferation effects as CUR and with several advantages over CUR. Firstly, it is colourless, and therefore the troublesome stain with CUR is not a problem with THC, which would allow its use in topical preparations. It is also known to be significantly more stable than the parent CUR especially at physiological pH and temperature, with probably improved bioavailability than CUR (Osawa et al., 1995).

![Tetrahydrocurcumin](image)

**Figure 1.7 Tetrahydrocurcumin.**

1.5.6.5  *Biological effects of tetrahydrocurcumin*

The biological effects of THC are similar to that of CUR as described above, although there were suggestions that THC had more potent antioxidant effects compared to CUR (Holder et al., 1978, Sugiyama et al., 1996, Nakamura et al., 1998). Recent reports suggested THC
induced G2/M cell cycle arrest and apoptosis involving p38 and MAPK activation in human breast cancer cells (Kang et al., 2014). Wu et al also suggested chemopreventative effects of THC on human degenerative diseases and cancer through prevention of oxidative stress and inflammation (Wu et al., 2014), again, confirming the common trend of biological effects of THC with CUR.

THC was also suggested to reduce melanogenesis through inhibitory effect on tyrosinase which is a rate limiting step in the melanogenesis, hence helpful in solar radiation induced dyspigmentation (Majeed et al., 2010).

These biological effects of THC with the advantage of its colour render THC potentially very useful in anti-ageing cosmetics or sunscreen formulations.

1.5.7 Curcuminoids at low concentrations behave like Vitamin D

As described earlier, VDR is an interesting receptor in that it can heterodimerize with retinoid X receptor for genomic actions and probably with ERp57 for non-genomic actions, and is expressed in almost all human tissues (Pike and Meyer, 2012) and upregulates around 2000 genes so far identified (Pike et al., 2010, Szeles et al., 2010). It is also known to have ligands other than 1,25(OH)₂D₃ such as lithocholic acid (LCA), a secondary bile acid with lower affinity (Makishima et al., 2002) and this suggested VDR may have other novel ligands and in particular, CUR and several polyunsaturated fatty acids (PUFA) were seen to promote dimerization of VDR and RXR suggesting they may be ligands for VDR (Jurutka et al., 2007). This was again, suggested from more recent study which has shown CUR induced expression of the VDR target genes CYP24A1, CYP3A4, TRPV6, and CDKN1A in human
colon cancer cells, Caco-2 (Bartik et al., 2010). However, there are other studies with inconsistencies when CUR was tested on human monocytes (U937), keratinocytes (HaCaT) and colon cancer (HT-29) cell lines. In these cells, human cathelicidin antimicrobial peptide (CAMP) was induced but not CYP24A1 (Guo et al., 2013).

Interestingly CAMP induction was observed to be persistent in the absence of VDRE (Guo et al., 2013). It is worthwhile to note the discrepancies may be due to firstly the difference in CUR dose tested. Traditional doses of CUR reported by others where it demonstrated affinity to VDR were around 10,000- fold greater than the dose tested in this current study, and the vitamin D like activity of CUR may be only seen in lower concentrations. Also the cell lines tested are different, and this may indicate the difference in the biological effect of CUR. Finally, it would also be worthwhile to note the two different binding sites of VDR, the genomic pockets and the alternative pockets may have a more complex relationship than traditionally known, which may trigger interest for further studies. In fact, Mizwiki et al identified CUR as a ligand which can bind to the alternative pocket of VDR (Mizwicki et al., 2004).
1.6 Aims of this project

It has been shown that 1,25(OH)\(_2\)D\(_3\) reduces several types of DNA damage in human keratinocytes in culture and in mouse skin but only TD in human subjects (Damian et al., 2010). Furthermore, in that study, 1,25(OH)\(_2\)D\(_3\) was applied topically before and after UV exposure, so there is no information concerning whether 1,25(OH)\(_2\)D\(_3\) protects human subjects if applied after UV.

The aims of this project were to:

(i) Examine the photoprotective effect of 1,25(OH)\(_2\)D\(_3\) against the various forms of UV-induced DNA damage in human skin explants ex vivo and in human participants in vivo.

(ii) Compare the photoprotective properties of the vitamin-D-like compound, tetrahydocurcumin with 1,25(OH)\(_2\)D\(_3\), to determine whether the more stable compound could be used as a photoprotective agent against UV-induced DNA damage.

(iii) Explore whether the photoprotective mechanism against UV-induced DNA damage is in part due to an upregulation of some DNA repair enzymes.

(iv) The following hypotheses were tested:

a. UV-induced oxidative DNA damage, 8-oxodG is reduced by 1,25(OH)\(_2\)D\(_3\) and THC in human skin ex vivo and in vivo.
b. UV-induced nitrosative DNA damage due to reactive nitrogen species is reduced by $1,25(\text{OH})_2\text{D}_3$ and THC in human skin ex vivo and *in vivo*.

c. The DNA repair enzymes, DDB2, XPC and OGG1 are upregulated by $1,25(\text{OH})_2\text{D}_3$ and THC in human skin ex vivo and *in vivo* and thus improve DNA repair.
Chapter 2

2 General Methods

2.1 Equipment

2.1.1 Solar simulator

UV source used for this study was an Oriel 1000W xenon-arc lamp solar simulator (Oriel, Stratford, CT, USA). The solar simulator is attached to a digital output controller, and a switch unit which controls the shutter within the solar simulator (Figure 2.1). Xenon-arc lamps produce a broad spectrum of high fluence light including UV, visible and infrared wavelengths. This light is collected and reflected through a set of collimated optics. There are 2 set of 280-400nm dichroic reflective mirrors to reduce the visible and infrared components and an atmosphere attenuation filter (Oriel, Cat. No 81017) is used to filter out UVC (<290nm) so the output is similar to natural solar radiation.
Electromagnetic radiation is produced when electric current is passed through xenon gas under pressure. Electrons flow between the lamp electrodes, collide with xenon atoms and excite xenon electrons. When the excited xenon electrons return to their original energy states, they release absorbed energy in the form of UV, visible and infrared radiation from the lamp. An ellipsoidal reflector surrounding the lamp collects over 70% of the lamp radiation, which is focused onto an optical integrator. It produces a uniform diverging beam, which is deflected 90 degrees by a mirror to a final collimating lens. The output is a uniform collimating beam of 7.5 centimetres squared. Two 280-400nm dichroic mirrors were used to
reduce visible and infrared components of the lamp emission. Also an atmospheric attenuation filter (Oriel, catalogue number 81017) was used to remove UVC (<290nm) and alter the UVB waveband so that the spectrum simulated sunlight more closely. Even though the solar simulator had two fan cooling systems for the lamp, the room temperature was kept around 18 degrees using air conditioning to prevent possible overheating. There was a thermal interlocking system which automatically shut down the lamp in case of overheating.

The solar simulator lamp was ignited and warmed up for 20 minutes prior to any irradiation to achieve stable spectral output. Volunteers were seated on an adjustable backless stool with their back against a 10 centimetres squared metal template, which was positioned 7.5
centimetres from the machine, in front of the collimated beam. Opaque plastic templates were taped to the backs of the volunteers to separate different UV dosage areas. Each of the UV dosage areas were progressively shielded using opaque covers between irradiations to deliver the desired dose of UV. Gloves and UV protective face shields were worn while the lamp was operating.

Figure 2.3 UV irradiation of subjects. Subjects lower back were marked with a surgical marker to fit a window and were individually irradiated with UV followed by topical treatments. Biopsies were taken from the centre of each square. The Oriel solar simulator was also used to irradiate ex vivo skin samples as described in chapter 3.
2.1.2 Spectroradiometer

The OL-754 portable UV-visible spectroradiometer (Optronics Laboratories Inc., Orlando, FL, USA) was used to measure the spectrum and intensity of the solar simulator. It consists of an OL 754-O-PMT optic head, an OL754-C controller, and OL 754 software installed in a laptop (Compaq) running on Windows XP operation system. (Figure 2.2) The optic head consists of an integrating sphere and a double monochromator. The integrating sphere conducted the radiation from the source to the monochromator. The monochromator had dual holographic gratings, and dispersed the radiation onto a detector. Absolute spectral output was measured at 1nm intervals.

Figure 2.4 OL752 spectroradiometer.
The spectroradiometer was initially calibrated against a National Institute of Standard and Technology traceable reference quartz-tungsten halogen lamp source, OL 752-10 (Optronics Laboratories Inc., Orlando, FL, USA). The wavelength and response of the spectroradiometer were regularly checked using a Dual Calibration and Gain Check Source Module OL 752-150 (Optronics Laboratories Inc., Orlando, FL, USA).

![Solar simulator calibration with Spectroradiometer and broadband radiometer.](image)

Figure 2.5 Solar simulator calibration with Spectroradiometer and broadband radiometer.

The spectroradiometer was warmed up for 1 hour prior to usage. The measurements were taken when the solar simulator output was 400 watts, due to the saturation of spectroradiometer at higher outputs. The integrating sphere was placed in the plane of
irradiation. The spectral output used for analysis was an average of 3 readings (Figure 2.2).

2.1.3 Broadband radiometer

Broadband radiometer readings were taken at the same time as from the spectroradiometer for mathematical calibration of the source.

An IL 1350 broadband radiometer (International light, Newburyport, MA, USA) was used prior to each irradiation to monitor the solar simulator output. It uses SED 038 (UVA) and SED 240 (UVB) detectors. It was calibrated against the solar simulator by measuring spectral output with the spectroradiometer at the same time. The broadband radiometer measurement ensured accurate and consistent UV doses, despite small changes in solar simulator output due to the lamp ageing and room temperature.
2.1.4 Reflectance erythema meter and melanin meter

The Dia-stron erythema meter and melanin meter (Dia-stron, Hampshire, UK) measures skin redness and skin pigmentation. It has a tungsten-halogen lamp which shines white light onto the skin through a fibre optic probe. This probe collects light scattered from within the skin. The collected light is analysed by narrow band interference filters at 546nm (green), 632nm (red/orange), and 905nm (near infra-red).
Figure 2.7 Dia-stron reflectance erythema and melanin meter.

Skin redness, expressed as erythema index (EI), was calculated by the ratio of reflectance at 546nm to the reflectance at 632nm. The waveband of 546nm coincides with a peak in the haemoglobin absorption spectrum, reflecting the blood flow in the superficial dermis. The 632nm waveband was used as a reference signal to compensate for skin tone and probe alignment.

\[ EI = \log_{10} \left( \frac{632\text{nm results}}{546\text{nm results}} \right) \times 1000 \]

Skin pigmentation, expressed as melanin index (MI), was calculated by the ratio of reflectance at 632nm to the reflectance at 905nm. The 632nm waveband is sensitive to the melanin content, and 905nm waveband was used as a reference signal.

\[ MI = \log_{10} \left( \frac{905\text{nm results}}{632\text{nm results}} \right) \times 1000 \]
Both EI and MI readings were the averages of 100 rapid measurements taken automatically over 2 seconds, with a dynamic range between -99 to +999 units. The EI of each test site was obtained by subtracting the average of 3 background readings adjacent to the test site, from the average of 3 readings at the test site. All readings were taken while volunteers were lying in the prone position.
Chapter 3

3 Human ex vivo skin study

3.1 Introduction

Ex vivo means “out of the living” which takes place outside an organism. It involves experimentation in tissues from an organism that is separated from the origin but the environment is kept close to the natural conditions. The advantage of ex vivo study is that experiments can be carried out without some of the ethical issues encountered in in vivo studies. The first ex vivo study reported was in 1964 when ex vivo pig liver was used for a perfusion study (Liem et al., 1964a). Human ex vivo study involves collection of skin explants, typically from surgeries where excess skin is excised and discarded. Once excised, it firstly needs to be kept cold in physiological solution such as phosphate buffered saline (PBS) for transportation, and then it needs to be processed as soon as possible before ischaemic changes take place which may influence the measurements.

3.2 Methods

3.2.1 Collection and Processing of human ex vivo skin.

The collection and handling of human ex vivo skin was approved by the Human Research Ethics Committee of the Royal Prince Alfred Hospital (Camperdown, NSW 2005), and the University of Sydney (Sydney NSW 2006). Human skin explants were collected from consenting patients undergoing elective surgery. Cold sterile phosphate buffered saline (PBS)
was used to transport the skin back to the laboratory. Skin was processed under aseptic conditions in a biohazard hood (Biological Safety Cabinet Class II, BTR Environmental, Email Airhandling) as soon as possible and within 4 hours of surgery.

Figure 3.1 **Biohazard hood** was sterilised with UV lamp and 70% ethanol spray before use. Human ex vivo skin was processed in this biohazard hood and the handling and disposal of tissue was in accordance with the University of Sydney guidelines.

Subcutaneous fat, blood vessels and debris were trimmed off to leave epidermis and dermis only for the study. Skin pieces were dissected into 4 mm pieces with a punch biopsy prior to UV irradiation. Three pieces of skin were prepared for each treatment and 3 independent experiments were performed for each study.
3.2.2 UV irradiation of ex vivo skin

The UV source used was an Oriel 1000W xenon-arc lamp solar simulator (Oriel, Stratford, CT, USA) with an atmospheric attenuation filter (Oriel,) to eliminate UVC (<290nm) as described in chapter 2.1.1. It was pre-calibrated using an OL754 spectroradiometer (Optronics Laboratories Inc., Orlando FL) and IL1350 broadband radiometer (International Light, Newburyport, MA, USA). A calculated total dose of 4000 mJ/cm² (326 mJ/cm² UVB (8.15%); 3674 mJ/cm² UVA) was delivered to skin samples incubated in sterile Martinez solution (145 mM NaCl, 5.5 mM KCl, 1.2 mM MgCl₂(H₂O)₆, 1.2 mM NaH₂PO₄(H₂O)₂, 7.5 mM NaHEPES, 7.5 mM HEPES, 10 mM D-glucose and 1 mM CaCl₂(H₂O)₂), in a volume that was just enough to surround the tissue without submersion. Spectroradiometer measurements of this simulated solar simulator showed comparable spectral characteristics to the UV spectrum from the sun (Damian 2002).

The UV dose delivered to ex vivo skin in this study was equivalent to around 5-10 minutes of exposure to the midday sun in a summer day in Australia, and is around 15% of total UV exposure from a typical tanning session in a tanning facility in the USA (Hornung 2003).
Figure 3.2 The solar-simulated UV (SSUV) spectrum of the Oriel solar simulator approximates solar UV. The solar spectrum here is the standard sun UV spectrum. The spectra have been normalised at 315nm. Spectral irradiance of the lamp, with its different combinations of filters, was measured at 2-nm intervals over the wavelength range 240–400 nm with a grating monochromators (Damian 2002).
3.2.3 Treatment of ex vivo skin and incubation

Immediately after UV irradiation, skins were treated with vehicle (0.01% spectrograde ethanol), or 1 nM 1,25(OH)₂D₃ (Cayman Chemical, MI USA) in 100% spectrographic grade ethanol and incubated in RPMI-1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 250 ng/ml amphotericin-B (Sigma Aldrich, NSW, Australia). This concentration of 1,25(OH)₂D₃ has previously been shown to produce near maximal protection from UV induced thymine dimers (Gupta 2007). Sham-irradiated skin samples were subjected to similar procedures but not irradiated. Skin samples were snap frozen in OCT compound (Tissue-Tek, Sakura Finetek USA) at time points of 0.5, 1, 3, 6, 24 and 48 hours and stored at -80°C. Cryosections (6μm thickness) were collected on microscope slides (Superfrost Ultra Plus, Menzel-Glaser, New Hampshire USA) and air dried at room temperature for 20 minutes, fixed in cold acetone at -20°C for 10 minutes, and air dried at room temperature for another 20 minutes and then stored at -80°C before immunohistochemical staining.

3.2.4 Immunohistochemistry

For detection of thymine dimers, 8-oxo-7,8-dihydro-2-deoxyguanosine, and 8-nitroguanosine, frozen skin sections previously fixed in acetone, dehydrated, and stored frozen, were allowed to thaw at room temperature, then rinsed and rehydrated in Tris Buffered Saline (137 mM Sodium Chloride, 20 mM Tris-base) with 0.5% Tween-20 for 10 minutes. Nuclear denaturation was achieved with 0.15N NaOH in 70% ethanol followed by neutralization in 100mM Tris-HCl in 70% ethanol. Proteolytic digestion was achieved with 5 μg/ml of Proteinase K in EDTA for 10 minutes at 37°C. Non-specific background staining was blocked with serum-free protein blocking solution (Dako, Denmark) for 1 hour at room
temperature. The samples were then incubated for 1 hour in the primary antibody diluted in background reducing antibody diluent (Dako, Denmark) at optimized concentrations. The primary antibodies used were a monoclonal antibody to thymine dimers (Sigma-Aldrich, USA) at 10 µg/ml (Roza 1988), a monoclonal antibody to 8-oxodG (Trevigen, MD, USA) at 2.5 µg/ml (Soultanakis 2000), and a monoclonal antibody to 8-nitroguanosine (Cosmo Bio Co., Japan) at 10 µg/ml protein concentration (Akaike 2003).

For detection of IL-10, primary antibody to IL-10 (Abcam, USA, ab34843 Rabbit polyclonal) was used at working protein concentration of 0.63µg/ml after 1 hour of protein block without the nuclear denaturation or proteolytic digestion steps, and incubated overnight at 4°C in air tight containers (Zhou 2010). The rest of the immunohistochemistry protocols were otherwise identical to those described above.

Sections were also incubated with an isotype control (Mouse IgG1, Isotype control, Dako Denmark) instead of the antibody to determine specificity of the antibody at the same protein concentration of each primary antibody equivalents. Staining was detected and visualized with a LSAB Plus Dako Real™ Detection System kit with AEC chromogen staining (red) to differentiate positive staining from melanin. Tissues were air dried overnight, and coverslips mounted with an aqueous mountant (Aquatex, Merck, Germany).

3.2.5 Image analysis and Statistical testing
Images were captured using a Nikon Eclipse E800 microscope (Nikon Instruments Inc. NY USA) and Leica software (version 3.1.0 Leica Geosystems, Switzerland) at x200 original magnification with fixed light and camera setting, and analyzed using Metamorph software
(version 7.7, Molecular Devices, USA) to quantify the proportion of positively stained nuclei in the epidermis.

![Microscope Image](image)

**Figure 3.3** Nikon E800 microscope with Leica DFC550 camera system were used to acquire images for analysis at Advanced Microscopy Facility (AMF) at the University of Sydney.

The quantified data were based on triplicate tissue samples and 3 images acquired from each sample (n=9). Significant differences between treatment groups were assessed by one way ANOVA and Tukey-Kramer test using Graphpad Instat statistical program (GraphPad Software Inc., San Diego, CA, USA).
3.3 Results: Photoprotection by 1,25(OH)\textsubscript{2}D\textsubscript{3} and THC against UV induced DNA damage in human ex vivo skin.

3.3.1 UV induced Thymine dimers are reduced by 1,25(OH)\textsubscript{2}D\textsubscript{3}, and THC.

UV irradiated ex vivo skin showed positive nuclear staining for TD in all time points, which includes 30 minutes, 1 hour, 3 hours, 6 hours, and 24 hours post irradiation while treatments with 1,25(OH)\textsubscript{2}D\textsubscript{3} or THC at 3 different doses immediately after UV irradiation reduced the nuclear staining with TD primary antibody at each time points (Figure 3.4c, 3.4d). No positive staining was observed in controls with no UV exposure with or without equivalent treatments (Figure 3.4a). Quantification by image analysis showed a significant increase in TDs from 30 minutes post-UV, which was the first time point tested, compared to the sham irradiated vehicle treated skin (Figure 3.5; p<0.001). It is interesting to note the proportion of nuclei positive for TD post-UV showed a gradual and progressive increase for the first 3 hours, which was statistically significant (p<0.01), followed by a slow decline, which may be due to DNA repair. Those skin samples exposed to UV, but treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} immediately post-UV showed a consistently reduced number of TD-positive stained nuclei at all time points (Figure 3.5; p<0.01 at 30 minutes, and p<0.001 at 3 hours). Treatment with THC post-UV irradiation showed a dose-dependent reduction in TD-positive staining (Figure 3.4d, Figure 3.5; 100nM compared to 1nm at 30 minutes p<0.05, and at 1 hour p<0.01).
Figure 3.4 Human ex vivo skin sections stained for TD.

Unirradiated skin showed no nuclear staining regardless of treatment (Figure 3.4a). Positively stained nuclei with primary antibody against TD appear red (arrow). Scale bar = 100μm.
Figure 3.5  **Time course and response of TDs after UV with vehicle, 1,25(OH)\(_2\)D\(_3\) at 1nM concentration, or 3 doses of THC.**

THC at concentration of 100nM showed significant reduction in UV induced TD from 30min, up to 3 hours post-UV in a similar manner to the reduction seen with treatment of 1,25(OH)\(_2\)D\(_3\) at 1nM concentration. There was no statistical difference between all treatment groups from 6 and 24 hours post-UV (p>0.05). Significantly different from THC @ 1nM at time points of 30min and 1 hour, # p<0.05; ### p<0.001; significantly different from UV Vehicle at 30min, && p<0.01; significantly different from UV vehicle at the corresponding time post-UV, * p<0.05; *** p<0.001. Proportion of positively stained nuclei refers to an arbitrary unit from image analysis. n=5 subjects.
Firstly, at THC concentration of 1nM, a significant degree of reduction in UV induced TD was only seen at 3 hour, unlike the 1,25(OH)$_2$D$_3$ treated skin where the significant reduction was seen from 30min post UV until 3 hours (Figure 3.5). At 10nM concentration of THC or higher, the reduction in UV induced TD was seen from 1 hr and at the subsequent time points although statistically significant reduction was only observed for the first 3 hours post-UV (Figure 3.5). There was no statistical difference in TD quantities in UV irradiated skin with treatment with 1,25(OH)$_2$D$_3$ or THC at 100nM concentration (p>0.05) at all time points tested.

![Figure 3.6 Reduction in TDs by THC at 100nM concentration compared with 1,25(OH)$_2$D$_3$ at 1nM.](image)

Treatment with THC at 100nM or 1,25(OH)$_2$D$_3$ at 1nm immediately post-UV caused highly significant degree of reduction in UV induced TD compared to the vehicle treated equivalent (p<0.001). The quantities of TD with THC 100nM treatment post-UV at all time points were not significantly different from the 1,25(OH)$_2$D$_3$ treated equivalents at each corresponding time point (p>0.05). There was no significant difference between all treatment groups at 6 and 24 hours post-UV (p>0.05). Significantly different from UV Vehicle at each corresponding time point, *** p<0.001. n=5.
3.3.2 UV induced 8-oxo-7,8-dihydro-2′-deoxyguanosine is reduced by 1,25(OH)\(_2\)D\(_3\) and THC.

UV irradiated skin showed positive nuclear staining with the antibody to 8-oxodG from 30 minutes post-UV. The number of positively-stained nuclei with the 8-oxodG antibody showed a progressive increase up to 6 hours post-UV but showed decline at 24 hours post-UV to the level similar to that seen in the sham irradiated skin (Figure 3.7). The sham irradiated skin at each corresponding time points showed some nuclear staining for 8-oxodG, however, this was much less than the UV irradiated skin at each corresponding time points (Figure 3.7). Treatment with 1,25(OH)\(_2\)D\(_3\) and THC immediately after irradiation reduced nuclear staining for 8-oxodG at 3hr and 6hr (Figure 3.7).

Quantification by image analysis showed that the first significant increase in UV-induced positive-staining with the 8-oxodG antibody compared to the sham irradiated skin was 3 hours post-UV (Figure 3.8). The progressive increase of 8-oxodG up to 6 hours post-UV was also significant (p<0.001). The level of 8-oxodG without UV irradiation gradually increased in time which reflects probable oxidative stress during the incubation period in culture. 1,25(OH)\(_2\)D\(_3\) treatments in sham irradiated skin showed apparent reduction of 8-oxdG, but this was not statistically significant (p>0.05).
Figure 3.7 Human ex vivo skin section for 8-oxodG 3 hours post UV and treatments. Positively stained nuclei with primary antibody against 8-oxodG appear red (arrow). Scale bar = 100µm.
Figure 3.8 **Dose related effect of THC in reducing UV-induced 8-oxodG.**

THC treatments at 3 different concentrations produced what appeared to be a dose related reduction in 8-oxodG at 3hr and 6hr. Skins treated with THC at 1nM concentration, showed a significant reduction of positive-stained nuclei with the 8-oxodG antibody at 3 and 6 hours post-UV compared to the vehicle treated skins. At this concentration of 1nM, the quantities of 8-oxodG were greater than that of 1,25(OH)2D3 at 3 and 6 hours post-UV, but still significantly less than the vehicle treated equivalents (p<0.01). With THC at higher concentrations, 10nM and 100nM, the degree of reduction in 8-oxodG was similar to that with 1,25(OH)2D3 and there was no statistical difference between these different treatments. The quantity of 8-oxodG at 24 hours post-UV showed no differences between all groups. Significantly different from the unirradiated (sham) vehicle treated skin at equivalent time, @@@ p<0.0001; significantly different from the UV-irradiated vehicle-treated equivalent, ### p<0.001, ## p<0.01. n=5.
3.3.3 UV induced 8-nitroguanosine sites are reduced by 1,25(OH)$_2$D$_3$ and THC.

UV irradiated skin showed strong positive nuclear staining with the antibody to 8-NG for all time points starting at 30 minutes post-UV (Figure 3.9). Treatment with 1,25(OH)$_2$D$_3$ immediately after irradiation reduced nuclear staining at all time points (Figure 3.10). There was a low level of positive staining in nuclei of sham irradiated skin explants (Figure 3.9a).

Quantification by image analysis showed a significant increase in 8-NG in UV irradiated vehicle treated skin explants by 30 minutes post-UV, the first time point tested, compared to sham irradiated skin (p < 0.001). This remained high up to 6 h after irradiation, the final time point tested (Figure 3.10). There was a significant reduction by 50% in 8-NG in UV irradiated skin treated with 1,25(OH)$_2$D$_3$ immediately after irradiation at 30 minutes (p<0.001), compared to the UV irradiated skin treated with vehicle at the same time point. The skin explants with 1,25(OH)$_2$D$_3$ treatment continued to show lower positive-staining with the 8-NG antibody compared with vehicle at all subsequent times tested. There was a statistically significant increase in 8-NG positive-staining in sham irradiated skin explants treated with or without 1,25(OH)$_2$D$_3$ at 1, 3 and 6 h, compared to sham irradiated skins at 0.5 h (p < 0.001), which again is suggestive that the ex vivo skins undergo nitrosative stress during the culture period (Figure 3.10).
Figure 3.9 Human ex vivo skin section stained for 8-NG 3hr after UV and treatments. Positively stained nuclei with primary antibody against 8-NG appear red (arrow). Scale bar = 100μm.
Figure 3.10 Reduction of UV-induced 8-NG by 1,25(OH)_{2}D_{3} or THC.

Treatment with THC immediately after irradiation also reduced nuclear staining with the 8-NG antibody from 30 minutes post UV and treatment (p<0.001) comparable to the reduction seen with 1,25(OH)_{2}D_{3} at concentration of 1nM THC. This concentration of THC seemed to be maximal since there was no concentration related changes observed at the different THC concentrations except for 1 hour post UV, where THC at 10 and 100nM showed greater reduction in 8-NG compared to 1,25(OH)_{2}D_{3} or THC at concentration of 1nM. Significantly different from unirradiated (sham), vehicle treated skin at each time points, *** p<0.001; significance from UV irradiated vehicle treated skin at each time points, ### p<0.001; significance from THC 1nm at 1hr, @ p<0.05. n=5.
3.3.4 IL-10 level was increased by $1,25(\text{OH})_2\text{D}_3$ but not with THC at the tested concentrations with or without UV.

Immunohistochemistry for IL-10 in skin samples examined 24 hours and 48 hours after irradiation, showed cytoplasmic staining (Figures 3.11). Sham irradiated skin with vehicle, $1,25(\text{OH})_2\text{D}_3$ or THC at 1nM, 10nM, or 100nM concentrations showed higher intensity of staining for IL-10 with $1,25(\text{OH})_2\text{D}_3$ compared with vehicle-treated skin at 24 hours after the treatments (Figure 3.11d). THC treatments at all 3 doses in the absence of UV seem to have a comparable degree of staining compared to the vehicle treated skin (Figure 3.11d).

UV irradiated skins showed significantly increased intensity of staining (Figure 3.11 e,f,g), where higher degrees of increased intensity were seen with vehicle or $1,25(\text{OH})_2\text{D}_3$ treatment, and THC at 3 doses showed a lower increase in intensity.

Quantification by image analysis revealed a significant rise in intensity of IL-10 staining in sham irradiated, $1,25(\text{OH})_2\text{D}_3$ treated skin compared to the sham irradiated vehicle treated skin (Fig 3.12). There was no difference between the sham irradiated vehicle treated skin and sham irradiated THC treated skin in all 3 doses THC tested. The highest intensity was seen in UV irradiated skin treated with vehicle or $1,25(\text{OH})_2\text{D}_3$, and there was no statistical difference between the two (Figure 3.12). THC treatments after UV did show increased IL-10 expression compared to the sham-irradiated vehicle-treated skin, but significantly lower than the UV irradiated vehicle or $1,25(\text{OH})_2\text{D}_3$ treated skin. There was no statistical difference between the 3 different concentrations of THC, indicating that dose dependent responses were not seen with the doses tested.
Figure 3.11 Human ex vivo skin sections stained with primary antibody against IL-10. Positively stained areas are mainly cytoplasmic (straight arrow) and the intensity of staining was quantified for analysis. Note significant degree of epidermal destruction after 48 hours of incubation (curved arrow) which made it difficult to acquire accurate and meaningful quantification beyond this time point. Scale bar = 100μm.
Relative intensity of IL-10 staining in human ex vivo skin demonstrating no significant difference amongst the non-UV irradiated skin (p>0.05) except for the skin treated with 1,25(OH)\(_2\)D\(_3\), with significantly increased IL-10 expression (p<0.05). In UV irradiated skin, 1,25(OH)\(_2\)D\(_3\) treatment did not show a significant difference compared to the vehicle treated skin (p>0.05). THC treated skin however, showed a significantly lower level of IL-10 expression compared to the vehicle or 1,25(OH)\(_2\)D\(_3\) treated skin (p<0.001). Significantly different from the sham irradiated vehicle treated skin, @@@ p<0.001; @ p<0.05; from UV irradiated vehicle treated skin, && p<0.01. n=5.

Figure 3.12 **Image analysis of IL-10 staining 24 hours post-UV and treatment.**
3.4 Discussion

The ex vivo study has shown that both 1,25(OH)$_2$D$_3$ and THC provide photoprotection in human ex vivo skin from 3 types of UV induced DNA damage. The major benefit of developing the ex vivo protocol was that most skin samples collected were big enough to yield hundreds of skin samples that could be used to test the photoprotective effects of not only 1,25(OH)$_2$D$_3$, but also of THC at 3 different concentrations, with 6 different time points post-UV, with sham irradiated controls for each group, and in triplicate. There were, however, limitations with the ex vivo protocol.

Firstly, the survival of ex vivo skin samples limited the study only up to 24 hours. Although none of the skin explant samples showed signs of infection during incubation, cryosectioning of skin samples older than 24 hours was difficult and significant degree of damage was noted especially at the dermoepidermal junction. As a result, detachment and severe distortion of the epidermis was observed in most cryosectioned slides which made meaningful analysis impossible. Secondly, the size chosen for skin explant samples was too small. Although the cryosections of the skin samples were 6µm in thickness, there was significant waste in skin sections due to inexperience, especially in earlier studies, due in part to malfunctioning cryotome, including inaccurate thickness, and faulty temperature control unit which had kept the temperature either too high or too low, which in either case, hindered production of optimal sectioned slides and wasting of the skin samples.

Despite the limitations, several interesting results were gained through this ex vivo study. Firstly, the dynamics of post-UV production and repair of several forms of DNA damage in
human ex vivo study were revealed. TDs are certainly one of the most important, hence most examined UV induced photolesion (Mizuno et al., 1991, Douki et al., 2000, Rochette et al., 2003, Mouret et al., 2008). Mouret et al. have shown that the repair efficiency of CPDs in human skin and cultured skin cells after UV depended on the type of CPD, and that TDs were the most abundant CPD to form after UV and the slowest to be repaired, with around 50% remaining in cultured keratinocytes, and 70% in human ex vivo skin after 24 hours post-UV (Mouret et al., 2008). Some of the results presented here are consistent with Mouret et al in that 68% of TDs present at 30 minutes post-UV were still present at 24 hours compared with 70% reported by Mouret et al (Mouret et al., 2008).

The current study showed that there was a significant (p<0.001) and progressive rise in TDs in vehicle treated UV exposed skin samples over the first 3 hours before the decline in TDs. This is similar to a recent report from the Mason group showing an increase in TD over 3 hour in human keratinocytes using either immunohistochemistry with primary antibody against TD and image analysis or enzymatic treatment of DNA with T4N5 endonuclease and comet assay (Gordon-Thomson et al., 2012). In that study, it was reported that nitric oxide generators such as SIN1 or sodium nitroprusside (SNP) in the absence of UV seemed capable of inducing CPD, despite the general consensus that these are produced by UV almost exclusively (Gordon-Thomson et al., 2012). It is also interesting to note the production of TD in keratinocytes by SNP without UV was significantly reduced when incubated at 4°C, but not abolished (Gordon-Thomson et al., 2012). This finding of temperature dependent chemical production of CPD suggests metabolic production of CPD may be possible. There have been reports of chemical production of CPDs observed in bacterial DNA, and isolated DNA, and it was proposed that this was due to a triplet-triplet energy transfer mechanism by
excited state species (Lamola, 1971, Lhiaubet-Vallet et al., 2007). In fact, Gupta et al made similar observation where increase in CPD was observed until 6 hours post-UV in cultured keratinocytes (Gupta et al., 2007). There were other reports with similar findings although no satisfactory explanations were proposed (Mullaart et al., 1989, Vink et al., 1993, Qin et al., 1994, Applegate et al., 1999, Lu et al., 1999, Cario-Andre et al., 2000). Despite similar results with 2 different detection methods in whole skin as well as keratinocyte cultures, these findings could be artefactual and will need to be reviewed by testing for CPD using liquid chromatography with tandem mass spectroscopy (Douki et al., 2000).

It was observed that in UV exposed skin samples treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} or THC, the rise in TD for the first 3 hours was not statistically significant. This observation may be due to the reduction in NO products with 1,25(OH)\textsubscript{2}D\textsubscript{3} as suggested by earlier studies (Gupta et al., 2007, Dixon et al., 2011). A decrease in NO products with 1,25(OH)\textsubscript{2}D\textsubscript{3} was also seen in this study as reduction in UV induced 8-NG. This reduction was not only seen with 1,25(OH)\textsubscript{2}D\textsubscript{3} but also with THC.

Indeed, UV is known to increase NO and its products (Bruch-Gerharz et al., 1998). Chronic inflammation is also known to increase NO (Kaneko et al., 2008) as well as sustained hypoxia (Tan et al., 2001). While the proposal that NO products such as peroxynitrite contribute to TD formation is controversial, there is acceptance that NO products are known to inhibit DNA repair (Bau et al., 2001) hence causing mutagenesis and carcinogenesis (Sawa and Ohshima, 2006, Kaneko et al., 2008). Earlier reports from our group have shown that 1,25(OH)\textsubscript{2}D\textsubscript{3} reduced the accumulation of the peroxynitrite degradation product, nitrite
Gupta et al., 2007), and 3-nitrotyrosine (Dixon et al., 2011) in UV irradiated cultured keratinocytes in vitro. Also the NO inhibitors, aminoguanidine and L-N-monomethylarginine (L-NMMA), reduced UV induced TDs in cultured keratinocytes to a similar level as 1,25(OH)2D3 (Gupta et al., 2007), which could be because DNA repair is not being inhibited. The current study also examined the dynamics of 8-NG which increased significantly in vehicle-treated sham-irradiated skin during incubation (p<0.001). 1,25(OH)2D3 or THC treatment did not affect this degree of rise in 8-NG in sham irradiated skin. In UV irradiated skin, however, 8NG levels were highly elevated as early as 30 minutes post-UV (p<0.001) and progressively increased until 3 hours post-UV (p<0.001) then declined. Treatment with 1,25(OH)2D3 significantly reduced post-UV 8-NG by 48% at 30 minutes (p<0.001), as well as other time points tested. This is in keeping with previous reports of the reduction in UV-induced nitrite by 1,25(OH)2D3 (Gupta et al., 2007), and 3-nitrotyrosine (Dixon et al., 2011), which probably would have contributed to the reduction in post-UV 8-NG production as well.

There are suggestions that the reactive nitrogen intermediate, peroxynitrite is also implicated in the production of 8-oxodG by oxidation of guanine (Niles et al., 2006) although this is controversial. Yerminov et al (Yermilov et al., 1995) and Douki et al (Douki and Cadet, 1996) failed to find increases in 8-oxodG in calf thymus DNA when treated with peroxynitrite, but others showed significant increases in 8-oxodG under similar conditions (Inoue and Kawanishi, 1995, Fiala et al., 1996, Spencer et al., 1996). Co-localisation of 8-oxodG and 8-NG was found in cervical biopsy specimens through immunohistochemistry (Hiraku and Kawanishi, 2009). The current study also demonstrated a similar trend where 8-oxodG and 8-NG both increased with statistically significant elevations with increased incubation time in sham irradiated skin, a significant elevation with UV irradiation and a significant degree of protection with 1,25(OH)2D3 or THC. Interestingly, the peak of 8-oxodG in this human ex
vivo skin study was seen at 6 hours post UV amongst the time points tested rather than 3 hours (p<0.001), as seen for 8NG and TD. It is unclear why the timing of the peak in 8-oxodG is later than other DNA lesions. It is speculated that the incubation environment may cause ongoing oxidative stress in human ex-vivo skin, which in turn may cause ongoing production of 8-oxodG even after the cessation of UV.

It is interesting to note that the protective effect of THC was near maximal at 1nM concentration with higher concentrations only associated with significantly less DNA damage in the case of TD at 1 hour and 8-NG at 1 hour.

Although this is the first time that a time-course for THC photoprotection against 3 types of UV-induced DNA damage in human skin has been reported, photoprotection by curcuminoids in cultured cells and *in vivo* mice has been shown by earlier studies from our group. Poliakov examined photoprotective effects of curcumin with or without DIDS (Poliakov, 2009) which is a chloride channel inhibitor which was reported to block nongenomic actions of 1,25(OH)₂D₃ in osteoblasts (Zanello and Norman, 1997) and inhibit photoprotective effects of 1,25(OH)₂D₃ in previous studies (Sequeira et al., 2013). In her study, 100nM concentration of curcumin reduced UV induced TDs, and addition of 50μM DIDS prior to UV and during treatment, had no effect on CPD on its own, but eliminated the protective effects of curcumin and 1,25(OH)₂D₃ (Poliakov, 2009), again suggesting the photoprotective mechanism of curcumin may be similar to 1,25(OH)₂D₃. Ryan showed THC, the *in vivo* metabolite of CUR, also reduced UV-induced TD formation in cultured keratinocytes comparable with 1,25(OH)₂D₃. Ryan also observed a reduction in UV induced 8-oxodG with both 1,25(OH)₂D₃ and THC in hairless mice (Skh:HR1), where the reduction
with THC was significantly greater than for 1,25(OH)₂D₃ (Ryan, 2010). Lastly, Sequeira found p53 was significantly upregulated with 1,25(OH)₂D₃ in UV irradiated keratinocytes, but not with curcumin, which suggested that the photoprotective effect of curcumin may not completely overlap with 1,25(OH)₂D₃ (Sequeira, 2011).

The human ex vivo study has also shown for the first time, the modulatory effects of 1,25(OH)₂D₃ and THC on IL-10 in human epidermis. IL-10 is known to contribute to UV-induced immunosuppression in skin (Loser et al., 2007), and its modulation is triggered by UV induced DNA damage (Kripke et al., 1992, Nishigori et al., 1996). The role of 1,25(OH)₂D₃ in UV induced immunosuppression is controversial. Animal studies have shown a protective role of 1,25(OH)₂D₃ in photoimmunosuppression which could be due to reduced DNA damage, a prime contributor to UV-immunosuppression (Kripke et al., 1992, Dixon et al., 2005, Dixon et al., 2007, Dixon et al., 2011). In human volunteers the Mason group reported that topical calcitriol, on its own caused immunosuppression (Damian et al., 2010). The current study in human ex vivo skin produced results which complement the latter, in that the expression of IL-10 was increased in sham irradiated skin treated with 1,25(OH)₂D₃. UV irradiation clearly increased IL-10 level but 1,25(OH)₂D₃ treatment did not affect the IL-10 expression in UV irradiated skin. THC treated skin, however, showed very different results. THC at nanomolar concentrations in sham irradiated skin did not affect the level of IL-10 expression, but significantly reduced IL-10 expression in UV irradiated skin compared to the vehicle treated skin. Curcumin was reported to induce IL-10 in lipopolysaccharide (LPS) stimulated macrophages at a concentration of 20μM but not at a lower concentration, 1μM (Sharma et al., 2007). This again suggests that curcuminoids have different effects at the nanomolar concentrations used in the current study. It is also worthwhile to note, the IL-10 detection was attempted at 2 time points in current study, 24
hours and 48 hours post UV. Nickoloff et al reported IL-10 expression was upregulated 6 hours post stimuli such as tape stripping or poison ivy dermatitis but not in psoriatic plaques (Nickoloff et al., 1994). Sharma et al detected increased IL-10 level in macrophages 48 hours after the stimuli (Sharma et al., 2007). Based on these reports, 24 hour and 48 hour post UV time points were tested, however, the measurements from 48 hour incubated skin samples were not accurate since the skin integrity had deteriorated by this time. The curcumin study by Poliakov used a contact hypersensitivity model in hairless mice with 2% oxazolone and demonstrated UV induced immune suppression was reduced by 3 different doses of CUR, 4.6, 23 and 115pmol/cm2, in a dose dependent manner (Poliakov, 2009). Ryan also demonstrated in the same setting that THC also produced protection from photoimmunosuppression (Ryan, 2010).

At this stage, the mechanism of photoprotection by 1,25(OH)2D3 or THC is not entirely clear. The presence of VDR is essential for reduction in TD, but VDR with a defective DNA binding domain or a mutation in helix H1 of the classical ligand binding domain (LBD) are still effective in mediating the response (Sequeira et al., 2012). A neutralizing antibody to ERp57, Ab099, or an ERp57 small interfering RNA completely abolished the protection against TD, and a co-IP study showed the interaction of VDR and ERp57, suggesting the presence of critical components for the protective action of 1,25(OH)2D3 against UV induced DNA damage (Sequeira et al., 2012). However, p53 upregulation by 1,25(OH)2D3 did not require VDR or ERp57 in human fibroblasts, suggesting a separate mechanism. While it is likely that a VDR/ERp57 complex would be the receptor responsible for the protection against UV induced ROS and NO as well, this has not yet been tested. The Mason group also reported recently, DIDS, a chlorine channel blocker previously shown to inhibit 1,25(OH)2D3 induced chloride currents, had no effect on its own on TD, but prevented the protective
effects of 1,25(OH)₂D₃ against TD production (Sequeira et al., 2013). DIDS was also seen to abolish the protective effects of both 1,25(OH)₂D₃ and CUR in cultured human keratinocytes post-UV against TD production (Poliakov, 2009) which suggest the involvement of the alternative ligand binding pocket of VDR, which is important in the non-genomic actions which could also explain the photoprotective effects seen with JN (Dixon et al., 2011).

1,25(OH)₂D₃ is not an antioxidant, but still is capable of reducing oxidative stress in human skin, most probably due to its ability to reduce NO and peroxynitrite, post-UV. While the reduction in NO products post-UV is likely to reduce production of 8-NG and probably 8-oxodG, and may reduce TD production, reduced NO would also be expected to enhance DNA repair (Bau et al., 2001) though this was not directly tested in the skin explants.

This study demonstrates for the first time, the dynamics of several types of UV induced DNA damage in human ex vivo skin, and confirms the photoprotective effects of 1,25(OH)₂D₃, as well as THC. THC, unlike 1,25(OH)₂D₃, may provide further photoprotection in human skin after UV exposure through reduced photoimmune suppression as suggested by its inhibition of UV induced IL-10 expression.
Chapter 4

4 In vivo Human Study

4.1 Introduction

1,25(OH)$_2$D$_3$ and THC both similarly reduced 3 types of UV induced DNA damage (TD, 8-oxodG, and 8NG) in human ex vivo skin. This study was repeated in healthy human volunteers in a randomised controlled trial to examine the in vivo effects of both 1,25(OH)$_2$D$_3$ and THC, and directly compare the degree of biological effects post-UV.

4.2 Methods

4.2.1 Recruiting volunteers for the human study

Healthy adult volunteers were recruited through noticeboard poster ads at the University of Sydney campus grounds (Sydney NSW, Australia), and Royal Prince Alfred Hospital (Camperdown NSW, Australia).

Twenty eight volunteers made enquiries regarding participation, and 20 eventually agreed to participate. Each volunteer was interviewed, (Appendix 6.6) their medical history was taken, and physical examinations and skin check were performed, which included measurements of the melanin index and erythema index with a reflectance melanin and erythema meter (Dia-ptron, Hampshire, UK) (Chapter 2.1.4). All participants were given detailed volunteer
information sheets which provided an explanation of the trial, and consent forms (Appendix 6.4, 6.5). The process of advertisement, recruitment, interview and informed consents were approved by the Royal Prince Alfred Hospital Human Research Ethic Committee and the University of Sydney Human Research Ethics Committee. This clinical trial was carried out in the Department of Dermatology, Gloucester House, Royal Prince Alfred Hospital (Camperdown, NSW Australia) in keeping with the human ethics guidelines. The exclusion criteria were as follows.

Exclusion criteria:

- Age < 18 years
- Pregnancy or lactation
- Recent sun exposure of mid-back (within the previous four weeks)
- History of photosensitivity
- Photosensitising medications such as, but not limited to, tetracyclines, or retinoids
- History of keloid scarring
- Treatment currently, or within the previous four weeks, with immunosuppressants (eg. corticosteroids either orally or inhaled)
- Abnormal skin on back (eg. vitiligo, dermatitis)
- People who are immunologically compromised.
- People who may be psychologically or psychiatrically unstable as well as those with an intellectual disability since their consents are not valid.
4.2.2 UV irradiation of human skin in vivo

Consenting volunteers were asked to have their lower back exposed and 4 squares 3x3cm were marked out using a surgical marker (Solmed skin marker, Multigate medical devices, Australia) by placing dots at 4 corners. The 4 squares were placed horizontally across the lower back where 2 squares were placed on each side (Figure 2.3).

The UV source used was an Oriel 1000W xenon-arc lamp solar simulator (Oriel, Stratford, CT, USA), which is the same model used for the human ex vivo study but not the identical unit in that there was an additional attachment at the output aperture so the UV light was emitted not vertically downwards, but horizontally, so the direction of UV light was perpendicular to the volunteer’s lower back when the volunteer was sitting down on a backless stool directly in front of the unit (Figure 4.1).
Unlike with the ex vivo study where 4 J/cm$^2$ of solar simulated UV was used, 7.5 J/cm$^2$ was delivered for the *in vivo* human study. This was because the average minimal erythemal dose gained from my preliminary testing in live volunteers was 5.6 J/cm$^2$ ranging from 3.7 J/cm$^2$ to 8.2 J/cm$^2$ which meant that 4 J/cm$^2$ was suberythemal, and this may not have been enough to elicit the DNA damage, particularly in volunteers with darker skin type. For the purpose of this clinical trial, it was decided to use 7.5 J/cm$^2$ for each site since it was mildly higher than the average MED, and also this dose of UV had been used in a clinical trial with human volunteers in the past (Damian 2010).
In our study, much attention was paid to ensure equal doses of UV irradiation in all 3 UV exposed skin sites in human volunteers. This was measured by broadband radiometer measurements on UV output through the square window prior to each irradiation and recalculated at the time of irradiation since the solar simulator output declined with time as shown by the decline in broadband radiometer readings (Figure 4.2).

The comfort of the volunteer was ensured before the commencement of UV irradiation and the volunteers were asked to minimise movements during irradiation as much as possible. The UV was delivered through a 3x3cm window template to match the square markings of the volunteer (Figure 2.2, Figure 4.3).

Figure 4.2 Oriel solar simulator’s output was gradually reduced with use: The UV output from the Oriel solar simulator gradually decreased over time according to the broadband meter readings made prior to each irradiation.
Figure 4.3 UV irradiation window: 3x3cm window was used to ensure irradiation of only the intended site at a time.
4.2.3 Topical treatments

Topical treatment lotions were prepared by Dr Clare Gordon-Thomson, Physiology Department on the day of testing and were coded before delivery.

Stock solutions for the production of the topical treatments included the base lotion (ethanol:milliQ water:propylene glycol in a ratio of 2:1:1), 1,25(OH)₂D₃ (1x10⁻⁴ M) in 100% spectrographic grade ethanol, and THC (1x10⁻⁴ M) in 100% ethanol. The topical treatment lotions (100µl each) were prepared, and were randomly coded with the batch in alphabet letters, and the number from 1-3 (eg. A1,A2,A3). One control solution (vehicle) was prepared from base lotion only and was labelled for use as a the negative control for the square which received no UV exposure, and the other squares received treatment from one of the randomised unknown treatments.

4.2.4 Preparation of working solutions:

1,25(OH)₂D₃:

11.5 µl of 1,25(OH)₂D₃ – stock (1x10⁻⁴ M) was mixed with 38.5 µl ethanol, and then mixed with 50 µl of base lotion to adjust the volume to 100 µl. This allowed a final concentration of 23 pmol/cm² on the back of the human subject.

THC:

34.5 µl of THC - stock (1x10⁻⁴ M) was mixed with 15.5 µl ethanol and then mixed with 50 µl base lotion to adjust the volume to 100 µl. This allowed a final concentration of 69 pmol/cm² on the back of the human subject.

Topical treatments were applied immediately post-UV. A 20 µl Gilson pipette was used to
apply 18 μl of the topical solution while the volunteer was lying prone. The solution was gently spread manually using a gloved fingertip within the 3 x 3 cm boundaries as marked before the UV irradiation. Vehicle solution was first applied over the non UV irradiated site, followed by solution number 1, 2 and then 3 from left to right (Figure general methods above). The solutions were allowed to air dry for a couple of minutes, and then the subjects were asked not to expose their back under the sun or refrain from exercise or any physical activity that may cause sweating for the next 2 hours. The subjects were asked to return in 2 hours for biopsy.

### 4.2.5 Harvesting human skin for analysis

Xylocaine 2% with adrenaline (AstraZeneca, UK), was injected subdermally in the centre of each of the sites followed by biopsies using a 4 mm punch biopsy tool (Kai Medical, Germany). The wounds were closed with 4.0 nylon sutures (Ethicon, Johnson and Johnson, USA) and then covered with waterproof dressings (Opsite, Smith and Nephew, USA). The sutures were removed after 12 days followed by application of adhesive skin closure strips (Steristrips, 3M, USA), which marked the end of volunteer contacts. The biopsy samples were immediately immersed in cryomoulds preloaded with OCT, and moved into liquid nitrogen filled metal platforms to be snap frozen as soon as possible.

### 4.2.6 Frozen section and immunohistochemistry

The frozen section method was identical to the human ex vivo protocol described earlier (Chapter 2). Immunohistochemical staining involved use of primary antibodies against the 3 types of DNA damage as used for the ex vivo study as well with the same protocol (Chapter 3). For the human *in vivo* study, primary antibodies against DNA repair proteins, DDB2
(Abcam, Cambridge, UK), XPC (Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA) and OGG1 (Novus Biologicals, USA) were also used. The working protein concentrations for the primary antibodies were 1µg/ml, 2µg/ml, and 10µg/ml respectively for DDB2, XPC and OGG1. Immunohistochemistry protocols for these were identical to the general protocol, except for the nuclear denaturation and proteolytic digestion steps which were omitted for these stains. Samples were stained and analysed as described in chapter 3.2.4 and 3.2.5. The code was only broken after analysis were completed for each set of experiments.

4.2.7 Pre-trial tests to confirm optimal parameters

One of the main objectives of the clinical trial was to compare the photoprotective effects of 1,25(OH)₂D₃ directly with THC in human subjects. The limitation, however, was that the human ethics only approved a maximum of 4 biopsies per subject which meant only one time point post UV exposure could be tested, and also non-UV irradiated controls for each treatment were not feasible. To get around this, pre-trial tests were performed with 2 objectives.

Firstly, the optimum time point after the UV irradiation which would show significant increases in all 3 types of DNA damage was assessed. The pre-clinical human ex vivo study had demonstrated the dynamics of UV-induced DNA damage and that the photoprotective effects of 1,25(OH)₂D₃ post UV irradiation were statistically significant for all 3 types of DNA damage at 3 hours post-UV. Despite this, it was assumed these dynamics seen in the human ex vivo study may be different in human in vivo skin since ongoing oxidative stress was also demonstrated in the ex vivo study. Considering this, it was decided to test shorter time intervals for the 3 types of UV induced DNA damage. For this study, 2 volunteers
received the measured UV dose on 2 separate sites and no UV on one site. All sites were treated with vehicle, and the volunteers were asked to return twice, in 1 hour for a biopsy from one of the UV irradiated sites, and again, in another hour for biopsies from the negative control site and the other UV irradiated site. The tissue samples were then processed as described above.

Secondly, the other pre-trial testing was designed in order to confirm that the three different topical treatments did not affect the 3 types of DNA damage in the absence of UV. Again, this was necessary since human ethics approved maximum of 4 biopsies per subject which meant adequate negative controls for 3 different topical treatments in a subject was impossible. In order to show this, volunteers were given the set dose of UV in one area and treated with vehicle. The other 3 areas were not irradiated with UV, but treated with 3 different topical treatments each. After 2 hours post UV and/or treatments, all the sites were biopsied and the tissues were processed as described earlier.

4.3 Minimal Erythemal Dose (MED) testing

Minimal erythemal dose (MED) testing was performed using the equipment described in Chapter 2 section 1, before commencement of the main study. The purpose of MED testing was to test the UV source which was to be used for the randomised clinical trial, as it had a capacity to cause erythema in healthy volunteers, and also to familiarise myself with the process of calibration, UV output measurements with a broadband radiometer, and the detailed procedures in delivering the exact amount of intended dose of UV to the volunteers which otherwise could potentially hinder the outcome of the randomised clinical trial.
Ten healthy volunteers were recruited from the University of Sydney and Royal Prince Alfred Hospital grounds in keeping with the exclusion criteria listed above. They were exposed to the solar simulator generated UV through a black plastic template with 10 x 1 cm$^2$ square holes which delivered UV doses in increments of 10-20 J/cm$^2$ on the subjects' mid/lower back. The subjects were reviewed 24 hours after the UV irradiation, and the UV dose delivered through the square that resulted in the first visible erythema was recorded for each subject (Figure 4.4).
Figure 4.4 **MED testing:** MED at the first square with perceivable redness (Damian 2007).
4.4 Results

4.4.1 MED and melanin index had a linear relationship.

The melanin index (MI) measured from the subjects ranged from -60 to -29.7. The minimal erythemal dose for the 10 volunteers tested ranged from 3.7-8.2 J/cm$^2$ and the average was 5.6 J/cm$^2$ ssUV. MED had linear relation with the MI of the volunteers (Figure 4.5), which was consistent with reports from Damian et al (Damian et al., 1997).

![Figure 4.5 MED vs Melanin index. There is linear relationship between the melanin meter index readings and MED. n=10 subjects.](image-url)
4.4.2 Topical treatments did not alter DNA damage in absence of UV

UV irradiated vehicle treated sites showed marked nuclear staining with antibodies against TD, 8-oxodG, and 8-NG after 2 hours. However, skin biopsies from the non-UV irradiated sites showed significantly lower degrees of nuclear staining regardless of the treatment. Quantification by image analysis and statistical testing showed that non-UV irradiated skin showed significantly lower degrees of all 3 types of DNA damage (p<0.001). There were no statistical differences between the 3 different topical treatments without UV irradiation.
Figure 4.6 **Topical treatments did not affect the 3 types of DNA damage.**

There were no significant differences in the quantity of 3 types of DNA damage in absence of UV (p>0.05). Significantly different from UV irradiated vehicle treated skin, *** p<0.001; %%% p<0.001; ### p<0.001. n=2 subjects.
4.4.3 Time course of DNA damage after UV in human subjects

Skin without UV exposure did not stain for TD. UV-irradiated vehicle-treated skin at 1 and 2 hours post UV showed a highly significant degree of positive staining for TD (Figure 4.7a). Quantification by image analysis showed that the rise in TD at 1 hour post-UV was significant (p<0.001), and also the difference between TD at 1 hour and 2 hours was highly significant (p<0.001). Both 8-oxodG and 8-NG showed mildly positive staining without UV exposure, but a significantly higher degree of nuclear staining was seen with UV exposure both at 1 and 2 hours (Figure 4.7b,c). Again, quantified data showed significant increases in both 8-oxodG and 8-NG level at 1 hour post UV compared to the negative control (p<0.01, p<0.05 respectively). The quantity of 8-oxodG at 2 hours showed a slight decline although statistically not significant (p>0.05). Nuclear staining for 8-NG at 2 increased from 1 hour post UV (p<0.05).
Figure 4.7 Effect of time after UV on three types of DNA damage.

Quantity of TD after 2 hours UV was significantly greater than 1 hour post-UV (P<0.001). There was no significant difference in 8-oxodG with UV between 1 and 2 hours (p>0.05). The difference in quantity of 8-NG between 1 and 2 hours was significant (p<0.05). n=2 subjects. Significantly different from no UV, ^^^ p<0.001; significantly different from UV 1hr, >>> p<0.001; > p<0.05. n=2 subjects.
4.4.4 Photoprotection by 1,25(OH)$_2$D$_3$ and THC from UV-induced DNA damage in human skin in vivo

All 3 types of UV induced DNA damage tested including TD, the most abundant form of CPD, 8-oxodG, and 8-NG showed strongly positive staining in UV-irradiated vehicle-treated skin (Figures 4.8b, 4.9b, 4.10b). Sham irradiated skin showed no nuclear staining for TD, but did exhibit a degree of positive staining for 8-oxodG and 8-NG, although significantly less than the UV irradiated vehicle treated skin (Figures 4.8a, 4.9a, 4.10a). UV irradiated and 1,25(OH)$_2$D$_3$ or THC treated skin showed less staining for TD in nuclei (Fig4.8c, 4.8d).

Quantification by image analysis followed by statistical analysis of results and subsequent revealing of the codes for different topical treatment solutions showed that both 1,25(OH)$_2$D$_3$ and THC significantly reduced TD with no significant difference between the active treatments (Figure 4.8e)
e. Proportion of positively stained nuclei for TD (+SEM)

- Sham Vehicle
- UV Vehicle
- UV 1,25(OH)2D3
- UV THC

Statistical significance:
- @@@: p<0.001
- @ &@@: p<0.01
- @: p<0.05

p>0.05
Figure 4.8 Reduction in UV-induced CPD by 1,25(OH)$_2$D$_3$ or THC.

TDs in sham irradiated vehicle treated skin were not observed with primary antibody for TD (Figure 4.8.a). UV irradiated skin showed marked degree of nuclear staining but reduced staining with topical treatments of 1,25(OH)$_2$D$_3$ or THC applied immediately after UV when compared with vehicle treated equivalent at 2 hours post-UV (Figures 4.8 b,c,d). Arrow points to a positively stained nuclei for TD. Quantification by image analysis showed very significant rise in TD with UV irradiation, and a highly significant degree of reduction when treated with either 11,25(OH)$_2$D$_3$ or THC. Significantly different from sham vehicle-treated at each corresponding time point @@@ p<0.001; from UV vehicle-treated at each corresponding time point &&& p<0.001. n=7 subjects. Scale bar = 100µm.
a. Sham Veh

b. UV Veh

c. UV 1,25(OH)2D3

d. UV THC

e. Proportion of positively stained nuclei for 8-oxodG (+SEM)

- $$$ p<0.001
- $$$ p<0.001
- p>0.05
Figure 4.9 Reduction of UV-induced 8-oxodG by 1,25(OH)₂D₃ or THC.

Sham irradiated vehicle treated skin still showed a degree of 8-oxodG (Figure 4.9a). UV irradiated skin showed marked degree of nuclear staining after 2 hours post-UV but 1,25(OH)₂D₃ or THC treated skin showed reduced degree of staining when compared with the vehicle treated equivalent (Figures 4.9 b,c,d). Arrow points to a positively stained nuclei for 8-oxodG. Quantification by image analysis showed very significant rise in 8-oxodG with UV irradiation, and a highly significant reduction in staining when treated with either 1,25(OH)₂D₃ or THC. Significantly different from sham vehicle-treated at the same time point, $$$ p<0.001; from UV vehicle-treated at the same time point ^^^ p<0.001. n=7 subjects. Scale bar = 100μm.
Proportion of positively stained nuclei for 8-NG (+SEM)

Sham Vehicle  UV Vehicle  UV 1,25D  UV THC

+++  ⌃&&&& p>0.05
Figure 4.10 Reduction of UV-induced 8-NG by 1,25(OH)₂D₃ or THC.

Sham irradiated vehicle treated skin still showed a degree of 8-NG staining (Figure 4.10a). UV irradiated skin showed a marked degree of nuclear staining but this was reduced with topical treatment with 1,25(OH)₂D₃ or THC when compared with vehicle treated equivalents at 2 hours post-UV (Figures 4.10 b,c,d). Arrow points to a positively stained nuclei for 8-NG. Quantification by image analysis showed very significant rise in 8-NG with UV irradiation, and highly significant degree of reduction when treated with either 1,25(OH)₂D₃ or THC. Significantly different from sham vehicle-treated at the same time point +++ p<0.001; from UV vehicle-treated at corresponding time point, %%% p<0.001. n=7 subjects. Scale bar = 100μm.
Quantitative analysis by image analysis showed that UV irradiated and 1,25(OH)₂D₃-treated skin showed at 2 hour post-UV, a significant reduction in TD staining by 57% (p<0.001), 8-oxodG by 50% (p<0.001), and 8-NG by 41% (p<0.001). THC treatment immediately after the same dose of UV showed reduction in TD by 61% (p<0.001), 8-oxodG by 54% (p<0.001), and 8-NG by 37% (p<0.001). When the reduction by THC treatment was compared to the reduction by 1,25(OH)₂D₃, there were no statistical differences between the two, for all 3 types of DNA damage tested (P>0.05).

4.4.5 DNA repair proteins are upregulated by 1,25(OH)₂D₃ and THC in human in vivo skin.

4.4.5.1 Effect of active treatments without UV exposure.

Sham irradiated skin treated with vehicle showed a mild degree of staining with primary antibody against DDB2 (Figure 4.11a). Treatment with 1,25(OH)₂D₃ without UV irradiation increased the degree of staining of DDB2, but this increase was not seen with treatment with THC (Figures 4.11e, 4.11f). Quantitative image analysis and statistical testing showed that the 1,25(OH)₂D₃ treatment without UV exposure significantly increased DDB2 by 2.1-fold, however, there was no statistical difference between the vehicle treated skin and THC treated skin without UV (Figure 4.12a, p>0.05), despite the degree of staining in THC treated skin appeared somewhat increased. THC only increased DDB2 level when applied after UV irradiation (0<0.001), but to a lesser degree than 1,25(OH)₂D₃ (p<0.05). UV irradiation alone did not increase DDB2.
Figure 4.11 *Sections of human skin stained for DDB2 2 hours post-UV and treatment.* Arrow points to a positively stained nuclei for DDB2. Scale bar = 100 µm.
Figure 4.12  **DDB2 modulation with topical treatments.**

UV did not increase DDB2. Topical treatments with 1,25(OH)\(_2\)D\(_3\) or THC increased DDB2 except THC alone did not significantly increase DDB2 unless accompanied by UV irradiation. Significantly different from UV vehicle, ** p<0.01; *** p<0.001; significantly different from UV 1,25(OH)\(_2\)D\(_3\) , # p<0.05. n=2 subjects.
XPC expression significantly increased with both 1,25(OH)₂D₃ and THC treatments in absence of UV, 2 hours after topical treatment (Figures 4.13e, 4.13f) compared with the vehicle treated skin without UV exposure (Figure 4.13a). The increase in XPC expression was by 1.9-fold (p<0.01) with 1,25(OH)₂D₃, and by 2.1-fold by THC (p<0.001) 2 hours after UV irradiation and treatment (Figure 4.14b).

OGG1 expression was also significantly increased after 2 hours with both 1,25(OH)₂D₃ or THC treatment compared with vehicle in the absence of UV (Figures 4.15e, 4.15f). The degree of increase in OGG1 expression was comparable between the two different treatments by 1.1-fold with 1,25(OH)₂D₃ (p<0.05), and by 1.2-fold with THC (Figure 4.16b, p<0.01). OGG1 staining was intense in the basal layer of epidermis but this did not significantly change with UV or different topical treatments, however, OGG1 expression in the upper epidermis showed significant change in expression with topical treatments only although the degree of staining was weaker than the basal layer (Figures 4.15, 4.16c).
Figure 4.13 Sections of human skin stained for XPC 2 hours post-UV and treatment. Arrow points to a positively stained nuclei for XPC. Scale bar = 100 μm.
a. XPC without UV

Proportion of positively stained nuclei for XPC (+SEM)

- UV Vehicle: 20 (SEM: 2)
- Sham Vehicle: 7 (SEM: 2)
- Sham 1,25D: 14 (SEM: 2)
- Sham THC: 13 (SEM: 2)

p > 0.05

b. XPC post UV

Proportion of positively stained nuclei for XPC (+SEM)

- Sham Vehicle: 5 (SEM: 2)
- UV Vehicle: 10 (SEM: 2)
- UV 1,25(OH)2D3: 22 (SEM: 2)
- UV THC: 20 (SEM: 2)

p > 0.05
Figure 4.14 XPC expression 2h after UV and/or active treatments.
XPC expression was increased with UV irradiation alone 2 hours post-UV. Treatment with 1,25(OH)\textsubscript{2}D\textsubscript{3} or THC, also significantly increased XPC level with or without UV irradiation. Significantly different from sham vehicle, @@ p<0.001; @ p<0.01; significantly different from UV vehicle, +++ p<0.001. n=2 subjects.
Figure 4.15 Sections of human skin stained for OGG1 2 hours post-UV and treatment. Arrow points to a positively stained nuclei for OGG1. Scale bar = 100 μm.
a. OGG1 without UV

![Graph showing relative intensity of staining for OGG1 without UV.]

b. OGG1 with UV

![Graph showing relative intensity of staining for OGG1 with UV.]

c. Upper epidermal OGG1 with UV

![Graph showing relative intensity of staining for upper epidermal OGG1 with UV.]

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UV Vehicle | Sham Vehicle | Sham 1,25D | Sham THC
---|---|---|---
60 ± 2 | 58 ± 3 | 64 ± 2 | 66 ± 3

p > 0.05

UV Vehicle | UV Vehicle | UV 1,25D | UV THC
---|---|---|---
10 ± 2 | 12 ± 3 | 16 ± 2 | 20 ± 3

p > 0.05

UV Vehicle | UV Vehicle | UV 1,25D | UV THC
---|---|---|---
8 ± 2 | 10 ± 3 | 14 ± 2 | 18 ± 3

p > 0.05
Figure 4.16 Changes in OGG1 expression in the upper epidermis of human skin 2 hour post UV and treatments.

Basal layers had high background staining for OGG1 in all skin samples without significant variation, but the upper epidermis showed significant increase in OGG1 expression from treatment with 1,25(OH)$_2$D$_3$ or THC (Figures 4.15, 4.16c). UV itself did not affect the epidermal OGG1 expression (Figures 4.16a,b). Significantly different from sham vehicle # p<0.05; ## p<0.01; ### p<0.001; significantly different from UV vehicle, ^^ p<0.01; significantly different from UV 1,25(OH)$_2$D$_3$, & & p<0.01. n=2 subjects for a, n=7 subjects for b,c.
4.4.5.2  Effect of UV exposure with vehicle treatment.

UV irradiated vehicle treated skin samples showed a similar degree of staining for DDB2 or OGG1 compared with the sham-irradiated vehicle-treated skin (Figures 4.11b, 4.15b), but a somewhat increased degree of staining for XPC in comparison to the sham irradiated equivalent (Figure 4.13b). Quantification by image analysis and statistical testing showed that there were no differences between the degree of DDB2 or OGG1 expression post-UV in vehicle-treated skin compared to the sham irradiated skin (Figures 4.12, 4.16, p>0.05), however, XPC expression was significantly increased by 1.5 fold compared to the sham irradiated skin (Figure 4.14, p<0.001).

4.4.5.3  Effect of active treatments on UV exposed skin samples.

When the UV irradiated skin samples were treated post-UV with either 1,25(OH)₂D₃ or THC, the degrees of staining for DDB2, XPC and OGG1 were all markedly increased (Figures 4.11c, 4.11d, 4.13c, 4.13d, 4.15c, 4.15d). Quantification by image analysis and statistical testing showed that DDB2 expression in UV irradiated skin was increased by 2-fold when treated with 1,25(OH)₂D₃ (Figure 4.12, p<0.001) and 1.6-fold with treatment with THC (Figure 4.12, p<0.001) when compared with UV irradiated vehicle treated equivalent. There was a statistical difference between the 1,25(OH)₂D₃ treated and THC treated skin (p<0.05). XPC was increased by 1.8 fold when 1,25(OH)₂D₃ was applied post-UV (Figure 4.14, p<0.001), and by 1.7 fold with THC (Figure 4.14, p<0.01). There was no statistical difference between the two different active treatments (p>0.05).
OGG1 showed an increase in expression by 1.6 fold when treated with 1,25(OH)$_2$D$_3$ post-UV which was statistically significant (Figure 4.16, p<0.05), and by 1.7 fold with THC treatment (Figure 4.16, p<0.01). There was no statistical difference between the two active treatments in OGG1 expression (p>0.05).

In summary, topical treatments with 1,25(OH)$_2$D$_3$ or THC without UV did not affect DNA damage, but significantly increased the expression of DNA repair proteins including DDB2, XPC and OGG1 with the exception of DDB2 which was not significantly increased with THC. UV irradiation alone did not affect the expression of DDB2 or OGG1, but caused a marginal increase in XPC expression. Active treatments post-UV caused all DNA repair proteins to increase in a comparable manner, except for DDB2 which increased to a lesser degree with THC compared to 1,25(OH)$_2$D$_3$. 
4.5 Discussion

This randomised controlled trial in human subjects has shown that topical application of 1,25(OH)$_2$D$_3$ or THC immediately after exposure to around 1 MED dose of UV, significantly reduced 3 types of UV induced DNA damage as shown in the ex vivo study. The initial design of this study was based on the results gained from the human ex vivo study especially for the UV dose used for the study, as well as the dynamics of DNA damage after UV irradiation. It was still kept in mind that the in vivo skin may respond differently to the same dose UV, or the repair dynamics may have been different, since Mouret et al had shown that the CPD repair efficiencies were different in different models (Mouret et al., 2008). Previous work from the Mason group demonstrated a significant reduction in UV induced TD seen in human volunteers treated with calcitriol 24 hours before UV, and then immediately after UV, with the TD assessed 24 hours after the UV exposure (Damian et al., 2010). It is worthwhile to note the MED testing showed widely varied MED with different individuals with different baseline melanin meter readings. However, the DNA damage data lacks such variability since the presented data represent the average of all tested subjects.

Our results from this clinical trial are consistent with previously reported in vitro and in vivo studies where 1,25(OH)$_2$D$_3$ reduced UV induced apoptosis in human keratinocytes in vitro (Wong et al., 2004), and protected the cells from UV induced DNA damage (Wong et al., 2004, Dixon et al., 2005). Studies in mice also showed that 1,25(OH)$_2$D$_3$ reduced DNA damage in mice irradiated with UV (Dixon et al., 2005, Dixon et al., 2011). Furthermore, previous studies also have shown that the photoprotective effects were comparable in human keratinocytes regardless of the timing of the treatment with 1,25(OH)$_2$D$_3$ either 24 hours
before and immediately after UV, or simply immediately after UV (Gupta et al., 2007), which indicates that the photoprotection by 1,25(OH)$_2$D$_3$ is not due to reduced UV exposure. The study showed for the first time in human volunteers that UV induced oxidative stress and the indirect generation of DNA damage, 8-oxodG is also reduced by 1,25(OH)$_2$D$_3$. Gordon-Thomson et al demonstrated that 1,25(OH)$_2$D$_3$ treatment immediately after UV significantly reduced T4 endonuclease V- and endonuclease IV-sensitive sites (TD) as well as hOGG-sensitive sites (8-oxodG) in UV irradiated keratinocytes compared to UV irradiated and vehicle treated keratinocytes, indicating a reduction in UV induced TDs and oxidative DNA damage (Gordon-Thomson et al., 2012). This was also demonstrated in a mouse in vivo study through immunohistochemistry and image analysis (Dixon et al., 2005, Dixon et al., 2007, Dixon et al., 2011). More recently, Jain et al suggested a possible mechanism of reduction in reactive oxygen species by 1,25(OH)$_2$D$_3$ due to upregulation of cellular glutathione (Jain and Micinski, 2013).

The current study shows for the first time, another vitamin D compound-induced mechanism that would enhance TD/CPD repair. Both 1,25(OH)$_2$D$_3$ and THC significantly increased expression of DDB2 and XPC post-UV, two of several proteins involved in NER. In particular, both DDB2 and XPC are known to be involved in the initial stage of recognition of DNA damage, and initiation of NER, and are thought to be important as rate determining proteins for NER (Yokoi et al., 2000, Wang et al., 2004, Fei et al., 2011, Bergink et al., 2012). DDB2, in particular, is thought to be recruited earlier than XPC to detect the DNA damage, hence thought to be a relevant marker for photodamage repair, even though no detectable increase was seen with UV irradiation alone without treatment. The current study looked at the level of expression of various DNA repair proteins at a single fixed time point, which is 2
hours post-UV and/or topical treatments which may be the reason why DDB2 or OGG1 did not show significant increase post-UV. Furthermore, this finding is in keeping with the suggestions that 1,25(OH)_{2}D_{3} may upregulate DDB2 and XPC genes as suggested in RNA array study by Moll et al, although this was never confirmed in a biological study (Moll et al., 2007).

This study also showed for the first time in human subjects, evidence of similar reductions of UV induced damage at micromolar concentrations by curcuminoids. In vivo studies are limited although there have been several reviews on possible photoprotective effects of curcuminoids. One study involving cultured keratinocytes which had suggested curcuminoids including THC at nanomolar concentrations regulate MAP kinase to inhibit keratinocyte growth while inducing p21 as well as enhancing p53 to protect DNA (Ayli et al., 2010).

Recently, Phillips et al reported an in vivo study involving hairless mice and suggested topically or orally administered curcumin at micromolar concentrations inhibited skin cancer formation, or prolonged tumour onset (Phillips et al., 2013), but did not suggest any experimental evidence on the mechanism of such photoprotection. Prior to this report, Chan et al had reported that curcumin could prevent UV induced apoptotic changes including c-Jun N-terminal kinase (JNK) activation, loss of mitochondrial membrane potential (MMP), mitochondrial release of cytochrome C, caspase 3 activation, and cleavage of PAK2 in keratinocyte-like A431 cells (Chan et al., 2003). Flow cytometric analysis showed that reactive oxygen species formation post-UV, was abolished by curcumin at micromolar concentration (Chan et al., 2003). The findings from this study which used THC at nanomolar concentrations extends these observations to show that the oxidative DNA damage, 8-oxodG generated by UV, was significantly reduced to a similar extent by 1,25(OH)_{2}D_{3} or THC in human subjects. Further to these suggestions, the current study has also demonstrated that the
OGG1 level is increased with 1,25(OH)_{2}D_{3} or THC. OGG1 is the enzyme responsible for BER of 8-oxodG, and it is known to be present in mitochondria and nuclei (de Souza-Pinto et al., 2001). Javeri et al reported OGG1 expression was higher in the superficial epidermis (stratum granulosum) than the more metabolically active basal layer, and the level of expression did not change with UV irradiation (Javeri et al., 2008). These authors suggested that the weaker expression of OGG1 in the basal layer may lead to lack of DNA repair and accumulation of UVA induced oxidative DNA mutations (Javeri et al., 2008). A report by Campalans et al presented evidences that OGG1 is capable of relocalising from mitochondria to nuclear speckles during UVA irradiation. This relocation of OGG1 was prevented by the presence of antioxidant compounds suggesting that this migration of OGG1 may be triggered by ROS signalling (Campalans et al., 2007). The current study demonstrated the level of OGG1 was increased by 1,25(OH)_{2}D_{3} or THC but not with UV irradiation. The lack of change of OGG1 after UV is consistent with the report from Javeri et al (Javeri et al., 2008). The increase in OGG1 by 1,25(OH)_{2}D_{3} or THC which occurred in sham- as well as UV-irradiated sites may explain the reduction in UV induced 8-oxodG by 1,25(OH)_{2}D_{3} or THC. UV did not increase the total OGG1 level in human skin, but it is unclear if there was any relocation of OGG1 from cytoplasm to nuclear speckles because the light microscopy examination and image analysis of the immunohistochemical staining of frozen sectioned human skin showed both cytoplasmic and nuclear staining (Figure 4.11).

The current study used the same antibody and protocol as Javeri et al but showed a different pattern of OGG1 staining, in that the staining for OGG1 was more intense in the basal layers than the superficial (Figure 4.11) and basal staining did not significantly change with active treatments. The increases in OGG1 expression were mainly seen in the upper half of the
epidermis when treated with active treatments (Figure 4.11). Javeri et al reported increased expression of OGG1 in the upper granular layer compared with basal when tested with immunohistochemistry and RT-PCR in adult skin, foreskin and reconstructed layers skin with or without UV irradiation (Javeri et al., 2008). It was concluded that this was more in keeping with the higher accumulation of oxidative stress post UV in the basal layer which may account for development of malignancies in this layer and also suggested that the higher OGG1 level was associated with the degree of differentiation in keratinocytes. The current study also demonstrated UV irradiation did not alter level of OGG1 expression in human skin, but treatment with 1,25(OH)2D3 or THC showed increased expression of OGG1 in epidermis, but again, not in the granular layer. The reason for these differences in two studies is not clear. The differences however, may be due to the difference in the collection of skin. Javeri took punch biopsies from buttocks 24 hours post UV, or used human skin explants which would have spent several hours in culture until the sample was snap frozen. In current study, the biopsy samples were taken 2 hours post-UV and were snap frozen with minimal time loss. Recent data from Lui et al suggested UVA irradiation induced NO release mainly in the upper epidermis, suggesting more oxidative stress may also exist in the upper epidermis as well (Inoue and Kawanishi, 1995, Fiala et al., 1996, Spencer et al., 1996, Liu et al., 2007).

Decreased DNA damage may be due to decreased inhibitors of DNA repair such as NO products as well as being due to increased expression of repair proteins including DDB2 and XPC. It is also reasonable to propose that these actions are due to activation of the non genomic pathway considering THC at nanomolar concentrations produced comparable protective effects to 1,25(OH)2D3.
Chapter 5

5 General discussion and future directions

5.1 Tetrahydrocurcumin as a Vitamin D alternative.

Numerous studies from the Mason group and others have now proven the photoprotective properties of 1,25(OH)$_2$D$_3$. Although the mechanism of this photoprotection is still not entirely clear, it is likely that the activation of a rapid response or non genomic pathway through the alternative binding pocket of VDR is responsible for the photoprotective effects. This may lead to enhanced DNA repair in part, through the reduction in nitric oxide products which inhibit repair of and in part through upregulation of initial early DNA damage recognition proteins (DDB2 and XPC) or DNA repair enzymes (hOGG1). The in vivo clinical trial has confirmed that the topical use of 1,25(OH)$_2$D$_3$ provides at least short term protection against acute UV exposure, which will probably reduce the risk of skin cancer development. Although daily use of topical 1,25(OH)$_2$D$_3$ or calcitriol may sound like an attractive idea, there are several problems associated with this.

Firstly, it is currently scheduled as S4 (prescription only) medicine, and not allowed to be sold over the counter. Secondly, the topical use of calcitriol carries the risk of hypercalciuria or hypercalcaemia which may cause renal or biliary stones, nephrocalcinosis, abdominal pain, nausea, vomiting, cardiac dysrhythmia, or neuropsychiatric disorders from raised threshold.
for depolarization in nerve and muscle fibres (Armstrong and Cota, 1999, Wesson et al., 2009).

Calcitriol is also expensive to produce and is unstable with a short half-life which makes it not ideal to be incorporated into topical preparation for the general public. However, curcuminoids, in particular THC, do not raise serum calcium even at high concentrations, and have few or no side effects.

THC is a known ligand for VDR alternative pocket and although it does not have any transactivating activity, the studies have shown that it is capable of mimicking non genomic actions of 1,25(OH)\(_2\)D\(_3\) including the photoprotective effects (Mizwicki et al., 2004). The human ex vivo study demonstrated possible dose dependent action of THC, where the higher concentration of 100nM amongst the tested concentrations, showed a comparable degree of photoprotection against UV induced DNA damage and upregulation of DNA repair enzymes to that of 1,25(OH)\(_2\)D\(_3\) .

Curcuminoids are generally considered safe since they have been consumed daily in large quantity for centuries without significant adverse effects. Topical application of THC is also regarded very safe since no adverse reaction has been reported other than a single report of contact dermatitis in a patient who developed allergic contact dermatitis where skin patch test confirmed he showed positive response to THC (Lamb and Wilkinson, 2003). There are numerous reports also suggesting CUR and THC act as photosensitisers (Chignell et al., 1994, Park and Lee, 2007, Nardo et al., 2009) but this may not be a problem at such low concentrations as used in the current study. The current study has provided evidence that
THC may be useful as a sunscreen formulation or an after-sun lotion to help minimize the solar damage from the residual UV which did not get absorbed by the UV filters in a sunscreen, hence provide additional benefit in further reducing solar ageing or skin cancer.
5.2 Future Directions

This study has confirmed both 1,25(OH)₂D₃ and THC provided photoprotection against UV induced direct and indirect forms of DNA damage, and suggested THC may also provide protection against photoimmunosuppression while 1,25(OH)₂D₃, when tested in human skin caused immunosuppression in part, through increase in IL-10. The mechanism of such protection may be, in part, mediated through enhanced DNA repair, with increase in DNA repair enzymes, and reduction in NO products. Immunohistochemistry and image analysis was the only method used for both the ex vivo and in vivo studies, as the studies used whole skin samples making endonuclease digestion and comet assay impractical. The group has confirmed that THC reduces DNA damage in human keratinocytes by endonuclease digestion and comet assay (Gordon-Thomson et al., 2012) although Western blot could be used to verify increase in repair proteins.

There were difficulties in detection of OGG1 through immunohistochemistry since the stained skin showed mixed mitochondrial and nuclear staining, but only the nuclear OGG1 is known to be important in repair of 8-oxodG (Hashiguchi et al., 2004)(Hashiguchi 2004). For this reason, it may be useful to separate nucleus and cytoplasm from human keratinocytes, and subject the nuclear extracts to western blot or immunoprecipitation so only the nuclear OGG1 may be accurately quantified. Other methods in quantifying DNA damage may also be considered and employed such as liquid chromatography or mass spectrometry (Douki et al., 2000).

The mechanisms of photoprotection by 1,25(OH)₂D₃ or curcuminoids may also involve other pathways such as haemoxigenase-I (HO-1) or glutathione. Reports have suggested HO-1
expression may be increased with 1,25(OH)$_2$D$_3$ (Oermann et al., 1999, Lu'o'ng and Nguyen, 2013), and also by CUR or curcuminoids (Balogun et al., 2003, McNally et al., 2007, Hsu et al., 2008, Jeong et al., 2009). Glutathione (GSH) is a well known intracellular antioxidant known to be depleted with UVB radiation through inactivation of cystine transporter system which makes keratinocytes more vulnerable to oxidative stress (Zhu and Bowden, 2004). It was suggested glutathione was increased with both 1,25(OH)$_2$D$_3$ and CUR (Biswas et al., 2005, Jain and Micinski, 2013), again suggesting another possible photoprotective mechanism of these compounds.

THC treatment resulted in photoprotection against UV induced DNA damage and photoimmunosuppression, which was also seen by earlier studies in cells and mice (Poliakov, 2009, Ryan, 2010). Other overseas groups have used CUR at 10-20 μM concentration, which is a significantly higher concentration compared to the doses tested in current study, to study its biological action or photoprotection (Guo et al., 2013, Phillips et al., 2013). It is likely that at higher concentrations, CUR may be acting as a chemical antioxidant whereas at much lower concentration, it is acting as a vitamin D-like compound (Mizwicki et al., 2004). It remains to be tested whether knockdown of VDR or ERp57 abolishes protective effect of THC as it does with 1,25(OH)$_2$D$_3$, since study in VDR knockout mice have shown no photoprotection by 1,25(OH)$_2$D$_3$ (Ellison et al., 2008, Quigley et al., 2009, Teichert et al., 2011).

The current study clearly demonstrated that various DNA repair proteins may be modulated with UV and/or topical treatments, although 2 hours post-UV did not increase expression of DDB2 or OGG1 when XPC showed significant elevation. Further human ex vivo skin study,
repeated, but inclusive of DNA repair protein expression may help in providing the dynamics of various DNA protein expression post different triggers including UV and/or different treatments.

There have been other compounds also suggested to be ligands for VDR alternative pocket (Haussler et al., 2008, Bartik et al., 2010). Duff et al reported that fluorescence polarization (FP) assay may be useful in identifying novel VDR ligands (Duff et al., 2006) which opens possibility we could also identify novel VDR ligands which may provide more potent photoprotective effects.

Finally, since the current study has demonstrated photoprotective effects in a randomised double blinded placebo controlled clinical trial, it may be plausible to suggest the addition of THC into a cosmetic products for daily use by the general public such as sunscreen may provide further protection against UV. To test this hypothesis, it would be worthwhile to conduct another human ex vivo study, and perhaps another clinical trial to test if a topically applied sunscreen with THC would provide more photoprotection than the same sunscreen but without THC. The design of the study will need to have the topical application of the sunscreen product prior to UV irradiation, with significantly higher doses of UV to be able to induce UV induced damage. The sunscreen with THC should be directly compared with the sunscreen without THC to see if the presence of THC would have significantly impact the degree of photoprotection.

It would be interesting to see if the addition of THC would impact on MED since topical calcitriol alone did not affect MED in human in vivo skin (Damian 2010). Amongst other
possible tests to test a sunscreen product, photostability testing with THC would be important to make sure it will resist UV induced degradation (Nash and Tanner, 2014). Furthermore, it may also be tested for the protection against UVA induced oxidative stress and dermal changes including metalloproteinases, intracellular GSH or immune suppression (Halliday, 2005, Vilela et al., 2013).
6 References


BYRNE, S., LIMON-FLORES, A. & ULLRICH, S. 2008. Mast cell migration from the skin to the draining lymph nodes upon ultraviolet irradiation represents a key step in the induction of immune suppression. *Journal of Immunology*, 180, 4648-4655.


DIXON, K. M., DEO, S. S., WONG, G., SLATER, M., NORMAN, A. W., BISHOP, J. E., POSNER, G. H.,
possible role of 1,25-dihydroxyvitamin D3 and its analogs. Journal of Steroid Biochemistry and
Molecular Biology, 97, 137-143.

Differential photoprotective effects of 1,25-dihydroxyvitamin D3 and a low calcaemic

DOUKI, T. & CADET, J. 1996. Peroxynitrite mediated oxidation of purine bases of nucleosides and

dimeric lesions within isolated and cellular DNA as measured by high performance liquid

identification of vitamin D receptor (VDR) ligands. American Chemical Society, 232nd
National Meeting and Exposition. San Francisco, CA.

ELLISON, T., SMITH, M., GILLIAM, A. & MACDONALD, P. 2008. Inactivation of the vitamin D receptor
enhances susceptibility of murine skin to UV-induced tumorigenesis. J Invest Dermatol, 128,
2508-17.

EPE, B. 2012. DNA damage spectra induced by photosensitization. Photochemical and
Photobiological Sciences, 11, 98-106.

during nucleotide excision repair provides a structure for cleavage by human XPG protein.
EMBO J, 16, 625-638.

excision repair by UV-DDB: Prioritization of damage recognition to internucleosomal DNA.
PLOS Biol, 9, e1001183.

FIALA, E. S., SODUM, R. S., BHATTACHARYA, M. & LI, H. 1996. (−)-Epigallocatechin gallate, a
polyphenolic tea antioxidant, inhibits peroxynitrite-mediated formation of 8-
oxodeoxyguanosine and 3-nitrotyrosine. Experientia, 52, 922-6.


Mechanisms of photoaging and chronological skin aging. Archives of Dermatology, 138,
1462-1470.

FITZPATRICK, T. 1998. The validity and practicality of sun-reactive skin types I through VI. Arch
Dermatol, 127, 869-871.

formation of 8-hydroxyguanine in isolated DNA. Archives of Biochemistry and Biophysics, 262,
266-272.


FOUSTERI, M., VERMEULEN, W., VANZEELAND, A. & MULLENDERS, L. 2006. Cockayne syndrome A
and B proteins differentially regulate recruitment of chromatin remodeling and repair
factors to stalled RNA polymerase II in vivo. Mol Cell, 23, 471-482.

FRIEDMAN, P. 2006. Vitamin D. In: BRUNTON, L. (ed.) Goodman and Gilman's the Pharmacological

GARLAND, C., COMSTOCK, G., GARLAND, F., HELSING, K., SHAW, E. & GORHAM, E. 1989. Serum 25-


HOLDER, G., PLUMMER, J. & RYAN, A. 1978. The metabolism and excretion of curcumin 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione in the rat. *Xenobiotica*, 8, 761-768.


MASON, R. S. 2000. Effect of Vitamin D on Melanocytes and Its Role in Melanogenesis,. New York, Marcel Dekker.


NASH, E. & NEWMAN, P. 2001. NASA confirms Artic Ozone Depletion Trigger. Image of the day. NASA.


POLIAKOV, S. 2009. Photoprotection by a Vitamin D-like Compound. Bachelor of Medical Science (Hons), The University of Sydney.


ZANELLO, L. & NORMAN, A. 1997. Stimulation by 1alpha,25(OH)2-vitamin D3 of whole cell chloride currents in osteoblastic ROS.


Introduction:
You are invited to take part in a research study aimed at understanding sun-induced skin damage and skin cancer development and ways to minimize these processes. Skin tissue, obtained at surgery undertaken for unrelated reasons, will be used to study the mechanisms by which sun damage and other forms of damage affect skin tissue and to examine ways of minimizing this damage, particularly the role that vitamin D compounds may play in this process. This research may lead to the development of methods or compounds which reduce skin damage and possibly skin cancers.
The study is being conducted in the University of Sydney and the Sydney South West Area Health Service by Prof Rebecca S Mason (Physiology), Prof Gary Halliday and A/Prof Diona Damian (Department of Dermatology, Royal Prince Alfred Hospital) and their associates, Dr Clare Gordon-Thomson and Dr Eric Song (Department of Physiology).

**Study Procedures:**
You will be having surgery, for reasons unrelated to this project, which will result in the removal of some skin. Your permission is sought to send the skin that is not needed for diagnostic or other clinical purposes to laboratories for use in research into the mechanisms of sun damage and ways to minimize this.

**Risks:**
As the study uses only skin already removed for unrelated reasons, there will be no additional risks to you, other than those related to your original procedure.

**Benefits:** While we intend that this research furthers medical knowledge and may improve sun protection in the future, it will not be of direct benefit to you.

**Costs:** Participation in this study will not cost you anything, nor will you be paid.

**Confidentiality:**
Your agreement to allow your skin tissue to be used for the above studies will be kept confidential and the skin samples will not be identified. No additional skin will be removed other than what is required for surgery.

**Voluntary Participation:**
Contributing tissue to this study is voluntary. You do not have to agree to it. However, if you do agree, it will not be possible to withdraw your skin sample from the study once it has been provided to the researcher, because the skin samples are not identified.

You may keep this information sheet: If you have further queries about this information or the study, now or at any stage, please contact Dr Eric Song on 9351 6524. This study has been approved by the Ethics Review Committees of the University of Sydney and the Sydney South West Area Health Service (SSWAHS).

Any person with concerns or complaints about the conduct of this study should contact the Secretary of the SSWAHS committee on 02 9515 6766 and quote protocol number X09-0069.
PARTICIPANT CONSENT FORM

I, .............................................................................................................. [name]

of .............................................................................................................. [address]

________________________________________________________

Date: ____________________________

Signature: _______________________

Print Name: ______________________

Witness Name: ___________________
I understand that my agreement to allow skin tissue to be used for the above studies will be kept confidential and the skin samples will not be identified. I further understand that no additional skin will be removed other than what is required for surgery. Permission to use the tissue is voluntary and I am permitted to withdraw permission without penalty or prejudice. As the samples are not identified in order to maintain confidentiality, it will not be possible to withdraw once the skin sample has been provided to the researcher.

Under these circumstances, I freely choose to allow the skin tissue removed at surgery to be included in this study. I also understand that the research study is strictly confidential.

I hereby agree to participate in this research study.

NAME :.................................................................
Does vitamin D-Like compounds protect from sun damage?

Volunteers are needed for RESEARCH at the Departments of Physiology and Medicine (Dermatology).

Some reimbursement for expenses and participation time is offered.

Please contact
7.4 Clinical in vivo Trial Patient information

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Vitamin D and photoprotection

(INFORMATION SHEET)

Introduction

You are invited to take part in a research study into the effects of the active metabolite of vitamin D (calcitriol), a compound which is normally present in skin, and vitamin D-like compounds or a component of fish oil on the skin’s response to ultraviolet light. The objective of this study is to investigate the ability of these compounds to reduce sunburn. We will treat skin with these compounds and measure the skin’s reaction to different doses of ultraviolet light (sunlight).

This research is being conducted by Professor Rebecca S Mason (Department of Physiology), Professor Gary Halliday and A/Professor Diona Damian (Dermatology, Royal Prince Alfred Hospital) and their associates Dr Clare Gordon-Thomson (Physiology) and Dr Eric Song (Physiology).

Study Protocol
If you agree to participate in this study, you will be asked to visit the hospital on three occasions, on day one, then either 3h later or 24h later and then 11 days after that. Each visit will last approximately 45 minutes. You will be asked to undergo the following procedures:

**Visit 1:** The protocol will be further explained. Medical history and examination will be carried out including assessment of skin type and melanin index measurement. This measurement will be used to estimate the UV dose required for your skin type. This is the minimum amount of UV to cause minimal redness (Minimal erythemal dose, or MED) which would be equivalent to 5-10 minutes of midday sun. Using this calculation your back will be exposed to ultraviolet light for a period equivalent to approximately ten minutes in the midday summer sun. In order to allow for the active vitamin D compounds to be absorbed and to work in the skin, you will have the active vitamin D compounds and/or the fish oil compound and the ointment base applied to the area on your back that has been exposed to ultraviolet light. The material will be left on until visit 2.

**Visit 2:** Either 3 or 24 hours later (you will be told which time point is relevant to you), a total of four small biopsies will be taken from these areas on your lower back after the area is numbed with injections of local anaesthetic. The local anaesthetic and biopsy procedure takes about 15 minutes in total and does not affect your ability to drive afterwards. Each biopsy measures 5mm in diameter, and you will have a suture (stitch) placed at each biopsy site. You should keep the biopsy sites dry for the first 24 hours, and then you may change the dressings and shower as normal. The sutures should be removed here in 11 days.
Visit 3: Return 11 days later for removal of the sutures.

Side Effects

You may experience some side effects as part of your participation in this study. Mild itching may be felt at the site of application of the vitamin D compounds or base. Most people, however, will develop only mild redness.

Potential side effects of the biopsies include infection (let us know if you notice any increasing redness, swelling, pain or discharge from the biopsy sites). In the longer term, you will have 4 small scars at the biopsy sites, each measuring about 5mm or less. Some people have a tendency to develop large, lumpy or painful scars after surgery or trauma – if you have a history of keloid scars, you must not participate in this study. Other reasons why you might not be eligible for the study include:

- if you have had any sunlight on your mid-back in the last 4 weeks
- if you are pregnant or breastfeeding
- if you are currently using immunosuppressive medications, such as prednisone
• if you are abnormally sensitive to sunlight or are taking photo-sensitising medications. You should tell us of any prescription or non-prescription medications you are taking.

Confidentiality

All aspects of the study, including the results, will be strictly confidential and only the investigators named above will have access to information on participants. A report of the study may be submitted for publication, but individual participants will not be identifiable in such a report.

Benefit

While we intend that this research study furthers medical knowledge and may improve our understanding of the development of skin cancers, it will not be of any direct benefit to you. You will be reimbursed $250 for the time you contribute to the study.

Participation

Participation in this study is entirely voluntary: you are in no way obliged to participate and – if you do participate – you can withdraw at any time. Whatever your decision, please be assured that it will not affect your relationship with Royal Prince Alfred Hospital or the University of Sydney. However, if you decide that you no longer wish to participate, it would be appreciated if
you were to contact me as soon as possible so that another volunteer can be booked in your place.

When you have read this information, Dr Eric Song will discuss it with you further, if required, and answer any questions you may have. If you would like to know more at any stage, please feel free to contact Dr Song on 93516524. This information sheet is for you to keep.

This study has been approved by the Ethics Review Committee of the Sydney South West Area Health Service. Anyone with concerns or complaints about the conduct of this research project can contact the Secretary on 02 9515 6766 and quote protocol number X09-0069.
PARTICIPANT CONSENT FORM

I, ........................................................................................................... [name]
of ............................................................................................................ [address] s,

"Vitamin D and photoprotection – Clinical Trial"
have read and understood the Information for Participants on the above named research study and have discussed the study with Dr Eric Song.

I understand that my agreement to participate will be kept confidential and the skin samples will not be identified. My intention to participate is voluntary and I am permitted to withdraw participation without penalty or prejudice. As the biopsied samples are not identified in order to maintain confidentiality, it will not be possible to withdraw once the skin samples have been provided to the researcher.

I have discussed and understand the possible side effects associated with participating in this study which includes scarring, bleeding, infection, and minor sunburn. These are also listed on the information printout provided in more detail.

I understand I am offered remuneration for the participation, which will be payable to me only after the full completion of my due participation schedule.

I hereby agree to participate in this research study.
Clinical Trial Patient Interview

Interview

1. Volunteer number

2. Full medical history including previous skin diseases and family history

3. Medication

4. Allergy

5. Examination including full skin check and site check

6. Psychosocial history

7. Preferred time/day of the week – allocation 3/24/48 hr group

8. Exclusion criteria

   8.1. Age

   8.2. Skin type – exclude I and V VI?

   8.3. Recent (within 4 weeks) sun exposure on the back

   8.4. History of MM (history of non-mm ? Gorlands)

   8.5. Pregnancy/Lactation

   8.6. Medication – vitaminD, corticosteroids, NSAIDs, immunosuppressives

   8.7. Photosensitivity – including medications

   8.8. Keloid

   8.9. Psychosocial - history

   8.10. Overseas visitors? (adverts at local backpackers etc)
7.7 Clinical Trial Volunteer Registration
The University of Sydney

Human Study X00-0264

Volunteer No:

1. Personal Details
   
   Name:
   Gender:
   Address:
   Contact No:

2. Bank account
   
   Acc Name:
   Acc Type (savings/cheque):
   BSB, Account No:

3. Background Health (including skin conditions):

4. Current medication/Dietary supplements (incl 4 weeks from now):

5. Recent Sun exposure
7.8 Clinical Trial Volunteer Records

The University of Sydney

Volunteer No:

1. Melanin index:

   4.2  
   4.9  
   4.9  

2. Erythema index:

   78  
   96  
   86  

3. Irradiation:

   BB UVA \[ 3.1 \times 10^{-1} \] 4.13 4.10

   Total UV (UVA+UVB): \( t = \frac{\text{Total}}{\text{BB} \times \text{CF}} \)

   (1000mJ->70mJ UVB, 2000mJ->140mJ UVB, 3000->210mJ UVB, 4000->280mJ UVB)

   Min: 150

   Max:

   Increments/Time: 20

4. MED (24 hours later):

   7

5. Treatment
1α,25-Dihydroxyvitamin D₃ reduces several types of UV-induced DNA damage and contributes to photoprotection.


AUTHOR CONTRIBUTIONS:

E.J. Song:

I was responsible for the preparation and formatting of the entire manuscript. For the experiments, I was responsible for the study design, patient consent, collection of human ex vivo skin, and other related work. I was also responsible for the cryosectioning, immunohistochemical staining, image analysis and statistical testing of the acquired data. I was responsible for showing evidences that 1,25(OH)₂D₃ reduced UV-induced CPD, 8-oxodG, and 8-NG in human ex vivo skin, and their dynamics after the UV irradiation.

C. Gordon-Thomson:

This author provided assistance with overall study design and methods including human skin cultures, immunohistochemistry and image analysis. This author also helped with preparation of the manuscript.
**L. Cole:**

This author provided assistance with microscope imaging and image analysis with Metamorph software.

**H. Stern:**

This author provided human ex vivo skin samples for the study.

**G.M. Halliday:**

This author provided general advice, facilitated the work and helped with manuscript review.

**D.L. Damian:**

This author provided general advice, facilitated the work and helped with manuscript review.

**V.E. Reeve:**

This author provided general advice, facilitated the work and helped with manuscript review.
**R.S. Mason:**

This author is the senior author on the manuscript, and was responsible for the coordination of the entire study. This author also had a major role in manuscript preparation and review.